Kishan Gopal Ramawat
Jean-Michel Mérillon
Editors

Natural Products

Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes

Springer Reference

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With 1569 Figures and 307 Tables

Springer Reference

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Preface

We are pleased to present a five volume treatise on “Natural products: phytochemistry, botany and metabolism”. Natural products are as diverse as plant biodiversity and it was a herculean task to bring together several hundred leading scientists distributed all over the world to contribute in this project.

This five volumes work on Natural Products is reference work providing state-of-the-art knowledge composed by highly renowned scientists in their field. The book is intended to serve the needs of graduate students, Ph.D. scholars, researchers in the field of phytochemistry, botany, agricultural sciences, pharmacy, nutrition, biotechnology and, industrial scientists and those involved in marketing phytochemicals, plants and their extracts. The present reference work will encompass the information about well established phytochemicals, biology and biotechnology of medicinal plants or their products, their biosynthesis, novel production strategies, demand and uses, metabolism and bioavailability. This book is a work of tertiary literature containing digested knowledge in an easily accessible format.

Use of medicinal plants is as old as human civilization and continuous efforts are being made to explore new and old medicinal plants for novel bioactive molecules or to produce these products in high amounts through modern technologies. About 200,000 natural products of plant origin are known and many more are being identified from higher plants and micro-organisms. Some plants based drugs are used since centuries and there are not many alternatives for some natural drugs as cardiac glycosides or morphine. Various facets of bioactive molecules have developed very rapidly in the last two decades particularly due to newer tools of isolation and identification as well as refined molecular techniques to establish the biological properties of isolated molecules. This endeavour is to timely compile this vast data generated in recent past. This is well reflected in 139 chapters running in over 4000 pages of text and vast literature cited in each chapter. The readers will find comprehensive information on almost all bioactive molecules. While planning this book our endeavour was to incorporate articles that cover the entire gamut of bioactive molecules of all the three major classes, viz., alkaloids, phenolics and terpenes. Each volume is further divided into sections such as General Biology and Biotechnology; Classes - Occurrence, Biosynthesis, Structure and Chemistry, Distribution; Methods of Analysis; (para) Pharmacology and Bioavailability; and Nutraceuticals and Functional Foods (in phenolics).

Some examples are sufficient to illustrate the spectrum of chapters in different sections such as: on alkaloids (Biotechnology and genetic engineering for alkaloid production, various classes of alkaloids e.g., Purine alkaloids, Ergot alkaloids, Terpenoid indole alkaloids, Pharmacological effects of ephedrine, Lycopodium alkaloids, Biological activities of pepper alkaloids, Neurotoxic alkaloids from cyanobacteria, Prevention of brain disorders by nicotine, Opioids and pain treatment, Ecological roles of alkaloids); on phenolics (Genetics of flavonoids, Functional foods: Genetics, metabolome and engineering, phytonutrient levels, Cocoa cultivation, directed breeding and polyphenolics, classes of phenolics, Polyphenols and anticancer activity, Tannins and anthocyanins of wine: phytochemistry and organoleptic properties, Polyphenols and beer quality, Wine polyphenols and vascular protective effects, Isoflavonoids and phytoestrogenic activity, Functional grapes, Potential neuroprotective actions of dietary flavonoids, Prospects of functional foods / nutraceuticals and markets), and on terpenes (Terpenes: Chemistry, biological role and therapeutic applications, Biotransformation of terpenoids and steroids, Production and genetic engineering of terpenoids production in plant cell cultures and organ cultures, Taxol-producing fungi, Metabolic engineering of isoprenoids biosynthesis, classes of terpenes, Cannabinoids: Chemistry and Medicine, Phytosterols: Beneficial effects, Ginsenosides: Biological activities, Ginkgolides and neuroprotective effects, Quassinoids: Anti-cancer and antimalarial activities, Phytoecdysteroids: phytochemistry and pharmacological activity, Brassinosteroids and their biological activity, and so on).

These compounds exhibit various ecological functions, provide protection against attack by herbivores and microbes, and serve as attractants for pollinators and seed-dispersing agents. Natural products are explored as sources of drugs, flavouring agents, fragrances and for a wide range of therapies. Rapid progress has been made in recent years in understanding natural product accumulation and synthesis, and regulation and functions. It is timely to bring this information together with contemporary advances in chemistry, plant biology, ecology, and pharmacology and metabolism of natural products in the form of a comprehensive treatise on natural products.

Because of the voluminous work for the treatise, this project was spread over almost two years, from concept to print. We would like to acknowledge cooperation, patient and support of our contributors who have put their serious efforts to ensure the high scientific quality of this book with up to date information.

This work could not be completed without active support of Springer team who took pains in streamlining the production process. We are particularly indebt to Drs. Marion Hertel, Lydia Muller, Sylvia Blago and Simone Giesler for their continuous support from very inception of the project.

March 2013 K. G. Ramawat
J.-M. Mérillon

About the Editors

Professor (Dr.) K. G. Ramawat, Former Professor & Head Botany Department, M.L. Sukhadia University, Udaipur, India

Professor K. G. Ramawat did his Ph.D. (1978, Plant Biotechnology) from the University of Jodhpur, India and joined as faculty member in January 1979. He joined M.L.Sukhadia University as Associate Professor in 1991 and became Professor in 2001. He served as Head, Department of Botany (2001-2004, 2010-2012), In charge, Department of Biotechnology (2003-2004), member task force on medicinal and aromatic plants, Department of Biotechnology, Government of India, New Delhi (2002-2005) and co-ordinator UGC-DRS and DST-FIST programmes (2002-2012). He did his Post-doctoral at the University of Tours, France (1983-85) and subsequently worked as visiting professor at University of Tours (1991) and University of Bordeaux2, France (1995, 1999, 2003, 2006, 2010). He visited Poland under INSA-PAN academic exchange programme (2005). During last 38 years of his career, he has published more than 170 peer reviewed papers and articles. He has edited books on Biotechnology of medicinal plants, secondary metabolites, Bioactive molecules, Herbal drugs, Plant defence: biological control, Desert plants; published by Science Publishers Inc, Enfield, USA and Springer Verlag, Germany. His research on recalcitrant woody legume trees of desert (*Prosopis, Zizyphus, Commiphora*), production of useful metabolites from woody plants (*Comiphora, Pueraria*) was funded by UGC, CSIR, ICAR, DBT and DST, New Delhi. Works related to use of novel growth modulators and elicitors on the production of guggulsterones, stibenes and isoflvonoids are well cited. He has supervised doctoral thesis of 25 students. He is member of several academic bodies, associations and editorial boards of journals.

Professeur Jean-Michel Mérillon, Directeur de l'EA 3675 (Groupe d'Etude des Substances Végétales à Activité Biologique + Polyphénols Biotech), Faculté de Pharmacie, Université de Bordeaux, Institut des Sciences de la Vigne et du Vin, Villenave d'Ornon, France

Professor J.M. Mérillon received his M.Pharma. (1979) and Ph.D. (1984) from the University of Tours in France. He joined the University of Tours as assistant professor in 1981, became associate professor in 1987, and a full professor in 1993 at the faculty of Pharmacy, University of Bordeaux, France. He is currently group leader of a “study group on biologically active plant substances” at the Institute of Vine and Wine Sciences, which comprises 25 scientists and research students. His group has worked for many years on phenolic compounds from vine and wine, mainly complex stilbenes and their involvement in health. He has supervised the doctoral theses of 18 students. He has published more than 125 research papers in internationally recognized journals. He has an H index of 29 according to the analysis of documents published between 1996 and 2013. He has co-edited four books on secondary metabolites and biotechnology (Science Publishers, USA; Springer, Germany). He is involved in developing teaching on plant biology, natural bioactive compounds and biotechnology. He has traveled widely as a senior professor. Scientists from several countries are working in his laboratory and his research is supported by funding from the Aquitaine Regional Government, the Ministry of Higher Education and Research, and various private companies. He founded a technology transfer unit in 2004, Polyphenols Biotech, providing support for R&D programs for SMEs and major groups from the cosmetic, pharmaceutical, agricultural and health-nutrition sectors.

Contents

Volume 1

Part I Alkaloids: General Biology and Biotechnology 1

1 **Microbial Production of Plant Benzylisoquinoline Alkaloids** . . . 3
Eitaro Matsumura, Motoki Matsuda, Fumihiko Sato, and Hiromichi Minami

2 **Alkaloids of Marine Macroalgae** 25
Kasım Cemal Güven, Burak Coban, Ekrem Sezik, Hüseyin Erdugan, and Ferda Kaleağasıoğlu

3 **Neurotoxic Alkaloids from Cyanobacteria** 39
Ralf Kellmann, Olivier Ploux, and Brett A. Neilan

4 **Bioproduction of Terpenoid Indole Alkaloids from *Catharanthus roseus* Cell Cultures** 85
Lorena Almagro, Mariana Sottomayor, and Maria Angeles Pedreño

5 **Bioactive Alkaloids from South American *Psychotria* and Related Rubiaceae** 119
Hélio Nitta Matsuura, Diogo Denardi Porto, and Arthur Germano Fett-Neto

6 **Ecological Role of Alkaloids** 149
Shaily Goyal

7 **Plant In Vitro Systems as Sources of Tropane Alkaloids** 173
Vasil Georgiev, Andrey Marchev, Strahil Berkov, and Atanas Pavlov

8 **Biotechnology and Genetic Engineering for Alkaloid Production** 213
Smita Srivastava and Ashok Kumar Srivastava

9 **Marine Pyrroloiminoquinone Alkaloids, Makaluvamines and Discorhabdins, and Marine Pyrrole-Imidazole Alkaloids** 251
Hiromichi Fujioka and Yasuyuki Kita

10 Internet Natural Product Trade, Business, and Risk of Damaged and/or Contaminated Products 285
Maria Laura Colombo, Maria Teresa Pinorini, and Ario Conti

Part II Alkaloids: Classes - Occurrence, Biosynthesis, Structure and Chemistry, Distribution **301**

11 Alkaloids Derived from Lysine: Piperidine Alkaloids 303
Éva Szőke, Éva Lemberkovics, and László Kursinszki

12 Alkaloids Derived from Ornithine: Tropane Alkaloids 343
Stefan Philipov and Tsvetelina Doncheva

13 Alkaloids Derived From Ornithine: Pyrrolizidine Alkaloids 359
Helmut Wiedenfeld

14 Alkaloids Derived from Lysine: Quinolizidine (a Focus on Lupin Alkaloids) 381
Giovanna Boschin and Donatella Resta

15 Alkaloids Derived from Tyrosine: Modified Benzyltetrahydroisoquinoline Alkaloids 405
Feroz Khan, Tabish Qidwai, Rakesh K. Shukla, and Vikrant Gupta

16 Alkaloids Derived from Tyrosine: Penethylisoquinoline (Autumnaline, Colchicine) 461
Pijush Paul, Mihir Halder, and Sumita Jha

17 Amaryllidaceae Alkaloids 479
Zhong Jin and Xiao-Hua Xu

18 Bioactive Alkaloids from Fungi: Psilocybin 523
Zafar Alam Mahmood

19 Alkaloids Derived from Tryptophan: Harmine and Related Alkaloids 553
T. D. Nikam, K. M. Nitnaware, and M. L. Ahire

20 Alkaloids Derived from Tryptophan: Terpenoid Indole Alkaloids 575
Shilpa Ramani, Nandadevi Patil, Suhas Nimbalkar, and Chelliah Jayabaskaran

21 Chemistry and Biology of Cinchona Alkaloids 605
Karol Michał Kacprzak

22 Chemistry and Biology of Camptothecin and its Derivatives 643
Karol Michał Kacprzak

23 Alkaloids Derived from Tryptophan: A Focus on Ergot Alkaloids 683
Peter Mai and Shu-Ming Li

24 Alkaloids Derived from Anthranilic Acid: Quinoline, Acridone, and Quinazoline 715
Maria Fátima das Graças Fernandes da Silva, João Batista Fernandes, Moacir Rossi Forim, Paulo Cezar Vieira, and Israel Cívico Gil de Sá

25 Alkaloids Derived from Histidine: Imidazole (Pilocarpine, Pilosine) 861
Ana Paula Santos and Paulo Roberto H. Moreno

26 Alkaloids Derived by Amination Reaction: Acetate-Derived (Coniine) 883
Rumen Binev

27 Ephedra Alkaloids-Alkaloids Derived by Amination Reaction: Phenylalanine Derived 909
Ali Parsaeimehr and Elmira Sargsyan

28 Terpenoid Alkaloids Derived by Amination Reaction 923
Krishna Prasad Devkota and Norbert Sewald

29 Purine Alkaloids, Cytokinins, and Purine-Like Neurotoxin Alkaloids 953
Hiroshi Ashihara, Takao Yokota, and Alan Crozier

30 Aziridine Alkaloids: Origin, Chemistry and Activity 977
Valery M. Dembitsky, Alexander O. Terent’ev, and Dmitri O. Levitsky

Volume 2

Part III Alkaloids: Methods of Analysis 1007

31 Methods of Analysis: Tropane Alkaloids from Plant Origin 1009
Philippe Christen, Stefan Bieri, and Strahil Berkov

32 Methods for Analysis of Pyrrolizidine Alkaloids 1049
Colin Crews

33 Methods of Analysis of Opium Alkaloids 1069
Carmen Cruces-Blanco, Diego Airado-Rodríguez, and Ana M. García-Campaña

34 **Analysis of Catharanthus Alkaloids** 1121
Qinhua Chen and Zilin Chen

35 **Methods for Analysis of Ergot Alkaloids** 1133
Colin Crews

36 **Analysis of Alkaloids by Capillary Electrophoresis** 1153
Roberto Gotti

Part IV Alkaloids: Pharmacology 1201

37 **Pharmacology of Macroalgae Alkaloids** 1203
Ferda Kaleağasıoğlu, Kasım Cemal Güven, Ekrem Sezik, Hüseyin Erdugan, and Burak Coban

38 **Pharmacological Effects of Ephedrine** 1217
Renata Pereira Limberger, Ana Laura Bemvenuti Jacques, Gabriela Cristina Schmitt, and Marcelo Dutra Arbo

39 **Lycopodium Alkaloids: Pharmacology** 1239
Elín Soffía Olafsdóttir, Elsa S. Halldorsdottir, N. M. Pich, and S. Omarsdottir

40 **Scope of Alkaloids in Antileishmanial Drug Discovery and Development** 1263
Bhuwan B. Mishra, Navneet Kishore, Rakesh K. Singh, and Vinod Kumar Tiwari

41 **Pharmacological/Biological Effects of Berberine** 1301
Eva M. Galvez, M. Perez, P. Domingo, D. Nuñez, V. L. Cebolla, M. Matt, and J. Pardo

42 **Alkaloids and Drug Discovery for Neurodegenerative Diseases** 1331
Melanie-Jayne R. Howes

43 **Opioids and Pain Treatment** 1367
Daniel Krashin, Andrea Trescot, and Natalia Murinova

44 **Pyrrolizidine Alkaloid-Induced Toxicity to the Liver** 1383
Manuela G. Neuman

45 **Biological Activities of Pepper Alkaloids** 1397
Krishnapura Srinivasan

46 **Antiangiogenic Alkaloids from Plants** 1439
Flávia Cristine Mascia Lopes, Luis Octávio Regasini, Manuel Nuno de Magalhães Pinheiro Alçada, and Raquel Soares

47 **Prevention of Brain Disorders by Nicotine** 1469
Karim Alkadhi, Samina Salim, and Karem Alzoubi

48 Aconitum Alkaloids and Biological Activities 1503
Ainura Chodoeva, Jean-Jacques Bosc, and Jacques Robert

49 Alkaloids and Inhibitory Effects Against Enzymes Linked to Neurodegenerative Diseases (Physostigmine, Galanthamine, Huperzine, etc.) 1525
Ilkay Erdogan Orhan and F. Sezer Senol

Part V Phenolics: General Biology and Biotechnology **1541**

50 Phenolic Compounds: Introduction 1543
Vincenzo Lattanzio

51 Metabolic Engineering for Functional Foods: Tomato Fruits and Stilbenes 1581
Giovanna Giovinazzo, Ilaria Ingrosso, Marco Taurino, and Angelo Santino

52 Cocoa Cultivation, Directed Breeding and Polyphenolics 1599
Reinhard Lieberei, Daniel Kadow, and David Seigler

53 Genetics of Flavonoids 1617
Ramesh Prasad Pandey and Jae Kyung Sohng

54 Isoflavonoid Production by Genetically Engineered Microorganisms 1647
Brady F. Cress, Robert J. Linhardt, and Mattheos A. G. Koffas

55 Bioproduction of *trans*-Resveratrol from Grapevine Cell Cultures 1683
Lorena Almagro, Sarai Belchí-Navarro, Ana Belén Sabater-Jara, Juan Carlos Vera-Urbina, Susana Sellés-Marchart, Roque Bru, and María Angeles Pedreño

56 Functional Foods: Genetics, Metabolome, and Engineering Phytonutrient Levels 1715
Tahira Fatima, Avtar K. Handa, and Autar K. Mattoo

Volume 3

Part VI Phenolics: Classes - Occurrence, Biosynthesis, Structure and Chemistry, Distribution **1751**

57 Flavanols: Catechins and Proanthocyanidins 1753
Joana Oliveira, Nuno Mateus, and Victor de Freitas

58 Anthocyanins 1803
Sonia de Pascual-Teresa, Maria Teresa Sanchez-Ballesta, and Cristina García-Viguera

59 Flavones and Flavonols: Phytochemistry and Biochemistry 1821
Qiang Zhang, Xinhuai Zhao, and Hongbin Qiu

60 Isoflavonoids 1849
Varsha Sharma and Kishan G. Ramawat

61 Minor Flavonoids (Chalcones, Flavanones, Dihydrochalcones, and Aurones) 1867
Masayuki Ninomiya and Mamoru Koketsu

62 Stilbenoids 1901
Tao Shen, Chun-Feng Xie, Xiao-Ning Wang, and Hong-Xiang Lou

63 Phenolic Acids 1951
Marta Goleniowski, Mercedes Bonfill, Rosa Cusido, and Javier Palazón

64 Hydrolyzable Tannins: Gallotannins and Ellagitannins 1975
Michaël Jourdes, Laurent Pouységu, Denis Deffieux, Pierre-Louis Teissedre, and Stéphane Quideau

Part VII Phenolics: Methods of Analysis **2011**

65 Preparative Extraction and Separation of Phenolic Compounds 2013
Winny Routray and Valérie Orsat

66 Mass Spectrometric Detection of Phenolic Acids 2047
Santi M. Mandal and Dipjyoti Chakraborty

67 Analytical Methods of Phenolic Compounds 2061
Ioana Ignat, Irina Volf, and Valentin I. Popa

68 Phenolic Compounds in *Catharanthus roseus* 2093
David M. Pereira, Patrícia Valentão, Mariana Sottomayor, Federico Ferreres, and Paula B. Andrade

69 Flavonoids by HPLC 2107
S. W. Annie Bligh, Olumuyiwa Ogegbo, and Zheng-Tao Wang

70 Polyphenol Purification by Solid Support-Free Liquid–Liquid Chromatography (CCC, CPC) 2145
Jane Hubert, Karen Plé, Mahmoud Hamzaoui, and Jean-Hugues Renault

Part VIII Phenolics: Biological Activity and Bioavailability **2173**

71 Cellular, Molecular, and Biological Perspective of Polyphenols in Chemoprevention and Therapeutic Adjunct in Cancer 2175
Sanjeev Banerjee and Paulraj Rajamani

72 **Tannins and Anthocyanins of Wine: Phytochemistry and Organoleptic Properties** 2255
Pierre-Louis Teissedre and Michael Jourdes

73 **Grapevine Stilbenoids: Bioavailability and Neuroprotection** 2275
Pierre Waffo-Téguo, Stéphanie Krisa, Alison D. Pawlus, Tristan Richard, Jean-Pierre Monti, and Jean-Michel Mérillon

74 **Proanthocyanidins of Cocoa: Bioavailability and Biological Activities** 2311
Manuel Rusconi, Maria Teresa Pinorini, and Ario Conti

75 **Polyphenols and Beer Quality** 2333
Sonia Collin, Vesna Jerkovic, M. Bröhan, and D. Callemien

76 **Wine Polyphenols and Vascular Protective Effects** 2361
Cyril Auger and Valérie B. Schini-Kerth

77 **Isoflavonoids and Phytoestrogenic Activity** 2381
Catherine Bennetau-Pelissero

78 **Phenolics in Human Nutrition: Importance of the Intestinal Microbiome for Isoflavone and Lignan Bioavailability** 2433
Thomas Clavel and Job O. Mapesa

79 **Bioavailability of Anthocyanins** 2465
Ana Faria, Iva Fernandes, Nuno Mateus, and Conceição Calhau

Volume 4

Part IX Phenolics: Nutraceuticals and Functional Foods 2489

80 **Prospects of Functional Foods/Nutraceuticals and Markets** 2491
Josep Valls, Nacho Pasamontes, Albert Pantaleón, Susana Vinaixa, Montse Vaqué, Arantza Soler, Silvia Millán, and Xavier Gómez

81 **Food Sources of Phenolics Compounds** 2527
Wende Li and Trust Beta

82 **Nutraceuticals and Antioxidants in Prevention of Diseases** 2559
Neelam Jain and Kishan G. Ramawat

83 **Functional Grapes** 2581
Maria Isabel Fernández-Marín, Raúl F. Guerrero, Belén Puertas, María Carmen García-Parrilla, and Emma Cantos-Villar

84 Potential Neuroprotective Actions of Dietary Flavonoids 2617
David Vauzour, Marcus Rattray, Robert J. Williams, and Jeremy P. E. Spencer

85 Improvement of Nutraceutical Value of Food by Plant Symbionts 2641
Manuela Giovannetti, Luciano Avio, and Cristiana Sbrana

Part X Terpenes: General Biology and Biotechnology **2663**

86 Terpenes: Chemistry, Biological Role, and Therapeutic Applications 2665
Priyanka P. Brahmkshatriya and Pathik S. Brahmkshatriya

87 Biosynthesis of Terpenoids 2693
Nelson L. Brock and Jeroen S. Dickschat

88 Biotransformation of Terpenoids and Steroids 2733
Wang-Yu Tong

89 Production and Genetic Engineering of Terpenoids Production in Plant Cell and Organ Cultures 2761
Mercedes Bonfill, Sonia Malik, M. Hossein Mirjalili, Marta Goleniowski, Rosa Cusido, and Javier Palazón

90 Taxol Producing Fungi 2797
Xiaoran Hao, Jiao Pan, and Xudong Zhu

91 Metabolic Engineering of Isoprenoid Biosynthesis 2813
Anne Pribat, Lisa Boureau, Anne Mortain-Bertrand, Linda S. Bert, Dominique Rolin, Emeline Teyssier, and Philippe Gallusci

92 Selection of Tomato Rich in Nutritional Terpenes 2853
Jaime Cebolla-Cornejo, Salvador Roselló, and Fernando Nuez

93 Volatile Oils from Marine Macroalgae 2883
Kasım Cemal Güven, Ekrem Sezik, Ferda Kaleağasıoğlu, Hüseyin Erdugan, Burak Coban, and Elif Karakaş

94 Ecological Functions of Terpenoids in Changing Climates 2913
Jarmo K. Holopainen, Sari J. Himanen, J. S. Yuan, F. Chen, and C. Neal Stewart Jr.

95 Hairy Root Culture: An Alternative Terpenoid Expression Platform 2941
Jocelyne Trémouillaux-Guiller

Part XI Terpenes: Classes - Occurrence, Biosynthesis, Structure and Chemistry, Distribution 2971

96 Regular Monoterpenes and Sesquiterpenes (Essential Oils) 2973
Remigius Chizzola

97 Monoterpenes: Iridoids 3009
Biswanath Dinda and Sudhan Debnath

98 Guaianolide Sesquiterpenoids: Pharmacology and Biosynthesis 3069
Henrik Toft Simonsen, Corinna Weitzel, and Søren Brøgger Christensen

99 Biosynthesis and Production of Artemisinin: A Novel Class of Molecule 3099
Athar Ali, Mather Ali Khan, and Mailk Zainul Abdin

100 Nivalenol (Fungal Sesquiterpenes) 3123
Yoshiko Sugita-Konishi and Makoto Kimura

101 Taxol and Related Compounds 3159
Hojjat Sadeghi-aliabadi and Afsaneh Yegdaneh

102 Diterpenes for Therapeutic Use 3173
Virginia Lanzotti

103 Diterpene Sweeteners (Steviosides) 3193
Akula Ramakrishna and Gokare Aswathanarayana Ravishankar

104 Triterpenoid Saponins: A Focus on Polygalaceae 3205
Marie-Aleth Lacaille-Dubois, Clément Delaude, and Anne-Claire Mitaine-Offer

105 Plant Steroidal Saponins: A Focus on Open-Chain Glycosides 3225
Victoria L. Challinor and James J. De Voss

106 Tetraterpenes: Carotenoids 3251
Shinichi Takaichi

Volume 5

Part XII Terpenes: Methods of Analysis 3285

107 Essential Oils: Analytical Methods to Control the Quality of Perfumes 3287
Alberto Chisvert, Marina López-Nogueroles, and Amparo Salvador

108 **Methods for Analysis of Triterpenoid Saponins** 3311
Jinlan Zhang and Feng Qu

109 **Methods of Isolation and Analysis of Forskolin from *Coleus forskohlii*** 3325
Mohamed Saleem A.

110 **Quassinoids: Chemistry and Novel Detection Techniques** 3345
Dipjyoti Chakraborty and Amita Pal

111 **Methods for Extraction and Analysis of Carotenoids** 3367
Siti Machmudah and Motonobu Goto

Part XIII Terpenes: Pharmacology and Bioavailability 3413

112 **Cannabinoids: Chemistry and Medicine** 3415
Giovanni Appendino and Orazio Taglialatela-Scafati

113 **Phytosterols: Beneficial Effects** 3437
Jonathan Santas, Rafael Codony, and Magda Rafecas

114 **Withanolides: Chemistry and Antitumor Activity** 3465
M. Iqbal Choudhary, Sammer Yousuf, and Atta-ur-Rahman

115 **Pharmacology of Ginsenosides** 3497
Kar Wah Leung

116 **Sesquiterpenes and Cytotoxicity** 3515
Ajay Sharma, Vivek K. Bajpai, and Shruti Shukla

117 **Tanshinones Against Cancer and Cardiovascular Diseases and their Biosynthesis** 3551
Lixin Zhang and Xueting Liu

118 **Strigolactones and Biological Activity** 3583
Koichi Yoneyama, Xiaonan Xie, and Kaori Yoneyama

119 **Biological Activity of Oleuropein and its Derivatives** 3605
Stefania Bulotta, Manuela Oliverio, Diego Russo, and Antonio Procopio

120 **Bacosides and Neuroprotection** 3639
Sukanya Majumdar, Amrita Basu, Pijush Paul, Mihir Halder, and Sumita Jha

121 **Garlic and Cardiovascular Diseases** 3661
Syed Haris Omar

122 **Ginkgolides and Neuroprotective Effects** 3697
Syed Haris Omar

123 **Cardiac Glycosides and Anticancer Activity** 3743
Yuan Lin, Dapeng Chen, Li Wang, and Dongmei Ye

124 **Terpenes and Improvement of Transdermal Drug Delivery** 3757
Lifeng Kang, Vaddi Haranath Kumar, Perry Fung Chye Lim,
Han Hui Cheong, and Sui Yung Chan

125 **Quassinoids: Anticancer and Antimalarial Activities** 3775
Emeline Houël, Didier Stien, Geneviève Bourdy, and
Eric Deharo

126 **Glycyrrhizic Acid: Biological Effects on Glucose and Lipid Metabolism** 3803
So Ha Ton, Chanchal Chandramouli, and Khalid BAK

127 **Phytoecdysteroids: Phytochemistry and Pharmacological Activity** 3827
Gert Laekeman and Arnold Vlietinck

128 **Brassinosteroids and their Biological Activities** 3851
Jana Oklešťková, Lucie Rárová, and Miroslav Strnad

Part XIV Terpenes: Parapharmaceutical Uses **3873**

129 **Lycopene in Cancer Prevention** 3875
Kazim Sahin and Omer Kucuk

130 **Xanthophylls and the Eye** 3923
Hannah E. Bartlett

131 **Triterpenoid Centellosides: Bioactivities and Uses in Healthcare Application** 3959
Puziah Hashim

132 **Essential Oils as Antimicrobial Agents** 3975
Vivek K. Bajpai, Shruti Shukla, and Ajay Sharma

133 **Menthol: Biological Effects and Toxicity** 3989
Demétrius A. M. Araújo, Darizy F. Silva, Humberto C. Joca,
José H. Leal-Cardoso, and Jader S. Cruz

134 **Eugenol as Local Anesthetic** 4001
Gehoon Chung and Seog Bae Oh

135 **Carotenoids as Colorants** 4017
Bernardo Dias Ribeiro and Raquel Grando de Oliveira

136 **Oleoresins from Pine: Production and Industrial Uses** 4037
Kelly Cristine da Silva Rodrigues-Corrêa, Júlio César de
Lima, and Arthur Germano Fett-Neto

137 Pyrethrins and Synthetic Pyrethroids: Use in Veterinary Medicine 4061
Arturo Anadón, Irma Arés, María Aránzazu Martínez, and María Rosa Martínez-Larrañaga

138 Essential Oils in Insect Control 4087
Catherine Regnault-Roger

139 Biological Activities of Selected Mono- and Sesquiterpenes: Possible Uses in Medicine 4109
Gerhard Buchbauer and Anja Ilic

Index 4161

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Part I

Alkaloids: General Biology and Biotechnology

Microbial Production of Plant Benzylisoquinoline Alkaloids

1

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Hiromichi Minami

Contents

1 Introduction 4
2 Metabolic Engineering of BIAs in Microbes 6
2.1 Design of BIA Production Pathways in Bacteria and its Enzymes 6
2.2 Construction of Reticuline-Producing *E. coli* and Reticuline Production 10
2.3 BIA Production Using *S. cerevisiae* 11
3 Fermentative Production of BIAs from Simple Sources of Carbon 11
3.1 L-Tyrosine Production from Simple Sources of Carbon 14
3.2 Conversion of L-Tyrosine to Dopamine 15
3.3 BIA Production from a Simple Source of Carbon in *E. coli* 17
4 Conclusion 21
References 21

Abstract

Higher plants produce diverse chemicals such as alkaloids, terpenoids, and phenolic compounds (phenylpropanoids and flavonoids) in secondary metabolism. Among these chemicals, benzylisoquinoline alkaloids (BIAs) are very important in medicine due to their high biological activities. However, extraction yields from plants are low because most of these metabolites accumulate at low levels in plant cells. There has been increasing interest in the microbial

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production of plant metabolites by reconstructing plant biosynthetic pathways in microorganisms. Advances in synthetic biology and metabolic engineering have enabled "tailored" production of plant secondary metabolites in microorganisms. Recently, a platform to produce BIAs was constructed using bioengineered *Escherichia coli* or *Saccharomyces cerevisiae*, which could be useful for bulk production. Here, we review the fermentation platforms for low-cost production of many diverse alkaloids in microbes.

Keywords

Benzylisoquinoline alkaloid • Metabolic engineering • Microbial production • Reticuline • Synthetic biology • Tyrosine

Abbreviations

3,4-DHPAA	3,4-Dihydroxyphenylacetaldehyde
4-HPAA	4-Hydroxyphenylacetaldehyde
4′OMT	3′-Hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase
6OMT	Norcoclaurine 6-*O*-methyltransferase
BBE	Berberine bridge enzyme
BIA	Benzylisoquinoline alkaloid
CM/PDH	Chorismate mutase/prephenate dehydrogenase
CNMT	Coclaurine-*N*-methyltransferase
CYP80G2	Corytuberine synthase
DAHPS	3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase
DODC	L-DOPA decarboxylase
E4P	Erythrose-4-phosphate
HPP	*p*-Hydroxyphenylpyruvate
L-DOPA	L-Dihydroxyphenylalanine
MAO	Monoamine oxidase
NCS	Norcoclaurine synthase
PEP	Phosphoenolpyruvate
PPA	Prephenate
PTS	Phosphotransferase system
SAM	*S*-Adenosyl-L-methionine
TH	Tyrosine hydroxylase
TKT	Transketolase

1 Introduction

The secondary metabolites of higher plants include diverse chemicals such as alkaloids, terpenoids, and phenolic compounds (phenylpropanoids and flavonoids), which are produced from primary metabolites such as amino acids or acetyl coenzyme A (acetyl-CoA). Although these compounds are widely used in the health and nutrition of humans, they are mainly obtained by plant extraction.

Table 1.1 Methods of BIA production

	Microbial production (*E. coli* or *S. cerevisiae*)	Chemical synthetic procedure	Genetically engineered plants and cultured plant cells
Advantages	High productivity	High productivity (increase in reaction steps reduce efficiency)	Moderate productivity but with large chemical diversity
	Short production time (within a few days)	Short production time (depends on number of steps)	Low environmental load (moderate reaction condition, such as neutral pH and medium temperature)
	Low environmental load (moderate reaction condition, such as neutral pH and medium temperature)	Space-saving	Space-saving for cultured plant cells
	Space-saving		
Demerits	Host-dependent enzyme expression; difficulty to express membrane-associated enzyme such as P450	High environmental load (dependence on starting chemical resources, specific catalysts, extreme reaction conditions, such as acidic or alkali, and high or low temperature)	Large culture field is needed for genetically engineered plants
	Limitation of available genes and substrates		Long production time (months to years)
			Extensive downstream purification of desired compound (except for some metabolic engineered plant/cells)

However, the yield is not high because of the low-level of accumulation of metabolites in plant cells. There have been many attempts to use metabolic engineering in plants to increase the amounts of alkaloids [1–8]. However, obtaining the desired products is very difficult because of the complicated and strict regulation of metabolic flows. Chemical synthesis has also been applied to obtain plant secondary metabolites. However, the complexity and chiral nature of these compounds have hampered the development of cost-effective methods.

Recently, microbial production of plant metabolites by reconstructing the plant biosynthetic pathway in microorganisms has garnered interest [9–12]. Microbial systems could improve not only the quantity but also the quality of secondary metabolites because they do not contain other plant metabolites. Microbial systems have a comparative advantage over chemical synthetic procedure or genetically engineered plants and plant cells (shown in Table 1.1). Although microbial systems offer several advantages for the biotransformation of chemicals, they also have disadvantages, such as limited availability of substrates (especially for plant metabolites), genes identified for biosynthesis, storage capacity, and tolerance to chemicals produced. Thus, the rational design of metabolic pathway using microbial- and plant-derived genes is needed for the establishment of efficient systems for the production of various compounds.

In this chapter, we have focused on current knowledge about the metabolic engineering of benzylisoquinoline alkaloids (BIAs) in microbes.

2 Metabolic Engineering of BIAs in Microbes

Among the secondary metabolites of higher plants, alkaloids are very important in medicine due to their high biological activities. Alkaloids are low-molecular-weight, nitrogen-containing compounds that are found in ~20% of plant species. Most alkaloids are derived from amines produced by the decarboxylation of amino acids such as histidine, lysine, ornithine, tryptophan, and tyrosine. One of the largest and most diverse groups of pharmaceutical alkaloids is the BIAs. These include the analgesic compounds morphine and codeine, and the antibacterial agents berberine and palmatine, which are produced via (*S*)-reticuline from L-tyrosine in Papaveraceae, Berberidaceae, Ranunculaceae, Magnoliaceae, and many other plant families. (*S*)-Reticuline is therefore a main branch-point intermediate in the biosynthesis of many types of BIAs. (*S*)-Reticuline also acts as a non-narcotic building block that is useful in the development of novel antimalarial and anticancer drugs.

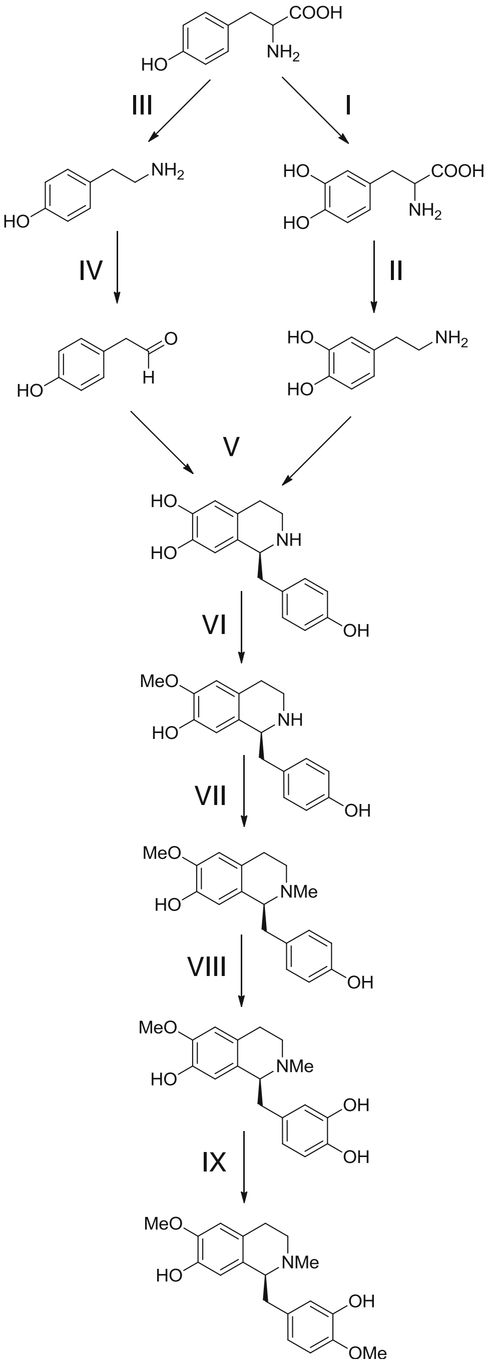
2.1 Design of BIA Production Pathways in Bacteria and its Enzymes

Various types of BIAs are produced from L-tyrosine via (*S*)-reticuline in plants. (*S*)-Reticuline is a key intermediate of the biosynthesis of BIAs. The biosynthetic pathway of (*S*)-reticuline from L-tyrosine is shown in Scheme 1.1 and has nine steps:

I. Hydroxylation of the C-3 group of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA)
II. Decarboxylation of L-DOPA to dopamine
III. Decarboxylation of L-tyrosine to tyramine
IV. Deamination of tyramine to 4-hydroxyphenylacetaldehyde (4-HPAA)
V. Condensation of dopamine and 4-HPAA via the Pictet–Spengler reaction to norcoclaurine
VI. 6-*O*-Methylation of norcoclaurine to coclaurine
VII. *N*-Methylation of coclaurine
VIII. Hydroxylation of the 3′-C in *N*-methylcoclaurine
IX. 4′-*O*-Methylation of 3-hydroxy-*N*-methylcoclaurine

The several upstream biosynthetic genes in these steps, tyrosine/DOPA decarboxylase and tyrosine aminotransferase, have been isolated and characterized [13, 14].

Scheme 1.1 Synthetic pathway of (*S*)-reticuline in plant


COOH
NH2
HO
III
I
NH2
HO
HO
COOH
NH2
HO
IV
II
O
H
HO
HO
NH2
HO
V
HO
NH
HO
OH
VI
MeO
NH
HO
OH
VII
MeO
NMe
HO
OH
VIII
MeO
NMe
HO
OH
OH
IX
MeO
NMe
HO
OH
OMe

BIA biosynthesis begins with the conversion of L-tyrosine to dopamine and 4-HPAA, which are condensed into (*S*)-norcoclaurine by norcoclaurine synthase (NCS) [15–21]. These first steps are difficult to reconstruct for the efficient production of divergent BIAs because the branched pathway from L-tyrosine to (*S*)-norcoclaurine is complicated to produce the desired product. Moreover, in many cases, producing active forms of plant cytochrome P450 enzymes in bacteria is difficult (even though the hydroxylation of 3′-C in *N*-methylcoclaurine (VIII) is catalyzed by P450 enzyme (CYP80B)). In fact, heterologous expression of CYP80B in *Escherichia coli* has not been reported. Therefore, these reactions involve the use of enzymes from other organisms or to design a "shortcut pathway" for the biosynthesis of compounds.

Bacteria do not possess the machinery to synthesize alkaloids like plants or fungi. Minami et al. first designed a shortcut pathway for the biosynthesis of (*S*)-reticuline via norlaudanosoline in bacteria based on providing dopamine (Scheme 1.2) [22]. The pathway has five reaction steps and is called the "NLS pathway":

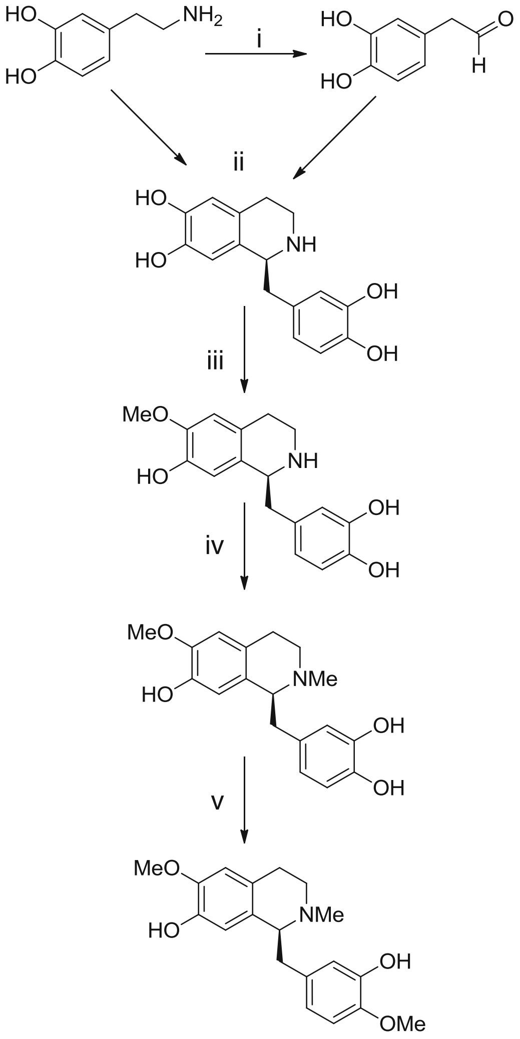
(i) Dopamine is converted to 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA) catalyzed by monoamine oxidase (MAO) from *Micrococcus luteus*.
(ii) Condensation of dopamine and 3,4-DHPAA into norlaudanosoline via the Pictet–Spengler reaction catalyzed by NCS.
(iii) The 6-*O*-methylation of norlaudanosoline to 6-*O*-methylnorlaudanosoline is catalyzed by norcoclaurine 6-*O*-methyltransferase (6OMT).
(iv) The *N*-methylation of 6-*O*-methylnorlaudanosoline is catalyzed by coclaurine *N*-methyltransferase (CNMT).
(v) The 4′-*O*-methylation of 6-*O*-methyllaudanosoline to reticuline is catalyzed by 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT).

The coupling of dopamine and 3,4-DHPAA permits bypassing cytochrome P450 hydroxylase (CYP80B). Moreover, deamination catalyzed by microbial MAO means that reticuline can be obtained from a single substrate: dopamine. The biosynthetic pathway of reticuline from L-tyrosine in plants requires nine enzymes. However, the shortcut biosynthetic pathway from dopamine in microbial production requires only five enzymes.

MAO catalyzing the deamination of dopamine (i) has been isolated from various organisms. Most eukaryotic MAOs exist as membrane proteins, so expression of this enzyme in *E. coli* is difficult. Therefore, Minami et al. selected microbial MAO from *M. luteus*. This enzyme exists as a soluble protein in the cytosol, and its expression in *E. coli* is readily achieved [23].

NCS catalyzes isoquinoline formation via an asymmetric Pictet–Spengler reaction (ii). The reaction entails an acid-catalyzed electrophilic addition of an iminium ion. The mechanism is a two-step process in which the iminium ion is generated first from the condensation reaction between the aldehyde carbonyl (such as 4-HPAA) and dopamine. Two types of NCS (CjNCS1 and CjPR10A) have been isolated from *Coptis japonica* cells and characterized [21]. CjPR10A has been sufficiently expressed in an active form in *E. coli*. Conversely, CjNCS1 forms a larger complex in plant cells. Therefore, the recombinant enzyme expressed in *E. coli* cells has considerably lower activity than that of the native enzyme.

Scheme 1.2 Synthetic pathway of (*S*)-reticuline reconstructed in *E. coli* (NLS pathway)



These results indicate that CjPR10A is more suitable than CjNCS1 for microbial production systems. CjPR10A has been used as an NCS enzyme in reticuline biosynthesis.

The three steps of methylation of norlaudanosoline (iii, iv, v) are catalyzed by three kinds of methyltransferases (6OMT, CNMT, and 4′OMT). These enzymes need *S*-adenosyl-L-methionine (SAM) as the methyl donor. The 6OMT and CNMT from *C. japonica* have broad substrate selectivity. Therefore, 6OMT can catalyze the 6-*O*-methlyation of not only norcoclaurine (VI) but also norlaudanosoline (iii). Similarly, CNMT can catalyze the *N*-methylation of not only coclaurine (VII) but also 6-*O*-methylnorlaudanosoline (iv).

In conclusion, the pathway has three features that enable the efficient production of reticuline.

1. The starting material is only dopamine.
2. The 3′-hydroxylation of (*S*)-*N*-methylcoclaurine is bypassed.
3. The biosynthetic pathway can be constructed in *E. coli* cells by using selected enzymes.

2.2 Construction of Reticuline-Producing *E. coli* and Reticuline Production

E. coli cells containing the genes in the NLS pathway have been constructed using two types of plasmid vector. The genes of NCS and MAO are inserted into pKK223-3 vector, and 6OMT, CNMT, and 4′OMT are inserted into pACYC184 vector. The gene of MAO from *M. luteus* has not been expressed by a T7 promoter. Therefore, only the gene of MAO has been cloned with a taq promoter, and other genes have been cloned with a T7 promoter. However, Nakagawa et al. reported that a codon optimization MAO gene has been expressed by a T7 promoter [24].

E. coli BL21(DE3) containing pKK223-3-NCS-MAO and pACYC184-6OMT-CNMT-4′OMT have been prepared as reticuline-producing strains. This strain had been cultured with 5 mM dopamine in a medium and was shown to produce mainly (*R*,*S*)-reticuline at a yield of 11 mg/L medium. The growth of *E. coli* was not inhibited by dopamine and reticuline. The overall yield of reticuline from dopamine was 2.9 %, which was attributed to the instability of dopamine and certain reaction intermediates. These compounds are readily oxidized to be a melanin-like pigment without being introduced to *E. coli* cells. Improvement of the utilization efficiency of dopamine will be needed for in vivo large-scale production. An advantage of fermentative production is that (*R*,*S*)-reticuline is produced without the addition of SAM because the regeneration of SAM in microbial cells is known to maintain in vivo methylation activity during bioconversion [25].

The reticuline produced in this strain is in a racemic form. However, NCS is known to produce the (*S*)-form in a stereospecific manner [21]. This fact suggests that NCS cannot function efficiently in this strain, and that a spontaneous condensation reaction occurred to form norlaudanosoline. This hypothesis is supported by the fact that *E. coli* cells expressing the reticuline biosynthetic genes without NCS also produce racemic reticuline at the same level.

For the dominant production of (*S*)-reticuline, crude enzymes from transgenic *E. coli* cells were prepared and reacted with dopamine and SAM. As a result, stereospecific (*S*)-reticuline was synthesized from dopamine with crude enzymes without "fine tuning" of the level of each enzyme or purification of enzymes. The in vitro biomanufacturing system produced 55 mg/L (the overall yield was 14.4 %) of (*S*)-reticuline from 5 mM dopamine within 1 h. Hence, this system can produce optically active (*S*)-reticuline much faster (i.e., 1 h) than other methods (month to years for cultured plant cells and transgenic plants).

2.3 BIA Production Using *S. cerevisiae*

The NLS pathway has enabled the production of reticuline without CYP80B. However, transformation of reticuline to diverse alkaloids (e.g., berberine, magnoflorine, morphine) often requires P450 enzymes, which are difficult to express in active forms in *E. coli*. To solve the problem, Minami et al. used a two-step synthesis of alkaloids with *E. coli* and *S. cerevisiae* cells [22]. They prepared *S. cerevisiae* containing the genes of the berberine bridge enzyme (BBE) or the diphenyl ring bridging enzyme (corytuberine synthase: CYP80G2) as well as CNMT to produce two BIAs: magnoflorine and scoulerine (Fig. 1.1). For magnoflorine production, transgenic *E. coli* cells expressing reticuline biosynthetic genes were cultured with 5 mM dopamine in the medium, and *S. cerevisiae* cells expressing CYP80G2 and CNMT (which has relatively broad substrate specificity and can *N*-methylate corytuberine to synthesize magnoflorine) were added to the culture medium after certain period of *E. coli* culture. Magnoflorine was synthesized at a yield of 7.2 mg/L culture (overall yield, 1.9 %) within 72 h. For a protoberberine-type alkaloid, scoulerine was produced by coculture of transgenic *E. coli* cells expressing reticuline biosynthetic genes and *S. cerevisiae* cells expressing BBE. Scoulerine was synthesized at a yield of 8.3 mg/L culture (overall yield, 2.2 %) within 48 h. The conversion efficiency of magnoflorine from reticuline in *S. cerevisiae* cells was 65.5 % and of scoulerine from reticuline was 75.5 %. Yeast cells are more effective at expressing eukaryote genes (e.g., cytochrome P450) than *E. coli* cells. These results suggest that this combination system may be useful for the synthesis of various BIAs.

Smolke et al. reported that (*R*,*S*)-norlaudanosoline could be converted to (*R*,*S*)-reticuline at a yield of 164.5 mg/L culture using *S. cerevisiae* containing the genes of types of methyltransferase (6OMT, CNMT, 4′OMT) from *Thalictrum flavum* and *Papaver somniferum* [26]. Moreover, three additional enzymes from *T. flavum* and *P. somniferum* and a reductase partner from *Arabidopsis thaliana* had been expressed and had produced (*S*)-scoulerine (65.4 mg/L), (*S*)-tetrahydrocolumbamine (68.2 mg/L), and (*S*)-tetrahydroberberine (33.9 mg/L) from (*S*)-reticuline in *S. cerevisiae*. Furthermore, intermediate in the biosynthesis of morphine, salutaridine had been produced from (*R*,*S*)-reticuline at a yield of 24.5 mg/L culture by using a human P450 enzyme (CYP2D6) (Fig. 1.2).

3 Fermentative Production of BIAs from Simple Sources of Carbon

The previous section demonstrated the microbial synthesis of BIAs from aromatic intermediate such as dopamine or simple BIA norlaudanosoline. The production cost of using an expensive material as a substrate is relatively high. Hence, developing a new process that can convert BIAs from less expensive material such as renewable sources (e.g., glucose, glycerol) is important.

As described above, reticuline is a key compound for the production of BIAs. Analgesic compounds (e.g., morphine, codeine) and antibacterial agents

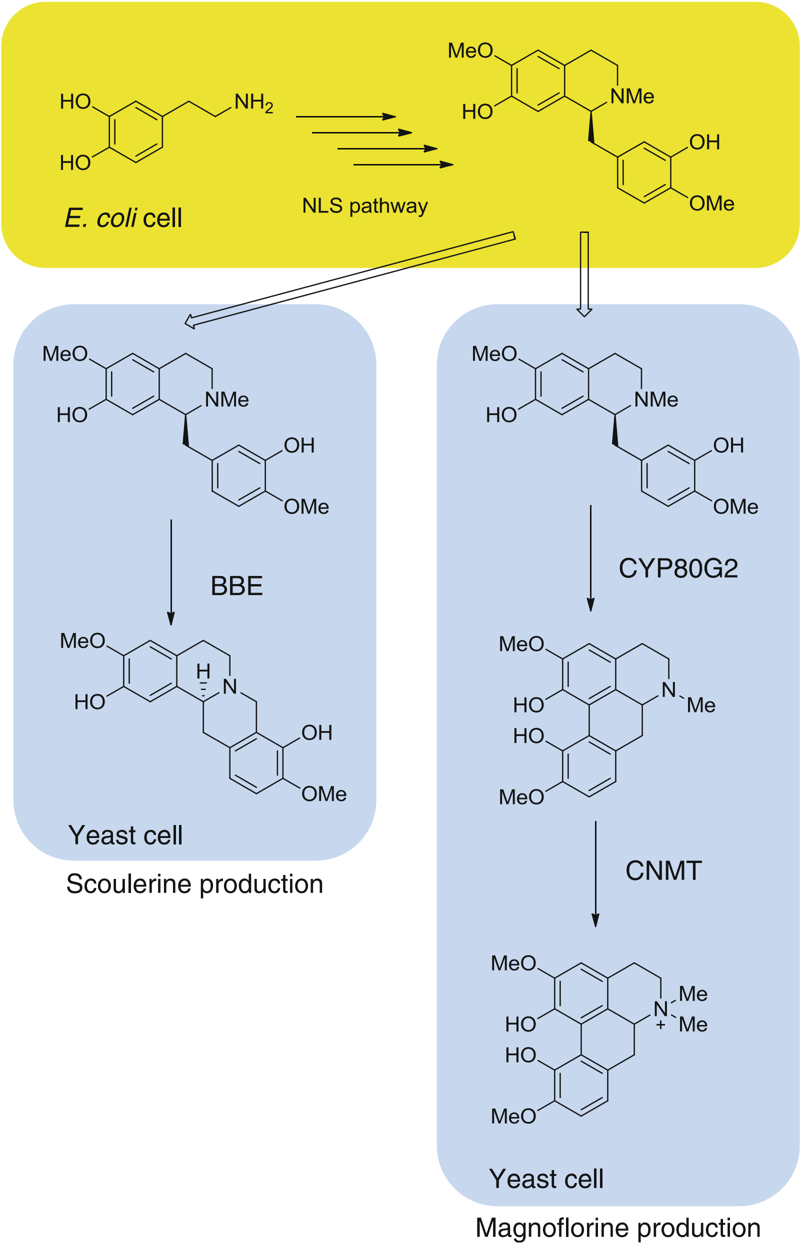

HO
HO
NH2
E. coli cell
NLS pathway
MeO
HO
NMe
OH
OMe
BBE
H
N
Yeast cell
Scoulerine production
CYP80G2
CNMT
N
Me
+
Yeast cell
Magnoflorine production

Fig. 1.1 Benzylisoquinoline alkaloid production in mixed culture of *E. coli* and *S. cerevisiae* cells

(e.g., berberine, palmatine) are produced mainly through (*S*)-reticuline with oxidation steps involved in cytochrome P450 oxidoreductases. For example, in morphine biosynthesis in plants, (*S*)-reticuline is converted to (*R*)-reticuline through two

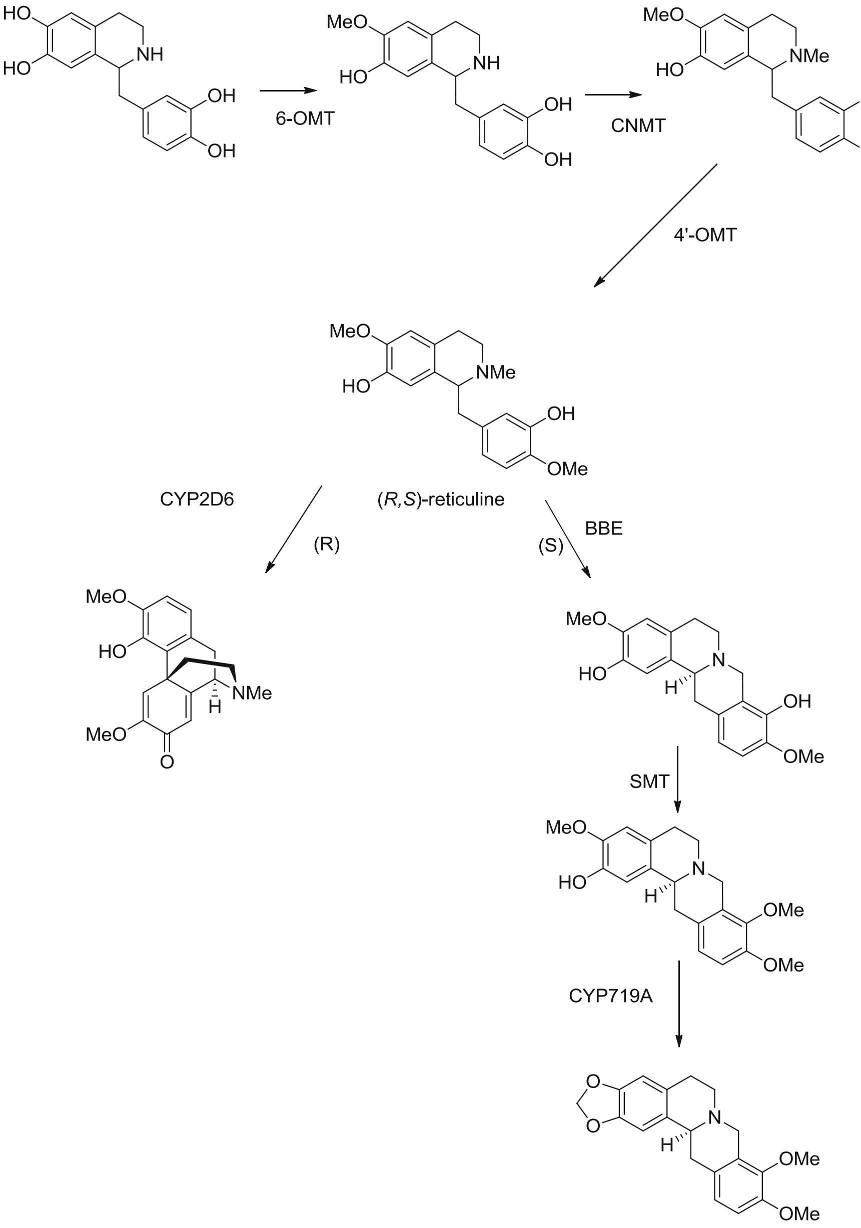

6-OMT
CNMT
4'-OMT
(*R,S*)-reticuline
CYP2D6
(R)
BBE
(S)
SMT
CYP719A

Fig. 1.2 Benzylisoquinoline alkaloid production in *S. cerevisiae* cells

enzymatic steps, and then a C-C- phenol linkage is formed by the oxidation of CYP719B1 to form salutaridine. The latter is converted to morphine with subsequent modification steps comprising five enzymatic and two spontaneous reactions [27, 28].

The P450-associated oxidations are important for the production of BIAs, but expressing P450 enzymes as active forms in microorganisms is still difficult [10, 29]. Therefore, P450-mediated oxidations are not suitable for the microbial production of plant alkaloids, whereas we need the future development of efficient expression systems for P450. Alternative pathways avoiding P450-mediated reactions should be employed. In this respect, the artificial synthetic pathway of BIAs described in the previous section should be employed for single microbial fermentation of BIAs from simple sources of carbon.

This section focuses on the strategy for the fermentation of dopamine (which is the starting compound for the artificial BIA synthetic pathway) from simple sources of carbon in *E. coli* cells. Dopamine fermentation is demonstrated in three steps: L-tyrosine fermentation, conversion of L-tyrosine to L-DOPA, and conversion of L-DOPA to dopamine.

3.1 L-Tyrosine Production from Simple Sources of Carbon

L-Tyrosine is used in various applications, including the synthesis of certain drugs, melanins, biodegradable resins, and phenylpropanoids [30]. Hence, the production of L-tyrosine has been promoted using traditional and genetic modification. Various microorganisms, such as *Corynebacterium glutamicum*, *Arthrobacter globiformis*, and *Brevibacterium lactofermentum*, were developed as overproducing strains of L-tyrosine using traditional mutagenesis methods [31–33]. On the other hand, plant BIAs are not naturally produced in microorganisms, so metabolic engineering for microbial production of BIAs needs the genes derived from plants producing BIAs and intensive genetic engineering of metabolism. Plant genes are now available from *P. somniferum* (opium poppy), *Eschscholzia californcia*, *Thalictrum* spp., and *C. japonica*. For the intensive genetic engineering, *E. coli* is an attractive host because more information is available on the biochemical pathway, the regulation mechanism, and the genetics, than any other microorganisms. Moreover, *E. coli* has been studied thoroughly and developed in molecular genetic methods to manipulate its genome, which can be readily constructed to the desirable mutants for overproduction of aromatic amino acids. Improvements in BIA productions are highly dependent upon increasing the carbon flow into the L-tyrosine pathway. L-Tyrosine-overproducing *E. coli* have been developed by metabolic engineering employing several strategies [30, 34–36].

L-Tyrosine biosynthesis starts with the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), the intermediates of the glycolytic pathway and pentose phosphate pathway, respectively, which is catalyzed by 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS: *aroF*/*aroG*/*aroH*). The resultant 3-deoxy-D-arabino-heptulosonate (DAHP) is converted into chorismate through the shikimate pathway with seven reactions. In plants, prephenate (PPA) is converted into L-arogenate by transamination whereas in *E. coli*, PPA is converted to *p*-hydroxyphenylpyruvate (HPP) by prephenate dehydrogenase, which is a bifunctional enzyme that behaves as chorismate mutase/prephenate dehydrogenase (CM/PDH: *tyrA*). The resultant HPP is transformed to L-tyrosine by a tyrosine aminotransferase (*tyrB*).

The first strategy is the elimination of *tyrR*, whose product is a "global regulator" of the shikimate pathway. The carbon flow of the L-tyrosine synthetic pathway is strictly regulated by a TyrR protein (Fig. 1.3). In *E. coli*, the TyrR protein acts as a repressor and activator in the transcription of several genes involved in the production of aromatic amino acids, including *aroF*/*aroG*/*aroH* (DAHPS), *aroL* (shikimate kinase), *tyrA*, and *tyrB* [37]. Therefore, disruption of the *tyrR* gene is expected to increase the production of aromatic amino acids. Furthermore, L-tyrosine and HPP inhibit the activity of three enzymes (DAHPS (*aroF*/*aroG*/*aroH*), 3-hydroquinate synthase (*aroB*), and CM/PDH (*tyrA*)) in the L-Tyr biosynthetic pathway of *E. coli*. There are reports that the mutation of the *tyrA* and *aroG* avoid the feedback inhibition. The feedback-resistant enzymes AroG$^{\mathrm{fbr}}$ (Asp$_{146}$ substituted with Asn [38]) and TyrA$^{\mathrm{fbr}}$ (Met$_{54}$ and Ala$_{354}$ substituted with Ile and Val, respectively [39]) were introduced into the *E. coli ΔtyrR* background, and resulted in an overall yield of ≤127 mg/L L-tyrosine [39].

Another strategy for overproduction of L-tyrosine is an increased supply of the precursors PEP and E4P to reduce the carbon flow for competitive pathways. Overexpression of phosphoenolpyruvate synthetase (PEPS: *ppsA*) and transketolase (TKT: *tktA*) is expected to improve the production of L-tyrosine. Using the *E. coli* transformant, which was eliminated in the TyrR regulation (by the deletion of *tyrR*) and overexpressing *aroG*^{fbr}, *tyrA*^{fbr}, *ppsA*, and *tktA*, up to 9.7 g/L L-tyrosine has been produced with a yield of 0.1 g/g glucose [30]. PEP is also used as a phosphate donor in the PEP: sugar phosphotransferase system (PTS) to induce the import of glucose in *E. coli*, thereby limiting the yield of aromatic compounds [40]. To avoid PEP consumption during glucose uptake, *E. coli* strains that lack PTS activity but consume glucose have been developed and improved by replacing PTS with galactose permease and glucokinase. These strains have accomplished higher yields and production capacities [41, 42].

3.2 Conversion of L-Tyrosine to Dopamine

The second step of dopamine production is hydroxylation of L-tyrosine to form L-DOPA. L-DOPA is a biologically important chemical employed for the treatment of Parkinson's disease, so L-DOPA has been produced employing chemical, enzymatic, or microbial processes [43–46]. In plants and animals, L-DOPA is mainly synthesized from L-tyrosine by tyrosine hydroxylase (TH). Since TH requires a tetrahydrobiopterin as a cofactor, TH cannot be utilized for the conversion of L-tyrosine to L-DOPA in *E. coli*. Therefore, tyrosinase or *p*-hydroxy phenylacetate 3-hydroxylase has been employed for the hydroxylation of L-tyrosine to form L-DOPA [43, 46, 47].

Tyrosinase is an enzyme belonging to major families of polyphenol oxidases, which contain the spectroscopic properties of metal ions. In general, tyrosinases catalyze the hydroxylation of monophenols to *o*-diphenols (monophenolase activity),

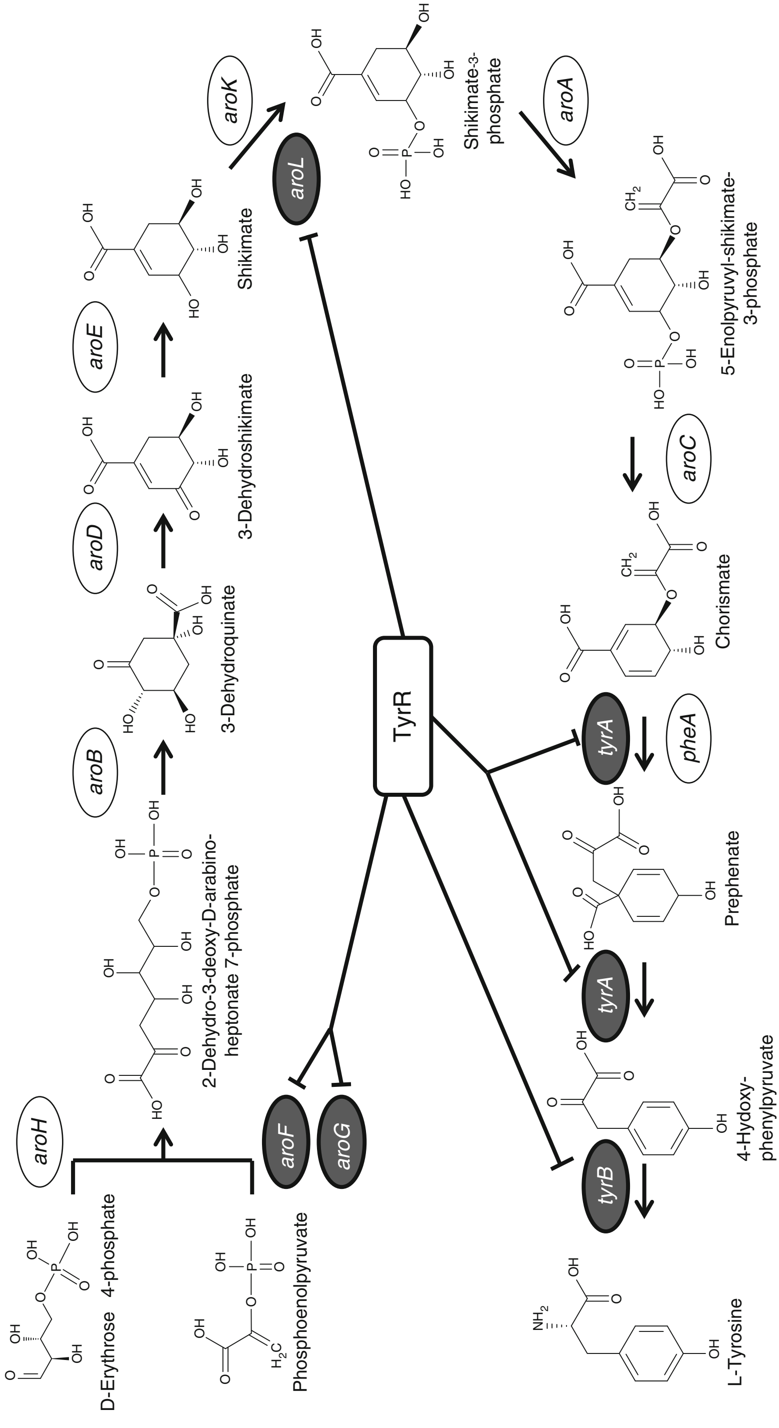
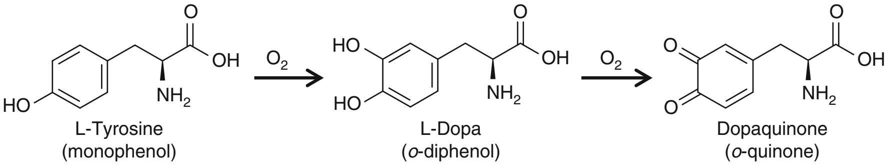

D-Erythrose 4-phosphate
Phosphoenolpyruvate
aroH
aroF
aroG
2-Dehydro-3-deoxy-D-arabino-heptonate 7-phosphate
aroB
3-Dehydroquinate
aroD
3-Dehydroshikimate
aroE
Shikimate
aroK
aroL
Shikimate-3-phosphate
aroA
5-Enolpyruvyl-shikimate-3-phosphate
aroC
Chorismate
TyrR
tyrA
pheA
Prephenate
tyrA
4-Hydoxy-phenylpyruvate
tyrB
L-Tyrosine

Fig. 1.3 TyrR regulon in L-tyrosine pathway of *E. coli* cell



Scheme 1.3 *o*-Diphenolase activity of tyrosinase

and also subsequently oxidize the resultant *o*-diphenols to *o*-quinones (*o*-diphenolase activity) (Scheme 1.3) [48]. The monophenolase activity of tyrosinase is usually coupled to *o*-diphenolase activity. Therefore, it is predicted that undesirable by-products (including L-dopaquinone and L-dopachrome) are produced during the conversion of L-tyrosine using a tyrosinase. However, the tyrosinase derived from *Ralstonia solanacearum* (RSc0337) has lower *o*-diphenolase activity than monophenolase activity [48]. Using RSc0337 for the conversion of L-tyrosine, effective production of L-DOPA without the formation of undesirable *o*-quinones (possibly melanin) is expected. In fact, when RSc0337 was overexpressed in an L-tyrosine-overproducing *E. coli*, 1.05 g/L of dopamine was produced with PpDODC as discussed below [24].

Production of L-DOPA using 4-hydroxyphenylacetate 3-hydroxylase encoded by *hpaBC* genes from *E. coli* was recently reported [46]. By using overexpressing *hpaBC* genes in the strain modified for L-tyrosine production, L-DOPA was produced ≤320 mg/L from glucose in minimal medium.

Dopamine is a key intermediate in the plant BIA biosynthesis pathway. It condenses with 4-HPAA, and forms a "BIA scaffold." In plants, tyrosine/DOPA decarboxylases catalyze the decarboxylation of L-tyrosine and L-DOPA to tyramine and dopamine, respectively [49]. Tyramine is an undesirable product for bacterial BIA synthetic pathways because its MAO product (i.e., 4-HPAA) combines with dopamine to form norcoclaurine, which needs CYP80B to be converted to reticuline. L-DOPA decarboxylase (PpDODC) from the *Pseudomonas putida* strain KT2440 exhibited a more than 10^3-fold preference for L-DOPA compared with other aromatic amino acids [unpublished data]. Therefore, conversion of L-DOPA with PpDODC is expected to reduce the formation of undesirable by-products, 4-HPAA, and the resultant norcoclaurine (Fig. 1.4). Using an L-DOPA-producing *E. coli* strain that overexpresses PpDODC, dopamine production reached 1.05 g. The conversion efficiency from L-tyrosine to dopamine was 29.1 % [24].

3.3 BIA Production from a Simple Source of Carbon in *E. coli*

The biosynthesis of reticuline from simple sources of carbon can be achieved by combining the artificial BIA synthetic pathway (which converts dopamine to reticuline) and the dopamine fermentation pathway (which consists of the three steps described in the subsections above). Only very recent study on microbial BIA fermentation has been reported using single *E. coli* cells [24]. In that report, reticuline

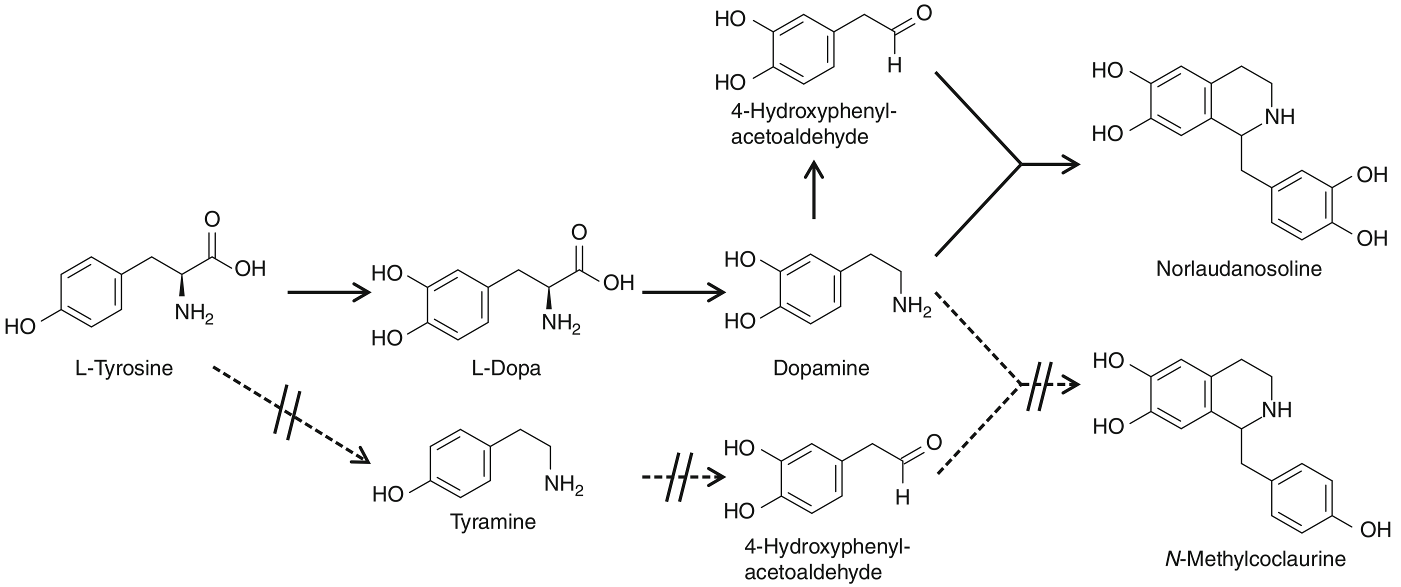
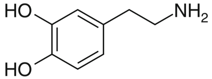
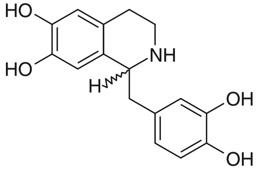
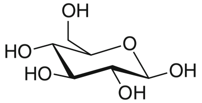
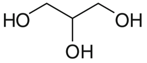
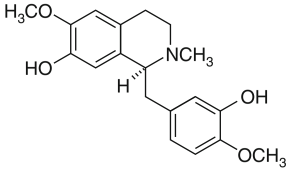
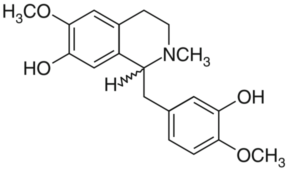
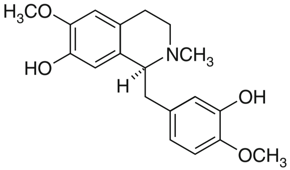
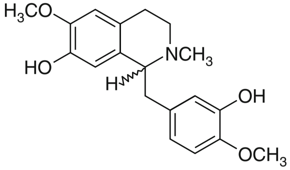
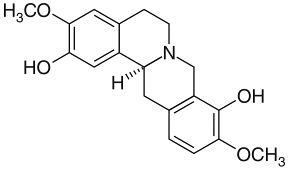

HO
NH2
O
OH
L-Tyrosine
HO
HO
NH2
O
OH
L-Dopa
HO
NH2
Tyramine
HO
HO
NH2
Dopamine
HO
HO
O
H
4-Hydroxyphenyl-
acetoaldehyde
HO
HO
O
H
4-Hydroxyphenyl-
acetoaldehyde
HO
HO
NH
OH
OH
Norlaudanosoline
HO
HO
NH
OH
N-Methylcoclaurine

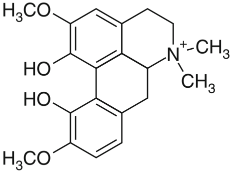
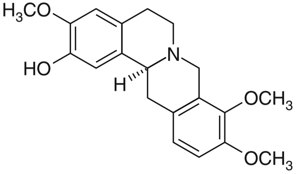
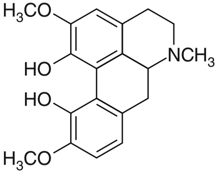
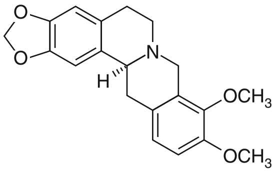
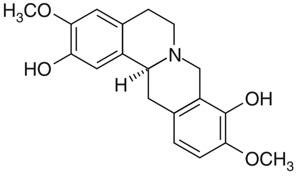
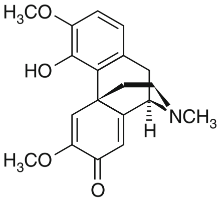
Fig. 1.4 The artificial BIA synthetic pathway in microbes

Table 1.2 Microbial production of BIAs

	Minami et al. (*PNAS* (2008) 105:7393)	Hawkins et al. (*Nat Chem Biol* (2008) 4:564)	Nakagawa et al. (*Nat Commun* (2011) 2:326)
Host	*E. coli* and *S. cerevisiae*	*S. cerevisiae*	*E. coli*
Starting material	Dopamine 	(*R*,*S*)-Norlaudanosoline 	Glucose  or glycerol 
Produced alkaloids	(*S*)-Reticuline 	(*R*,*S*)-Reticuline 	(*S*)-Reticuline 
	(*R*,*S*)-Reticuline 	(*S*)-Scoulerine 	

(*continued*)

Table 1.2 (continued)

	Minami et al. (*PNAS* (2008) 105:7393)	Hawkins et al. (*Nat Chem Biol* (2008) 4:564)	Nakagawa et al. (*Nat Commun* (2011) 2:326)
Produced alkaloids	Magnoflorine  H₃CO HO HO H3CO N+-CH3 CH3	(*S*)-Tetrahydrocolumbamine  H3CO HO N H OCH3 OCH3	
	Corytuberine  H3CO HO HO H3CO NCH3	(*S*)-Tetrahydroberberine  O O N H OCH3 OCH3	
	(*S*)-Scoulerine  H3CO HO N H OH OCH3	Salutaridine  H3CO HO H3CO O H NCH3	

production was achieved from a simple source of carbon (e.g., glycerol, glucose) using modified *E. coli* BL21 (DE3), which had been adopted in the artificial BIA synthetic pathway (NLS pathway in Sect. 2.1) and the dopamine fermentation pathway (in this section). The reticuline-producing strain was developed to delete the *tyrR* gene and to introduce eleven genes (*aroG^{fbr}*, *tyrA^{fbr}*, *ppsA*, *tktA*, *RsTYR*, *DODC*, *MAO*, *NCS*, *CNMT*, *4′OMT*, *6OMT*) using three plasmid vectors. Reticuline has been produced from simple sources of carbon such as glucose and glycerol in the strain, and reached a maximum yield of 46.0 mg/L from glycerol in fed-batch cultures [24]. Interestingly, reticuline was mainly produced in the (*S*)-isomer but not the (*R*)-isomer in reticuline-producing *E. coli*, unlike observations in the dopamine-adding system [22]. Further analyses showed that the (*S*)-isomer was the main product in the strain, and that only small amounts of the (*R*)-isomer were formed when a low concentration of norlaudanosoline was used. Conversely, (*S*)-reticuline was produced with high stereoselectivity under almost all culture conditions.

The above production system is manipulated by a jar fermenter system but it is difficult to manipulate this system with numerous samples in parallel. Many samples can be cultured in parallel using shake flask cultures, allowing optimization of production conditions. Using shake flask cultures, bench-top production of (*S*)-reticuline and optimization of culture conditions are examined [50]. Using this bench-top method, a sufficient amount of (*S*)-reticuline (a yield of 33.9 mg/L in 60 h, a similar yield to that obtained using the jar fermenter culture) can be readily prepared without a jar fermenter system. Thus, the bench-top method for production of (*S*)-reticuline may facilitate research on BIAs.

4 Conclusion

In this chapter, three kinds of multistep BIA production systems are reviewed (shown in Table 1.2). These microbial systems should open a new field in which microbial cells can be given the ability for low-cost production of many diverse alkaloids. The bacterial platform for BIA fermentation has been established, but further applications face problems. Further metabolic engineering (such as optimization and modification of the pathway) may overcome the productivity of alkaloids and enhance the field of applications for microbial alkaloid fermentation. The widespread application may lead to further progress with microbial systems for use in the pharmaceutical industry, which needs a diverse chemical library to develop more advanced tools for chemical therapy.

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2 Alkaloids of Marine Macroalgae

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Contents

1 Introduction 26
2 Classification and Natural Occurrence of Alkaloids 27
2.1 PEA and Related Amines and HORD 28
2.2 PEA Group Amines 28
2.3 DOP 29
2.4 PEA Alkaloid 29
2.5 Indole Derivatives 29
3 Halogenated Indole Alkaloids 31
3.1 BRI 32
3.2 Sulfur-Containing Bromoindole Alkaloids 32
3.3 Polyhalogenated Bisindole 33
3.4 Polyhalogenated Indoles 34
3.5 2,7-Naphthyridine Derivatives 34
4 Conclusion 34
References 35

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Abstract

Marine alkaloids have their origins from marine organisms including marine macroalgae. On the other hand, some marine algal alkaloids such as phenylethylamine derivatives were previously isolated from terrestrial plants. The other groups of alkaloids like indole derivatives and halogenated alkaloids are specific for algae. Most of the indole group alkaloids are concentrated in red algae. Green algae are rich of bromine- and chloride-containing alkaloids. These halogenated alkaloids of algae are not present in terrestrial plants and are specific for algae and sea organisms. Marine algae contain 44 alkaloids, consisting of 1 phenylethylamine, 41 indole, and 1 naphthyridine derivates. In the halogenated alkaloid group, there are 25 bromine-containing compounds, among which 7 have chlorine and 5 have sulfur, additionally.

Abbreviations

ALM	Almazolone
BRI	Bromoindole
CAL A	Callophycin A
CLP	Caulerpin
CLS	Caulersin
DOP	Dopamine
DTC	Denticin
FRG	Fragilamide
HAİ	3-(Hydroxyacetyl)indole
HLI	Halogenated indoles
HORD	Hordenine (anhaline)
ICA	Indole-3-carboxaldehyde
LO A B	Lophocladine A
MRF A	Martefragin A
MRT A B	Martensine A, B
N-ACPEA	*N*-Acetylphenylethylamine
N-ACTYR	*N*-Acetyltyramine
PEA	Phenylethylamine
SBRI	Sulfur-containing bromo indole alkaloids
TYR	Tyramine

1 Introduction

Alkaloids are a large group of naturally occurring organic compounds. The first alkaloid was isolated from opium, terrestrial plant *Papaver somniferum*, by

Serturner in 1805 [1] and 164 years later, in marine algae by Guven et al. [2, 3]. The term "alkaloid" was proposed by Meissner in 1819 [4], which originated from the words of Arabic *al kaly* and the Greek *eidos*, meaning alkali-like. Alkaloid definition has evolved through years. Initially, alkaloid was defined as a nitrogen-containing heterocyclic compound. Later, a compound with a biological amine group was included, and recently, Br-, I-, Cl-, and S-containing heterocyclic nitrogenous compounds were also defined as alkaloids. Alkaloids possess important pharmacological activities, which are presented in another chapter. Alkaloids are produced by large variety organisms, basically terrestrial plants, including bacteria, fungi, and marine algae and animals.

Most of the alkaloids, around 30,000, have been isolated from terrestrial plants, but marine algae contain only 44 alkaloids. The origins of some alkaloids in plants are oxidation products of amino acids, whereas others' origins remain unknown, especially halogenated alkaloids in marine algae.

Alkaloids can be extracted from the materials by acid–base extraction technique, then various purification techniques were applied. The obtained pure marine algal alkaloids were identified by IR, HPLC and MS, GC-MS, and NMR and comprehensively reviewed time to time [5, 6].

An important feature of an algal alkaloid is that it is either a natural component or a host product. Some alkaloids were produced by host organisms on algae. For example, communesin was isolated from the mycelium of a strain of *Penicillium* sp. on the *Enteromorpha intestinalis* [7], citrinadin A was isolated from *Penicillium citrinum* separated from a marine red algae [8], and Nb-acetyltryptamine and oxaline were isolated from unidentified fungus collected from the surface of the red alga *Gracilaria verrucosa* [9]. *N*-hydroxy-2-pyridone was isolated from *Penicillium* sp. on brown alga *Xiphophora gladiata* [10]. Ascosalipyrrolidinone was isolated from fungus *Ascochyta salicornia* on green alga *Ulva* sp. [11]. These host alkaloids are not presented in this chapter.

TYR, DOP, and HORD are biological amines, and among these, only HORD is defined as an alkaloid. These amines were included in this chapter because all are derived from the parent compound PEA and have important pharmacological activities.

2 Classification and Natural Occurrence of Alkaloids

The alkaloids nomenclature has not been systematized. The commonly used systems for alkaloid classification are designed according to the plant genera or to the basis of molecular structure similarities.

In this chapter, the alkaloids were classified as follows:

1. 2-Phenylethylamine derivatives
2. Indole derivatives
3. Halogenated indoles
4. 2,7-Naphthyridine derivatives

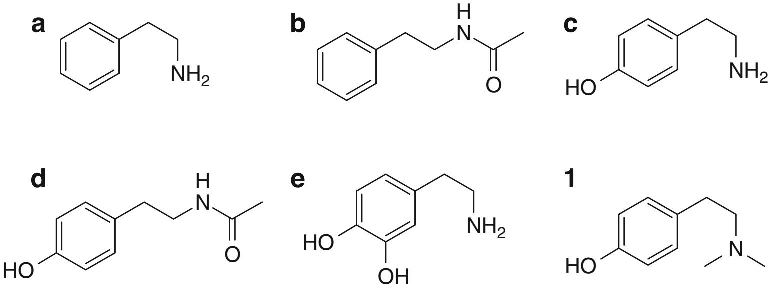


Fig. 2.1 PEA and related amines (**a**–**e**) and alkaloid (**1**)

2.1 PEA and Related Amines and HORD

2.1.1 PEA

PEA (a) consists of a benzene ring and an ethylamine side chain. Its structure permits substitution on the aromatic ring α (1-) and β (2-) carbon atoms. Among PEA derivatives (5 amines) in algae are *N*-ACPEA, TYR, *N*-ACTYR, and DOP (Fig. 2.1). Phenylethylamine is a decarboxylation product of phenylalanine. They were firstly found in terrestrial plants and later in marine algae. The distribution of PEA in plants and eight algae was reviewed by Smith [12]. PEA-contained algae are brown (*Desmerestia aculeata*, *Desmerestia viridis*) and red (*Ceramium rubrum*, *Cystoclonium purpureum*, *Delesseria sanguinea*, *Dumontia incrassata*, *Polysiphonia urceolata*, *Polyides rotundus*) [13]. PEA content was investigated in two green, five brown, and ten red algae, and it was reported to be present only in six red algae as *Gelidium crinale*, *Gracilaria bursa-pastoris*, *Halymenia floresii*, *Phyllophora crispa*, *Polysiphonia morrowii*, and *Polysiphonia tripinnata*. PEA amount varied in these algae as 0.34–30.42 $\mu g/g^{-1}$, and the highest amount was found in *Gelidium crinale* and trace in *Phyllophora crispa* [14].

2.2 PEA Group Amines

2.2.1 *N*-ACPEA (b)

N-ACPEA is *N*-2-phenylethylacetamide. *N*-ACPEA was first isolated from the red alga *Gelidium crinale* [15].

2.2.2 TYR (c)

TYR is 4-hydroxyphenylethylamine. TYR occurs in many plants, fungi, and animals but is rare in algae. It was found in brown alga *Laminaria saccharina* and in red algae *Chondrus crispus* and *Polysiphonia urceolata* [16].

2.2.3 *N*-ACTYR (d)

It was isolated for the first time from marine algae *Phyllophora crispa* [15].

2.3 DOP

DOP (e) is 3,4-dihydroxyphenethylamine. DOP is a catecholamine, carrying two hydroxyl groups in the position 3 and 4 of the phenyl ring. DOP was found in animals and several terrestrial plants [12]. It was found in the green alga *Monostroma fuscum* [17] and recently in *Ulvaria obscura*, a prominent component of green tide blooms in Washington. Green tide is stimulated as a result of dopamine release to the sea by *U. obscura* [18].

2.4 PEA Alkaloid

2.4.1 HORD

HORD (1) is 4-(2-dimethylaminoethyl)phenol, *N*-dimethyl derivative of tyramine. Exceptionally, hordenine contains a quaterner amine group. HORD was first isolated from red algae *Phyllophora nervosa* [*Phyllophora crispa*] [2, 3] and later from *Ahnfeltia paradoxa* [19], from *Gigartina stellata (Mastocarpus stellatus)* [20], and from *Gelidium crinale* [21]. HORD amount was determined in *Gelidium crinale* [21] and *Phyllophora nervosa* [22].

2.5 Indole Derivatives

Indole Derivatives are shown in Fig. 2.2.

2.5.1 ICA (2) and HAI (3)

ICA and HAI were extracted from the marine red alga *Peyssonnelia* sp. [23].

2.5.2 ALM *E* (4) and *Z* (5)

ALM is a disubstituted oxazolindole derivate and was isolated from the red alga *Haraldiophyllum* sp. collected in Dakar (Senegal) and synthesized *E* and *Z* stereoisomers [24].

2.5.3 FRG (6)

FRG is a 3-substituted indole and corresponds to an *N*-methylhomoisoleucyl unit and a *p*-hydroxybenzyl group attached to the indole unit C-3. The amide NH was connected to a *cis* disubstituted carbon-carbon double bond and was extracted from the red alga *Martensia fragilis* [25].

2.5.4 MRF A (7)

MRF A displays a 3-oxazolylindole structure. It was isolated from the red alga *Martensia fragilis* [26] and was also synthesized [27].

2.5.5 MRT A (8), B (9)

MRT A (**8**) is a 3-substituted indole bound to a 5-membered lactam ring, and MRT B (**9**) contains two carbonyl as a γ-lactam and a heterocyclic ketone group. MRT A and B were extracted from the red algae *Martensia fragilis* [25].

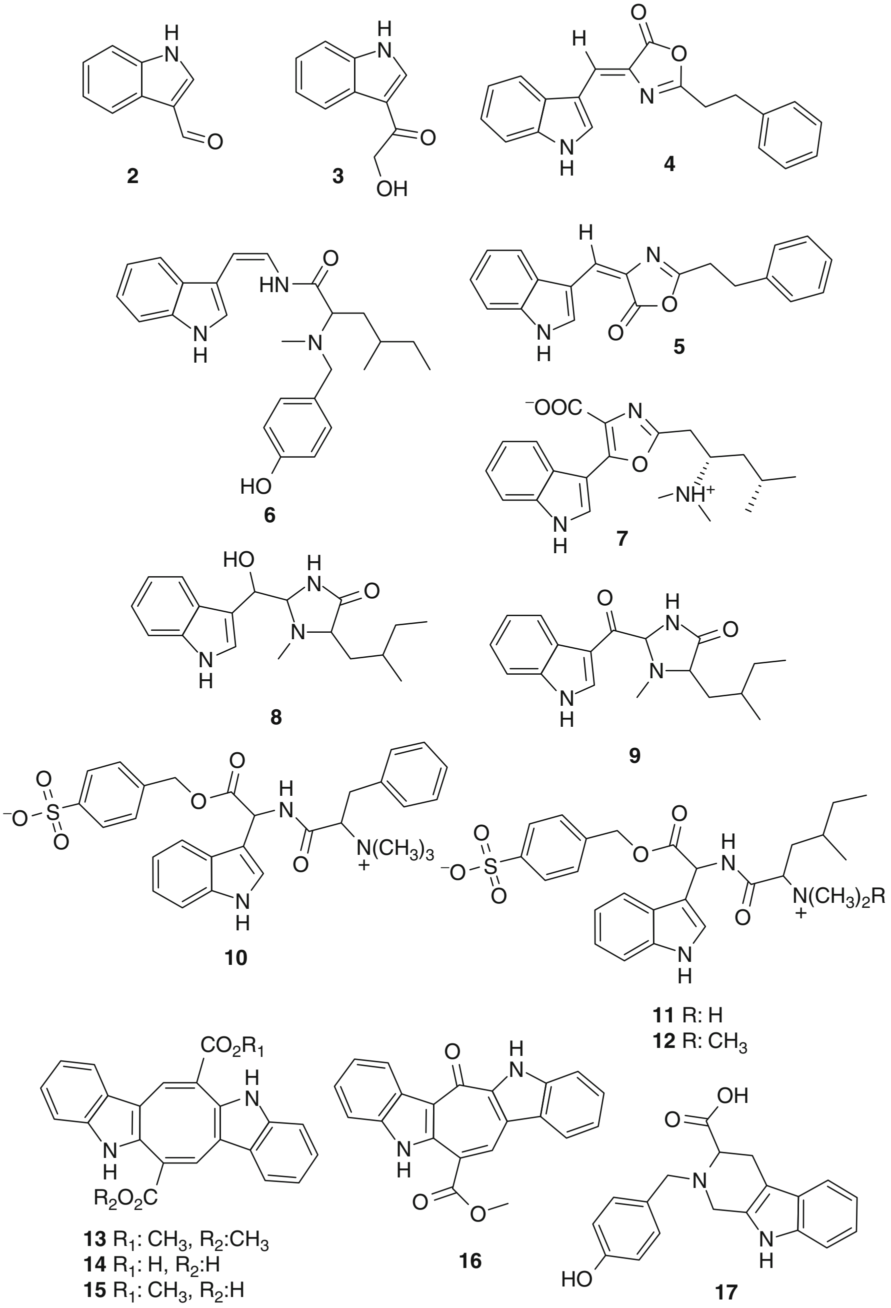

2
3
4
6
5
7
8
9
10
11 R: H
12 R: CH_3
13 R_1: CH_3, R_2:CH_3
14 R_1: H, R_2:H
15 R_1: CH_3, R_2:H
16
17

Fig. 2.2 Indole derivatives, alkaloids

2.5.6 DTC A (10), B (11), and C (12)

DTCs are 3-subsituted indole derivates named DTC A, B, and C. These alkaloids contain sulfonic acids which are rarely found in alkaloids. DTCs were isolated from *Martensia denticulate* [28].

2.5.7 CLP (13)

CLP (**13**) is a dimethyl-6,13-dihydrodibenzo[b,i]phenazine-5,12-dicarboxylate methyl ester. CLP (I) **(13)** contains two indole groups linked by a cyclic ring containing eight carbons with two carboxy groups. CLP (I) was first isolated from *Caulerpa racemosa* var. *clavifera*, C. *sertularioides*, and *C. serrulata* [29], and it was also synthesized [30]. Its two analogues CLP (II) (**14**) and CLP (III) (**15**) were also isolated from *Caulerpa racemosa*. CLP contains two COOMe, but CLP analogue I contains two COOH and CLP II one COOH and COOMe [31].

CLP-containing algae are listed as follows: in green algae *C. lamourouxii* [32], *C. prolifera*, *C. ashmeadii*, *C. racemosa var. macrophysa*, *C. racemosa var. laetevirens* [33], C. *paspaloides, C. microphysa* [34], *C. decorticatum* [35], *C. taxifolia* [36], *C. peltata* [37], *C. scalpelliformis* [38], and *Halimeda incrassate* [39], and in red algae, *Chondria armata* [40], *Laurencia majuscula, Hypnea concornis, Caloglossa leprieurii* [41], *Laurencia cartilaginea, Eucheuma muricatum*, and *Colpomenia sinuosa* [42].

2.5.8 CLS (16)

CLS is a bisindole alkaloid with central ring of seven members and two antiparallel indole cores. CLS was isolated from *Caulerpa serrulata* [43]. CLS has three isomers A, B, and C and was also synthesized [44].

2.5.9 CAL A (17)

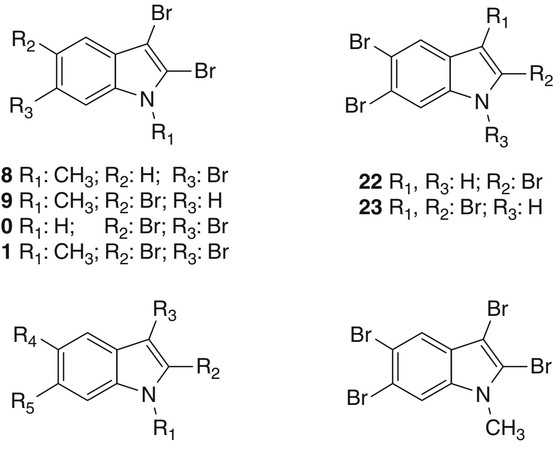
CAL is a 2-(4-hydroxybenzyl)-2,3,4,9-tetrahydro-1 H-pyrido[3,4-b]indole-3-carboxylic acid.

CAL is a tetrahydro-*β*-carboline. It was isolated from the red algae, *Callophycus oppositifolius* [45]. The similar steroidal alkaloid was found in tomato (*Lycopersicum esculentum*) [46].

3 Halogenated Indole Alkaloids

Halogens, bromine, and chlorine were found in the same compound. Some of them contained Br, and some of halogenated alkaloids contain two different halogens as Br and Cl.

HLI alkaloids were isolated only from marine organisms and algae. Many HLI alkaloids were isolated from red algae and only one from a green alga. These alkaloids contain an indole group substituted by bromine and chlorine atoms. Sulfur-containing bromo alkaloids were also extracted from red algae.



18 R_1: CH_3; R_2: H; R_3: Br
19 R_1: CH_3; R_2: Br; R_3: H
20 R_1: H; R_2: Br; R_3: Br
21 R_1: CH_3; R_2: Br; R_3: Br

22 R_1, R_3: H; R_2: Br
23 R_1, R_2: Br; R_3: H

24 R_1: R_2: H; R_3, R_4, R_5: Br
25 R_1: CH_3, R_2: H; R_3, R_4, R_5: Br
26 R_1, R_4: H; R_2, R_3, R_5: Br

27

Fig. 2.3 Bromoindole alkaloids

3.1 BRI

The BRI isolated from algae are shown in Fig. 2.3.

From red alga *Laurencia brongniartii* collected from Caribbean Sea [47] and Okinawan Sea [48], 2,3,6-tribromo-1-methyl indole (**18**) [47], 2,3,5-tribromo-l-methyl indole (**19**) [49], 2,3,5,6-tetrabromo-l*H*-indole (**20**) [47], 2,3,5,6-tetrabromo-l-methyl indole (**21**) [47], 2,4,6-tribromo-l*H*-indole (**22**) [48] and 2,3,4,6-tetrabromo-l*H*-indole (**23**) [48] were isolated. Compounds **20** and **23** were also identified in the red alga *Laurencia similis* collected from Pulau Gaya, Malaysia [49], and compound **19** was isolated from a red alga *Laurencia decumbens* collected from Weizhou Island (South China Sea) [50]. 3,5,6-tribromo-l*H*-indole (**24**) [51], 3,5,6-tribromo-l-methylindole (**25**) [51] and 2,3,6-tribromo-1*H*-indole (**26**) [51] were isolated from red alga *Laurencia similis* collected from Sanya, China [51]. 2,3,4,6-tetrabromo-l-methylindole (**27**) was isolated from the red alga *Laurencia decumbens* collected from Weizhou Island (South China Sea) [50].

3.2 Sulfur-Containing Bromoindole Alkaloids

The formulas of sulfur-containing bromoindole alkaloids are given in Fig. 2.4. Thiobromoindoles 3-thiomethyl 2,4,6-tribromo-1 *H*-indole (**28**), 3-thiomethyl 2,4,5,6-tetrabromo-1 *H*-indole (**29**), 2,3-dithiomethyl-4,6-dibromo-1 *H*-indole (**30**),

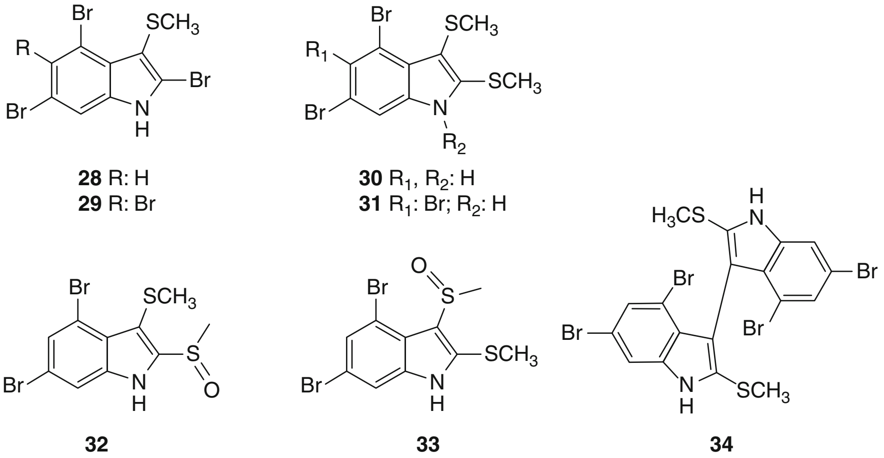


Fig. 2.4 Sulfur-containing bromoindole alkaloids

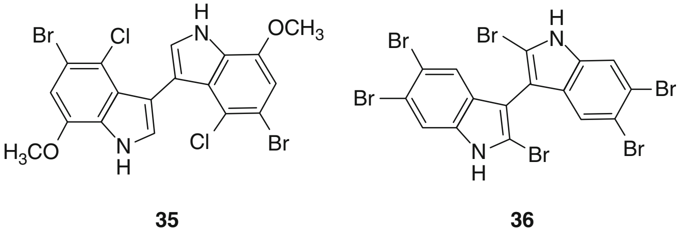


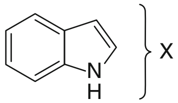
Fig. 2.5 Polyhalogenated bisindoles

and 2,3-dithiomethyl-4,5,6- tribromo-1 *H*-indole (**31**) were isolated from *Laurencia brongniartii* [47, 48].

Thiomethyl and sulfoxide-containing bromoindoles 2-thiomethyl-3-sulfoxymethyl-4,6-dibromoindole (**32**) and 2-sulfoxymethyl-3-thiomethyl-4,6-dibromo-1 *H*-indole (**33**) were also isolated from *Laurencia brongniartii* [47]. 3,3′-Bis(4,6-dibromo-3-methylthio) indole (**34**) was isolated from *Laurencia brongniartii* collected in Okinawa Sea [48].

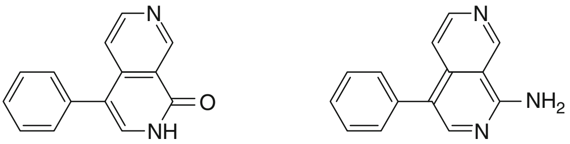
3.3 Polyhalogenated Bisindole

4,4′-Dichloro-5,5′-dibromo-7,7′-dimethoxy-3,3′-bis-1 *H*-indole (**35**) was identified from the green alga *Chaetomorpha basiretorsa* [52]. 2,2′,5,5′,6,6′-Hexabromo-3,3′-bis-1 *H*-indole (**36**) was identified from *Laurencia similis* collected from the coast of Sanya, Hainan Island (China) [42] (Fig. 2.5).



37 Br_3, Br_2Cl, $BrCl_{2,}$ Cl_3
38 Br_4, Br_3Cl, $Br_2Cl_{2,}$ $BrCl_3$, Cl_4
39 Br_5, Br_4Cl, $Br_3Cl_{2,}$ Br_2Cl_3
40 Cl_3, $BrCl_2$, Br_2Cl
41 Br_6, Br_5Cl, $Br_4Cl_{2,}$ Br_3Cl_3
42 Br_5, Br_4Cl, $Br_3Cl_{2,}$ Br_2Cl_3, Br_2Cl_2, Br_4, Br_3, Br_3Cl, $BrCl_3$

Fig. 2.6 Polyhalogenated indoles



43 Lophocladine A

44 Lophocladine B

Fig. 2.7 Lophocladines

3.4 Polyhalogenated Indoles

Many polyhalogenated indoles (**37–42**) were identified in *Rhodophyllis membranacea* collected from the Kaikoura coast (New Zealand). The fractions (**37**, **38**, **39**, **40**, **41**, and **42**) obtained from the extract of *R. membranacea* contain polychlorinated and polybrominated alkaloids [53] (Fig. 2.6).

3.5 2,7-Naphthyridine Derivatives

3.5.1 LO A (43) and LO B (44)

There are two derivatives, lophocladine A (4-phenyl-[2, 7]-naphthyridine-1(2 *H*)-one) and lophocladine B (4-phenyl-[2, 7]-naphtyridine-1-amine), which were isolated from a red alga *Lophocladia* sp. [54] (Fig. 2.7).

4 Conclusion

Alkaloids are very important compounds. They have been isolated exclusively from plants and marine organisms and rarely from macroalgae. The main difference between the alkaloids present in terrestrial plants and algae is that indoles and halogenated alkaloids are found specifically in algae. The highest number of alkaloids is present in the indole group, followed by the phenylethylamine group.

In total, marine algae contain 44 alkaloids, consisting of 1 phenylethylamine, 41 indole, and 1 naphthyridine derivates. In the halogenated alkaloid group, there are 25 bromine-containing compounds, among which 7 have chlorine and 5 have sulfur, additionally. Alkaloids are more abundant in red than green algae but are found rarely in brown algae.

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Neurotoxic Alkaloids from Cyanobacteria

3

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Contents

1 Introduction 41
2 Anatoxin-a and Homoanatoxin-a 42
2.1 History 42
2.2 Chemistry 43
2.3 Distribution and Isolated Producing Strains 45
2.4 Pharmacology and Toxicology 47
2.5 Biosynthesis and Degradation 49
3 Anatoxin-a(s) 51
3.1 History, Chemistry, and Toxicology 51
3.2 Occurrence and Detection 52
3.3 Biosynthesis 52
4 The Saxitoxin Family 53
4.1 History 53
4.2 Chemistry 54
4.3 Biological and Biochemical Assays for PSTs 57
4.4 Chemical Analysis of PSTs 58
4.5 Phylogenetic Distribution of Paralytic Shellfish Toxins 58

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4.6 Biosynthesis of Paralytic Shellfish Toxins 59
4.7 Molecular Genetics of PSTs 61
4.8 Toxicology and Pharmacology 63
5 Conclusion 66
References 68

Abstract

Cyanobacteria produce a wealth of secondary metabolites that are very diverse in chemical structure and in biological activity. A number of neurotoxins have been identified in strains of cyanobacteria including anatoxin-a and its higher homologue homoanatoxin-a, anatoxin-a(S), saxitoxins, and related paralytic shellfish toxins. This chapter focuses on these neurotoxic alkaloids from cyanobacteria, with emphasis on the chemical, biochemical, toxicological, and biosynthetic aspects. Anatoxin-a and homoanatoxin-a are two deadly potent agonists of the nicotinic acetylcholine receptor at the neuromuscular junction, provoking the rapid death of animals after respiratory failure. The chemistry and the pharmacology of these neurotoxins have been well studied, while their biosynthesis was recently deciphered. Our knowledge concerning anatoxin-a(S), an irreversible inhibitor of acetylcholine esterase, lies far behind and future work will be needed. Saxitoxin and its analogues, the paralytic shellfish toxins, have been associated with massive intoxication episodes in marine environment. They act as blockers of voltage-gated sodium channels provoking death in animals at low doses. The chemistry, pharmacology, and biosynthesis of saxitoxins have been well described. The genetic origin of the biosynthetic genes of this ancient toxin has been studied by detailed phylogenetic analysis.

Keywords

Acetylcholine esterase inhibitor • Anatoxin-a • Anatoxin-a(s) • Cyanobacteria • Homoanatoxin-a • Neurotoxins • Nicotinic acetylcholine receptor activator • Saxitoxin • Voltage-gated sodium channel blocker

Abbreviations

ACP	Acyl carrier protein
AOP	Advance oxidation process
AT	Acyltransferase
ATX	Anatoxin-a
CM	Methyltransferase
Cy	Cyclase
DH	Dehydratase
ER	Enoyl reductase
GC-MS	Gas chromatography coupled to mass spectrometry
HATX	Homoanatoxin-a
HPLC	High pressure liquid chromatography

i.p.	Intraperitoneal
KR	Keto reductase
KS	Ketosynthase
LC-FLD	Liquid chromatography coupled to a fluorescence detector
LC-MS^2	Liquid chromatography coupled to tandem mass spectrometry
LC-UV	Liquid chromatography coupled to a UV detector
LD	Lethal dose
nAChR	nicotinic acetylcholine receptor
PKS	Polyketide synthase
PSP	Paralytic shellfish poisoning
PST	Paralytic shellfish toxin

1 Introduction

The cyanobacteria produce a plethora of natural products with a wide variety of bioactivities. Many of these compounds are potent toxins and pose a significant hazard to human health and the environment. In freshwater systems, blooms of hepatotoxic cyanobacteria are of greatest concern, while in marine environments neurotoxic species are more problematic. More than 65 neurotoxins have been isolated from cyanobacteria to date. Most notably, these include the highly potent alkaloids anatoxins and saxitoxins (also known as paralytic shellfish toxins, PSTs); however, a variety of other neurotoxic substances have also been isolated from cyanobacteria including the lipopeptides, jamaicamide, antillatoxin, and kalkitoxin, as well as the nonproteinogenic amino acid, β-*N*-methylamino-L-alanine (BMAA).

Cyanobacterial neurotoxins primarily target cholinergic synapses or voltage-gated ion channels. For example, anatoxin-a and homoanatoxin-a are nicotinic acetylcholine receptor agonists [1–3], while anatoxin-a(s) irreversibly inhibits acetylcholinesterase activity [4, 5]. The saxitoxins, on the other hand, exert their toxicity via the inhibition of voltage-gated sodium, calcium, and potassium channels [6–8]. Despite their different modes of action, when ingested in acute doses, the alkaloid neurotoxins impart similar symptoms of paralysis and respiratory failure.

The lipopeptides jamaicamide and kalkitoxin also inhibit voltage-gated sodium channels [9, 10]; however, cases of acute human poisonings are rare. Conversely, antillatoxin is a sodium channel activator and one of the most potent ichthyotoxins isolated from nature [11, 12]. The neurotoxic amino acid BMAA activates AMPA/kainate receptors [13], which in chronic cases may contribute to the development of amyotrophic lateral sclerosis-Parkinsonism dementia (for review see [14]).

The cyanotoxins display remarkable structural diversity, however, most can be classified as cyclic peptides, depsipeptides, lipopeptides, or alkaloids [15–17]. Among the alkaloid neurotoxins, which are the focus of this chapter, anatoxin-a and homoanatoxin-a can be described as low molecular weight secondary bicyclic amine alkaloids [18, 19]. While anatoxin-a(s) is a guanidine methyl phosphate ester [20]. Saxitoxin is a tricyclic perhydropurine alkaloid containing guanidinium moieties [21], and is the parent compound of more than 57 naturally occurring derivatives differing at four positions that may be hydroxylated, sulfated, or carbamylated [22].

The enzymes responsible for cyanotoxin biosynthesis, modification, and transport are usually encoded within large gene clusters ranging in size from around 25 to 58 kb. A common theme in cyanotoxin biosynthesis is the occurrence of nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs). NRPSs and/or PKSs constitute the core biosynthesis enzymes in the production of several cyanobacterial hepatotoxins [23–25], as well as in the production of the neurotoxins anatoxin-a, homoanatoxin-a, and jamaicamide [9, 26]. Saxitoxin biosynthesis also involves a novel PKS-like enzyme [27]. Other enzymes associated with cyanobacterial neurotoxin gene clusters include those that transfer functional groups (e.g., methyl-, sulfo-, carbamoyltransferases), cyclases, and transporters. Transposase genes are also commonly associated with cyanotoxin gene clusters including the anatoxin-a and saxitoxin clusters [26, 27], and may play a role in their genetic mobilization via horizontal transfer.

This chapter provides an overview of the current status of cyanobacterial alkaloid neurotoxin research, with specific focus on the chemistry, toxicology, and biosynthesis of these potent compounds.

2 Anatoxin-a and Homoanatoxin-a

2.1 History

(+)-Anatoxin-a (systematic name: (1*R*,6*R*)-1-(9-azabicyclo[4.2.1]non-2-en-2-yl)) ethanone, Fig. 3.1, which was first coined Very Fast Death Factor (VFDF) because it provoked the rapid death of animals after ingestion, was isolated from *Anabaena flos-aquae* strains by Gorham and coworkers in the 1970s (as reviewed in [28]). The structure and configuration of the toxin was elucidated by X-ray crystallography of its *N*-acetyl derivative [29], and confirmed by other spectroscopic means [28] and by synthesis from (−)-cocaine [30]. Carmichael and coworkers rapidly showed that anatoxin-a provoked the rapid death of animals by acting as a depolarizing neuromuscular blocking agent [31]. Homoanatoxin-a, the higher homologue of anatoxin-a (Fig. 3.1), was synthesized as a potent analogue of anatoxin-a [3], and detected in cultures of *Oscillatoria formosa* [32] and of other strains. In the years following the discovery of anatoxin-a, several syntheses of anatoxin-a, homoanatoxin-a, and analogues were published, and numerous pharmacological studies allowed defining the pharmacology and toxicology of these deadly natural neurotoxins. The biosynthesis of anatoxin-a and homoanatoxin-a, by cyanobacteria, was only deciphered recently when the biosynthetic cluster of genes was identified and sequenced [33].

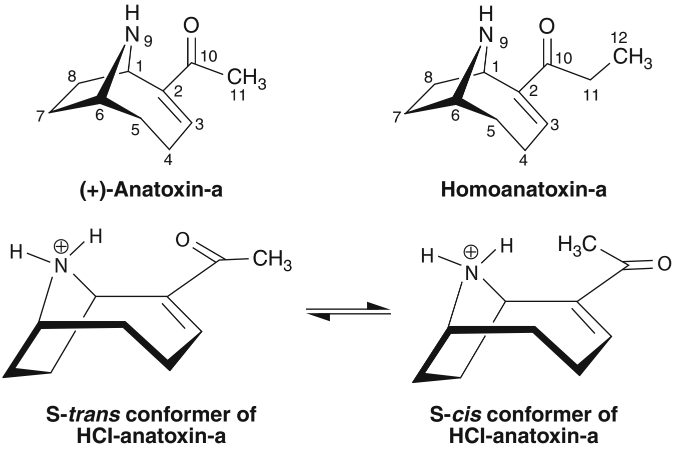


Fig. 3.1 Chemical structure of (+)-anatoxin-a and of homoanatoxin-a and representation of the two major side chain conformers proposed for HCl-anatoxin-a (adapted from [34])

2.2 Chemistry

2.2.1 Synthesis

Synthetic chemists have long been attracted by the structure and the pharmacological properties of anatoxin-a, and have thus proposed numerous syntheses of anatoxin-a and analogues, including homoanatoxin-a. The group of Rapoport has proposed several approaches and has synthesized the two enantiomers of anatoxin-a as well as a number of analogues that were used as pharmacological probes [34–37]. The group of Gallagher has prepared anatoxin-a, homoanatoxin-a, and other analogues [3, 38, 39], while the group of Parsons has proposed a simple and efficient synthesis of racemic anatoxin-a and analogues, from cyclooctadiene [40]. The reader is referred to the report by Mansell, 1996 [41], covering this literature before 1996, and to more recent synthetic approaches [42–49].

2.2.2 Molecular Properties

Anatoxin-a is a homotropane alkaloid with a monoisotopic mass of 165.11536 Da ($C_{10}H_{15}NO$). Its hydrochloride salt is soluble in water, methanol, and chloroform, and its amino group has a pKa of 9.36 ± 0.06, thus being almost fully protonated at physiological pH [34]. The natural enantiomer shows a specific rotation of $[\alpha]_D = + 43.2$ (c 0.676, ethanol) [36]. Due to its conjugated ketone, anatoxin-a shows specific absorptions in the UV range ($\lambda_{max} = 227$ nm, $\varepsilon = 10\,700\ \text{cm}^{1}\ \text{M}^{-1}$) and in the IR range (1 665 cm^{-1}) [36]. Its ^{1}H and ^{13}C NMR spectra have been fully interpreted [34]. The conformation of anatoxin-a as a solid has been elucidated, by X-ray crystallography, and in solution by NMR, as well as by calculations [34, 50]. The molecule seems to adopt the same conformation in solution and in the crystal with the seven-membered ring in a twist chair form. However, the side chain adopts two possible orientations, s-*cis* and s-*trans*, in solution (Fig. 3.1). Unfortunately, homoanatoxin-a has not been fully characterized even if its synthesis has been described, and its molecular properties have not yet been reported.

2.2.3 Reactivity and Stability

The secondary amine of anatoxin-a and homoanatoxin-a has similar chemical properties as other amines, and can form various salts, such the fumarate salt, which is a commercially available form of anatoxin-a. Modification of the amine has been used to derivatize these alkaloids for their detection, using diverse techniques (see below), as their *N*-acetyl- [51, 52], *N*-butyl- [53], *N*-(7-nitro-2,1,3-benzoxadiazole)-4- [54] or *N*-fluorenylmethyloxycarbonyl derivatives [55]. The double bond can easily be reduced by hydrogen in the presence of a catalyst, leading to a mixture of *cis*- and *trans*-dihydroderivatives [56] (Fig. 3.2). Epoxidation of this double bond is readily achieved by oxidation with hydrogen peroxide, probably yielding to a mixture of diastereoisomers [56] (Fig. 3.2). The reactivity of the methyl ketone of anatoxin-a has been largely used by Gallaghers and coworkers for the preparation of numerous side chain analogues [39]. Anatoxin-a, in aqueous solution, is photosensitive and degrades to unknown compounds, with a half-life of about 100 min, when irradiated with sunlight. This process is oxygen independent but pH dependent and light intensity dependent [57]. In this study, it was also noted that the degradation of anatoxin-a, at pH 9 in the dark, has a half-life of about 4 days. This dark degradation process is accelerated in the presence of oxygen and Cu^{2+} ions. Unfortunately, the degradation compounds have not been characterized but it was shown that they were nontoxic [57]. The oxidation of anatoxin-a by Advanced Oxidation Processes (AOPs), such as VUV photolysis or combination of UV and H_2O_2, or by other oxidant (chlorine, permanganate, ozone, etc.) has been studied, in view of detoxifying contaminated water. Anatoxin-a is oxidized very rapidly by ozone ($k = 9.42\ s^{-1}$, at pH 7 and 20 °C), while chlorine oxidation is slow. However, while the kinetics of this process is well characterized, the by-products formed by oxidation have not yet been identified [58–65].

2.2.4 Detection and Quantification Methods

Because the presence of anatoxin-a and homoanatoxin-a in the environment represents a risk for animals and humans, several analytical methods have been designed to detect these toxins as well as their natural derivatives (Fig. 3.2). GC-MS was first used to detect and quantify anatoxin-a or its *N*-acetyl derivative [51, 52, 66]. High Pressure Liquid chromatography (HPLC) coupled to UV detection was also used, although this detection method is not very sensitive [67]. Thus, to improve the sensitivity, several authors have used pre-derivatization with a fluorophore, which reacts on the amine of anatoxin-a or of homoanatoxin-a, followed by separation by HPLC coupled to a fluorescence detector [56, 68, 69]. However, the derivatization might lead to false positives even if the technique was improved to remove primary amines present in the sample [70] or by concentration by extraction of anatoxin-a prior to analysis [71, 72]. It is now accepted that the best analytical technique relies on the use of HPLC coupled to tandem mass spectrometry (LC-MS^2, or even LC-MS^n) without derivatization to avoid

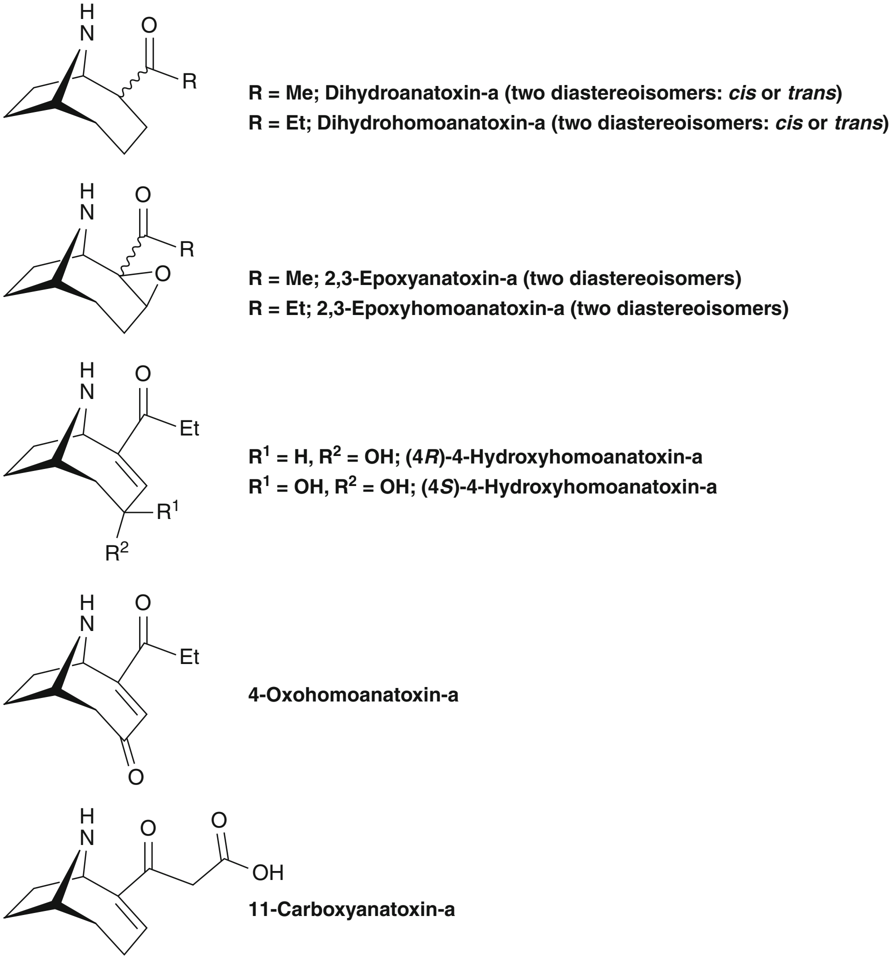

H
N
O
R
R = Me; Dihydroanatoxin-a (two diastereoisomers: *cis* or *trans*)
R = Et; Dihydrohomoanatoxin-a (two diastereoisomers: *cis* or *trans*)
H
N
O
R
O
R = Me; 2,3-Epoxyanatoxin-a (two diastereoisomers)
R = Et; 2,3-Epoxyhomoanatoxin-a (two diastereoisomers)
H
N
O
Et
R1
R2
R1 = H, R2 = OH; (4*R*)-4-Hydroxyhomoanatoxin-a
R1 = OH, R2 = OH; (4*S*)-4-Hydroxyhomoanatoxin-a
H
N
O
Et
O
4-Oxohomoanatoxin-a
H
N
O
O
OH
11-Carboxyanatoxin-a

Fig. 3.2 Chemical structure of the natural derivatives of anatoxin-a and homoanatoxin-a, so far identified

false positives [68, 73–76]. Improvement of this technique with preconcentration of the toxins has also been described [77]. Table 3.1 summarizes the major techniques published to date. Surprisingly, the use of specific antibodies against anatoxin-a in analytical methods has not yet been described, although the synthesis of an analogue of anatoxin-a for monoclonal antibody production was recently proposed [78].

2.3 Distribution and Isolated Producing Strains

Since the discovery of anatoxin-a in Canada, this toxin and its higher homologue, homoanatoxin-a, have been detected in bodies of water (lakes, reservoirs, rivers,

Table 3.1 Selected analytical methods for the detection and quantification of anatoxin-a, homoantoxin-a, and their natural derivatives (see Fig. 3.2)

Method	Extraction (matrice)	Derivatization	LOD	LOQ	Comments	Reference
GC-MS	Methanol/HCl (cyanobacteria or water)	Formation of *N*-acetyl-anatoxin-a and preconcentration on Sep-Pack silica cartridge	100 ng L^{-1}	n.r.	Good selectivity	[51]
HPLC-UV	50 mM acetic acid (cyanobacteria)	No derivatization	20 ng	n.r.	Low sensitivity (estimated from the calibration curve) and low selectivity	[67]
HPLC-FLD	1% HCl 1 M in methanol and weak cation exchange SPE (cyanobacteria or water)	Derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole	10 ng L^{-1}	n.r.	High sensitivity. Determination of anatoxin-a, homoanatoxin-a, and their natural derivatives. See also Azevedo et al., 2011 for improvements [79].	[56]
LC-MS	NH_4OH, pH 10 and reversed phase SPE (water)	Online derivatization using fluorenylmethyloxycarbonyl chloride	2.1 ng L^{-1}	15.2 ng L^{-1}	High sensitivity. Automated process.	[55]
LC-MS^n	Water, pH 7, purification with weak cation exchange SPE (water)	No derivatization	600 ng L^{-1}	2 500 ng L^{-1}	Mass fragmentation pathways for anatoxin-a, homoanatoxin-a, and their natural derivatives. Low sensitivity.	[68]
LC-MS^2	Filtration (water)	No derivatization	8 ng L^{-1}	13 ng L^{-1}	High sensitivity.	[75]
LC-MS^2	50% acetic acid in methanol (cyanobacteria)	No derivatization	0.7 ng	1.15 ng	Very high sensitivity. Analysis of anatoxin-a and derivatives.	[80]
LC-MS^2	Aqueous NH_4OH, pH 10.5, porous graphitic carbon SPE (water)	No derivatization	0.65 ng L^{-1}	1.96 ng L^{-1}	Very high sensitivity. Use of d_5-phenylalanine as internal standard.	[76]

SPE solid phase extraction, *LOD* limit of detection, *LOQ* limit of quantification, *n.r.* not reported.

estuaries, etc.) all over the world. The literature published up to 2006 has already been reviewed [81] and we have summarized in Table 3.2 the reports published since 2006. Clearly, anatoxin-a and homoanatoxin-a have been detected in various part of the world suggesting that cyanobacteria producing these neurotoxins have probably colonized the entire surface of the globe. However, it is difficult to compare the data because the detection methods differ and the units used are not always consistent (values are reported in gram per liter or per gram of dry weight or wet weight). Nevertheless, the important point is that the concentration of anatoxin-a or homoanatoxin-a is sometimes very high (1.7 mg L^{-1}; 1.3 mg g^{-1} dry weight), thus confirming the importance of surveying these neurotoxins in bodies of water. From the toxic blooms, researchers have isolated strains and identified the cyanobacteria producing these neurotoxins. The producers belong to different genera such as *Anabaena*, *Aphanizomenon*, *Oscillatoria*, *Phormidium*, *Cylindrospermum*, and *Raphidiopsis* [81]. Selected examples are summarized in Table 3.4 together with the concentration of anatoxin-a or homoanantoxin-a found in the cells. Interestingly, the strains can be classified into two groups according to the neurotoxin produced, but the concentration of the toxin varies greatly from strain to strain. There are currently no explanations for these observations.

2.4 Pharmacology and Toxicology

The toxicology and pharmacology of anatoxin-a and homoanatoxin-a have been extensively studied and reviewed [39, 81], and we only present here the main facts and review the relevant literature since 2006. Anatoxin-a is highly toxic and provokes the rapid death of animals after ingestion or intraperitoneal (i.p.) injection [31]. Several animals have been tested (rats, mice, calves, ducks, fish) and they all show the same type of toxicity although with different sensitivity [81]. The mouse has been thoroughly used as a model in toxicological studies, and it shows a minimal lethal dose (LDmin i.p.) of 0.25 mg kg^{-1} and death occurs in 2–7 min. Homoanatoxin-a shows the same toxicological profile as anatoxin-a [39]. These alkaloids act as potent agonists of the muscular and neuronal nicotinic acetylcholine receptor (nAChR). The acute toxicity of anatoxin-a and homoanatoxin-a is the result of their binding to the nAChR at the neuromuscular junction, leading to the opening of the channel of this ionotropic receptor and thus to the flow of cations (K^+, Na^+, Ca^{2+}), provoking the depolarization of the cell membrane. Because these agonists are not degraded, their prolonged presence sustains the desensitized state of the receptor and provokes muscle paralysis and respiratory failure. Anatoxin-a binds to the muscular nAChR with Kd of 0.1–0.2 μM, and to the neuronal nAChR with Kd from 0.05 to 0.6 μM depending on the receptor subtype [39, 102]. Homoanatoxin-a shows similar binding capacities. Because these alkaloids have their conformation restricted, due to the aza-bicyclic moiety, they have been used as pharmacological probes to study the nAChR. It has been shown that (−)-anatoxin-a, the nonnatural enantiomer, does not bind to the receptor and that methylation of the amine decreases affinity by a 1,000 fold [39]. Many analogues with different

Table 3.2 Occurrence of anatoxin-a or homoanatoxin-a in bodies of water as reported since 2006

Country	Type of body of water	Sampling year	Intoxication episodes	Method of detection	Toxin concentration	Reference
Kenya	7 lakes	2001–03	Flamingoes died	HPLC-UV, MS	ATX 0.3–1,260 μg g^{-1} dry weight	[82]
New Zealand	3 water bodies	2001–04	n.r.	HPLC-FLD	ATX detected	[83]
New Zealand	Hutt River	2005–06	5 dogs died	LC-MS^2	ATX 27 μg kg^{-1} wet weight HATX 4.4 mg kg^{-1} wet weight	[84]
New Zealand	Lake Hakanoa	n.r.	n.r.	LC-MS^2	ATX detected	[85]
New Zealand	Lake Okaro	2006	n.r.	LC-MS^2	ATX detected in sediment	[86]
New Zealand	7 rivers	2009	n.r.	LC-MS^2	ATX detected	[87]
New Zealand	2 rivers	2007–08	n.r.	LC-MS^2	ATX 1.7 mg kg^{-1} HATX 23.5 mg kg^{-1}	[88]
New Caledonia	Ocean	2005	n.r.	GC-MS	HATX detected	[89]
USA Florida	6 lakes	2002–04	n.r.	LC-MS^2	ATX 0.05–7.0 μg L^{-1}	[90]
USA Wisconsin	421 samples	2004–05	1 young boy died (?)	LC-MS^2	ATX 0.7–1.750 μg L^{-1}	[91]
USA East Coast	Chesapeake Bay	2000–06	Fish and birds died	HPLC-FLD or LC-MS^2	ATX 0.003–3 μg L^{-1}	[92]
USA Midwest	23 lakes and reservoirs	2006	n.r.	LC-MS^2	ATX 0.02–9.5 μg L^{-1}	[93]
USA Florida	6 sites	2007	n.r.	LC-MS^2	ATX 0.01–0.04 μg g^{-1} dry weight	[94]
Portugal	9 water bodies	2000–05	n.r.	HPLC-FLD	ATX 0.06–24.6 μg g^{-1} dry weight	[95]
France	River Tarn	2005–06	20 dogs died	GC–MS	ATX and HATX detected	[96]
Spain	14 reservoirs	2002–04	n.r.	HPLC-UV	ATX 0.31 μg L^{-1}	[97]
Italy	28 lakes	1989–2006	n.r.	GC-MS	ATX 0.1–12.1 μg g^{-1} wet weight	[98]
Poland	Dam reservoir, Zemborzycki	2007	n.r.	HPLC-FLD	ATX 1 mg L^{-1}	[99]

(*continued*)

Table 3.2 (continued)

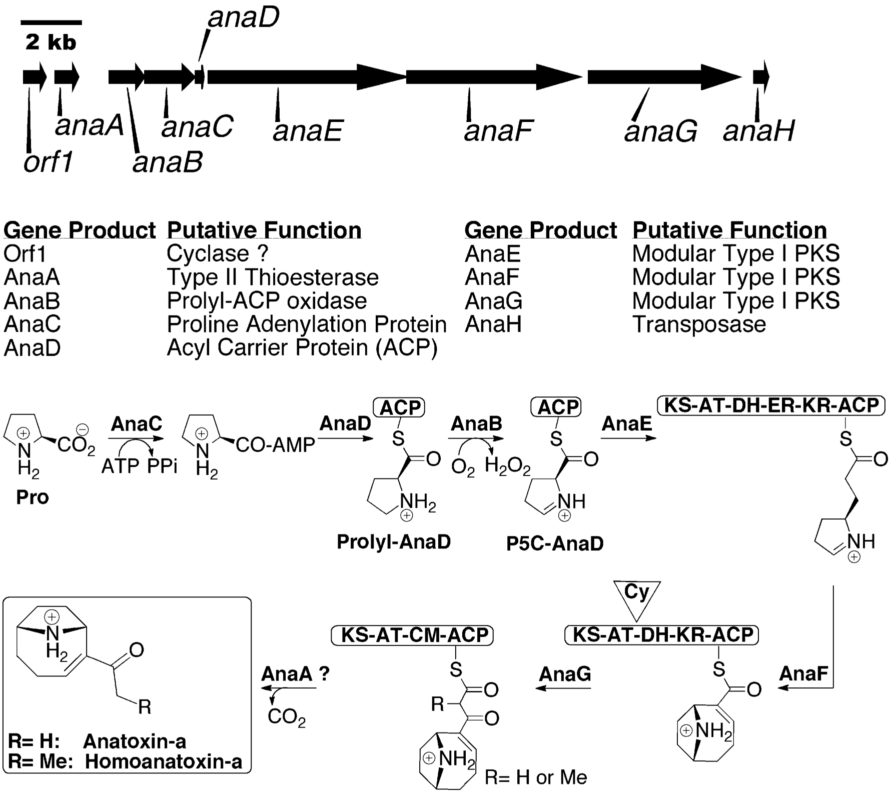
Country	Type of body of water	Sampling year	Intoxication episodes	Method of detection	Toxin concentration	Reference
Germany	Lake Stolpsee	2007–08	n.r.	LC-MS^2	ATX 0.01–0.12 μg L^{-1}	[100]
China	Yanghe reservoir	2007	n.r.	LC-MS^2	ATX 0.1 μg L^{-1}	[101]

n.r. not reported, *ATX* anatoxin-a, *HATX* homoanatoxin-a

side chains have been described. They have helped to define the bioactive conformation of anatoxin-a, which appears to be the s-*trans* congener (Fig. 3.1) [39]. Other toxic effects of anatoxin-a have been studied such as developmental [103], genotoxic [104], or apoptopic [105] but the observed effects were limited.

2.5 Biosynthesis and Degradation

The first conclusive experiments concerning anatoxin-a and homoanatoxin-a biosynthesis were reported by Hemscheidt and colleagues in 1995 [106]. They used acetate and glutamate labeled with ^{13}C at different positions, in feeding experiments using *Anabaena flos-aquae* 37 and *Oscillatoria formosa* CYA-92 strains, the former producing anatoxin-a and the latter homoanatoxin-a. By purifying the toxins and recording their ^{13}C NMR spectra, these authors proposed a biosynthetic route starting from glutmate that would be reduced to (*S*)-pyrroline-5-carboxylate, which in turn would serve as the starter of a polyketide synthase that would incorporate three units of acetate. A decarboxylation and further unspecified steps were proposed to lead to anatoxin-a. It was proposed that homoanatoxin-a was formed by methylation of anatoxin-a on position C11. If the polyketide starter is in fact proline rather than (*S*)-pyrroline-5-carboxylate, the implication of PKS involvement would be correct (see below). The origin of the methyl group of homoanatoxin-a was also confirmed by feeding experiments, with *Raphidiopsis mediterranea*, using deuterium labeled methionine on its methyl group [107]. Later on, Méjean and coworkers used degenerate primers to amplify, by PCR, sequences coding for ketosynthase domain of PKSs, in the genome of *Oscillatoria* PCC 6506, a homoanatoxin-a producer [108]. Three sequences were identified and designated *ks1*, *ks2*, and *ks3*, all coding for KS-AT domains of PKS of unknown function. However, using a panel of 50 axenic strains taken from the PCC collection, these authors showed that the presence of *ks2* in the genome of a particular strain correlated perfectly with its ability to produce anatoxin-a or homoantoxin-a. This specific sequence was then identified in a cluster of nine genes that was identified in the genome sequence of *Oscillatoria* PCC 6506 [109]. This cluster, designated *ana*, was thus proposed to be responsible for the biosynthesis of anatoxin-a and homoanatoxin-a. Then, Ploux and colleagues proposed a complete biosynthetic scheme for anatoxin-a and


2 kb
anaD
orf1
anaA
anaB
anaC
anaE
anaF
anaG
anaH
Pro
AnaC
ATP PPi
CO-AMP
AnaD
ACP
Prolyl-AnaD
AnaB
O_2 H_2O_2
P5C-AnaD
AnaE
KS-AT-DH-ER-KR-ACP
Cy
KS-AT-DH-KR-ACP
AnaF
AnaG
KS-AT-CM-ACP
R= H or Me
AnaA ?
CO_2
R= H: Anatoxin-a
R= Me: Homoanatoxin-a

Gene Product	Putative Function	Gene Product	Putative Function
Orf1	Cyclase ?	AnaE	Modular Type I PKS
AnaA	Type II Thioesterase	AnaF	Modular Type I PKS
AnaB	Prolyl-ACP oxidase	AnaG	Modular Type I PKS
AnaC	Proline Adenylation Protein	AnaH	Transposase
AnaD	Acyl Carrier Protein (ACP)		

KS: ketosynthase; AT: acyltransferase; KR: ketoreductase; DH: dehydratase; ER: enoylreductase; ACP: acyl carrier protein; Cy: cyclase; CM: methyltransferase

Fig. 3.3 Biosynthesis of anatoxin-a and homoanatoxin-a in cyanobacteria: genes and biosynthetic pathway

homoanatoxin-a by using a combination of bioinformatic analysis, feeding experiments as well, as in vitro biochemical experiments on isolated enzymes (Fig. 3.3) [33]. The biosynthesis starts from free proline, which is tethered to an acyl carrier protein (ACP) and oxidized to pyrroline-5-carboxyl-ACP. It is proposed that this molecule is then transformed by three consecutive PKSs. The first PKS, AnaE, should add one acetate unit that is completely reduced. The second PKS, AnaF, should add one acetate unit and should catalyze a Mannich cyclization that forms the azabicyclic homotropane structure of anatoxin-a and homoanatoxin-a. It has been proposed, on the basis of bioinformatics, that the protein coded by *orf1* participates in the cyclization reaction. The last PKS, AnaG, should add one acetate unit without any reduction, to yield 11-carboxylanatoxin-a or 11-carboxylhomoanatoxin-a, tethered to the ACP domain as thioesters. 11-Carboxylhomoanatoxin-a thioester would be formed by methylation on AnaG, which contains a methylation domain. The last step would be the hydrolysis of the thioesters to give 11-carboxyanatoxin-a or 11-carboxyhomoanatoxin-a that would give the corresponding toxin by decarboxylation. It is not yet clear if AnaA, a type II thioesterase, is implicated in these steps, but 11-carboxyanatoxin-a has actually been detected in extracts of *Aphanizomenon issatchenkoi*, an anatoxin-a producer [110]. The first steps of this biosynthesis, catalyzed by AnaC and AnaB, have been completely reconstituted in vitro, thus validating the predicted functions [111, 112]. AnaC is an adenylation protein very specific for proline, and AnaB is a flavoprotein oxidase homologous to isovaleryl-CoA dehydrogenase in terms of reaction mechanism and structure. The *ana* cluster has been recently identified in *Anabaena flos-aquae* 37 an anatoxin-a producer [113]. In that *ana* cluster, the genes *anaB, C, D, E, F, G* share strong sequence identity with those of *Oscillatoria* PCC 6506 and are arranged in the same manner. However, the gene *orf1* and *anaA* are located 7 kilobases downstream of the *ana* cluster. Thus, the *ana* cluster seems to be conserved in *Anabaena* and *Oscillatoria* genera, suggesting a common origin. The *ana* genes, or part of them, have been successfully used as genetic markers to track anatoxin-a or homoantoxin-a producers, in some reports [87, 100, 108, 113]. No transporter has been identified for anatoxin-a, and it is thus assumed that the toxin is released when the cells lyse during senescence. Several derivatives of anatoxin-a and homoanatoxin-a have been detected in cultures of cyanobacteria or in environmental samples (Fig. 3.2) [56, 66, 67, 107, 114]. The oxidized compounds, epoxy- hydroxyl-, or oxo-derivatives are thought to be degradation products after oxidation, although nothing is known about the reactions (catalyzed or not) leading to these derivatives. These compounds are not toxic. The reduced compounds, the dihydroderivatives, have been detected in environmental samples and are also assumed to be degradation products, although we still do not know how they are formed. However, it has been shown that dihydroanatoxin-a as a mixture of diastereoisomers, is quite toxic, retaining 10% of the toxicity of anatoxin-a [35, 37]. There is thus a need to determine these dihydroderivatives in the environment. Very little is known on the biodegradation of anaatoxin-a in the environment [115].

3 Anatoxin-a(s)

3.1 History, Chemistry, and Toxicology

Anatoxin-a(s) (Fig. 3.4) was discovered by Mahmood and Carmichael as a metabolite of *Anabaena flos-aquae* NRC 525-17 [4, 5]. This toxin was implicated in the death of wild animals (ducks, birds, swine) after ingestion of contaminated water. This cyanotoxin is a neurotoxin, but different from anatoxin-a, and this unique alkaloid is an irreversible inhibitor of acetylcholine esterase with a toxicology similar to that of known anticholinesterases such as paraoxon [116–119]. Anatoxin-a(s) provokes salivation (thus the suffix (s)), chromodacryorrhea, fasciculation, and urinary incontinence, and death within 7–20 min, on rats treated with 0.25–1.0 mg kg^{-1} (i.p.). The lethal dose LD_{50} (mouse, i.p.) is 50 μg kg^{-1} making

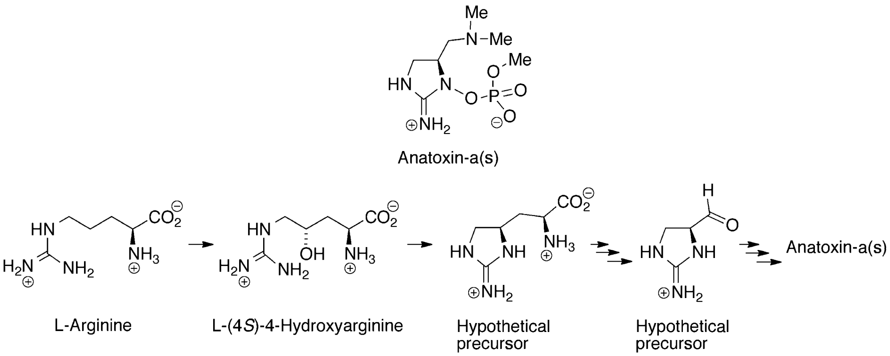


Fig. 3.4 Chemical structure of anantoxin-a(s) and the proposed biosynthetic pathway leading to this neurotoxin (Adapted from [122])

this toxin a very powerful poison. However, anatoxin-a(s) does not cross the blood-brain barrier and acts on peripheral acetylcholine esterase. The structure and the configuration of this compound were elucidated by Moore and colleagues [120]: anatoxin-a(s) is a unique guanidine methyl phosphate ester with a molecular weight of 252.0987 ($C_7H_{17}N_4O_4P$). Based on this structure, these authors proposed that anatoxin-a(s) inactivates acetylcholine esterase by reacting with the active-site serine that would be phosphorylated. Anatoxin-a(s) is dephosphorylated in basic solution but quite stable at neutral or acidic pH [120]. The total synthesis of anatoxin-a(s) has not yet been described but the synthesis of the cyclic moiety of anatoxin-a(s) has been reported [120, 121].

3.2 Occurrence and Detection

Anatoxin-a(s) has been detected in toxic blooms in lakes in Canada [4], Denmark [123, 124], and Brazil [125, 126]. So far, only a few cyanobacterial strains producing anatoxin-a(s) have been characterized: *Anabaena flos-aquae* [4], *Anabaena crassa* [126], and *Anabaena lemmermannii* [124]. The detection methods used to assay anatoxin-a(s) rely on the colorimetric assay for acetylcholine esterase [5], or on an electrochemical biosensor based on acetylcholine esterase activity [127, 128], or more recently on the use of LC-MS^2 [129].

3.3 Biosynthesis

Not much is known regarding the biosynthesis of anatoxin-a(s). Using radiolabeled arginine and deuterium labeled arginine and (4*S*)-4-hydroxy-arginine, Moore and coworkers have proposed a pathway starting from arginine and involving (4*S*)-4-hydroxy-arginine and further steps to yield to anatoxin-a(s), as shown in Fig. 3.4 [122, 130].

4 The Saxitoxin Family

4.1 History

Saxitoxin and its analogues are the causative agents of paralytic shellfish poisoning (PSP), which is one of the five major phycotoxin poisoning syndromes with a global impact. The paralytic shellfish toxins (PSTs) have historically been associated with blooms of saxitoxin-producing dinoflagellates in marine environments. Toxic mass-proliferating dinoflagellates can have devastating effects, such as mass mortalities of marine organisms, and cause substantial financial damage to aquaculture industries and fisheries. One of the most important aspects of these toxins is that they can accumulate to very high levels in marine invertebrates, which are utilized as seafood. PSP is usually caused by marine shellfish, which filter feed on toxic dinoflagellates, and thus accumulate the toxins [131]. Shellfish are highly resistant to the toxins [132], and may accumulate levels that can be fatal for humans, and a range of shellfish predators. Marine invertebrates other than shellfish [133, 134], as well as some fish species [135–138], may also accumulate PSTs to high levels that can cause illness or death when they are consumed [139]. The global incidence of PSP has been estimated at 2,000 cases per year globally with a mortality rate of 15% [140].

PSP is the longest known form of poisoning by phycotoxins. The earliest medical record dates back over 300 years [141, 142], and outbreaks of PSP with, at times, epidemic proportions, have been regularly reported from the eighteenth to the twenty-first century [143–145]. For a long time, the reason for the unpredictable and sporadic occurrence of shellfish toxicity was a mystery, until Sommer and coworkers identified certain species of dinoflagellates as the toxin producers [146, 147]. Since that discovery, it took almost 40 years until the chemical structure of saxitoxin, which was purified from the giant butter clam, *Saxidomus giganteus*, and cultured dinoflagellates could be determined by X-ray diffraction [21, 148], and a further decade before a highly unusual biosynthesis pathway for saxitoxin was inferred from isotope-labeled precursor feeding studies in conjunction with NMR [149, 150].

Although PSP is generally associated with marine dinoflagellates, they are not the only organisms that produce saxitoxin analogues. The first observation of a PSP-toxic cyanobacterial bloom of *Aphanizomenon flos-aquae* was made in the late 1960s in a freshwater body in New Hampshire (USA). A water-soluble toxin that was purified from this bloom, labeled aphantoxin, had similar chemical and pharmacological properties as saxitoxin [151–153]. Additional studies confirmed the identity of aphantoxin as saxitoxin and neosaxitoxin [154, 155]. Like dinoflagellates, cyanobacteria may form extensive blooms, and their toxins may accumulate in filter-feeding invertebrates, such as fresh water shellfish [156, 157], and their predators. PST-producing blooms of cyanobacteria can threaten the safety of drinking water supplies, potentially causing mass mortalities of aquatic organisms, livestock, and other animals consuming the water [158, 159].

4.2 Chemistry

4.2.1 Structural Diversity of Paralytic Shellfish Toxins

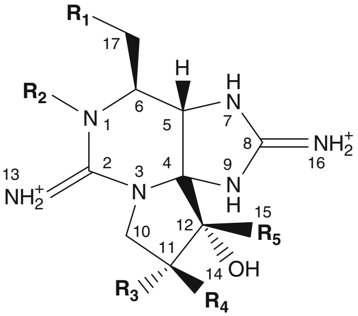
Saxitoxin is the principle component of more than 57 chemically related compounds that form the PST family [22]. They share a tricyclic perhydropurine backbone [21, 148], which can carry different functional groups at positions R1–R5 (Table 3.3). The carbamate toxins have a carbamoyl side chain at R1, and comprise saxitoxin (STX), neosaxitoxin (neoSTX), and gonyautoxins-1 to -4 (GTX-1 to -4). neoSTX differs from STX by an N1-hydroxylation (R2). GTX-2/-3 are the corresponding C-11-sulfated analogues of STX, whereas GTX-4/-1 are the corresponding sulfated C-11 analogues of neoSTX. The sulfated C-11 becomes a chiral center, where GTX-1 and GTX-4, and GTX-2 and GTX-3 are epimers of each other, respectively. *N*-sulfocarbamoyl toxins carry an N-linked sulfate group at the carbamate side chain. GTX-5 and GTX-6, which are also sometimes referred to as B-1 and B-2 toxins, are the corresponding *N*-sulfocarbamoyl toxins to STX and neoSTX, respectively. C-1/2 to C-3/4 toxins are the corresponding *N*-sulfocarbamoyl toxins to GTX-2/3 and GTX-1/4, respectively. Further adding to the diversity, toxins may have a hydroxyl group instead of a carbamoyl side chain (decarbamoyl analogues), or lack the hydroxyl group (deoxy toxins). Most of the aforementioned analogues have been detected in both cyanobacteria and dinoflagellates [160–162]. Some more unusual derivatives have been described and are listed in Table 3.4. In addition to the usual carbamate and decarbamoyl toxins, six new saxitoxin derivatives have been isolated from the cyanobacteria *Lyngbya wollei*, including derivatives that provide an acetyl side chain instead of the typical carbamate, or lack one of the C-12 hydroxyl moieties [163]. *N*-sulfocarbamoyl toxins that were C-11 hydroxylated were isolated from mussels [164]. Unusual modifications, such as the presence of a C-11 ethanoic acid side chain [165], or the *N*-hydroxylation of the carbamoyl side chain [166], have also been described for saxitoxin derivatives from several species of xanthid crabs, and from the freshwater puffer fish, where the carbamoyl side chain is *N*-methylated [167]. Three PSTs isolated from *Gymnodinium catenatum* possessed a hydroxybenzoate side chain, instead of a carbamoyl side chain [168]. The most unusual saxitoxin analogue is zetekitoxin AB (Scheme 3.1) from the Panamanian golden frog *Atelopus zeteki*. Its chemical structure has been a mystery for over 30 years and was recently confirmed to be a novel analogue of saxitoxin [169].

Like saxitoxin, zetekitoxin AB is a selective sodium channel blocker; however, its sodium channel blocking activity was 580-fold higher than that of saxitoxin [169]. Sadly, the Panamanian golden frog is now extinct.

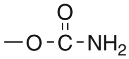
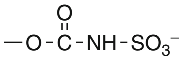
4.2.2 Chemical Properties and Stability

PSTs are highly polar molecules. STX has two pKa values of 8.22 and 11.28 in aqueous solution, which belong to the 7,8,9 and 1,2,3 guanidinium groups, respectively [171]. neoSTX has an additional pKa of 6.75 due to its N-1 hydroxylation. The 1,2,3 guanidinium group is fully protonated at physiological pH, whereas the 7,8,9 guanidinium group is partially protonated. The C-12 hydroxyls are in

Table 3.3 The molecular structure of saxitoxin and common analogues [170]



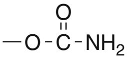
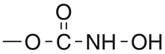
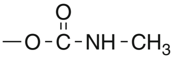
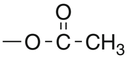
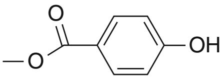
T3-S1

R-1	*R-2*	*R3*	*R-4*	*Compound name*
Carbamates	-H	-H	-H	STX
	-OH	-H	-H	neoSTX
	-OH	$-OSO_3^-$	-H	GTX-1
	-H	$-OSO_3^-$	-H	GTX-2
	-H	-H	$-OSO_3^-$	GTX-3
	-OH	-H	$-OSO_3^-$	GTX-4
N-sulfocarbamates	-H	-H	-H	GTX-5
	-OH	-H	-H	GTX-6
	-OH	$-OSO_3^-$	-H	C-3
	-H	$-OSO_3^-$	-H	C-1
	-H	-H	$-OSO_3^-$	C-2
	-OH	-H	$-OSO_3^-$	C-4
Decarbamoyl toxins	-H	-H	-H	dcSTX
	-OH	-H	-H	dcneoSTX
	-OH	$-OSO_3^-$	-H	dcGTX-1
-OH	-H	$-OSO_3^-$	-H	dcGTX-2
	-H	-H	$-OSO_3^-$	dcGTX-3
	-OH	-H	$-OSO_3^-$	dcGTX-4
Deoxy toxins	-H	-H	-H	doSTX
	-OH	$-OSO_3^-$	-H	doGTX-2
-H	-OH	-H	$-OSO_3^-$	doGTX-3

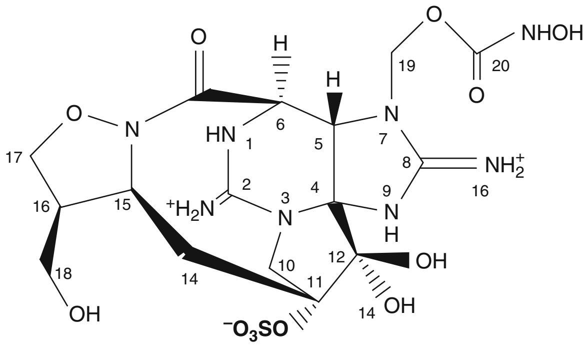
Abbreviations used are, *STX* saxitoxin, *GTX* gonyautoxin, *C* C-toxin; prefixes mean: *dc* decarbamoyl, *do* deoxy.

equilibrium between the hydrated and the keto form [21, 172]. At pH 7.7, only 1% of STX molecules are in the keto form, whereas that proportion rises to 24% at pH 8.4, and 36% at pH 9.8 [173]. Saxitoxin is highly stable under physiological conditions, but degrades slowly under alkaline conditions [171, 174]. The *N*-sulfocarbamoyl toxins are more labile, and readily hydrolyze the N-linked sulfate group. N-sulfocarbamoyl toxins, such as GTX-5, GTX-6, and the C-toxins, are thereby transformed to the corresponding carbamate toxins.

Table 3.4 Substituents of unusual saxitoxin derivatives

R-1	R2	R3	R4	R5	Compound
11-STX-ethanoate from xanthid crab *Atergatis floridus* [165]					
	-H	$-CH_2COO^-$/-H		-OH	
Carbamoyl-*N*-hydroxy-STX from xanthid crab *Zosimus aeneus* [166]					
	-H	-H	-H	-OH	
	-OH	-H	-H	-OH	
Carbamoyl-*N*-methyl-STX from freshwater puffer *Tetraodon cutcutia* [136]					
	-H	-H	-H	-OH	
De-amino-STX-analogues from *Lyngbya wollei* [163]					
	-H	-H	$-OSO_3^-$	-H	LTX-1
	-H	-H	$-OSO_3^-$	-OH	LTX-2
	-H	$-OSO_3^-$	-H	-OH	LTX-3
	-H	-H	-H	-OH	LTX-5
	-H	-H	-H	-H	LTX-6
-H	-H	-H	-H	-H	LTX-4
Hydroxybenzoate STX from *Gymnodinium catenatum* [168]					
	-H	-H	$-OSO_3^-$	-OH	GC1
	-H	$-OSO_3^-$	-H	-OH	GC2
	-H	-H	-H	-OH	GC3

Abbreviations used are, *LTX* Lyngbya wollei toxin, *GC* Gymnodinium catenatum toxin.



Scheme 3.1 Zetekitoxin AB

4.2.3 Synthesis

Chemical synthesis of saxitoxin analogues is challenging, in part because the unusual heterocyclic backbone is highly functionalized and susceptible to oxidation, and in part because of the high polarity of synthetic intermediates, which limits the range of suitable solvents for the chemical reactions [175]. The first chemical synthesis involved 20 steps, and had a total yield of only 0.25% [176]. Saxitoxin synthesis was improved in a later study [177]. Further studies focused on the selective synthesis of the natural (+)-enantiomers of STX and dcSTX [178–180], and the unnatural (−)-enantiomer of dcSTX [175]. These studies revealed that only the natural (+)-enantiomers had biological activity [181]. Other studies achieved the synthesis of GTX-3 [182]. The initial successful synthesis of saxitoxin triggered much debate with regard to potential misuses of this substance, and it was therefore scheduled as a chemical warfare agent of the highest category in the international chemical weapons convention, which came in effect in 1997.

4.2.4 Detection and Quantification

The detection and quantification of PSTs is challenging, because of the naturally low concentrations at which they occur, the high diversity of saxitoxin analogues, and some of their chemical properties, such as a small molecular weight, and high water solubility.

4.3 Biological and Biochemical Assays for PSTs

The Association for Official Analytical Methods (AOAC) has approved the mouse bioassay [183] as a standard method for the screening of PSTs in seafood. It involves the intraperitoneal injection of 1 ml acid extract into a 20 g mouse, and the recording of symptoms and time to death. One mouse unit (MU) is defined as the toxin amount that kills a 20 g mouse in 15 min, which is equivalent to 0.18 mg saxitoxin. An obvious drawback of this method is animal cruelty. In addition, this method is not specific for PSTs, and it is prone to interference from substances that are unrelated to PSTs. Also the dose-response relationship is not always linear.

Other biological assays have been developed that avoid the sacrifice of animals. The mouse neuroblastoma assay measures PSTs by the survival of cultured neuroblastoma cells after addition of extract. In this assay, Na^+/K^+-ATPase inhibitor ouabain and the sodium channel activator veratridine are added to neuroblastoma cells prior to the addition of toxin extract. PSTs that are present in the extract prevent the veratridine-induced influx of sodium ions into the cells, and thus prevent cell death. A commercially available kit version of this assay, the MIST shippable cell bioassay kit [184], showed good agreement in a comparative study to the mouse bioassay [185]; however, it performed unsatisfactorily in an AOAC international collaborative study in 1999, and there have been quality problems related to the shipping of the kit. The MIST kit was eventually replaced by the MIST Alert kit, which is an immunological assay that utilizes antibodies to STX, neoSTX, GTX1-4 [186]. Various other immunoassays exist [187].

The reversible nature of the binding of STX to voltage-gated sodium channels and the PST-binding protein, saxiphilin, is utilized in radioreceptor assays. The competitive displacement of tritiated saxitoxin from voltage-gated sodium channels or saxiphilin by PSTs from extracts is measured by a decrease in radioactivity in these assays [188–190].

4.4 Chemical Analysis of PSTs

A rapid and sensitive fluorescence spectroscopy method has been described that may be useful for applications, such as screening chromatographic fractions for the presence of PSTs [191].

Chemical methods that are based on high-pressure liquid chromatography (HPLC) with either fluorescence (HPLC-FLD), or tandem mass spectroscopic (LC-MS^2) detection are the methods of choice with regard to sensitivity and specificity, and have been used extensively in routine biotoxin monitoring programs [192]. The fluorometric detection of PSTs requires their oxidation, as only the oxidation products are fluorescent. Prechromatographic oxidation can be carried out in PSTs extracts prior to injection into the HPLC system [193, 194]; however, the chromatograms are not easily interpreted. Different PSTs may produce the same oxidation products, whereas some PSTs produce multiple oxidation products [195]. In HPLC methods with post-column oxidation, the toxins are oxidized after they have been separated and eluted from the column, but before they enter the detector [160, 192, 196]. Post-column oxidation methods require additional instrumentation, and careful control of flow rates and chemical composition of reagents and mobile phases. Separation of all saxitoxin analogues, including that of stereoisomers, can be achieved by using three separate runs with different isocratic elution conditions [160], or by using a solvent gradient [196]. Although HPLC-FLD is regarded as highly specific, there are cases where fluorescent compounds that are unrelated to PSTs can interfere [197]. Mass spectroscopic detection of PSTs can confirm the chemical identity, as well as quantify measured compounds. Several methods for the mass spectroscopic measurement of PSTs have been described [198–202]. Most of these methods are based on HPLC and electrospray ionization (ESI) in the positive mode. The most promising LC-MS^2 methods use hydrophilic interaction chromatography (HILIC) for the separation of PSTs prior to detection [199]. HILIC has high resolving power, and involves mobile phases with high concentrations of organic solvent, which favors the ionization of analytes in ESI, and thus the sensitivity of the method. Triple quadrupole-type MS^2 mass spectrometers are particularly suitable for the quantitative determination of known compounds [200]; however, the low mass resolution of these instruments prevents the detection of unknown compounds. Benchtop orbitrap mass spectrometers have become affordable, and their high mass resolution, sensitivity, and speed make them prime instruments for the quantitative measurement of PSTs, as well as for the unambiguous confirmation of known and novel toxins [198].

4.5 Phylogenetic Distribution of Paralytic Shellfish Toxins

Most cyanobacteria that produce PSTs belong to the order Nostocales, and include *Anabaena* spp. [161, 203], *Aphanizomenon flos-aquae* [154, 204–206], *Cylindrospermopsis raciborskii* [159, 207], and *Scytonema* [208]. There is also at least one PST-producing species, which belongs to a phylogenetic order that is distant from the Nostocales, such as the Oscillatoriales species, *Lyngbya wollei* [163, 209]. Furthermore, different isolates of the same species variably produce PSTs. A geographic segregation of toxin production has been observed for the species *Anabaena circinalis*, of which only Australian strains produce PSTs [210], while strains from America and Europe produce anatoxin-a [1, 5, 211].

In light of the biochemical uniqueness of PSTs, it is intriguing to find that cyanobacteria as well as dinoflagellates, which are not related to one another, are capable of producing these toxins. As with cyanobacteria, the capacity for PST production among dinoflagellates is distributed in an irregular manner. The majority of PSP-toxic dinoflagellates belong to the order Gonyaulacales; however, at least one representative from the distantly related order Gymnodiniales, *Gymnodinium catenatum*, is also known to produce PSTs [168, 212]. Species that include toxic strains also include strains that are nontoxic, whereas the toxin profile, which is considered to be constant in clonal isolates, differs between strains [213].

The divergent and irregular phylogenetic distribution of PST producer organisms sparked much debate regarding the primary source for these toxins, and their evolutionary origin. Proposals included that endo- or ectosymbiotic bacteria may be the original toxin producers [214–218]. A bacterial origin of PSTs may also explain the occurrence of PSTs in organisms, where cyanobacteria or dinoflagellates are unlikely the source. Examples include the calcareous red alga, *Jania* sp. [219, 220], xanthid crabs from coral reefs [166, 221], freshwater as well as marine puffer fish [135, 167, 222], and the Panamanian golden frog *Atelopus zeteki* [169]. The chemical identity of bacterial compounds suspected to be PSTs has not been determined conclusively, and a bacterial production of PSTs remains controversial.

4.6 Biosynthesis of Paralytic Shellfish Toxins

The structural resemblance between saxitoxin and purines of primary metabolism confounded efforts to determine the biosynthetic pathway for saxitoxin. Isotope-labeled precursor feeding studies revealed that saxitoxin is produced from arginine, acetate, and *S*-adenosylmethionine (SAM) by a unique pathway [149, 150, 223].

Arginine, minus its carboxylate group, the guanidino group of a second molecule of arginine, and an intact acetate unit form the tricyclic backbone. The methyl side chain is derived from SAM, whereas the origin of the carbamoyl side chain was uncertain but proposed to be derived from the guanidino group of a third molecule of arginine.

The loss of the arginine carboxyl suggested that acetate is linked to arginine by a Claisen-condensation in the initial step of biosynthesis (Fig. 3.5). The resulting intermediate would receive a guanidino group at its α-amine, whereby three heterocycles are formed. It was further proposed that the methyl side chain is introduced after cyclization, which gets subsequently epoxidized, opened to an aldehyde, and dehydrogenated to a hydroxyl. The incorporation of methionine

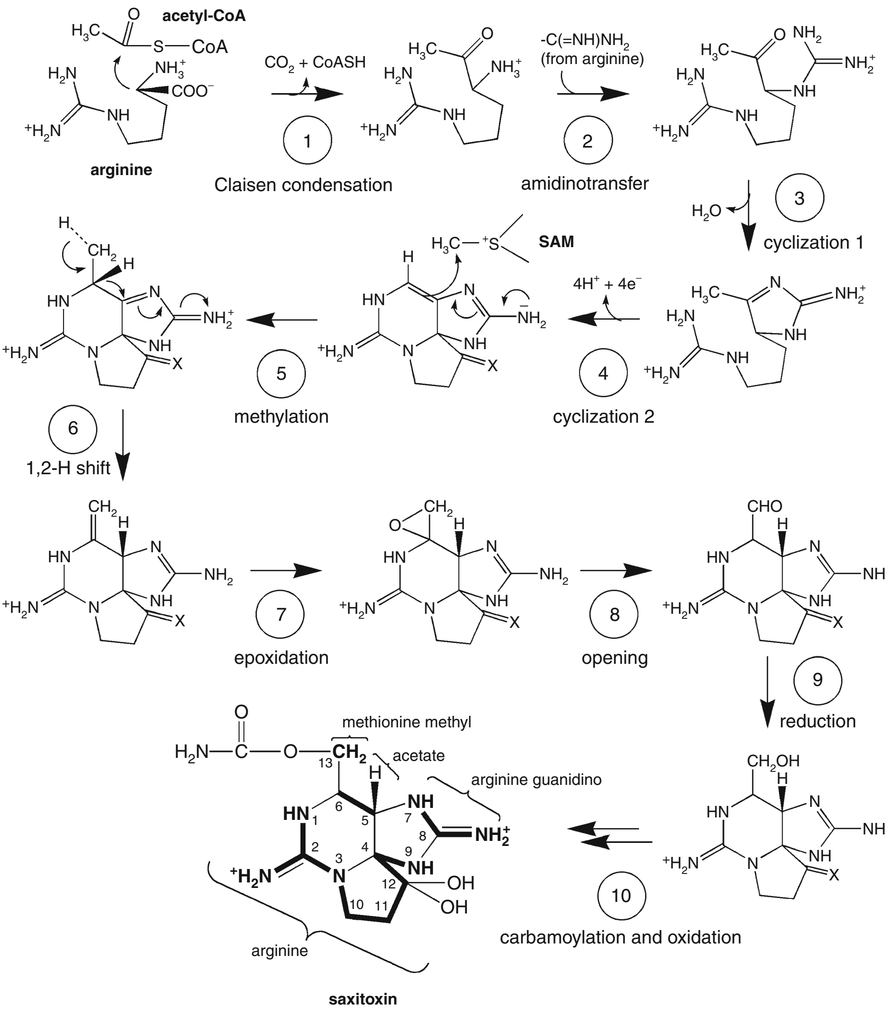

acetyl-CoA
arginine
CO_2 + CoASH
1
Claisen condensation
-C(=NH)NH_2
(from arginine)
2
amidinotransfer
H_2O
3
cyclization 1
SAM
$4H^+ + 4e^-$
4
cyclization 2
5
methylation
6
1,2-H shift
7
epoxidation
8
opening
9
reduction
methionine methyl
acetate
arginine guanidino
arginine
saxitoxin
10
carbamoylation and oxidation

Fig. 3.5 Biosynthesis pathway of saxitoxin as proposed by Shimizu et al. [224]. The reaction steps are: (1) Claisen-condensation between acetate and arginine; (2) amidino transfer from a second arginine to the α-amino group of intermediate B; (3) and (4), cyclization; (5), introduction of S-adenosyl methionine (SAM) methyl-derived side chain, involving the loss of one methionine methyl hydride; (6) 1,2-H shift; (7) epoxidation of methylene side chain; (8) opening of epoxide to an aldehyde; (9) reduction of the aldehyde to hydroxyl; (10) carbamoyl transfer and dihydroxylation of C-12

methyl and its hydroxylation has been studied in detail. Only one methionine methyl-derived hydrogen is retained in saxitoxin, and a 1,2-hydride shift was observed between acetate-derived C-5 and C-6. Shimizu [224] proposed that this incorporation pattern resulted from an electrophilic attack of methionine methyl (see step 5 in Fig. 3.5) on the double bond between C-5 and C-6, which would have formed during the preceding cyclization (see step 4 in Fig. 3.5). Subsequently, the new methylene side chain would be epoxidated, followed by opening to an aldehyde and subsequent reduction to a hydroxyl (see steps 7–9 in Fig. 3.5). This scheme should also lead to a 1,2-H shift between C-1 and C-5; however, this has not been observed [224]. Finally, the saxitoxin precursor is di-hydroxylated at ring carbon C-12, and receives a carbamoyl group at its hydroxymethyl side chain. Modifications at other positions may produce a great diversity of saxitoxin derivatives (Table 3.3).

4.7 Molecular Genetics of PSTs

The unique biochemistry of PSTs, combined with the divergent phylogenetic distribution of PST-producer organisms, made the identification of biosynthesis genes and enzymes an attractive and challenging target [225–229]. Kellmann and coworkers discovered the saxitoxin biosynthesis (*sxt*) genes in cyanobacteria [27, 230–232]. Saxitoxin biosynthesis is initiated by a novel modular polyketide synthase (SxtA), which has four catalytic domains that are related to *S*-adenosylmethionine-dependent methyl transferase (SxtA1), GNAT-acetyl transferase (SxtA2), acyl carrier protein (ACP) (SxtA3), and class II aminotransferase (SxtA4). The predicted function of SxtA was that SxtA2 would load SxtA3 with acetate from acetyl-CoA or malonyl-CoA, followed by a methylation of the distal methyl group of ACP-bound acetate by SxtA1. SxtA4 would catalyze a subsequent Claisen-condensation of the resultant ACP-bound propionate to arginine, and the first intermediate metabolite, 4-amino-3-oxo-guanidinoheptane, is released [27]. This compound was the predicted substrate for the amidinotransferase, SxtG, which would transfer an amidino group from arginine to its α-amine. Following the transfer, the SAM-derived methyl group gets desaturated by SxtD. The resulting double bond becomes epoxidated, and opened to an aldehyde with concomitant formation of two heterocycles. This scheme is consistent with the lacking 1,2-hydride shift between C-1 and C-5 as observed by Shimizu et al. [149]. The latter sequence of reactions is catalyzed by the α-ketoglutarate-dependent dioxygenase SxtS, which produces succinate from α-ketoglutarate. The aldehyde side chain becomes reduced to a hydroxymethyl side chain by SxtU, a short-chain alcohol dehydrogenase, and carbamoylated from carbamoylphosphate by the *O*-carbamoyltransferase, SxtI. Two highly similar dioxygenases, SxtH and SxtT, finalize biosynthesis by introducing two hydroxyl groups at C-12. SxtH and SxtT require regeneration after each catalytic cycle, which may be performed by the succinate dehydrogenase-like enzyme SxtV, with the involvement of a ferredoxin (SxtW). The revised scheme for saxitoxin biosynthesis is depicted in Fig. 3.6. In addition to these core biosynthesis enzymes, the *sxt* gene cluster encodes proteins with functions that are related to the modification of saxitoxin or its intermediates to produce the various analogues, to cellular transport of PSTs, transcriptional regulation, and other functions.

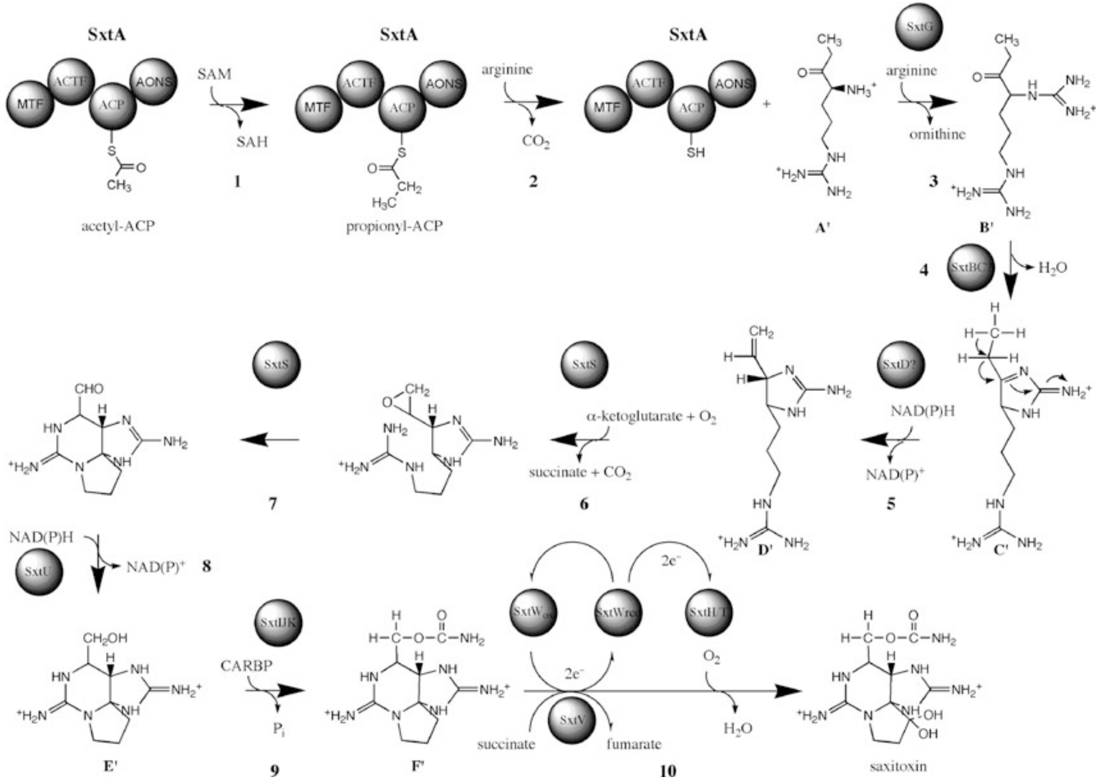

SxtA
MTF
ACTF
ACP
AONS
acetyl-ACP
SAM
SAH
1
propionyl-ACP
arginine
CO_2
2
SH
A'
SxtG
arginine
ornithine
3
B'
4
SxtBC
H_2O
C'
SxtD?
NAD(P)H
NAD(P)$^+$
5
D'
SxtS
α-ketoglutarate + O_2
succinate + CO_2
6
SxtS
7
NAD(P)H
SxtU
NAD(P)$^+$
8
E'
SxtIJK
CARBP
P_i
9
F'
SxtW$_{ox}$
SxtWred
SxtH/T
2e$^-$
2e$^-$
SxtV
succinate
fumarate
O_2
H_2O
10
saxitoxin

Fig. 3.6 Revised pathway for saxitoxin biosynthesis and the putative functions of *sxt* genes (adapted from Kellmann et al. [27]). Abbreviations used were: *ACP* acyl carrier protein, *ACTF* acetyltransferase, *AONS* 8-amino-7-oxononanoate synthase, *CARBP* carbamoyl phosphate, *MTF* methyltransferase, *NAD(P)* nicotinamide adenine dinucleotide (phosphate)

The new molecular genetic data indicated that saxitoxin biosynthesis proceeded in a different sequence than previously proposed [149], and would consequently involve different intermediate metabolites. Such metabolites were detected by LC-MS^2 analysis in cyanobacterial extracts, which supported the putative functions of *sxt* genes [27].

Phylogenetic analysis revealed that the majority of *sxt* genes have evolutionary origins from within cyanobacteria, whereas the remainder originated in other bacteria [27, 230]. More detailed phylogenetic analysis suggests that saxitoxin is an ancient metabolite that may have evolved in cyanobacteria early in the evolutionary divergence of the Nostocales at least 2,100 Ma ago [233]. The apparent cyanobacterial roots of PSTs are intriguing and raise the question how dinoflagellates, which are eukaryotic organisms, have evolved or acquired the capacity to produce the same toxins. Mass sequencing of expressed sequence tags have revealed *sxt* homologues in the genomes of PST-producing dinoflagellates [234]. The study by Stüken et al. suggests that dinoflagellates have acquired an entire metabolic pathway from cyanobacteria in one or multiple ancient horizontal gene transfer events. One can only speculate that the ancient cyanobacterial ancestors to extant chloroplasts might have been the donors for *sxt* genes in dinoflagellates.

4.8 Toxicology and Pharmacology

Saxitoxin and its analogues are potent blockers of voltage-gated sodium channels that reside in the membranes of excitable cells [235]. They also block or modulate cardiac ion channels, such as L-type calcium channels [7], and hERG potassium channels [8], although at a much lower potency. The intraperitoneal LD_{50} of saxitoxin is 10 μg/kg body weight in mice, while human death has occurred following the ingestion of as little as 1 mg of the toxin [236]. The toxicity of saxitoxin derivatives varies greatly. The carbamate toxins (STX, neoSTX, GTX-1 to -4) are 10–200 times more potent than corresponding *N*-sulfocarbamoyl derivatives (GTX-5/-6 and C-toxins). *N*-sulfocarbamoyl analogues are labile, however, and may easily be converted to their corresponding carbamates [237, 238]. Structure-activity relationships of PSTs are reviewed in detail by Llewellyn [239].

The clinical effect of saxitoxin is restricted to acute symptoms, which generally occur within minutes of ingestion. Poisoning invariably begins with a tingling or numbness of the lips, tongue, and throat [240]. This altered sensation may spread to the neck and extremities, and progress to muscular weakness, loss of motor coordination, and an ascending paralysis of the body. Gastrointestinal symptoms, such as nausea, vomiting, and diarrhea, may sometimes occur during the early stages. PSP may also cause severe hypertension in victims [241], and animal experiments indicated that PSTs alter dopamine levels in the brain [242]. In severe cases, PSP may lead to sudden death of previously fully conscious victims, due to cardiopulmonary failure. So far, there is no clinically approved antidote to saxitoxin. The drug, 4-aminopyridine, is in experimental stages, and produced a striking reversal of saxitoxin-induced cardiopulmonary depression in guinea pigs without serious side effects [243–245]. Treatment of PSP remains symptomatic until a drug has been approved. Removal of unabsorbed toxin by emesis or lavage, and treatment with activated charcoal, which effectively absorbs saxitoxin at gastric pH, are important during early stages of poisoning. Artificial respiration is the most vital treatment in moderate cases; however, it may not be sufficient to save the victim's life, if the poisoning is more severe [246]. The first 12h are most critical for the survival of a victim [247]. Permanent or chronic effects of PSP have not been reported; however, muscular weakness may persist for several weeks.

4.8.1 Voltage-Gated Sodium Channel

Various isoforms of voltage-gated sodium channels exist. They are encoded by nine genes in the human genome as reviewed by Catterall [248]. $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$ are primary sodium channels of the central nervous system, whereas $Na_v1.7$, $Na_v1.8$, $Na_v1.9$ are primarily expressed in peripheral neurons. $Na_v1.4$ and $Na_v1.5$ are primarily expressed in skeletal and heart muscle, respectively.

Voltage-gated sodium channels are multi-subunit proteins, where the ion channel consists of a 260 kDa large α-subunit, which may bind β-subunits that modify the gating behavior [249, 250]. The α-subunit consists of four homologous domains. Each domain provides six membrane-spanning regions, and three loops that protrude from the membrane into the extra- and intracellular space. The three-dimensional structure of voltage-gated sodium channels has been examined by X-ray crystallography and NMR [251], and by cryo-electron microscopy [252]. The membrane-spanning regions are arranged circumferentially around a central pore, whereas the loops form a bell-shaped structure that provides a network of channels, which connects the central pore to the intra-, and extracellular spaces. The loop that connects segments 5 and 6 of each of the four domains is part of the selectivity filter. It protrudes into the outer vestibule of the central pore, and is also termed the pore (P-) loop. The P-loop represents toxin-binding site 1, which binds site 1 toxins, such as saxitoxin, tetrodotoxin, and μ-concotoxins [253]. Specific interactions of saxitoxin with amino acids of the P-loop have been mapped by mutational studies, homology modeling, and ligand docking [254–260]. Each P-loop has a conserved sequence motif with saxitoxin-binding residues. The 7,8,9 guanidinium group of saxitoxin binds to D400 of domain I, and E755 of domain II via salt bridges, whereas the 1,2,3 guanidinium group makes a salt bridge to D1532 of domain IV. Hydrogen bonds are formed between the two hydroxyls at C-12 and E758 of domain II, as well as between the carbamoyl amine and E403. Hydrophobic interactions between M1240 of domain IV and hydrophobic regions of saxitoxin also make an important contribution to the binding of saxitoxin in the channel [257]. A schematic drawing of the orientation of gonyautoxin-4/1 in the outer vestibule is shown in Fig. 3.7.

The various functional groups of saxitoxin analogues have different effects on their affinity to the channel. The N-1 hydroxyl of neoSTX, a lacking carbamoyl

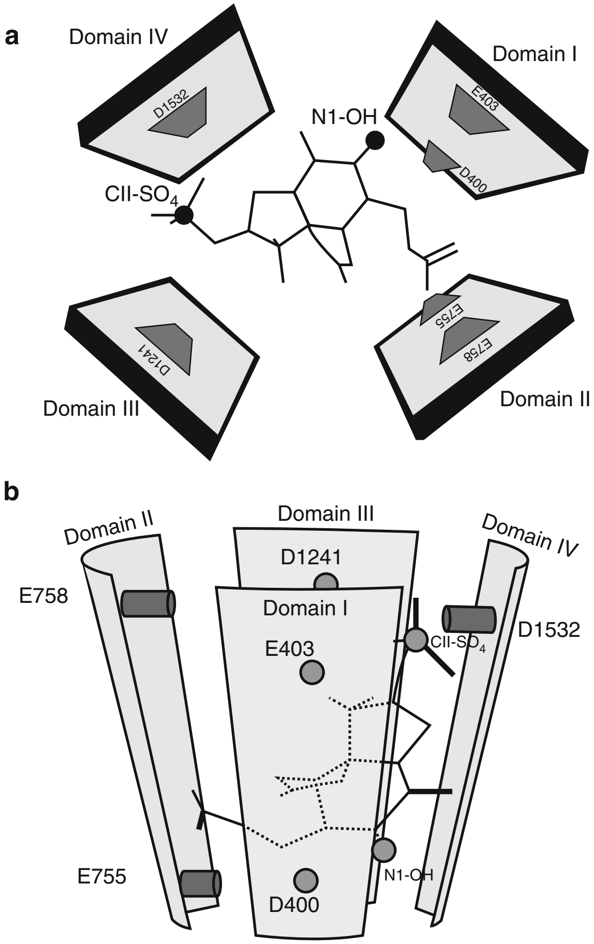

a
Domain IV
D1532
Domain I
E403
D400
N1-OH
CII-SO$_4$
Domain III
D1241
E755
E758
Domain II
b
Domain II
Domain III
Domain IV
D1241
Domain I
E758
E403
D1532
CII-SO$_4$
E755
D400
N1-OH

Fig. 3.7 Localization of the saxitoxin analogue GTX1,4 in the outer vestibule of the sodium channel (adapted from Choudhary et al. [254])

side chain, or its replacement by a bulky hydroxybenzoate has little effect on toxicity. On the other hand, the *N*-sulfocarbamoyl side chain reduces toxicity over 200-fold. The sulfate at C-13 in turn decreases toxicity only slightly, whereas there is a notable difference in the response between α- and β−epimers [239, 261, 262].

4.8.2 The Pharmacological Potential of PSTs

The useful pharmacological properties of PSTs for medical treatment have been recognized for over three decades, and motivated the development of a variety of patented medical and cosmetic treatments (Table 3.5). They include local injection of PSTs to treat chronic anal fissures [263–267], tension headaches [268], and local anesthesia [269–271]. A major advantage in using site 1 sodium channel blocking toxins is that they lack central nervous system, cardiac, and

Table 3.5 Examples of patented medical applications of PSTs

Title	Patent or application number (date)	Type of PST analogue	Application
Sensory-specific local anesthesia and prolonged duration local anesthesia	US 044549 (2009)	STX, neoSTX, dcTSX, GTXs	Local anesthesia
Phycotoxins and uses thereof	US 0021051 A1 (2008)	GTX-1 to -5	Muscle spasm, facial cosmetics
Transdermal administration of phycotoxins	US 0045553 A1 (2008)	STX, dcSTX, neoSTX, GTXs	Muscle relaxation facial cosmetics
Methods of treating wounds with gonyautoxin	US 0280970 (2007)	GTXs	Chronic anal fissures
Prolonged suppression of electrical activity in excitable tissues	US 0202093 A1 (2005)	STX, neoSXT, dcSTX, GTXs	Epilepsy
Method of local anesthesia and analgesia	US 6599906 (2003)	STX	Local anesthesia
Method of anesthesia	US 6030974 (1998)	STX, neoSTX, GTXs	Local anesthesia (veterinary medicine)
Local anesthetic formulations	US 6326020 B1 (1998)	STX, dcSTX, neoSTX	Local anesthesia
Pharmaceutical local anesthetic composition employing saxitoxin	US 4001413 (1977)	STX	Local anesthesia
Spinal anesthesia using small amount of saxitoxin	US 3892847 (1975)	STX	Local anesthesia

Abbreviations used were *STX* saxitoxin, *neoSTX* neosaitoxin, *dcSTX* decarbamoylsaxitoxin, *GTXs* gonyautoxins

local neurotoxicity [271–273]. Local injection of saxitoxin has been shown to provide a prolonged nerve block with minimal systemic and local toxicity that can be utilized in periprocedural care and pain management [269].

5 Conclusion

More than five decades of research have revealed the chemistry, toxicology, and distribution of the most clinically relevant cyanobacterial neurotoxins. More recently, the genetic basis for the biosynthesis of the alkaloid neurotoxins, anatoxin-a, homoanatoxin-a, and saxitoxin have been elucidated [27, 33]. Advances in analytical techniques and molecular genetic methods are continuing to reveal novel toxin isoforms in a wide range of producing organisms. In particular, PCR-based methods exploiting conserved regions of cyanotoxin biosynthesis genes are being used to screen environmental samples for candidate toxin pathways. These techniques are also being developed for the routine monitoring of water bodies and seafood destined for human consumption [274, 275], and to determine the environmental conditions under which favorable toxin production occurs (for review see [276]).

While the gene clusters responsible for the biosynthesis of several of the most toxicologically important cyanobacterial neurotoxins have been identified and sequenced (with the notable exception of anatoxin-a(S)), most of the individual genes encoded therein have not been functionally verified. Conventional characterization via mutagenesis is hampered by the fact that most of the producing organisms are difficult to culture and/or are not readily amenable to genetic manipulation. Therefore, most of the experimentally characterized steps in these neurotoxin biosynthesis pathways have been investigated using a biochemical/enzymatic approach. Nonetheless, detailed characterization of these enzymes will broaden our understanding of the mechanisms of neurotoxin biosynthesis, modification, and transport and may additionally provide us with a means to bioengineer these pathways to produce novel compounds for the treatment of neurological disease. For example, anatoxin-a has been identified as a good core structure for the symptomatic treatment of Alzheimer's disease as it activates nicotinic acetylcholine receptors and readily crosses the blood-brain barrier [37]. Bioengineering of the anatoxin-a pathway could potentially yield a novel anatoxin-a analogue for the treatment of patients with reduced nicotinic acetylcholine receptor function.

Another interesting field of research is the evolution of neurotoxin biosynthesis. The presence of transposase genes in or proximal to neurotoxin gene clusters suggests that neurotoxicity may be transferred horizontally between cyanobacteria and perhaps to other organisms. The recent discovery of saxitoxin biosynthesis gene clusters in eukaryotic dinoflagellates provides evidence in support of this theory [234]. Remarkably, this is the first and only example of a secondary metabolite pathway shared across two kingdoms of life.

While the anatoxins and saxitoxins are generally considered to be the most toxicologically significant of the cyanobacterial neurotoxins, recent interest in other neurotoxin pathways has generated some interesting data. For example, the gene cluster responsible for the biosynthesis of the lipopeptide jamaicamides was recently characterized in *Moorea (Lyngbya) majuscula* [9]. This remarkable set of collinear genes includes novel biosynthesis and tailoring enzymes that create the various unusual functional groups in jamaicamide A, such as the rare alkynyl bromide moiety. Another cyanobacterial neurotoxin that has generated recent interest as well as controversy is the nonproteinogenic amino acid BMAA [14]. While a biosynthesis pathway for BMAA in cycads has been postulated [277], research by Cox et al. revealed that it is the cyanobacterial endosymbionts rather than their plant hosts that are the original producers of BMAA [278]. The discovery of a BMAA pathway in cyanobacteria is anticipated in the near future.

In conclusion, while many of the details pertaining to the chemistry and toxicology of the cyanobacterial neurotoxins have been elucidated, there are still significant knowledge gaps relating to their biosynthesis and evolution. Furthermore, we are yet to discover the true function of these mysterious compounds in their native blue-green algal hosts.

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Bioproduction of Terpenoid Indole Alkaloids from *Catharanthus roseus* Cell Cultures

4

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Contents

1 Introduction 87
2 Distribution of Terpenoid Indole Alkaloids in Plants 89
3 The Biosynthetic Pathway of Terpenoid Indole Alkaloids 89
3.1 The Biosynthesis of the Terpenoid Indole Alkaloid Precursors, Tryptamine and Secologanin 89
3.2 The Biosynthesis of Strictosidine, a Universal Glucoalkaloid Precursor of all Terpenoid Indole Alkaloids 92
3.3 The Biosynthesis of Terpenoid Indole Alkaloids from Strictosidine 92
4 Compartmentalization and Cellular Differentiation in the Biosynthesis of Terpenoid Indole Alkaloids 98
5 Biotechnology Approaches to Improve Terpenoid Indole Alkaloid Production from *Catharanthus roseus* In Vitro Cultures 100
5.1 Importance of Screening and Selection of *Catharanthus roseus* In Vitro Culture Lines and Level of Cell Differentiation to Improve the Production of Terpenoid Indole Alkaloids 101
5.2 Optimization of Culture Conditions to Improve the Production of Terpenoid Indole Alkaloids 103
5.3 Feeding Strategies to Improve the Production of Terpenoid Indole Alkaloids 104
5.4 Immobilization Strategies to Improve the Production of Terpenoid Indole Alkaloids 105
5.5 Elicitation Strategies to Improve the Production of Terpenoid Indole Alkaloids 105

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5.6 Large-Scale Production of Terpenoid Indole Alkaloids 107
5.7 Metabolic Engineering as a Strategy to Improve the Production of Terpenoid Indole Alkaloids 108
6 Conclusions 111
References 111

Abstract

Catharanthus roseus is an ornamental plant belonging to the Apocynaceae family which produces flowers of different color for most of the year. Besides its importance as an ornamental plant, its interest today is centered on its capacity to biosynthesize a great variety of terpenoid indole alkaloids (TIAs), which have a high added value due to their wide spectrum of pharmaceutical applications. The most important TIAs are the two antitumoral alkaloids, vinblastine and vincristine. Likewise, *C. roseus* also produces ajmalicine used as antihypertensive and serpentine used as sedative. The high cost of these alkaloids is due to the very small amounts that occur in *C. roseus* and the difficulty of their extraction which is carried out in the presence of many other compounds. This problem has created the need to find alternative sources to produce these compounds. In this respect, plant tissue/cell cultures could be a useful alternative source of pharmacologically active *C. roseus* alkaloids, but, even so, these have only been obtained in very low concentrations and after a substantial amount of research. This problem has stimulated intense research into the biosynthesis of TIAs and in the regulation of its pathways, with the aim of increasing the production of these high-value compounds by biotechnological approaches.

The aim of this chapter is centered on different strategies to improve TIA production which have been developed, including screening and selection of high-yield cell lines, optimization of culture conditions, feeding and elicitation strategies, and the metabolic engineering of TIA biosynthetic pathway. An up-to-date view on the biosynthesis of TIAS is also given. Although not yet successful, metabolic engineering offers the most promising perspective for improving TIA production in the future, as increases the knowledge of the genetic determination and regulation of the TIA pathway.

Keywords

Bioproduction • *Catharanthus roseus* • in vitro cultures • terpenoid indole alkaloids • biosynthetic pathways

Abbreviations

16OMT	16-hydroxytabersonine 16-*O*-methyltransferase
2,4-D	2,4-dichlorophenoxyacetic acid
AVLBS	Anhydrovinblastine synthase
BA	Benzyladenine

CR	Cathenamine reductase
CS	Cathenamine synthase
D4H	Desacetoxyvindoline-4-hydroxylase
DAT	Deacetylvindoline-4-*O*-acetyltransferase
DMAPP	Dimethylallyl diphosphate
DW	Dry weight
FW	Fresh weight
G10H	Geraniol-10-hydroxylase
GD	Geissoschizine dehydrogenase
GPP	Geranyl diphosphate
GPP synthase	Geranyl diphosphate synthase
IAA	Indole-3-acetic acid
IPP isomerase	Isopentenyl-diphosphate isomerase
IPP	Isopentenyl diphosphate
LAMT	Loganic acid methyltransferase
MeJa	Methyl jasmonate
MEP	2-methyl-erythritol 4-phosphate
NMT	*N*-methyltransferase
PNAE	Polyneuridine aldehyde esterase
Prx	Peroxidases
SBE	Sarpagan bridge enzyme
SGD	Strictosidine β-glucosidase
SLS	Secologanin synthase
STR	Strictosidine synthase
T16H	Tabersonine 16-hydroxylase
TDC	Tryptophan decarboxylase
TIAs	Terpenoid indole alkaloids
VH	Vinorine hydroxylase
VS	Vinorine synthase

1 Introduction

Alkaloids are secondary metabolites containing nitrogen in their molecules, usually in a heterocyclic configuration. The nitrogen atom is usually basic conferring alkalinity to the molecule and hence the name alkaloids. Alkaloids were initially isolated exclusively from higher plants, and until the middle of this century, they were referred to as typical plant products. Later, they were also found in bacteria, fungi, algae, and even in animals. Nevertheless, flowering plants, especially dicotyledonous species, are the main source of alkaloids. As secondary metabolites, alkaloids are thought to play a defensive role against herbivores and pathogens. This group of natural compounds exhibits some of the most complicated molecular structures and also strong physiological activity in humans and animals. Owing to their potent biological activity, alkaloids have been exploited as pharmaceuticals, stimulants, narcotics, and poisons. Alkaloid biosynthesis in plants involves many steps, catalyzed by enzymes that belong to a wide range of protein families. The characterization of alkaloid biosynthetic enzymes in terms of biochemistry, molecular biology, and biotechnological applications has been the focus of extensive research in recent years.

C. roseus is an ornamental and medicinal plant of enormous pharmaceutical interest because it is nothing less than a chemical factory, producing more than 130 TIAs, some of which exhibit strong and important pharmacological activities [1]. The most important TIAs are the two antitumoral alkaloids, vinblastine (used in the treatment of Hodgkin's disease, non-Hodgkin lymphomas, and several kinds of carcinomas) and vincristine (used against acute children leukemia, Hodgkin's disease, non-Hodgkin lymphomas, some types of sarcomas, and breast cancer). In fact, these two alkaloids were the first natural products used in anticancer chemotherapy and, together with a number of semisynthetic derivatives, are universally known as the vinca alkaloids. Likewise, *C. roseus* also produces ajmalicine used as an antihypertensive to combat heart arrhythmias and to improve blood circulation in the brain and serpentine used as a sedative [1, 2]. The great pharmacological importance of vinblastine and vincristine, allied with their low availability in *C. roseus* (approximately half a ton of dry leaves is needed to obtain 1 g of vinblastine), has stimulated intense research into the biosynthesis of TIAs and in the regulation of its pathways, with the aim of increasing the production of these high-value compounds by biotechnological approaches. At present, much is known about TIA biosynthesis, despite the length and the complexity of the pathway involved. Starting from the amino acid tryptophan and the monoterpene geraniol, TIA biosynthesis in *C. roseus* involves at least 35 intermediates and 30 enzymes [2]. All TIAs in *C. roseus* are derived from the central precursor strictosidine, which is a fusion product of the shikimate-pathway-derived tryptamine moiety and the plastidic nonmevalonate pathway-derived secologanin moiety. After deglycosylation of strictosidine, the TIA biosynthetic pathway splits into several branches, among them, a short pathway leading to ajmalicine, and two long branches leading to vindoline and catharanthine, the monomeric precursors of vinblastine and vincristine. Quite a few enzymes have been isolated, and several genes are now well identified. TIA biosynthesis also involves at least seven different intra- and intercellular compartments indicating that extensive transport of intermediates is required, as evidenced from the cell-type-specific expression of biosynthetic genes [3, 4]. Likewise, TIA biosynthesis is under strict developmental and environmental control. Lastly, transcription factors have been identified in *C. roseus* as being responsible for coordinating gene expression in response to external and internal signals [5].

The application of genomics to alkaloid metabolism has accelerated the discovery of cDNAs encoding enzymes implicated in their biosynthetic pathway [6, 7]. Also, large-scale gene expression analyses and metabolic engineering approaches with transgenic plants have provided new insights into the regulatory architecture of the alkaloid metabolism. The integration of genome-wide transcript and metabolic profiles will enable not only to visualize most of the genes involved in TIA biosynthesis but also to draw the nondescribed gene-to-gene and gene-to-metabolite networks and so to design biotechnological strategies to improve the production of these compounds [7]. In this chapter, we discuss recent advances toward understanding the complex TIA biosynthetic pathway, its regulation, and how to gain knowledge from biotechnological, genomic, and metabolic approaches in order to improve the production of these much desired compounds.

2 Distribution of Terpenoid Indole Alkaloids in Plants

The TIAs form a family of more than 3,000 representatives. These alkaloids, which are unique in plants, have been found in several plant families such as Apocynaceae, Loganiaceae, Nyssaceae, and Rubiaceae, which belong all to the Gentianales order. The most important and well-known plants that produce TIAs are *Tabernaemontana divaricata*, *Rauvolfia serpentina*, and *C. roseus* [1].

Catharanthus is a perennial plant belonging to the Apocynaceae family and comprises eight species, seven endemic to Madagascar (*C. coriaceus*, *C. lanceus*, *C. longifolius*, *C. ovalis*, *C. roseus*, *C. scitulus*, *C. trichophyllus*), and one, *C. pusillus*, from India. Specifically, *C. roseus* has been extensively cultivated in subtropical and tropical areas of the world [1] as an ornamental plant and now has a pantropical distribution. It is naturalized in continental Africa, the North and South America, Asia, Australia and southern Europe, and on some islands in the Pacific Ocean. Today, more than 100 cultivars are commercially available.

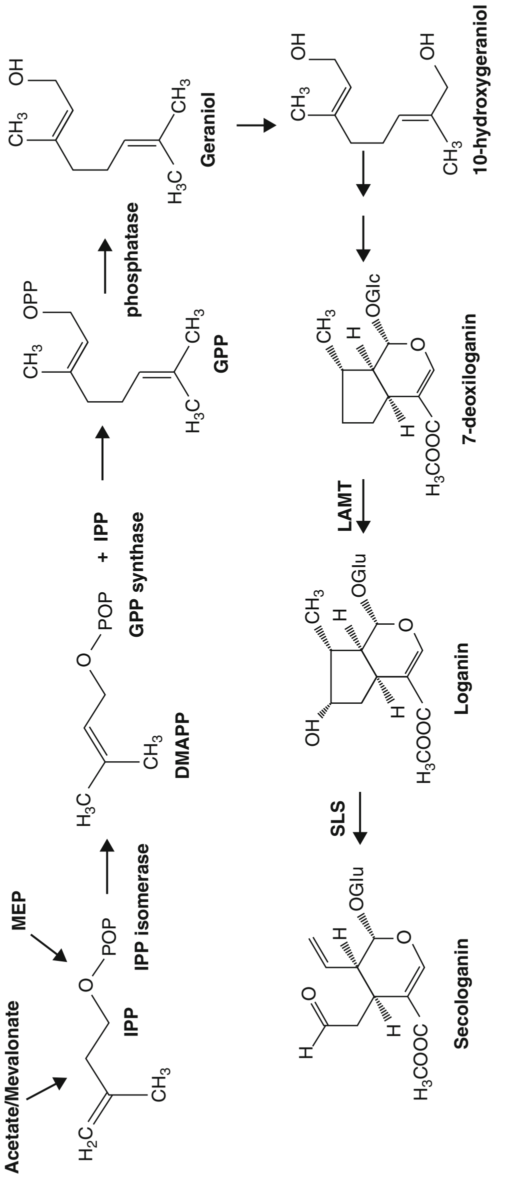
3 The Biosynthetic Pathway of Terpenoid Indole Alkaloids

The biosynthesis of TIAs in *C. roseus* is extraordinarily complex, involving more than 50 metabolic steps to synthesize the most important alkaloids. To date, only about 20 of the 50 enzymes required for their biosynthesis have been determined and characterized [2]. Thus, there are still a number of important enzymes that need to be characterized, which can only be done after the isolation and cloning of the relevant genes. It is also of fundamental importance to elucidate the regulatory aspects of TIA biosynthesis, both at the cellular and the molecular level, in order to address the question of their function in the plants and to improve their production by biotechnological approaches.

3.1 The Biosynthesis of the Terpenoid Indole Alkaloid Precursors, Tryptamine and Secologanin

The two main metabolites from which the TIAs found in *C. roseus* are biosynthesized are tryptamine and secologanin. They arise from two independent metabolic pathways and require the coordination of the intermediate levels supplied by the TIA pathway. The indole precursor, tryptamine, is derived from the shikimate pathway, whereas the monoterpene glucoiridoid precursor, secologanin, involves a much more complex branching route than the shikimic acid pathway used to supply tryptophan for tryptamine biosynthesis. Plants have two independent routes to biosynthesize the precursor used for monoterpene formation, isopentenyl diphosphate (IPP), which in turn, work in different cellular compartments: the classical pathway of acetate/mevalonate in the cytoplasm and the route of 1-deoxy-D-xylulose-5-phosphate in the plastids. According to Contin et al., secologanin mainly comes from the IPP produced via the plastidic pathway, which involves the formation of 2-methyl-erythritol 4-phosphate (MEP) [8]. However, data in the literature suggest that the mevalonate pathway also participates in the biosynthesis of this monoterpene which is essential to the biosynthesis of secologanin, although to a lesser degree [9]. IPP is turned into its isomer, dimethylallyl diphosphate (DMAPP) by the isopentenyl-diphosphate isomerase (IPP isomerase) (Scheme 4.1). The condensation of DMAPP with one molecule of IPP generates geranyl diphosphate (GPP) in a reaction catalyzed by geranyl diphosphate synthase (GPP synthase), initiating the biosynthesis of monoterpenes. The next step of the iridoid pathway is the conversion of GPP into geraniol by a phosphatase. Geraniol is the first product to channel the carbon skeleton toward the biosynthesis of monoterpenes. Then, geraniol is converted to 10-hydroxygeraniol by geraniol-10-hydroxylase (G10H), which is a P450 monooxygenase [10]. It has been suggested that this enzyme compromises the geraniol carbon skeleton to the biosynthesis of secologanin and represents an important regulatory point. Accordingly, G10H activity is induced under conditions that produce an increase in the alkaloid content and is inhibited by the addition of phosphates, which causes a decrease in the alkaloid content. Also, G10H is the only enzyme that is inhibited by the accumulation of catharanthine, showing a feedback inhibition. From 10-hydroxygeraniol to the biosynthesis of secologanin, there are at least 10 enzymatic steps. However, only six are known, and most enzymes have not been fully characterized. In fact, the steps after G10H in the secologanin pathway have not been well characterized, although it is known that the formation of loganin is produced from 7-deoxyloganin acid in a reaction catalyzed by loganic acid methyltransferase (LAMT). The last step to the conversion of loganin into secologanin is catalyzed by secologanin synthase (SLS), a second P450 monooxygenase (Scheme 4.1) [2].

The amino acid tryptophan produced by the shikimate pathway is decarboxylated to tryptamine by tryptophan decarboxylase (TDC) using pyridoxal phosphate as cofactor. TDC has been purified from suspension cultures and hairy roots of *C. roseus* and was shown to be a cytosolic enzyme whose activity is modulated by a variety of factors [11, 12]. In fact, TDC activity increases in cultures of *C. roseus* in response to fungal elicitors and the addition of hydrolytic enzymes, by increasing both gene transcription and mRNA levels [13]. However, auxins repress *tdc* gene expression [14]. In addition to being regulated by external factors including UV light, TDC is also tissue-specific and developmental regulated [15]. The *tdc* gene occurs in a single copy in *C. roseus*. The amounts of


Acetate/Mevalonate
MEP
IPP
IPP isomerase
DMAPP
GPP synthase
+ IPP
GPP
phosphatase
Geraniol
10-hydroxygeraniol
7-deoxiloganin
LAMT
Loganin
SLS
Secologanin
POP
OPP
OH
OGlc
OGlu
H₃COOC
CH₃
H₃C
H₂C
H
O

Scheme 4.1 Biosynthesis of secologanin

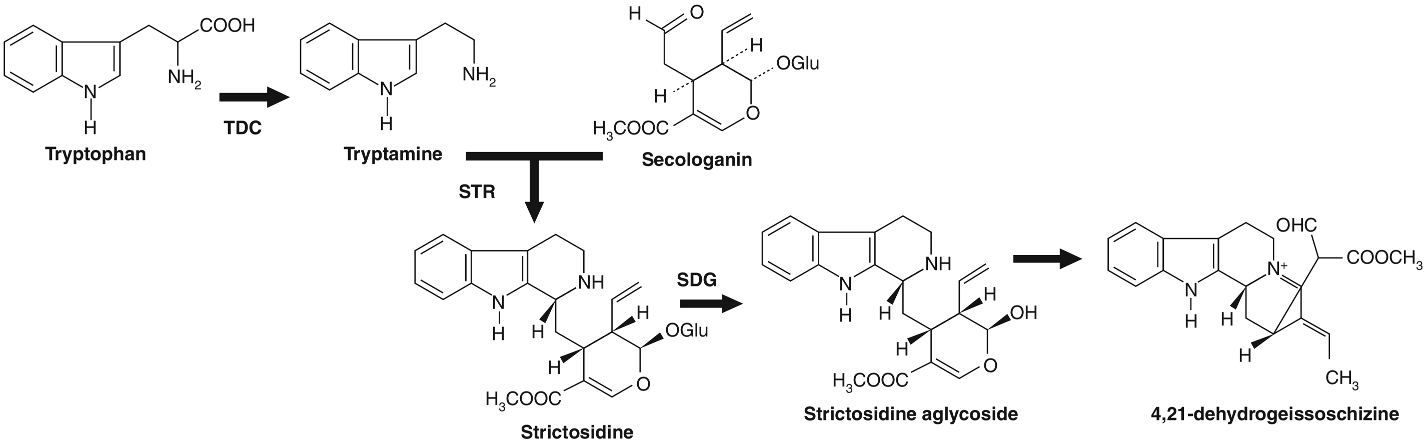
TDC in seedlings and cell suspensions show that both gene expression and enzymatic activity are induced in a transient form, suggesting that TDC activity is regulated at transcriptional, translational, and posttranslational levels [2, 16].

3.2 The Biosynthesis of Strictosidine, a Universal Glucoalkaloid Precursor of all Terpenoid Indole Alkaloids

Tryptamine and secologanin are condensed to form strictosidine (Scheme 4.2), the universal glucoalkaloid precursor of all the TIAs isolated from *C. roseus*. The enzyme that catalyzes this condensation is strictosidine synthase (STR). This enzyme is highly specific for its two substrates, and it is inhibited by the end products of this metabolic route [17]. STR is a soluble enzyme and has different isoenzymes distinguishable by their catalytic activities and their pI. The occurrence of these isoenzymes is not related to the developmental stage or to tissue-specific regulation since they are expressed equally in suspension cultures, leaves, stems, and roots [18]. These data suggest that these isoenzymes could be the result of different patterns of glycosylation of the protein, although the types of carbohydrates present have not yet been characterized. The function of these STR isoforms is not known. STR is encoded by a single gene, suggesting that isoforms are the result of posttranslational modifications of a single precursor [19]. In *C. roseus*, the s*tr* gene is regulated by a wide variety of signals including auxins, methyl jasmonate (MeJa), and fungal elicitors in cell cultures and by tissue-specific control in plant organs [16]. However, it is not yet clear if this enzyme is regulated by the developmental process. Some evidence suggests that STR does not constitute a limiting step in TIA biosynthesis in seedlings of *C. roseus*. In fact, when *C. roseus* suspension cells were transferred from a maintenance medium into an induction medium in order to produce alkaloids, the increase in the accumulation of alkaloids does not correlate with an increase in STR activity. On the other hand, in *C. roseus* callus transformed with s*tr* gene, a direct correlation between the increase of the enzymatic activity and the accumulation of alkaloids was observed [20]. These results suggest the possibility that the STR may indeed be a rate-limiting step of the TIA pathway. Nevertheless, the information is imprecise and sometimes contradictory, which emphasizes the necessity to expand the investigation that allow us to establish the real role of STR in TIA biosynthesis [2, 9, 16].

3.3 The Biosynthesis of Terpenoid Indole Alkaloids from Strictosidine

The following step in the biosynthesis of the TIAs consists in the deglycosylation of strictosidine by strictosidine β-glucosidase (SGD) to form the strictosidine aglycone, which is an unstable product rapidly transformed into 4,21-dehydrogeissoschizine (Scheme 4.2). SGD is a glycoprotein localized in the endoplasmic reticulum and is highly specific for strictosidine [21]. This enzyme


Tryptophan
TDC
Tryptamine
Secologanin
STR
Strictosidine
SDG
Strictosidine aglycoside
4,21-dehydrogeissoschizine

Scheme 4.2 Biosynthesis of strictosidine and 4, 21-dehydrogeissoschizine

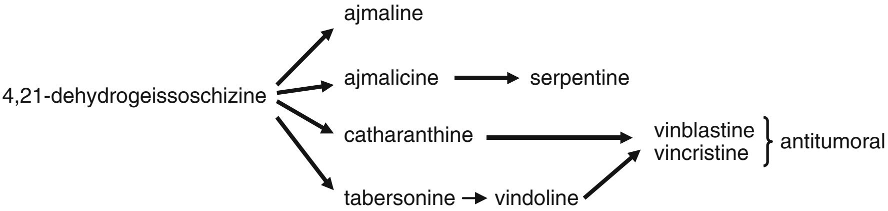


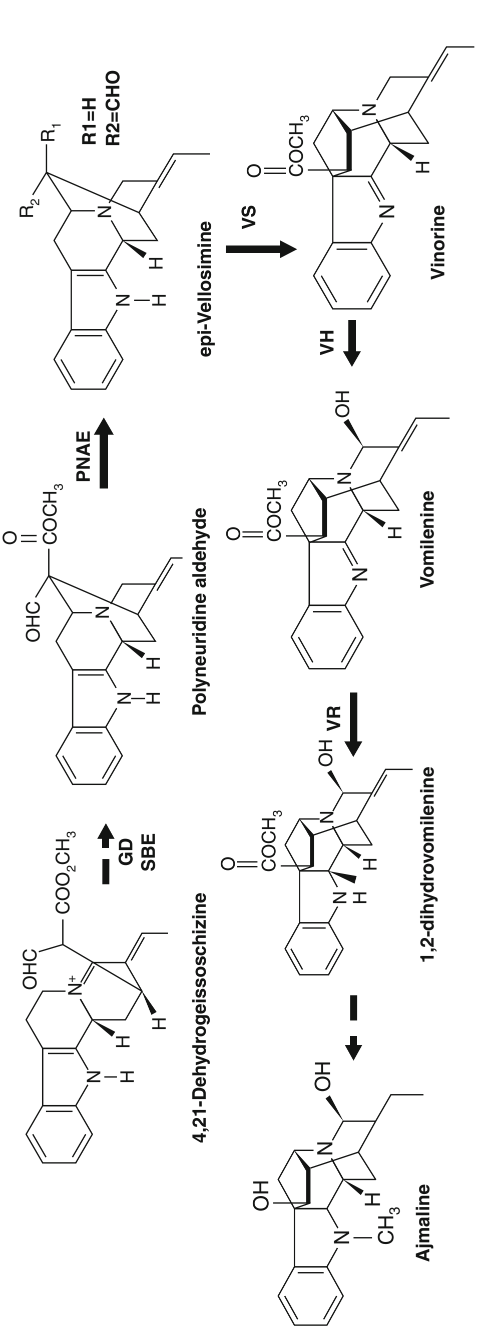
Fig. 4.1 Biosynthesis of several TIAs from 4,21-dehydrogeissoschizine

is codified by a single gene that has recently been cloned, and it is expressed at different levels in flowers, stems, leaves, and roots, suggesting a tissue-specific regulation [9]. In addition, the enzymatic activity in each organ is proportional to the mRNA amount detected. Since the level of SGD expression is induced by MeJa, and its activity is increased in cultures that accumulate TIAs, this enzyme is considered a key step in the biosynthesis of these compounds [2, 9, 19]. On the other hand, 4,21-dehydrogeissoschizine represents a key intermediate that leads to the biosynthesis of several TIAs including catharanthine, tabersonine, ajmalicine, and ajmaline (Fig. 4.1).

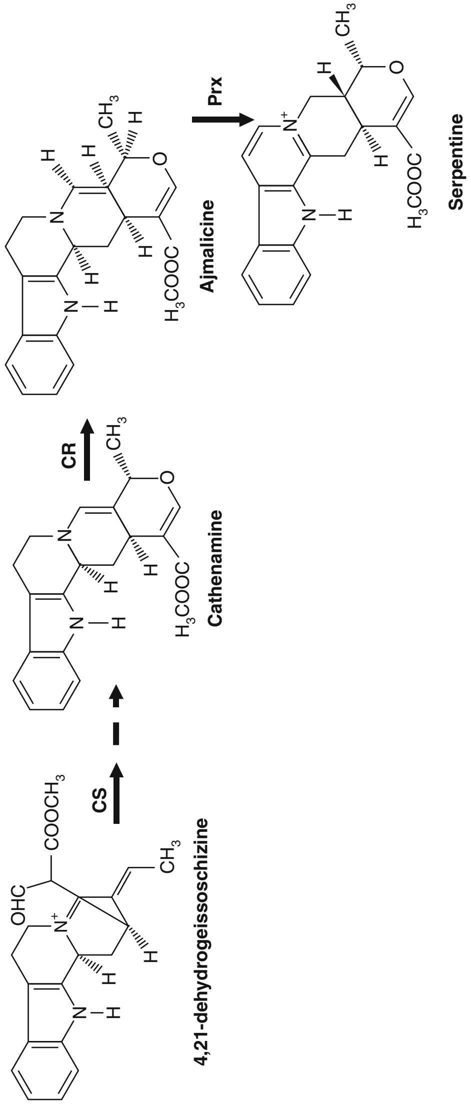
The biosynthesis of ajmaline involves several enzymatic steps (Scheme 4.3) [2, 9]. The first metabolic step is the oxidation of 4,21-didehydrogeissoschizine to geissoschizine in a reaction catalyzed by geissoschizine dehydrogenase (GD), followed by the formation of polyneuridine aldehyde through the action of sarpagan bridge enzyme (SBE). The resulting polyneuridine aldehyde is transformed by polyneuridine aldehyde esterase (PNAE) into epi-vellosimine, which is acetylated to form vinorine by vinorine synthase (VS). Then, vinorine is hydroxylated by vinorine hydroxylase (VH) to form vomilenine. VH is completely dependent of NADPH and oxygen, and it is located in the microsomal fraction [22]. VH is inhibited by typical inhibitors of cytochrome P450 proteins so that this monooxygenase is surely a cytochrome P450 protein. The last known step in ajmaline biosynthesis is the saturation of the indolamine double bond of vomilenine to produce 1,2-dihydrovomilenine by 1,2-dihydrovomilenine: NADP$^+$ oxidoreductase (VR) which is a NADPH-dependent enzyme [23]. The last steps leading to ajmaline biosynthesis are not well known.

4,21-Dehydrogeissoschizine may also be converted to cathenamine in a reaction catalyzed by cathenamine synthase (CS) [24]. This compound is the basis for the formation of ajmalicine by the action of the enzyme cathenamine reductase (CR), and then, ajmalicine is thought to be oxidized to serpentine by class III peroxidases (Prx) localized in the vacuoles, with its production being strongly regulated by light (Scheme 4.4, [25]).

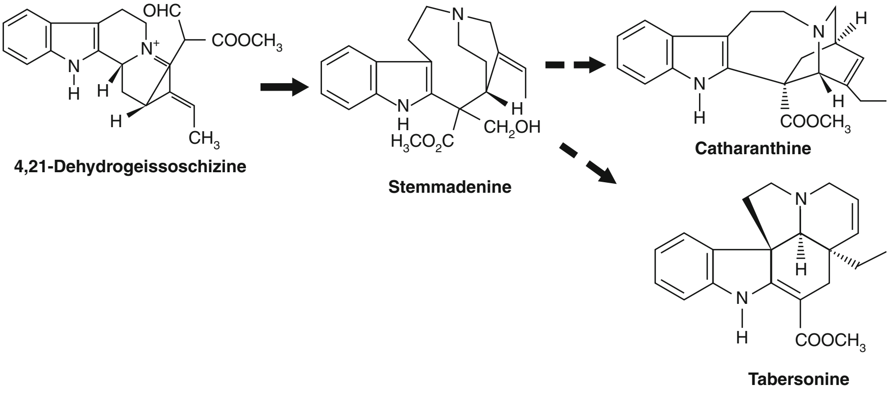
While the biosynthesis of catharanthine and tabersonine is poorly characterized (Scheme 4.5), the biosynthetic pathway from tabersonine to vindoline has been extensively studied [2]. Thus, the hydroxylation of tabersonine at position 16 represents the first reaction leading to vindoline (Scheme 4.6), and it is catalyzed by tabersonine 16-hydroxylase (T16H), a cytochrome P450-dependent enzyme


4,21-Dehydrogeissoschizine
GD
SBE
Polyneuridine aldehyde
PNAE
epi-Vellosimine
R1=H
R2=CHO
VS
Vinorine
VH
Vomilenine
VR
1,2-dihydrovomilenine
Ajmaline

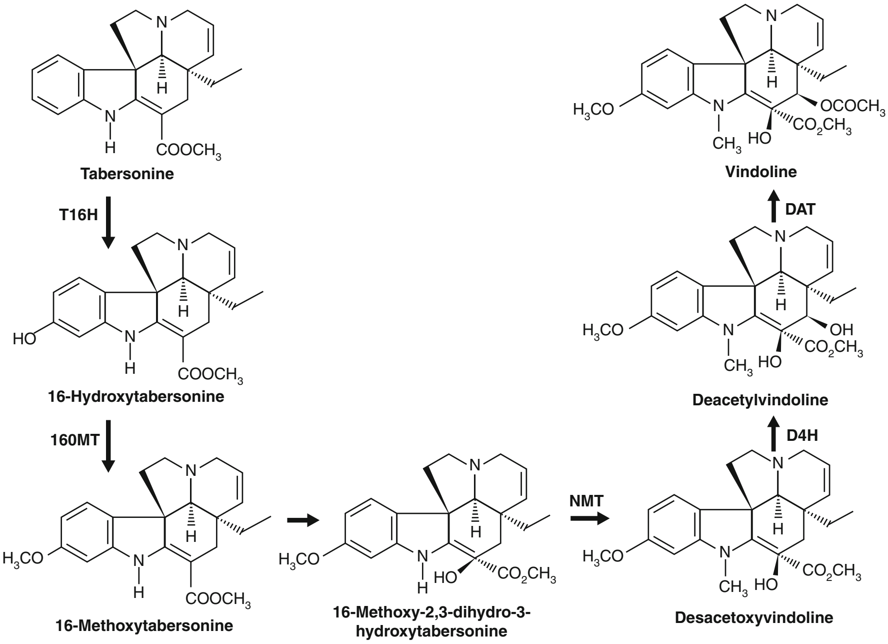
Scheme 4.3 Biosynthesis of ajmaline from 4,21-dehydrogeissoschizine


4,21-dehydrogeissoschizine
CS
Cathenamine
CR
Ajmalicine
Prx
Serpentine
OHC
COOCH3
CH3
H3COOC
N
H
O

Scheme 4.4 Biosynthesis of ajmalicine and serpentine from 4,21-dehydrogeissoschizine


4,21-Dehydrogeissoschizine
Stemmadenine
Catharanthine
Tabersonine

Scheme 4.5 Biosynthesis of catharanthine and tabersonine from 4,21-dehydrogeissoschizine


Tabersonine
T16H
16-Hydroxytabersonine
16OMT
16-Methoxytabersonine
16-Methoxy-2,3-dihydro-3-hydroxytabersonine
NMT
Desacetoxyvindoline
D4H
Deacetylvindoline
DAT
Vindoline

Scheme 4.6 Biosynthesis of vindoline from tabersonine

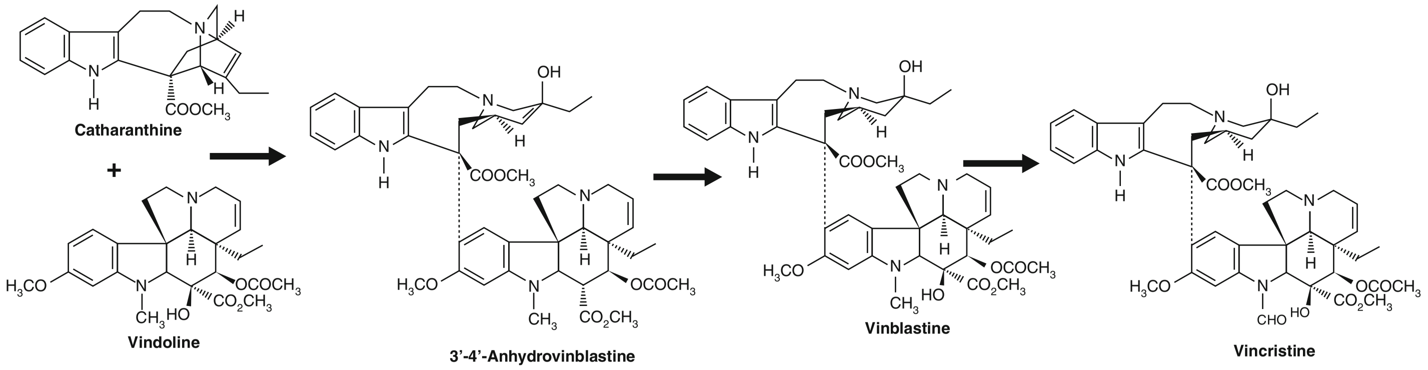
localized in the endoplasmic reticulum [26]. Then, 16-hydroxytabersonine is *O*-methylated by 16-hydroxytabersonine 16-*O*-methyltransferase (16OMT), which has been found both in plants and cell cultures of *C. roseus* [27]. The third reaction is a hydration of the 2,3 double bond, and the subsequent step involves a *N*-methylation of 16-methoxy-2,3-dihydro-3-hydroxytabersonine by a partially characterized *N*-methyltransferase (NMT) to produce desacetoxyvindoline. This enzyme, which is localized in the thylakoids, requires *S*-adenosyl-methionine as methyl donor and is highly specific for its substrate [28]. Desacetoxyvindoline is then converted to deacetylvindoline by the cytosolic enzyme desacetoxyvindoline-4-hydroxylase (D4H), and the last step consists in the acetylation of deacetylvindoline by deacetylvindoline-4-*O*-acetyltransferase (DAT) to form vindoline [29].

The biosynthesis of vindoline is regulated by developmental processes and is restricted to the young leaves and cotyledons of adult plants. In these tissues, high mRNA levels as well as enzymatic activity have been detected [30]. In cell cultures of *C. roseus*, enzymatic activity of T16H and 16OMT, but not NMT, D4H, and DAT, has been detected [31, 32]. Also, in plantlets of *C. roseus*, the effect of light on D4H and DAT activity is mediated by phytochrome since these proteins are activated by a pulse of red light (660 nm), and this activation is reverted by a pulse of far red light (710 nm [33]). In fact, after exposing plantlets to light, the transcript levels, the protein amount, and the activity of D4H and DAT increased significantly. MeJa also induces the vindoline accumulation and increases the enzymatic activity of D4H and DAT, STR, and TDC [34].

Finally, the monomeric precursors vindoline and catharanthine are coupled in a reaction thought to be catalyzed by a class III Prx, namely, CrPrx1, to yield the dimeric alkaloid 3′,4′-anhydrovinblastine, which is converted into vinblastine and then into vincristine (Scheme 4.7) [35, 36].

4 Compartmentalization and Cellular Differentiation in the Biosynthesis of Terpenoid Indole Alkaloids

In *C. roseus*, TIA biosynthesis is a highly dynamic, complex, and compartmentalized process in which different branching routes are expressed in a tissue-specific form. The enzymes that have been characterized are associated to different cell types and found in different subcellular compartments [4]. The compartmentalization and localization involved in the TIA pathway is considered another regulatory mechanism, since this localization requires the transport of different metabolic intermediates from one point to another, for their transformation. TDC, SLS, and STR are localized in the epidermal cells of aerial organs and root apical cells, while D4H and DAT are restricted to the laticifer cells and idioblasts of leaves, stems, and floral buds, and G10H is found in the internal phloem parenchyma of aerial organs [3, 37]. This means that, at least during vindoline biosynthesis, alkaloid intermediates must be translocated from one leaf cell type to another. In fact, vindoline biosynthesis is organ-tissue-cell regulated, since it is detected in the aerial organs, mainly in juvenile tissues, but not in roots, while catharanthine as well as strictosidine, ajmalicine, and serpentine are isolated from both roots and leaves. Moreover, enzymes involved in TIA biosynthesis in *C. roseus* are localized in different subcellular compartments (Fig. 4.2). Thus, the cytochromes P450 as G10H, SLS, and T16H are located in membranes, most likely from endomembrane systems, specifically from endoplasmic reticulum (ER) similar to the localization has been determined for SGD, a soluble enzyme associated to the cytoplasmic face


Catharanthine
+
Vindoline
3'-4'-Anhydrovinblastine
Vinblastine
Vincristine

Scheme 4.7 Biosynthesis of vinblastine and vincristine from catharanthine and vindoline

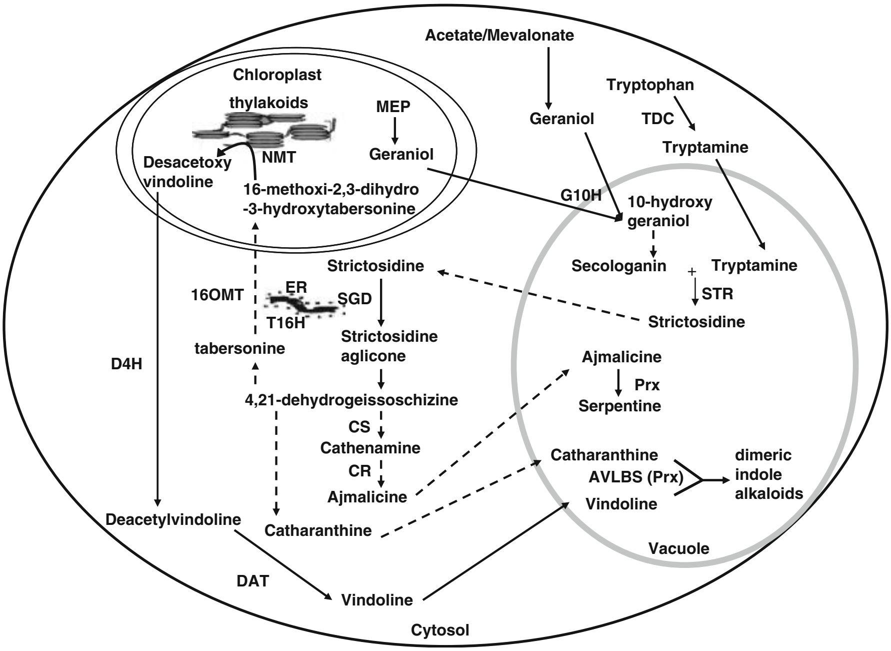

Acetate/Mevalonate
Chloroplast
thylakoids
MEP
Geraniol
Tryptophan
TDC
Tryptamine
Geraniol
Desacetoxy vindoline
NMT
16-methoxi-2,3-dihydro -3-hydroxytabersonine
G10H
10-hydroxy geraniol
Secologanin
+
Tryptamine
STR
Strictosidine
16OMT
ER
SGD
T16H
Strictosidine
tabersonine
Strictosidine aglicone
D4H
Ajmalicine
Prx
Serpentine
4,21-dehydrogeissoschizine
CS
Cathenamine
CR
Ajmalicine
Catharanthine
AVLBS (Prx)
Vindoline
dimeric indole alkaloids
Deacetylvindoline
Catharanthine
Vacuole
DAT
Vindoline
Cytosol

Fig. 4.2 Cellular localization of both enzymes and intermediates, during TIA biosynthesis

of the ER [4]. The reactions catalyzed by the enzymes TDC, 16OMT, D4H, and DAT are carried out in the cytoplasm, while STR and class III Prx implicated in AVLB and serpentine synthesis are located in the vacuoles, and NMT in the thylakoids [4, 36, 38]. The complex cellular and subcellular compartmentalization of the TIA pathway indicates a transfer of intermediates among different types of cells and cell compartments, revealing a high complexity that may hinder its biotechnological manipulation.

5 Biotechnology Approaches to Improve Terpenoid Indole Alkaloid Production from *Catharanthus roseus* In Vitro Cultures

Higher plants are a rich source of bioactive compounds, many of which are used in the pharmaceutical industry. Some plant-derived bioactive products include drugs such as morphine, codeine, TIAs, tropane alkaloids, among others. Many of these pharmaceuticals are still in use today, and no synthetic substitutes have been found that possess the same pharmacological efficacy. The most common strategies used to produce these natural compounds are their direct extraction from plants and their chemical synthesis. However, such strategies are not exempt of problems. Production by plants is not always satisfactory as may be restricted to one species or genus and might be activated only during a particular development stage or under specific seasonal, stress, or nutrient availability conditions, and, in some cases, it may even lead to the risk of the plant's extinction [39]. In the case of chemical synthesis, the complexity of the pathways, the stereospecificity, the strict conditions of the reactions involved, and high costs represent the main difficulties for the production of these high-value metabolites. For these reasons, much effort has been devoted to in vitro cultures as an attractive biotechnological method to produce secondary metabolites of commercial interest. The advantages of such a system for the industrial production of plant secondary metabolites are also the uniform quality of the products, the independence from disease and climatic changes, the stability of supply, and a closer relationship between supply and demand. In some cases, the production of secondary metabolites using large-scale in vitro cultures is technically feasible, when the generation and maintenance of the cell biomass is achieved together with a high yield of these metabolites. However, the use of in vitro cultures is limited in most cases by the economy of the process determined by the low yields of secondary metabolite obtained.

In this sense, *C. roseus* and TIAs have become an important model system in plant biotechnological research. In fact, the low levels of the TIAs with anticancer activity found in the plant stimulated intense research, aiming to obtain *C. roseus* in vitro cultures with high production of these TIAs. Technologically, Zhao and Verpoorte showed that although *C. roseus* cells can be cultured in large-scale, the levels of TIAs produced are too low to favor commercial production [40]. To increase the production of TIAs using in vitro cultures, different approaches have been tried, such as the screening and selection of high-producing cell lines, the optimization of culture conditions and feeding, elicitation, and cell immobilization. Genetic modification or metabolic engineering is also promising biotechnological approach to improve the production of these compounds [1].

5.1 Importance of Screening and Selection of *Catharanthus roseus* In Vitro Culture Lines and Level of Cell Differentiation to Improve the Production of Terpenoid Indole Alkaloids

The selection of genotypes starts with the selection of the plant from which cell and tissue cultures are to be initiated. Deus and Zenk showed that TIA levels in *C. roseus* plants could vary from almost undetectable to more than 1% in a dry weight (DW) basis [41]. High alkaloid-producing plants gave rise to cell cultures with higher alkaloid content. Kurz et al. investigated over 2,000 cell lines obtained from three *C. roseus* cultivars and observed significant cultivar-dependent differences between TIA-producing cell lines [42]. Differences observed were not only on the alkaloid levels but also in the TIA types produced. Naaranlahti et al. analyzed the leaves of 70 plants of *C. roseus* belonging to three cultivars and selected three plants with high vindoline levels for inducing callus [43]. One third of the 60 established cultures when screened with a vindoline-specific radioimmunoassay showed vindoline contents of about 10^{-5}% DW. Suspension cultures derived from the five most immunopositive calli, and cultivated in an alkaloid production medium, seemed to contain vindoline. Suspension cells also present a high degree of variability in alkaloid levels, no matter if they are derived from a single plant or even a single mother colony, so the selection of cell lines with suitable and uniform genetic, biochemical, and physiological characteristics is also an important approach to improve the production of TIAs. Screening of high-yielding cell lines with selection along successive subculture has enabled the selection of cell lines producing higher levels of TIAs [41, 42, 44]. However, the selection of high-yielding cell lines is hindered by the same variability that enables it. Actually, selected cell lines are usually unstable and tend to lose their productivity during long-term subcultures. The reason for such variability and instability is not known, and so far the process to overcome this problem has been the preservation of cell lines by cultivation as slow-growing callus and by techniques of cryopreservation and storage under mineral oil. Otherwise, continuous selection for high yields has to be performed over successive subcultures [1]. Several studies carried out using whole plants, shoot, callus, and cell cultures of *C. roseus* have demonstrated that the level of cell differentiation and organogenesis plays an important regulatory role in the pattern of TIA production [16, 45]. Endo et al. observed that the pattern of TIA production in root and shoot cultures of *C. roseus* was similar to that found in roots and leaves from intact plants [46]. However, many TIAs cannot be produced by *C. roseus* cell cultures since the activation of biosynthetic pathways is tissue-specific and developmentally regulated. In fact, as observed for many other plant species, *C. roseus* cultures present a much simpler alkaloid pattern than intact plants, with vindoline and anticancer TIAs detected very seldom, and in extremely low levels, in accordance with the high organ-cell-developmental regulation of these TIAs. Naaranlahti et al. reported the presence of vindoline ($10^{-5}\%$ on a DW basis) in suspension cultures newly established [43], and O'Keefe et al. reported a stable production of vindoline for a period of 6 years, in undifferentiated suspension cell lines of *C. roseus* transformed with *Agrobacterium tumefaciens* and *A. rhizogenes*, but also in low levels (of about $10^{-5}\%$ DW) [47]. Otherwise, vindoline and anticancer TIAs were only detected in organ cultures. Shukla et al. demonstrated that vindoline and catharanthine production increased in *C. roseus* in vitro cultures as the level of cell differentiation increased (suspension-cultured cells < callus culture < shoot/leaf tissue) [45]. However, these compounds were only detected in elicited conditions, the highest amounts of catharanthine and vindoline occurring in shoots (0.0039% and 0.0013% on a DW basis, respectively) compared with the levels found in callus (0.00019% and 0.00015% on a DW basis, respectively). Although catharanthine was detected in trace amounts in the suspension-cultured cells, vindoline was not detected in the cells or the medium in any conditions. Miura et al. found that the vinblastine content was higher in shoot cultures (around 0.015 mg g DW^{-1}) than in young callus cultures, although vinblastine was mostly found in leaf tissue cultures, suggesting that the production of this compound is closely associated with morphological and cellular differentiation phenomena [48]. In fact, Shukla et al. did not detect vinblastine in either control or elicitor-treated in vitro cultures [45].

The culture of hairy roots is an attractive alternative to the use of undifferentiated cell cultures to obtain secondary metabolites, since roots grow rapidly in hormone-free liquid medium, they are genetically stable and are differentiated cells potentially able to produce more secondary metabolites than undifferentiated cells. Hairy root cultures produce a broad spectrum of TIAs. Production of ajmalicine, serpentine, and catharanthine has been repeatedly reported by different authors, sometimes with levels superior to nontransformed root cultures (around 0.2–0.4% DW) [49, 50]. Production of vindoline has been surprisingly detected in hairy roots, in levels which are not so low (0.04–0.08% DW) [49, 51]. Very recent work with *C. roseus* hairy roots include elicitation treatments, overexpression of TIA genes, and regulatory transcription factors, but although vindoline is reported in some cases, the occurrence of anticancer TIAs was never reported, and vindoline levels were always below 0.08% DW [52–55].

5.2 Optimization of Culture Conditions to Improve the Production of Terpenoid Indole Alkaloids

Composition of the culture medium, as well as the physical environmental conditions, and the addition of specific factors have been proved to be able to increase TIA yields in *C. roseus* cultures, sometimes in a dramatic way. In fact, reducing or even deleting nitrate and/or phosphate from the medium strongly enhances TIA accumulation but inhibits cell growth [56]. On the other hand, the addition of KCl (4 g L^{-1}) did not inhibit cell biomass accumulation and increased the production of ajmalicine (33.5 mg L^{-1}), serpentine (11.9 mg L^{-1}), and catharanthine (12.3 mg L^{-1}) to levels that were threefold those of the control in compact callus cluster cultures of *C. roseus* The highest levels of alkaloids in these cultures were obtained by adding 250 mM mannitol (four- to fivefold higher compared with the controls) [57]. The production of TIAs from *C. roseus* cell cultures is also associated with the carbon source concentration. Schlatmann et al. showed that the presence of a low glucose concentration (40 g L^{-1}) correlated with a high ajmalicine production level [58]. Zhao et al. observed an increase of ajmalicine, serpentine, and catharanthine in both cells and medium (a total alkaloid yield of over 43 mg L^{-1}), when the concentration of sucrose (40–60 g L^{-1}) increased in the culture medium of compact callus clusters of *C. roseus* [59]. In addition, Jung et al. observed an increase in catharanthine accumulation when the carbon source was changed, using 3% sucrose instead of 3% fructose in *C. roseus* hairy root cultures [60].

It is well established that inoculum size greatly affects secondary metabolite production in plant cell cultures. Several authors have investigated the production of ajmalicine from *C. roseus* cell cultures at different cell inoculum densities, observing that ajmalicine production using a low cell inoculum density is significantly lower than when high cell densities are used [61, 62]. Plant hormones affect both culture growth and TIA production in *C. roseus* in vitro cultures. The addition of auxins to the culture medium had a negative influence on the TIA biosynthetic pathway. In fact, the addition of 2,4-dichlorophenoxyacetic acid (2,4-D) inhibits TIA production especially during the growth phase [63], even when *C. roseus* cell cultures are fed with the precursors, loganin and tryptamine [64]. Zhao et al. also observed a decrease in TIA production when the culture medium of compact callus clusters of *C. roseus* were supplemented with 2,4-D [65]. However, in those cultures, the combination of benzyladenine (BA) or kinetin with indole-3-acetic acid (IAA) led to the highest TIA production (total alkaloid yield of over 45 mg L^{-1}). Moreover, Satdive et al. analyzed the effects of different concentrations of both IAA and BA on ajmalicine production in *C. roseus* shoot cultures [66]. They found that in the presence of a high concentration of BA (8.90 μM) and a low concentration of IAA (2.85 μM), shoot cultures released high levels of ajmalicine (over 0.85 g L^{-1}) into the culture medium after 15 days of cultivation, whereas the addition of a high concentration of IAA (11.42 μM) and a low concentration of BA (2.22 μM) resulted in high levels of ajmalicine (over 0.40 g L^{-1}) being accumulated in the shoots. In addition, exposure to light, together with IAA and BA, improved TIA biosynthesis in *C. roseus* callus, especially vindoline (0.19 mg g DW^{-1}) and serpentine (0.53 mg g DW^{-1}) compared with callus that was not exposed to light [65].

On the other hand, some gaseous growth regulators may play a critical role. Indeed, the accumulation of ethylene in *C. roseus* cell cultures seemed to inhibit the production of ajmalicine [67].

Physical factors like aeration, gas-phase composition, and light (mentioned above) may promote an enhancement of the biosynthesis of TIAs. Lee-Parsons and Shuler studied the effect of O_2 and CO_2 on the production of ajmalicine in immobilized cell cultures of *C. roseus* [68]. They observed that low and high O_2 concentrations (10%, 90% and 95%) had either inhibitory or toxic effects to cell growth and decreased ajmalicine production. The effects of CO_2 depended on O_2 concentration. At low O_2 concentrations (21%), increasing the CO_2 concentration decreased both cell growth and ajmalicine production, while at high O_2 concentrations (78%), increasing the CO_2 concentration decreased cell growth, although ajmalicine production was not affected. The extracellular ajmalicine concentration was maximized with a gas mixture of 50% O_2 + 0.03% CO_2.

5.3 Feeding Strategies to Improve the Production of Terpenoid Indole Alkaloids

Sometimes, precursor feeding has proved to be a successful approach to increase the levels of TIAs in in vitro cultures. In particular, feeding with loganin, secologanin, and tryptamine has been extensively studied [64, 69]. El-Sayed and Verpoorte demonstrated that feeding *C. roseus* cell cultures with tryptamine and loganin resulted in a high accumulation of strictosidine (95.6 ng g DW^{-1}) [64]. Furthermore, El-Sayed et al. observed that adding stemmadenine to *C. roseus* cell cultures resulted in the accumulation of catharanthine and tabersonine, suggesting that stemmadenine is an intermediate precursor in the biosynthetic pathway of these TIAs [69]. Zhao et al. also carried out feeding experiments in compact callus clusters cultures of *C. roseus* [57]. They found that the addition of succinic acid, tryptamine, and tryptophan to the culture medium significantly increased the production of ajmalicine (41.5, 36.9, and 31.8 mg L^{-1}, respectively) and catharanthine (21.1, 17.2, and 18 mg L^{-1}, respectively), while geraniol feeding inhibited cell biomass and alkaloid accumulation. Zhao et al. also showed a maximal production of ajmalicine (over 50 mg L^{-1}), catharanthine (over 10 mg L^{-1}), and serpentine (over 20 mg L^{-1}) in *C. roseus* cell cultures treated with 0.5 mM malic acid for 3 days. Succinic and malic acids are involved in the citric acid cycle, and at the concentration tested, they greatly improved TIA production but inhibited cell growth [70].

Precursor feeding studies with *C. roseus* hairy root cultures also pointed to a limitation of the TIA pathway by the monoterpenoid precursors. In fact, after feeding geraniol, 10-hydroxygeranioll or loganin, a significant increase of tabersonine was observed. Thus, the addition of 80 mg L^{-1} geraniol to the culture medium provoked a tabersonine increase of around 1.4 mg g DW^{-1} [71].

5.4 Immobilization Strategies to Improve the Production of Terpenoid Indole Alkaloids

Another approach is to immobilize plant cells since it is thought that improved cell-to-cell interaction may increase TIA productivity. In fact, cells of *C. roseus* have been immobilized into various carriers such as alginate, agarose, agar, and polyurethane foam [1]. Immobilization leads to diffusional resistance and could lead to higher local concentrations of TIAs, particularly in high-density cell cultures. For example, Asada and Shuler reported a significant enhancement of ajmalicine production and excretion to the medium when *C. roseus* cells were immobilized with calcium alginate [72]. It was observed that the effects of immobilization adsorption and elicitation were synergistic, with the amount of ajmalicine in the medium reaching 90 mg L^{-1}, compared to 2 mg L^{-1} for suspension cultures under identical conditions. Also, Lee and Shuler observed that a high inoculum density in *C. roseus* cultures in which cells are immobilized on alginate beads increased ajmalicine concentration throughout the experiment, the maximum production of ajmalicine reaching more than 120 mg L^{-1} [62]. Lee-Parsons and Shuler also studied the effect of O_2 and CO_2 on the production of ajmalicine in immobilized cell cultures of *C. roseus* observing an increase of ajmalicine production (over 160 mg L^{-1}) on day 33 using 21% O_2 + 1.5% CO_2 [68].

5.5 Elicitation Strategies to Improve the Production of Terpenoid Indole Alkaloids

Several strategies have been adopted to enhance the levels of TIAs in cultures and produce them on an industrial scale. One of the most effective strategies to increase their productivity in a short period of time has been the use of biotic or abiotic stimuli as elicitors. The elicitors most frequently used include fungal extracts, metal ions, jasmonic acid and its derivative, MeJa, and irradiation with UV light [73, 74]. It is well documented that *C. roseus* cell cultures respond to jasmonates by increasing the production of TIAs. Lee-Parsons et al. showed that *C. roseus* cell cultures respond to MeJa by increasing the extracellular accumulation of ajmalicine in a way that was dependent on the MeJa dose and elicitation time [73]. In fact, the maximum ajmalicine production (10.2 mg L^{-1}) was obtained with 100 μM MeJa after 6 days, a 300% increase over that obtained with nonelicited cultures. Almagro et al. also described the high production of ajmalicine (137.2 mg L^{-1}) on day eight when *C. roseus* cell cultures were elicited with 100 μM MeJa using a high cell density (200 g fresh weight (FW) L^{-1}) [61]. Furthermore, in these cultures, the extracellular accumulation of ajmalicine increased to over 200 mg L^{-1} in the presence of cyclodextrins, which are modified cyclic oligosaccharides derived from starch with seven glucose residues linked by α 1→4 glucosidic bonds. The treatment of *C. roseus* cell cultures with a fungal preparation derived from *Penicillium citrinum* resulted in increased catharanthine production, reaching the highest level (20.1 mg L^{-1}) after 1 day of treatment, which was threefold the production of control cells [74]. In this case, too, treatment with cyclodextrins provoked an increase of catharanthine production (84.9 mg L^{-1}).

On the other hand, the addition of 1 mM of acetylsalicylic acid, which is an important plant signaling derivate of salicylic acid, provoked an important enhancement of total alkaloids released to the culture medium and accumulated in tumor *C. roseus* cells transformed with *A. tumefaciens* [75]. However, in nontransformed *C. roseus* cell cultures, acetylsalicylic acid also stimulated ajmalicine and serpentine accumulation, but the effects were not significant.

It has also been shown that UV light induces an enhancement of TIAs in *C. roseus* cell cultures. Ramani and Jayabaskaran observed an enhanced production of catharanthine (0.12 mg g DW^{-1}) and vindoline (0.06 mg g DW^{-1}) from *C. roseus* cell cultures irradiated with UV-B light [76]. Also, Almagro et al. used UV-C light irradiation to promote the production of TIAs in *C. roseus* cell cultures. A production of 90 mg L^{-1} ajmalicine and 49.5 mg L^{-1} catharanthine was obtained when high-cell-density cultures were exposed to 15 min of UV-C light, and these production levels of ajmalicine and catharanthine were maintained for 96 h [61].

The combined effect of elicitors is a widely used strategy to increase the production of TIAs. Indeed, the use of fungal and chemical elicitors separately or in combination is considered an effective strategy for enhancing TIA production in *C. roseus* cell cultures. Zhao et al. observed a synergistic effect on alkaloid accumulation in *C. roseus* cell cultures when *Aspergillus niger* mycelium and tetramethylammonium bromide were jointly used as elicitors, since they gave the highest levels of ajmalicine (63 mg L^{-1}) and high levels of catharanthine (17 mg L^{-1}). Also, the combination of malate and sodium alginate resulted in a high ajmalicine (41 mg L^{-1}) and catharanthine (26 mg L^{-1}) yield [57, 59, 65].

Recently, Almagro et al. described a new strategy for enhancing ajmalicine and catharanthine production from *C. roseus* cell cultures, combining the addition of cyclodextrins and MeJa. The addition of 50 mM cyclodextrins and 100 μM MeJa to high-cell-density cultures led to a release of ajmalicine and catharanthine to the culture medium of 450.1 and 155.5 mg L^{-1}, respectively. This effect was even greater when these chemical compounds were combined with short UV light exposure [61]. In fact, Almagro et al. demonstrated that *C. roseus* cell cultures irradiated with UV light (A or C) and elicited with cyclodextrins in combination with MeJa increased the production of ajmalicine to 1,040.5 mg L^{-1} (85 mg g DW^{-1}) and of catharanthine to 196.3 mg L^{-1} (10 mg g DW^{-1}) [77].

5.6 Large-Scale Production of Terpenoid Indole Alkaloids

Large scaling is the last step for the development of an industrial process for the production of the medicinal TIAs of *C. roseus*. Although present levels of TIAs still do not make such a process commercially viable, some research on large scaling of *C. roseus* cultures has already been performed, in view of possible future achievements. The major factors affecting TIA production in *C. roseus* cell cultures during a scale-up process are the components of the medium, growth regulators, pH, dissolved O_2 and gas composition, and culture conditions including temperature, light, aeration, and stirring regime [40]. Elicitation is also very important to consider, since it not only improves TIA biosynthesis in a short time but also causes excretion of the products into the medium, enabling fast isolation of the TIAs and the possibility of a continuous process. The combination of two or more elicitors can synergistically induce metabolic fluxes toward TIAs, as observed by Almagro et al. and even further improving the productivity of target compounds and the performance of bioreactor processing [61]. However, large scaling may affect negatively TIA production. Godoy-Hernández et al. observed that TIA total production in *C. roseus* cell cultures maintained in 250-ml batch cultures was around 60 mg L^{-1} [78]. These values rapidly decreased in a 14-L bioreactor (15 mg L^{-1}), especially when cultures entered in the active growth phase. In addition, the exposure to *A. niger* mycelium homogenates did not result in any increase of TIA production, but the combination of osmotic stress (2.50% sucrose plus 1.25% mannitol) and 1 mM cinnamic acid restored the original TIA levels. Zhao et al. studied ajmalicine production in elicited *C. roseus* cell cultures both in stirred flasks and bioreactors [70]. By using a combined elicitor treatment consisting of a homogenate of *A. niger* with tetramethylammonium bromide, the yield of ajmalicine was 48, 52, and 33 mg L^{-1} in 0.5-L flasks, 1-L flasks, and a 20-L airlift bioreactor, respectively. In the same conditions and same flasks, catharanthine production was 25, 32, and 22 mg L^{-1}, respectively. These results reflect by 3.1-, 3.5-, and 2.2-fold increase in ajmalicine levels over the controls without elicitation and a 3.1-, 3.9-, and 2.7-fold higher production of catharanthine, respectively.

In another experiment, the treatment of 7-day-old *C. roseus* cell cultures with abscisic acid produced an 82.25 mg L^{-1} accumulation of catharanthine after 3 days of cultivation in a 30-L airlift bioreactor [79].

5.7 Metabolic Engineering as a Strategy to Improve the Production of Terpenoid Indole Alkaloids

Successful metabolic engineering in plants requires an extensive knowledge of the whole biosynthetic pathway and a detailed understanding of the regulatory mechanisms controlling the flux of the pathways. Such information is not yet available for the vast majority of plant secondary metabolites, which is why only limited success has been achieved by metabolic engineering. Nowadays, only a few pathways in plants are well understood, following many years of classical biochemical research [80]. Usually, enzymes of the biosynthetic pathway can be selected as targets for gene cloning and then manipulated by genetic engineering, e.g., to increase the metabolic flux to a desired secondary metabolite and thus improve its productivity in the whole plant or in plant cell cultures. Despite our limited knowledge on the TIA pathway and genes involved in *C. roseus*, the information that is available has provided the tools to carry out metabolic engineering of TIA biosynthesis [40]. Therefore, among the strategies used to enhance the production of TIAs in *C. roseus* in vitro cultures, the overexpression of transcription factors and enzymes and also the incorporation of TIA biosynthetic pathway genes in other organisms may be highlighted.

The molecular cloning of a number of TIA biosynthetic pathway genes has been achieved using several approaches. STR is one of the key enzymes of this pathway that has been studied by many research groups. Indeed, it was the first gene involved in alkaloid biosynthesis to be cloned. The pathway leading to several TIAs (ajmalicine, serpentine, ajmaline, tabersonine, vindoline, catharanthine, and the dimeric anhydrovinblastine) has been partially elucidated, and several of the responsible genes have been cloned and their enzymes characterized, more specifically TDC, G10H, LAMT, STR, SGD, T16H, PNAE, 16OMT, DAT, D4H, and a class III Prx implicated in the condensation of vindoline and catharanthine into anhydrovinblastine [36, 81]. Several transcription factors implicated in the regulation of TIA biosynthesis genes have also been isolated and studied, and their genetic manipulation was performed in some cases [5].

5.7.1 Overexpression of Enzymes Involved in Terpenoid Indole Alkaloid Biosynthesis

Several attempts have been published stating the successful genetic transformation in cell and tissue cultures of *C. roseus*, resulting in higher yields of some TIAs [40, 82]. In the past decades, several transformation methods have been developed for the genetic transformation of cell and tissue cultures of *C. roseus* [83]. Although efficient direct transformations using particle bombardment have been carried out in *C. roseus* cell cultures, indirect transformations using genetic vectors such as *A. tumefaciens* have also been successfully developed. The genes mostly overexpressed in *C. roseus* include *tdc*, *str*, *dat*, *g10h*, and those expressing apoplastic Prx [40, 82, 84–86]. Along these lines, extensive research has been conducted in *C. roseus* cell cultures overexpressing *tdc* and/or *str* [17]. Cell cultures overexpressing *str* showed a tenfold higher STR activity than nontransformed cell cultures, leading to an increase in total TIAs to 300 mg L^{-1}, strictosidine, ajmalicine, serpentine, catharanthine, and tabersonine, being the major alkaloids found in all the transgenic *C. roseus* cell cultures studied. However, the overexpression of *tdc* did not increase TIA levels. Whitmer et al. also studied the overexpression of *tdc* in *C. roseus* cell cultures and only observed an increase in tryptamine levels over the levels recorded in nontransformed cell cultures. In addition, when loganin or secologanin was added to these transgenic cultures, the production of serpentine, catharanthine, and strictosidine increased, resulting in a total alkaloid production of 1,250 μM (ca. 600 mg L^{-1}) [84].

In a similar way, Peebles et al. constructed *C. roseus* transgenic hairy root cultures overexpressing genes of the monoterpenoid and indole precursors pathway, as well as *g10h*, alone or in combination, and obtained increases and decreases of different TIAs, pointing to the need for overexpressing multiple genes within the TIA pathway to obtain significant results [54]. In recent studies, *g10h* was introduced independently or cointroduced with ORCA3(octadecanoid-responsive Catharanthus AP2/ERF domain) in *C. roseus* hairy roots cultures [85]. The results showed that catharanthine production increased (1,250 μg g DW^{-1}) through the overexpression of *g10h* alone or in combination with ORCA3, representing a 6.5-fold increase when compared with nontransformed cultures. Similarly, Magnotta et al. described that the overexpression of *dat* in *C. roseu*s hairy root cultures provoked an increase in the production of hörhammericine (8 μg g DW^{-1}) [87]. Jaggi et al. studied the impact of overexpression of a Prx gene in *C. roseus* hairy root cultures [86]. Through metabolite analysis, higher levels of ajmalicine (350 μg g DW^{-1}) and serpentine (3,700 μg g DW^{-1}) were detected in these overexpressed cultures. These results indicated the involvement of Prx gene expression in the regulation of TIA biosynthesis.

5.7.2 Overexpression of Transcription Factors Involved in Terpenoid Indole Alkaloid Biosynthesis

The identification and subsequent manipulation of transcription factors has become a very attractive research tool for the metabolic engineering of TIAs. As mentioned above in Sect. 5.5 concerning elicitation strategies, jasmonic acid and MeJa induce the biosynthesis of TIAs. Both are capable of activating the gene expression that codifies enzymes which catalyze the formation of TIAs, including TDC, SGD, G10H, and STR. ORCA3 was isolated as jasmonate-responsive AP2-domain transcription factors that could promote the transcription of several TIA biosynthetic genes [5, 88]. In *C. roseus* cell cultures, the overexpression of ORCA3 led to an increase in the levels of the genes *tdc*, *str*, *sgd*, and *d4h*. However, the gene expression of *g10h* and *dat* was not affected by ORCA3 overexpression [88]. In addition, tryptamine and tryptophan levels increased upon overexpression of ORCA3, but no TIAs were detected, suggesting that the monoterpenoid branch of the TIA pathway remained limiting. This was confirmed by the fact that when cells were fed with the monoterpenoid precursor loganin, ORCA3 overexpression indeed caused an increase in TIA production.

The overexpression of ORCA3 has also been carried out in hairy root cultures of *C. roseus* by Peebles et al. [89]. This study showed that ORCA3 overexpression did not significantly increase the accumulation of ajmalicine, serpentine, catharanthine, tabersonine, hörhammericine, or lochnericine, although the mRNA transcript levels of *str* and *sls* increased, the levels of *tdc*, *g10h*, and *ORCA2* remained constant, and the levels of *sgd* decreased. The increase of *str* transcript levels and the constant levels of *g10h* transcripts agree with the results observed in *C. roseus* cell cultures overexpressing ORCA3 [88]. The decrease in *sgd* transcripts and the maintenance of *tdc* transcripts detected in hairy root cultures of *C. roseus* differ from the results observed in *C. roseus* cell cultures, where both transcripts increased [88]. Therefore, differences in mRNA accumulation between hairy roots and cell cultures seem to indicate the existence of different control mechanisms of the TIA pathway between culture types. In any case, the levels of TIAs in the manipulated cultures did not suffer a remarkable increase, meaning that the regulation of TIA accumulation must involve other key transcription factors, yet to be discovered.

5.7.3 Incorporation of Genes from the Terpenoid Indole Alkaloid Biosynthesis in Other Plants and Microorganisms

Some genes of the TIA pathway have been expressed in other plants apart from *C. roseus*. Hallard et al. obtained a transgenic cell culture of tobacco expressing *tdc* and *str* genes from *C. roseus* [90]. The TDC activity was constant in transgenic tobacco cells, while a two- to sixfold increase in STR activity was observed in comparison with nontransformed tobacco cells. This increase in enzymatic activity correlated with an increase in the production of tryptamine (12.6 μg g FW^{-1}). Similarly, *tdc* and *str* genes were overexpressed in *Morinda citrifolia* cell cultures, resulting in an increase of strictosidine when cells were fed with tryptamine and secologanin [91]. In addition, *tdc* and *str* genes obtained from *C. roseus* were introduced in *Cinchona officinalis* hairy root cultures. The results showed that *C. officinalis* transgenic hairy root cultures accumulated high amounts of tryptamine (1,200 μg g DW^{-1}), strictosidine (1,950 μg g DW^{-1}), quinine, and quinidine (around 500 and 1,000 μg g DW^{-1}, respectively) [92]. Unfortunately, such production proved not to be a stable trait.

On the other hand, as the slow growth of plant cells is an important constraint for an economically feasible TIA production, their production in microorganisms would be an interesting alternative [39]. In this sense, Geerlings et al. tried overexpressing *str* and *sgd* obtained from *C. roseus* in yeast (*Saccharomyces cerevisiae*) [93]. The STR activity was mainly found in the medium of the transgenic yeast, whereas the SGD activity was mainly found in cells. When this transgenic yeast was fed with tryptamine and secologanin, a greater amount of strictosidine (2 g L^{-1}) was secreted into the medium in 3 days. By breaking up the yeast cells, strictosidine was converted into cathenamine by the action of SGD. The transgenic yeast was able to grow on a juice of snowberries, which is a rich source of secologanin, confirming the interest of this approach as an alternative for TIA production. On the other hand, when *str* was overexpressed in *E. coli*, the strictosidine produced was converted to vallesiachotamine and isovallesiachotamine, which are TIAs that have been isolated in plant extracts of the Apocynaceae family [94].

6 Conclusions

The commercial production of valuable metabolites by in vitro culture techniques largely depends on the economics of the production process, which in turn, depends on productivity. In the case of TIA production in *C. roseus* in vitro cultures, the productivity obtained is not sufficient for the economically feasible large-scale production. Since the low productivity of TIAs in *C. roseus* in vitro cultures is one of the bottlenecks for their commercial production, different strategies to improve TIA productivity have been developed, such as the selection of high-yield cell lines, elicitation of *C. roseus* in vitro cultures, feeding strategies using precursors, and the metabolic engineering of TIA biosynthetic pathway and regulatory transcription factors. Although not yet successful, metabolic engineering offers the most promising perspectives for improving TIA production in the future, as knowledge of the genetic determination and regulation of the pathway increases. Therefore, improvement of such strategies should continue to be investigated, using TIA high-yield cell lines and transformation by stable genetic overexpression of genes or regulators of the biosynthetic pathway, together with inhibition of competitive pathways. Moreover, there is still a need of improving bioreactor performance, namely, by using elicitation and precursor feeding and by performing optimization of growth of engineering *C. roseus* in vitro cultures, as well as optimization of the release of TIAs into the medium for efficient recovery by processing technologies.

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Bioactive Alkaloids from South American *Psychotria* and Related Rubiaceae

5

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Contents

1 Introduction 120
2 Biodiversity and Drug Discovery Potential in the Neotropics 121
3 Bioactive Alkaloids from South American Rubiaceae Plants 122
3.1 *Uncaria* Genus 122
3.2 *Cinchona* Genus 124
3.3 Brazilian Medicinal Plants 124
4 The *Psychotria* Genus 125
4.1 Bioactive Alkaloids from South American *Psychotria* 126
4.2 Regulation of *Psychotria* Alkaloid Biosynthesis 137
5 Conclusions 138
References 139

Abstract

The largest fraction of global plant diversity is located in the Neotropics, with the Atlantic Forest and the Amazon being a rich untapped reservoir of species that may lead to new drug discovery. Bioactive molecules are often isolated from Rubiaceae species. Ethnobotanic and chemotaxonomic studies may provide clues to guide the prospection of bioactive molecules of interest. In South America, three genera are of special interest due to the bioactivities of their phytochemicals along with their importance to local human populations: *Uncaria*, *Cinchona*, and *Psychotria*. The numerous bioactivities of alkaloids from species in these genera

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include emetic, cytotoxic, analgesic, anxiolytic, antidepressant, psychotropic, antipsychotic, antipyretic, anti-inflammatory, antioxidant, antimutagenic, immunomodulatory, vasorelaxant, antiviral, antimicrobial, and antiprotozoal. Indole alkaloids are widely represented in these plant groups. Detailed studies *in planta* and in vitro on the dynamics of biosynthesis, accumulation, and distribution of some of these metabolites in *Psychotria* of Southern Brazil have revealed complex regulatory controls, encompassing responses to both developmental and environmental signals. In addition, even closely related alkaloids may present distinct dynamic profiles of accumulation, varying from constitutively produced to highly responsive to environmental signals.

Keywords
Bioactivity • biodiversity • monoterpene indole alkaloid • *Psychotria* • Rubiaceae

Abbreviations

5-HIAA	5-Hydroxyindole-3-acetic acid
5-HT	Serotonin
AADC	Aromatic amino acid decarboxylase
CNS	Central nervous system
DA	Dopamine
DMT	*N*,*N*-dimethyltryptamine
DNA	Deoxyribose nucleic acid
DOPAC	3,4-Dihydroxyphenyl acetic acid
DW	Dry weight
GPV	*N*,*β*-D-glucopyranosyl vincosamide
HVA	Homovanillic acid
INMT	Indolethylamine *N*-methyltransferase
IpeGlu	Ipecac *β*-glucosidase
IpeOMT	Ipecac *O*-methyltransferase
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MDR	Multidrug resistance
MIA	Monoterpene indole alkaloid
NMDA	*N*-Methyl-D-aspartate
PMN	Polymorphonuclear leukocytes
ROS	Reactive oxygen species
STR	Strictosidine synthase
UV	Ultraviolet

1 Introduction

Plants are a rich source of bioactive molecules, partly as a result of their immobile nature. A driving force for evolution of higher photosynthetic organisms is the need to respond *in situ* promptly and effectively to a variety of stimuli, both biotic and abiotic, as well as to attract pollinators, seed dispersers, and microbial/fungal mutualists. Metabolic pathways leading to final compounds have several steps, yielding a number of intermediates. One or more of these metabolites may confer adaptive advantage, fixing the pathway in populations. Environments that are prone to host a high biodiversity are often also conducive to higher selective pressures for chemical diversification. This combination may explain why the Neotropics and some genera of Rubiaceae converge to be a rich reservoir of bioactive alkaloids. This chapter focuses on the alkaloids of *Psychotria*, *Uncaria*, and *Cinchona* and their structural diversity, pharmacological properties, and applications.

2 Biodiversity and Drug Discovery Potential in the Neotropics

The largest fraction of global biodiversity is located in the Neotropics, with the Atlantic Forest and the Amazon being a rich untapped reservoir of plants that may lead to new drug discovery [1]. The Atlantic Forest biome is considered one of the key global hotspots of biodiversity, hosting thousands of endemic species [2], spread along 194,524 km^2 in three countries (Brazil, Paraguay, and Argentina), which corresponds to only 12.9% of the original forest cover [3–5]. Amazon is one of most biodiverse biomes in the world [6] comprising eight countries, among which Brazil holds the largest forest area; deforestation and forest fires, however, are major concerns for the integrity of its biodiversity. The Atlantic Forest is also under high levels of pressure, sharing space with the most populated areas of the South American Atlantic Coast; deforestation of this biome may reach annual rates of 0.5% [7].

Reducing Amazon deforestation, besides biodiversity conservation, is linked to a possible and reachable reduction in global greenhouse-gas emissions (2–5%) [8], making this biome an important target in the worldwide scenario. One billion US dollars from the Amazon Fund, pledged by Norway, are committed to reducing deforestation through reduced greenhouse-gas emission in sustainable fashion [9] and also by excluding Amazon deforesters from the beef- and soy-industry markets, mainly by stimulating environmental and social certification of these products [10].

Unexplored biodiversity and high levels of endemism [2, 11], combined with ethnopharmacological knowledge from traditional communities [12], lead to high potential for drug discovery in this region. Some plants from the Neotropics are already booming in the international scenario, and some examples are the fruit pulp from *Euterpe oleracea* (Arecaceae) and *Myrciaria dubia* (Myrtaceae) with antioxidant effects after consumption [13, 14], being a rich source of anthocyanins and vitamin C, respectively; *Peumus boldus* leaves (Monimiaceae) with the alkaloid boldine conferring hepatoprotection [15]; the resin extracted from *Copaifera officinalis* (Fabaceae) with anti-inflammatory properties [16, 17]; *Bertholletia* *excelsa* (Lecythidaceae) as fatty acids and selenium source in food supplementation [18]; stimulant effects from caffeine-rich drinks prepared with *Paullinia cupana* (Sapindaceae) fruits and *Ilex paraguariensis* (Aquifoliaceae) leaves [19, 20]; *Annona muricata* (Annonaceae) with antitumor activity conferred by the presence of acetogenins [21]; and *Stevia rebaudiana* (Asteraceae) as a stevioside source, a low calorie sweetener used as safe substitute for sugar [22].

Despite capable of controlling core technologies in drug discovery and development in a geographical region of megabiodiversity, South American scientists and government officials face significant delays in finding and developing new bioactive compounds from the Neotropical flora. This scenario is at least in part due to a lack of cooperation among South American organizations as well as among those with more industrialized countries [1]. There are many barriers for natural products of the Neotropical region to reach international markets, including historical, cultural, regulatory, scientific, and economic [1].

3 Bioactive Alkaloids from South American Rubiaceae Plants

South American biomes are among the most diverse of the world. Besides the well-known Amazonian biodiversity, other tropical and subtropical ecosystems contain very rich plant communities. One of these is the Brazilian Atlantic Forest, today reduced to a fraction of its original distribution, making it very important to engage in cataloguing, evaluating, and prospecting its species in a sustainable way [23].

One plant family with considerable chemical potential is the Rubiaceae. It is a large plant family, with 13,143 registered species distributed in 611 genera [24]. Rubiaceae species are distributed mostly in tropical and humid locations. Plants belonging to this family can be easily recognized in the field by their opposite leaves, interpetiolar stipules, and inferior ovary (Fig. 5.1). The most widely known Rubiaceae representative is the coffee plant, *Coffea arabica*. Its main active constituent, caffeine, is one of the most extensively studied plant compounds [25].

Bioactive molecules are often isolated from Rubiaceae species [26–33]. In South America, two genera are of special interest due to the bioactivities of their phytochemicals along with their importance to local human populations: *Uncaria* and *Cinchona*.

3.1 *Uncaria* Genus

The *Uncaria* genus includes 34 species of vines and shrubs and is a rich source of medicinal natural products. *Uncaria* plants are traditionally used to heal wounds, headaches, and ulcers and to fight infections [34]. Over 150 compounds have been isolated from *Uncaria* tissues, and the most prominent of those are alkaloids.

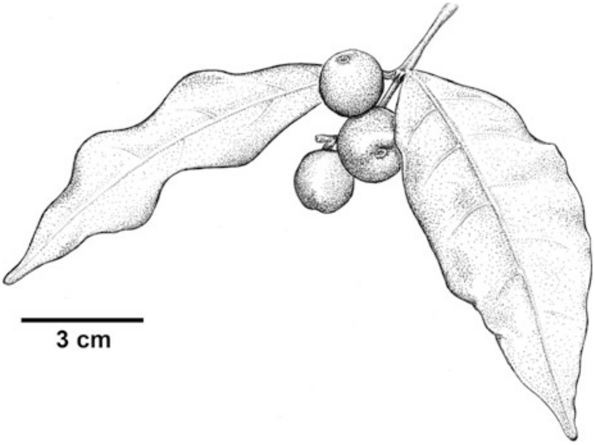


Fig. 5.1 Fruit-bearing shoot of *Psychotria brachyceras*

Numerous bioactive alkaloids have been isolated from *Uncaria* [35–47], but two species yielded a considerable number of active compounds: *U. rhynchophylla* and *U. tomentosa*.

Extracts of *U. rhynchophylla* have been shown to be anticonvulsive, antioxidant, neuroprotective, and cytotoxic [34, 38–40]. Some of these activities are attributed to the tetracyclic oxindole alkaloids rhynchophylline and isorhynchophylline. They have been shown to inhibit NMDA receptors [41]. These receptors are transducers of postsynaptic signals in the central nervous system of mammals. Inhibitors of NMDA receptors could be used to treat disorders caused by excessive activation of this pathway, like cerebral ischemia, Parkinson's, and Huntington's diseases [42].

U. tomentosa is the richest source of molecules among *Uncaria* plants, with over 50 compounds isolated [43]. The species is endemic to Central and South America and is known as "uña-de-gato" (cat's claw) by Peruvian populations, which use extracts from *U. tomentosa* bark for a variety of illnesses, from viral infections to arthritis.

Extracts of *U. tomentosa* have been shown to have anti-inflammatory, antioxidant, antiviral, immunomodulatory, and cytostatic activities [43–50]. Some of these effects appear to be derived from alkaloids. Kurás et al. [48] tested alkaloid-rich and alkaloid-free extracts from *U. tomentosa* for mitosis inhibition and observed that both fractions were cytostatic, with a stronger activity of alkaloid-rich extracts. In a similar study, *U. tomentosa* alkaloids were shown to have antiviral properties on dengue virus infections, as well as immunomodulatory activity [46]. However, alkaloids seem not to participate on antioxidant and anti-inflammatory activity of cat's claw extract [47]. In fact, immunostimulant activity of the alkaloid fraction can even counteract the anti-inflammatory polar fraction [51].

3.2 *Cinchona* Genus

Cinchona plants yielded a crucial phytochemical resource to nineteenth century European people involved in trade and colonization of tropical lands – quinine [52]. Before quinine, malaria was a major health issue in the new world, lowering quality of life by causing severe discomfort and even death. Quinine is an alkaloid present in the bark of several *Cinchona* species known as "quina quina" by native populations. It is most concentrated in *Cinchona calisaya*; however, the most widely known "quina quina" plant became *Cinchona officinalis* (*officinalis* means "medical herb"). Besides quinine, *Cinchona* plants produce other antimalarial alkaloids, namely, quinidine, cinchonine, and cinchonidine. *Cinchona* alkaloids can be found in other Rubiaceae genera as well, as described for *Remijia peruviana* [53].

Cinchona alkaloids have other bioactivities besides antimalarial. Quinine has been used to treat cramps [54]. Cinchonine is an inhibitor of MDR [55]. MDR is a detoxification mechanism present in certain cancer cell lines that renders them less sensitive to chemotherapeutic medications. Administration of cinchonine along with chemotherapeutic agents would result in better efficiency of treatment on MDR cancer cells.

3.3 Brazilian Medicinal Plants

Traditional medicine in Brazil relies on hundreds of plant species, and for a great number of them, standardized preparations are recorded in at least one of the five editions of Brazilian Official Pharmacopeia [56–60]. The first edition, published in 1929, presents 713 monographs about medicinal plants. This number falls to 193 in the second edition, published in 1956, and then to 23 in the third edition (1977). This decrease in the number of monographs about medicinal plants coincides with increased numbers of monographs on synthetic drugs and with a time of accelerated industrialization of the country [61]. The fourth edition published in 1996 has 44 monographs on medicinal plants and the fifth edition, published in 2010, has 53.

Eight Rubiaceae species appear in the first four editions as medicinal plants: *Remijia ferruginea, Borreria centranthoides, Chiococca brachiata, Cephaelis ipecacuanha, Cephaelis acuminata, Cinchona calisaya, Cinchona succirubra*, and *Cinchona pubescens*. The first four species are native of the Brazilian flora, and the others are common in South American tropical forests. In the fifth edition, the only Rubiaceae is *C. calisaya*, for which the powder from bark tissues is characterized and protocols for its chemical analysis (mainly the alkaloids) are described. The bark from *Cinchona* species and *R. ferruginea* is used for treatment of fever and malaria [62]. *B. centranthoides* root decoction is used to treat problems of the urinary tract [63]. *C. brachiata* root preparations have diuretic properties and ameliorate cutaneous infections [64]. Finally, both *C. ipecacuanha* (also known as *Psychotria ipecacuanha*) and *C. acuminata* dried roots yield the drug "ipecac," which is a potent emetic, used in the treatment of gastrointestinal diseases, intoxications, and also as expectorant and vasoconstrictor [65, 66].

4 The *Psychotria* Genus

Psychotria is the largest Rubiaceae genus and one of the most species-rich genera of flowering plants, with 1,834 species [24, 67, 68]. *Psychotria* species are mostly understory shrubs, i.e., small woody plants growing in lower strata of dense forests. Taxonomy of *Psychotria* plants is complicated since the genus is a grouping of Rubiaceae species, many of which lacking distinctive features that would otherwise classify them in other genera [67]. Since morphological traits alone do not provide enough basis for classification, data from DNA [68] and phytochemicals [69] have been useful for the systematic analysis of the taxa.

South American *Psychotria* belongs to the subgenus Heteropsychotria that comprises Neotropical species, being the presence of MIAs a constant; with exception of *P. colorata* and *P. glomerulata*, polyindoline-type alkaloids are not found in Neotropical P*sychotria*. Southern Brazilian species MIAs have the peculiar characteristic of retaining a glucoside residue. *P. borucana* and *P. ipecacuanha* are separated from the Heteropsychotria clade, and both share the presence of dopamine-iridoid alkaloids instead of tryptamine-iridoid (MIAs) [67], similarly to *P. klugii* [70]. *P. stachyoides* MIAs isolated from leaves show unusual skeleton formed by the condensation through N-1, and not N-4 [71], similar to *P. correa* [72].

Ethnobotany studies may provide clues to direct the prospection of bioactive molecules of interest. Among reports of traditional uses, *P. papantlensis* in Mexico is used for treating inflammatory diseases [73]; *P. tenuicaulis*, *P. pilosa*, *P. poeppigiana*, *P. marcgraviella*, and *P. carthagenensis* in Peruvian Amazon are used for different purposes such as uterine hemorrhage, vomits, fever, diarrhea, spasms, painkiller, sedative, and health tonic [74, 75]; in a local community in Papua New Guinea, *P. microlabastra* is used for malaria treatment, stomachache, boils, sores, and venereal diseases [76]; and in a West Indonesian archipelago, *P. malayana* is used for skin diseases [77].

Some *Psychotria* species, such as *P. microlabastra* [76], *P. capensis* [78], *P. rostrata* [79], and several species from the Atlantic Forest [80], have known antibiotic properties. Antiviral (*P. serpens*) [81], antiviral/antifungal, anti-inflammatory (*P. hawaiiensis* [82] and *P. insularum* [83]), and antiparasitical activities (*P. camponutans* [84] and *P. klugii* [70]) have also been reported. Other bioactivities include cytotoxic properties (*P. prunifolia*) [85] and regulation of human growth hormone release (*P. oleiodes*) [86]. The active molecules produced by *Psychotria* species include pigments [87], peptides [88], naphthoquinones [89], benzoquinones [90], and alkaloids [91].

Psychotria species that are well known for their bioactive compounds include *P. ipecacuanha*, producing the isoquinoline alkaloid emetine, with well-characterized emetic effect (inducer of vomiting) [92]. Isolated emetine is also effective in the treatment of amoebiasis [93]. The alkaloid, however, has severe side effects, like muscle weakening and cardiac damage.

Another *Psychotria* species worth noting is *P. viridis*. Leaves of this plant, along with the vine *Banisteriopsis caapi*, are used in the preparation of ayahuasca, a hallucinogenic beverage that is consumed in some indigenous-derived religious rituals [94]. The decoction made from these two species is rich in the β-carboline alkaloid harmine, produced by *B. caapi*, and in the alkaloid DMT, from *P. viridis*. Harmine is thought to inhibit the action of monoamine oxidases, enzymes that process DMT in the liver and brain. DMT itself is a strong psychoactive, and harmine also has activity by itself. When in combination, the effect is amplified through increased availability of DMT caused by monoamine oxidase inhibition by harmine.

Plant extracts are continuously being tested for bioactivity, and in cancer research, major enterprises for screens of antiproliferative activity are sometimes carried out. In one of these screens, nearly 70,000 species of terrestrial plants were tested against leukemia cell lines [95]. The authors labeled plant genera as “hot” if they yielded three or more plant species that showed activity in the screens. Six *Psychotria* species gave active extracts, so *Psychotria* earned the title of “hot genus” [95]. This type of approach, extensive analysis of plant extracts in the search of bioactive phytochemicals, is very expensive, time-consuming, and labor-intensive, but can unveil precious new drug leads, as was the case with paclitaxel [96].

Another type of approach, the ethnopharmacological, can abbreviate a considerable amount of effort by, instead of testing extracts of all plant materials available, concentrating the experimentation on plants used by traditional communities. The knowledge accumulated by these people, who lived for hundreds, even thousands of years in contact with native plants and often depending on them for health care and other needs, is taken as a starting point for bioactive phytochemical prospecting. This approach has resulted in several important discoveries, including the antitumoral alkaloids vincristine and vinblastine from *Catharanthus roseus*, the antimalarial artemisinin from *Artemisia annua*, and the neuroactive reserpine from *Rauwolfia serpentina* [97].

An ethnopharmacological survey was conducted in order to chemically investigate a *Psychotria* species, *P. colorata*, for analgesic activity. The plant is used by the “caboclos,” local rural communities in the state of Pará, northern Brazil, to alleviate pain. The molecules discovered in this plant were the first of a fruitful endeavor: phytochemical prospection of *Psychotria* species.

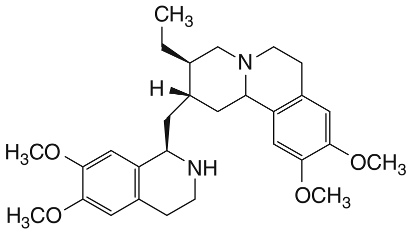
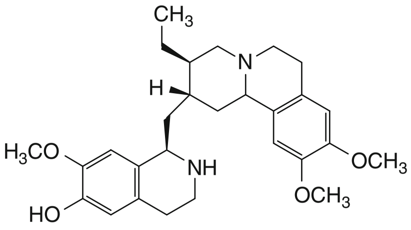
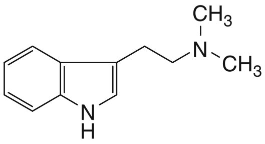
4.1 Bioactive Alkaloids from South American *Psychotria*

South American *Psychotria* are a rich source of alkaloids displaying various bioactivities. Examples are shown in Table 5.1.

4.1.1 *Psychotria ipecacuanha* (Brot.) Stokes: Emetine and Cephaeline

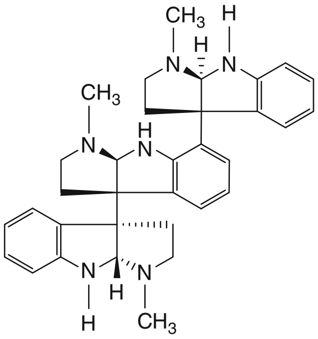
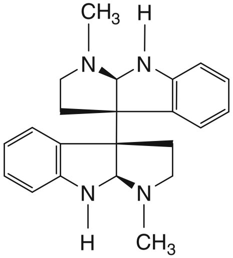
P. ipecacuanha, native to South and Central America, is a well-known species used in therapy since the early seventeenth century due to emetic and expectorant activity and later as a medicine for amebic dysentery [98], being widely used as ingredient in pharmaceutical industry. Traditional communities use this plant for intoxication treatment due to its emetic effect [99], as well as stimulant and “antidote to opium” [100].

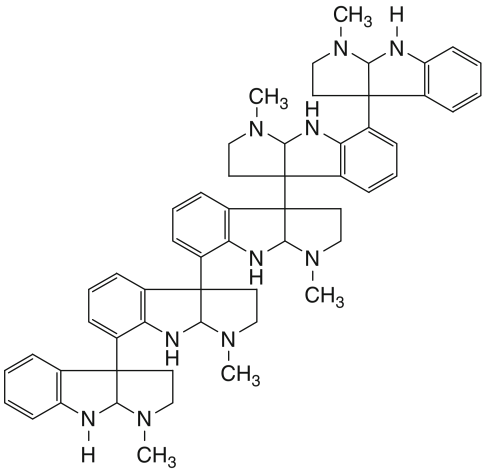
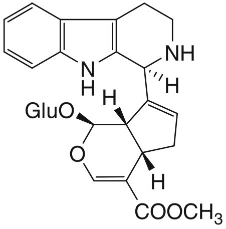
Table 5.1 Bioactive alkaloids from South American *Psychotria*

Alkaloid	Structure	Plant	Pharmacological properties	References
Emetine	 CH₃ H N H₃CO H₃CO NH OCH₃ OCH₃	*P. ipecacuanha*	Emetic, cytotoxic	[92, 101]
Cephaeline	 CH₃ H N H₃CO HO NH OCH₃ OCH₃	*P. ipecacuanha*	Emetic	[101]
DMT		*P. viridis*	Psychotropic	[94]

(*continued*)

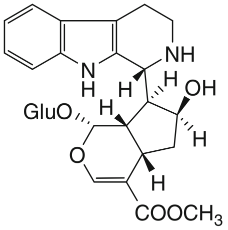
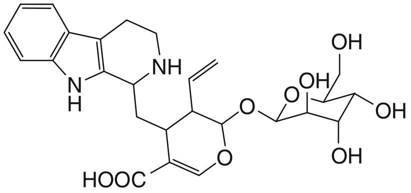
Table 5.1 (continued)

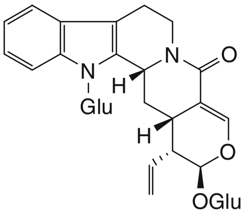
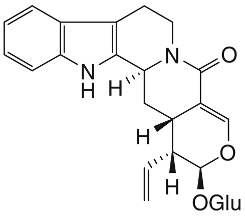
Alkaloid	Structure	Plant	Pharmacological properties	References
Hodgkinsine	 CH$_3$ H N CH$_3$ H N H N N H H N CH$_3$	*P. colorata* *P. malayana*	Analgesic	[118, 119]
Chimonanthine	 CH$_3$ H N N N N H CH$_3$	*P. colorata*	Analgesic	[120]

Psychotridine		*P. colorata*	Analgesic	[121]
Psychollatine		*P. umbellata*	Anxiolytic, antidepressant, antipsychotic, amnesic, antioxidant, analgesic	[125–128]

(*continued*)

Table 5.1 (continued)

Alkaloid	Structure	Plant	Pharmacological properties	References
Brachycerine	 NH N H H H OH GluO H H O H COOCH3	*P. brachyceras*	Antioxidant	[131–133]
Strictosidinic acid	 NH N H OH OH O O OH O OH HOOC	*P. myriantha*	Analgesic, antipyretic, anti-inflammatory	[136, 138]

GPV		*P. leiocarpa*	Antioxidant	[Unpublished]
Strictosamide		*P. bahiensis* *P. nuda* *P. myriantha* *P. suterella*	Anti-inflammatory	[138]

This plant contains ipecac alkaloids, comprising the monoterpene isoquinoline alkaloids emetine, cephaeline, and psychotrine being the main components responsible for emetic properties [101]. In addition, emetine is also used for amebic dysentery treatment [102], displaying cytotoxic activity, inhibiting protein synthesis, and with potential applications in drug-induced apoptosis [92]. Currently, synthetic analogues of emetine with less adverse effects are used in the treatment of amoebiasis [93]. These ipecac alkaloids are present in all parts of the plant but in a greater quantity in the roots [92, 93, 98–103]. Initial ipecac alkaloid biosynthesis is analogous to other MIA, with secologanin and dopamine, instead of tryptamine, condensation. After some methylations through *O*-methyltransferases, cephaeline is generated; and downstream, with the addition of another methyl group, emetine is formed (Scheme 5.1) [104].

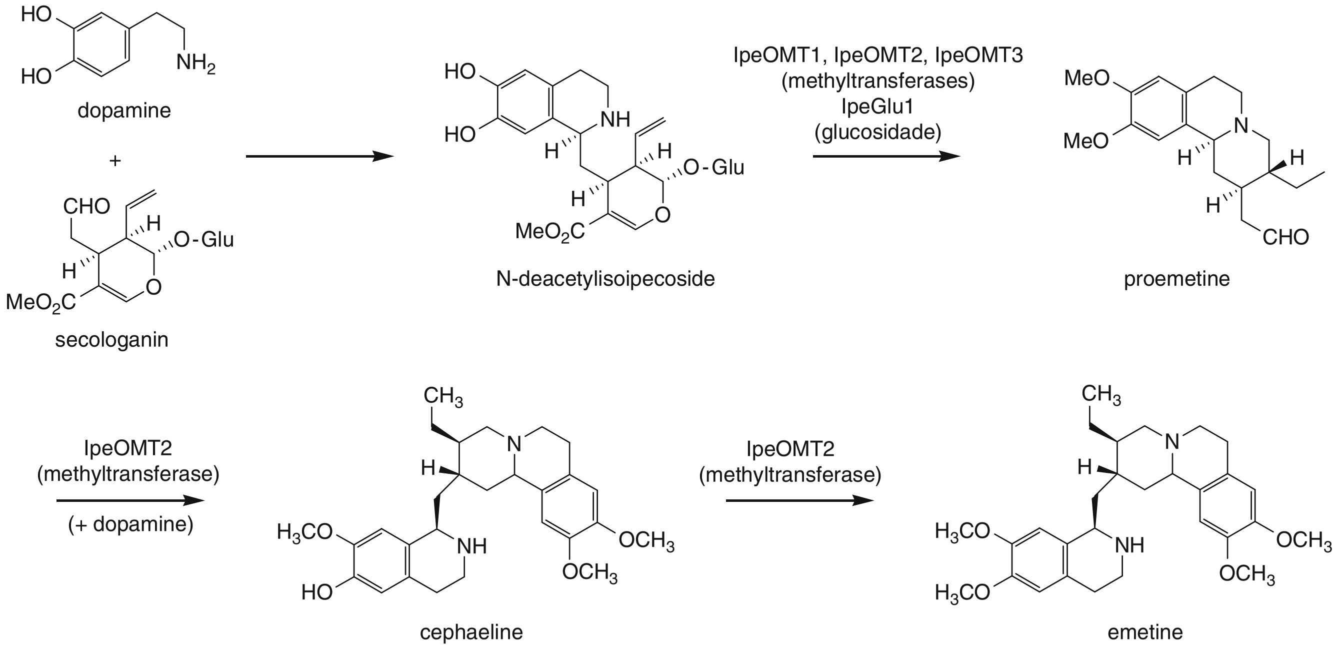
4.1.2 *Psychotria viridis* (Ruiz & Pav.): DMT

P. viridis grows naturally in Amazonian tropical forests in Central and South America, and the leaves are used as one of the main components in the preparation of the hallucinogenic drink "ayahuasca." This plant is a rich source of the psychedelic indole-alkaloid DMT [94] in the mixture that also contains *β*-carboline alkaloids provided by *Banisteriopsis caapi* (Malpighiaceae), mainly harmine, harmaline, and tetrahydroharmine, that besides psychoactive properties, act as reversible MAOIs. MAO acts as a detoxifying enzyme [105], and when inhibited by MAOIs, DMT inactivation is prevented in the gut [106] enabling it to reach the CNS site of action, affording significant psychotropic effect. DMT biosynthesis is relatively simple and is summarized in Scheme 5.2.

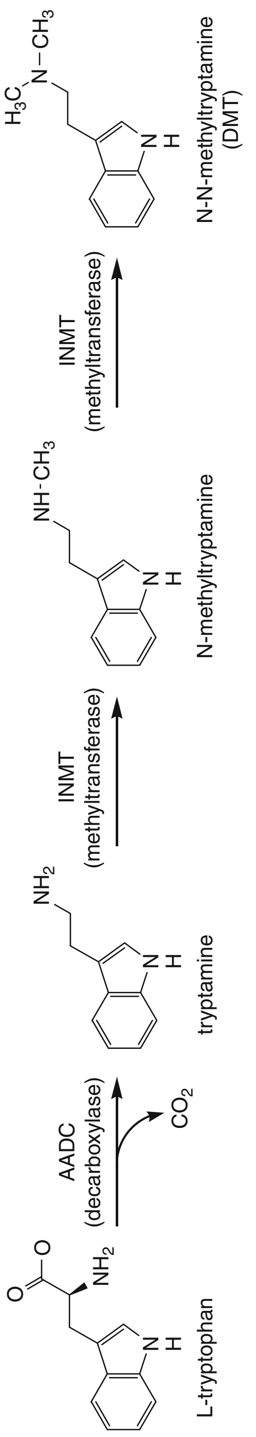
Ayahuasca recent popularization led to several debates including mental health issues and drug abuse, as well as religious freedom [107, 108]. In Brazil, the religious use of the ayahuasca drink is free since January 2010 [109]. Interestingly, *P. carthagenensis* is regarded as a substitute for *P. viridis*, although no evidence of alkaloids in plants [110] or activity of key enzymes of secondary metabolism in cell cultures were detected in *P. carthagenensis* from Southern Brazil [111]. However, the possibility of its presence in individuals from other geographical origins cannot be ruled out before extensive analyses are carried out.

4.1.3 *Psychotria colorata* (Willd. ex Roem. & Schult.) Müll. Arg.: Hodgkinsine, (+)-Chimonanthine, meso-Chimonanthine, and Psychotridine

An ethnobotanical survey identified several species used as painkillers among traditional rural communities from Brazilian Amazon, and different parts of *P. colorata* were reported for therapeutical uses among the Amazonian traditional rural communities, being flowers used for earache treatment and roots/fruits for abdominal pain [112]. Similar qualitative composition is present in flowers and leaves of *P. colorata* [113, 114], being the highest content of alkaloids found in flowers (0.7% DW) followed by leaves (0.6% DW) and lower concentrations in roots (0.05% DW) [115].


HO
HO
NH_2
dopamine
+
CHO
H
O-Glu
H
MeO_2C
O
secologanin
HO
HO
NH
H
H
O-Glu
H
MeO_2C
O
N-deacetylisoipecoside
IpeOMT1, IpeOMT2, IpeOMT3
(methyltransferases)
IpeGlu1
(glucosidade)
MeO
MeO
N
H
H
H
CHO
proemetine
IpeOMT2
(methyltransferase)
(+ dopamine)
CH_3
H
N
H_3CO
HO
NH
OCH_3
OCH_3
cephaeline
IpeOMT2
(methyltransferase)
CH_3
H
N
H_3CO
H_3CO
NH
OCH_3
OCH_3
emetine

Scheme 5.1 Emetine biosynthesis


L-tryptophan
O
O
NH2
N
H
AADC
(decarboxylase)
CO2
tryptamine
NH2
INMT
(methyltransferase)
N-methyltryptamine
NH-CH3
INMT
(methyltransferase)
N-N-methyltryptamine
(DMT)
H3C
N
CH3

Scheme 5.2 *N*,*N*-dimethyltryptamine biosynthesis

Preliminary tests with *P. colorata* extract from flowers indicated alkaloids as the major responsibles for its analgesic effect, probably with opioid-like activity [113], and later, hodgkinsine, a pyrrolidine indole alkaloid, was identified as the major component among several pyrrolidine indole and quinoline alkaloids present in the flowers of this species [116]. Hodgkinsine was first isolated from *Hodgkinsonia frutescens* (Rubiaceae) [117], and it is a potent analgesic, with results comparable to morphine in hot-plate and tail-flick models [118, 119]. Both tests measure the animal response time to heat stimulus. Hodgkinsine is also present in *P. muscosa* [115] and in the Indonesian *P. malayana* (also as the major alkaloid) [77].

The pyrrolidine indole alkaloids (+)-chimonanthine, *meso*-chimonanthine, and psychotridine also showed analgesic activity [120, 121], but the *P. colorata* quinoline alkaloids did not present positive results to the nociceptive tests, suggesting strong structure-activity relationship [115]. Capsaicin (the active component in pepper)-induced pain models suggest alkaloid action on opioid and glutamate receptors [119–121].

4.1.4 *Psychotria umbellata* Vell.: Psychollatine

P. umbellata is a woody species that occurs in the Brazilian Southern Coast Atlantic Forest [122]. Due to promising results with *P. colorata*, investigations of Southern Brazilian subtropical species of *Psychotria* were performed, including *P. umbellata*; a dose-dependent opioid-like analgesic activity was described for the foliar ethanolic extract from the latter species [123].

Psychollatine is a monoterpene indole alkaloid, the major alkaloid from *P. umbellata*, being restricted to shoots (nearly 4% of dry weight in leaves). The alkaloid structure suggests an alternative biosynthetic pathway, STR-independent, by the condensation of tryptamine and a geniposide derivative [124].

The alkaloid accumulation seems to be regulated by developmental stage with the highest levels in inflorescences and the lowest in seeds, followed by fruit [125], suggesting a defense role.

In light/dark and hole-board models for anxiety (which basically measure inhibition on mice exploratory behavior in the presence of different stimuli), the alkaloid presented anxiolytic activity (7.5 and 15 mg kg^{-1}); in a forced swimming test, the antidepressant effect (3 and 7.5 mg.kg^{-1}) was comparable to that of imipramine (15 mg kg^{-1}) and fluoxetine (20 mg kg^{-1}). Antipsychotic and amnesic effects were also observed in mice models presumably by alkaloid modulation of serotonergic (5-$HT2_{A/C}$) [126] and NMDA glutamate [127] neurotransmitter systems. Administration of higher doses of the alkaloid (100 mg kg^{-1}) impaired the acquisition of learning and memory consolidation [126].

Other properties of psychollatine include antimutagenic properties in *Saccharomyces cerevisiae* [128] and antioxidant activity against a variety of ROS [125, 128]. The alkaloid was also capable of improving survival of antioxidant defense-deficient strains of yeast upon oxidative stress [128].

Due to its properties, psychollatine has high pharmacological potential. Postharvest stability experiments showed high stability of the molecule in temperatures below 65°C [73], highlighting an extra useful feature in future alkaloid applications.

4.1.5 *Psychotria brachyceras* Müll. Arg.: Brachycerine

P. brachyceras is native from tropical and subtropical forests of Brazil [129]. Brachycerine is a MIA, present only in shoots with levels generally varying from 0.02% to 0.1% of DW [130] reaching up to 1.8% DW under UV radiation stress [131]. Its biosynthetic pathway differs from usual MIAs, being the terpenic fraction derived from epiloganin instead of secologanin. Epiloganin is condensed with tryptamine through a putative STR-like enzyme. Antioxidant activity against most physiologically relevant ROS in plants is a feature of this alkaloid [131–133]. Unlike *Nicotiana sylvestris*, in which nicotine is whole-plant-induced [134], brachycerine is accumulated only in damaged sites. In vitro assays showed that brachycerine has antimutagenic activity in *S. cerevisiae* [132]. Other interesting results were nonspecific analgesic activity of *P. brachyceras* leaf ethanolic extract [135].

4.1.6 *Psychotria myriantha* Müll. Arg.: Strictosidinic Acid

P. myriantha is common in Southern Brazilian forests [129] and produces the MIA strictosidinic acid, being the major alkaloid present in *P. myriantha* leaves. Oral administration of strictosidinic acid in mice has analgesic and antipyretic effects [136], and it is also an efficient inhibitor of PMN chemotaxis in vivo [137], having anti-inflammatory properties [138].

Opioid-like analgesic activity was observed after intraperitoneal injection of this alkaloid in hot-plate and tail-flick models [137]. Also, intraperitoneal injection of 10 mg kg^{-1} reduces 5-HT and DA levels (followed up by an increase of 5-HIAA, DOPAC, and HVA levels), showing relevant effect in the central nervous system [139]. Opioid-like denomination suggests morphine-similar effect, being blocked with naloxone-like antagonists.

4.1.7 *Psychotria leiocarpa* (Cham. & Schltdl.): GPV

P. leiocarpa is native to Argentina, Paraguay, and Brazil [129]. The MIA GPV is the major alkaloid present in the ethanolic extract of this plant, and it is detected only in shoots being the highest levels in leaves, reaching up to 2.5% DW [140]. Nonspecific analgesic activity from foliar crude ethanolic extract was reported in tail-flick test [135]. An uncommon configuration for MIAs, with a glucose residue attached to the indole ring nitrogen, was first described in GPV.

4.1.8 *Psychotria suterella* Müll. Arg.: Lyaloside, Naucletine, and Strictosamide

P. suterella is found in the coast of tropical forests in Southern Brazil [122]. Lyaloside, naucletine, and strictosamide are MIAs present in *P. suterella* leaves, being absent in roots. In the tail-flick assay, no analgesic effects of the alkaloid extract obtained from leaves (100 or 300 mg kg^{-1}), or isolated lyaloside (10 or 30 mg kg^{-1}) were observed; in addition, the higher doses of extract and lyaloside lead to convulsions followed by death [141].

4.1.9 *Psychotria nuda* (Cham. & Schltdl.) Wawra: Strictosamide

P. nuda is common in tropical forests from Southern Brazil [142]. The MIA strictosamide is the major alkaloid from *P. nuda* [143], being previously isolated from other plants [144]. It is considered an important biosynthetic precursor of the antitumor alkaloid camptothecin, from *Camptotheca acuminata* [145]. From South American *Psychotria* species, strictosamide was isolated from *P. leiocarpa*, *P. suterella*, *P. bahiensis*, and *P. myriantha* [137, 141, 146, 147].

The alkaloid-rich extract obtained from *P. nuda*, as well as isolated strictosamide (major alkaloid from *P. nuda*), shows in vitro antichemotactic activity on PMN in vivo [138]. PMNs are recognized as the first cells to arrive at the sites of inflammation [148], these results being related to anti-inflammatory effects.

4.2 Regulation of *Psychotria* Alkaloid Biosynthesis

Alkaloid biosynthesis can be a very complex and highly regulated biological process. It may encompass various cell types [149], intensify in certain developmental stages, and be influenced by circumstantial factors, like nutritional status or stress [150–152]. Some alkaloid biosynthetic pathways, due to their importance, are meticulously studied, as is the case with *Catharanthus roseus, Nicotiana tabacum, Papaver somniferum*, and many others. The main goal of alkaloid (and other phytochemicals) research is to establish the genes and enzymatic steps needed for building a valuable compound, as well as what influences its production rate. This information could be used, for example, to manage phytochemical exploration of intact plants or to engineer controlled bioprocesses for their synthesis (e.g., bioreactors) [153].

Despite progresses in alkaloid research, some of the most demanded alkaloids are still produced in large scale by land-cultivating alkaloid-containing plants and purifying the compounds, in a very expensive and laborious process. The commonest first attempt for switching production into a more efficient and controlled way is to establish in vitro tissue-culture protocols for the species of interest. There are many examples of strong inhibition or total lack of biosynthesis of alkaloids and other phytochemicals in such conditions [154], including for *Psychotria* species [125], reinforcing the evidence for tight regulation of alkaloid biosynthesis and requirement of specialized tissues for its completion. This underlines the need to investigate distribution of alkaloids among tissues of adult plants as well as to determine the influence of various treatments on alkaloid production.

Seedlings of in vitro cultured *P. leiocarpa* were exposed to different sucrose concentrations in the medium along with presence or absence of white light. GPV production, restricted to shoots, was strongly induced by light and inhibited by sucrose supply, indicating that photoautotrophic metabolism plays a major role in alkaloid accumulation [140]. GPV also seems to be developmentally regulated since its content in older (150 days) seedlings is significantly higher than in younger ones (100 days). The alkaloid is most concentrated in leaves and reproductive structures [140].

Brachycerine accumulation in *P. brachyceras* also is restricted to shoots. It is most concentrated in inflorescences (0.3% over dry weight) but is also present in fully expanded leaves (0.18%), stems (0.18%), younger leaves (0.12%), and fruit pulp (0.04%). Leaf brachycerine concentration in adult field-grown plants varies seasonally, with lower concentrations in summer months. In vitro germinated seeds yield seedlings with alkaloid accumulation as observed in leaves of adult plants [155].

Brachycerine accumulation is highly responsive to environmental signals. Mechanic damage and also jasmonic acid, a hormone related to signaling of herbivory [156], induce brachycerine production in cuttings [155]. The same happens with UV radiation but with higher fold induction. Brachycerine is a strong antioxidant, and its up regulation in stress situations suggests a protective role for it *in planta* [131].

For *P. umbellata*, the opposite dynamic profile is observed. The plant alkaloid content does not respond to almost any environmental stimuli. Psychollatine, however, accumulates constitutively at high levels in leaves (1–4% of dry weight). The alkaloid is strongly accumulated in inflorescences and fruit [125]. An in vitro somatic embryogenesis protocol was established for *P. umbellata*. It employs a hormonal balance and physical variable manipulation, notably light, that is capable of driving direct embryo development from somatic cells of rhizogenic calli, resulting, after further growth and acclimation to ex vitro conditions, in fully differentiated plants. *P. umbellata* plants established in in vitro cultures had psychollatine concentrations similar to field-grown shrubs [153].

5 Conclusions

Taken together, data to date indicate that a number of common general features can be identified in MIAs from *Psychotria* of Southern Brazil. These alkaloids are relatively simple structures, which probably represent primitive forms of this class of metabolites, compared to the more elaborate MIAs of *Catharanthus roseus*, for example. The MIAs of Southern Brazilian *Psychotria* species usually retain glucose residues, a characteristic that is not common in most MIAs, but can be useful for pharmacological applications, considering solubility and bioavailability issues. Other features often associated with these alkaloids include antioxidant and antimutagenic properties and high concentration in plant reproductive structures. A shoot-specific distribution and strong dependence on differentiated photosynthetic tissue for biosynthesis are probably connected with the plastid-located source of isopentenyl diphosphate which is needed for synthesizing the terpene moieties required for alkaloid production. Some of these MIAs are formed by condensing tryptamine with unusual non-secologanin terpene moieties (epiloganin derivatives in brachycerine and geniposide aldehyde in psychollatine). This observation implies that new STR-like enzymes are likely involved in the biosynthesis of these particular alkaloids. In addition, different strategies of accumulation are found in these plants and alkaloids, ranging from highly constitutive to strongly inducible upon environmental stimuli. Therefore, it is likely that distinct transcription factors orchestrate the expression of genes encoding enzymes leading to the production of these related alkaloids. Clearly, in spite of significant progress, large efforts are still required to fully understand *Psychotria* alkaloid production and its multitude of applications.

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Ecological Role of Alkaloids

6

Shaily Goyal

Contents

1 Introduction 150
2 Ecological Role of Alkaloids on Plants 151
3 Ecological Role of Alkaloids on Herbivores and Insects 154
4 Ecological Role of Alkaloids on Microorganisms 160
5 Ecological Role of Endophytic Alkaloids 163
6 Conclusion 166
References 167

Abstract

Plant secondary metabolites are well recognized to provide protection against harmful organisms. Alkaloids are one of the most diverse groups of secondary metabolites. They are produced by a large variety of organisms, including bacteria, fungi, plants, and animals. Positive or negative effects of alkaloids can be seen among different herbivores and insects. Alkaloids being toxic in nature help the plants, herbivores, and insects to ward off their enemies or competitors and facilitate their own survival in the ecosystem. Some herbivores and insects sequester them to defend against their own enemy illustrating the flow of alkaloids through different trophic levels. Among plants, alkaloids work as allelopathic compounds, and this property makes some of the plants a potential natural weedicide. This chapter highlights the ecological roles of alkaloids and their possible role in chemical management of pests.

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Keywords
Alkaloids • allelopathy • endophytes • pyrrolizidine alkaloids • quinolizidine alkaloids

Abbreviations

BIA	Benzylisoquinoline alkaloids
MLA	Methyllycaconitine
PA	Pyrrolizidine alkaloids
QA	Quinolizidine alkaloids

1 Introduction

Plant secondary metabolites are structurally highly diverse chemical compounds. They exert various biological effects even at very low concentration and, hence, play an important role in ecological communications. Unlike in the twentieth century, ecologists now are well aware of the impact secondary metabolites (SM) can have through their interaction with the biotic and abiotic environment in which they exist [1]. SM provides protection against harmful organisms like herbivores, insects, and microbes and helps the plant to tolerate stress and to survive in the ecosystem [2, 3]. Such roles of SM are important as the plants have to compete with their competitor plants for light, water, and nutrients. In nature, SM play different ecological functions, e.g., they serve as signal compounds to attract pollinating or seed dispersing insects and animals and can mediate the interactions between symbiotic bacteria and their plant hosts (e.g., rhizobia) [4]. They can also perform some physiological functions such as nitrogen storage, nitrogen transport, and UV protection. As secondary metabolites have group-specific distribution in the plant kingdom, these physiological functions are not common to all but are unique and additional to certain compounds.

Plants are attacked by herbivores and pathogens. They deter both by direct and indirect defenses. These organisms often share the same individual plant, mainly when feeding insects are vectors or their feeding wounds allow establishment of pathogens. Thus, there are many opportunities for direct interactions to affect the fitness and ecology of plants [5, 6]. Plants themselves are not simply passive hosts. They participate truly in these three-way interactions. As part of direct defenses morphological structures or SM are used to inhibit attack by insects and pathogens [7]. With indirect defenses, against herbivores, damaged plants emit volatile compounds that attract the enemies of their enemies [8].

Variations in plant nutrients and herbivore-deterrent plant secondary metabolites (PSMs) across a landscape create a chemically complex environment that may influence the distribution and abundance of animal species [9]. The defensive and nutritional chemistry of not majority, but many plant populations are highly variable [10]. Spatial hotspots of highly palatable or highly unpalatable plants are ecologically important, because the probability of a plant being eaten depends not only on its own characteristics but also on the environment in which it occurs [11]. Plants experience associational defense or associational resistance from unpalatable neighboring plants when herbivores make foraging decisions at the scale of a patch of plants, or by association with palatable neighbors if they act as attractant-decoys when herbivores forage at an individual-plant scale. Some plants can experience associational susceptibility if the association is disadvantageous [12] whether such associational effects occur, and whether spatial patches of trees of similar palatability are of any significance to foraging herbivores thus depends upon the scale of the herbivore's foraging movements [13].

In comparison with flavonoids, the distribution of alkaloids is more restricted, with only 20–30 % of plants (mostly angiosperms) producing this major class of metabolite. A subdivision of the alkaloids with respect to their biosynthetic origin from a particular amino acid precursor (usually phenylalanine/tyrosine, tryptophan, ornithine, lysine, histidine, or anthranilic acid) further restricts the distribution of each subclass. More information about alkaloids types and distribution is available in other chapters of the book. Most alkaloids are physiologically active compounds having a variety of toxic effects on animals and allelopathic effects on plants. Alkaloids with allelopathic properties include aconitine, berberine, caffeine, cinchonine, colchicine, cytisine, ergometrine, gramine, harmaline, hyoscyamine, lobeline, lupanine, narcotine, nicotine, papaverine, quinidine, quinine, salsoline, sanguinarine, sparteine, strychnine, theophylline, and yohimbine [14, 15]. These compounds are originated from aerial parts of plants and released into the environment through foliar leaching by rain, residue decomposition, and debris incorporation. However, some alkaloids of plant origin are produced by roots and are released directly into the soil and have marked effects on positive and negative interactions between plants and soil-borne organisms [16].

2 Ecological Role of Alkaloids on Plants

The absence of secondary metabolites generally does not immediately kill plants but can have profound effects on a species' ecology and evolution. It is often found that particular secondary metabolites are restricted to individual species or narrow sets of species within a phylogenetic group, providing the potential for biochemically mediated behavior almost as diverse as the plant kingdom. Alkaloids strengthen the competitiveness of invasive plant species by inhibiting the growth of neighboring plants (this phenomenon is known as allelopathy). Most of the structural types of alkaloids can exhibit allelopathic effect. Allelopathy in crop plants can help in warding off weeds. In plants, allelopathy is often related to harmful effects on germination and seedling growth [17]. Some authors suggest that allelopathic effects can also contribute to promote shifts in density, dominance, and spatial patterns of plant populations [18, 19]. Thus, allelopathic plants may have a differential role in species coexistence and in forest succession [20, 21].

Soil is arguably the most complex and difficult system to choose for the study of ecological functions of secondary metabolites. However, soil is also the ecosystem in which chemical interactions play the most substantial role, and from where major insights into the role of leaching alkaloids on neighboring plants can be revealed. There are many examples which show the effect of alkaloids on other plants. Some of the earlier studies showed numerous examples of allelopathic effect of Solanaceae family members, e.g., the germination of *Medicago sativa* L. (lucerne) was inhibited by *Solanum tuberosum* extract; the germination of *Triticum aestivum* L. (wheat) was inhibited by seeds of *Solanum melongena* L. (eggplant) and *Nicotiana tabacum*, while a mixture of 200 g of dried plant material from *Lycopersicon esculentum* in 100 mL of water inhibited the germination of *T. aestivum* by 42 %, and significantly reduced radicle and coleoptile growth. Studies on *Solanum rostratum* Dun. showed that fresh, alkaloid containing extract of *S. rostratum* had inhibitory action against symbiotic nitrogen-fixing bacteria (*Rhizobium* species), with a slightly less marked inhibition of the free-living nitrogen-fixing bacterium *Azotobacter*. The inhibition of symbiotic nitrogen-fixing bacteria by this weed could aid in its competition with plants relying on nodulation for their nitrogen supply [22, 23].

Alleopathic studies by Hussain and coworkers [24] showed that *Datura innoxia* significantly inhibited germination and growth of test species by root exudates, aqueous extracts from various aboveground parts, and substances volatilizing from the shoots. Gressel and Holm [25] found that aqueous extracts of ground seeds of *Datura stramonium* (thornapple) inhibited the germination of many crop species. It is generally believed that alkaloids of *Datura* often leach from seeds into the surrounding soil and make the surrounding environment toxic to some plants and may be favorable to others due to decreased competition. *D. stramonium* inhibits germination and root growth of *Helianthus annuus*; the strength of its effects depends on the amount of alkaloid adsorbing clay in the soil [26]. As compared to leached alkaloids, the inhibitory effect can be absent for the endogenously produced alkaloids, as was reported for quinolizidine alkaloids in lupins [27] and colchicines in *Colchicum autumnale*. However, in case of alkaloid sensitive plants, both high and low concentration of alkaloid in soil is harmful. At lower concentration they inhibit or stimulate the growth of radicles and hypocotyls and at higher concentration marked reduction in the germination of seeds can be seen. The presence of *Datura* near wild cucurbits and other members of the complex of starchy seeds used by prehistoric occupants of the Eastern Woodlands [5] suggest that not all crop plants would be deleteriously affected by its proximity. By poisoning weeds in agricultural fields, *D. stramonium* instead might have had a positive effect on the growth of certain crop plants that can tolerate leached alkaloids. Depending on the susceptibility of various cultigens in the Eastern Woodlands to alkaloid poisoning and its deleterious effects on harmful invaders, prehistoric Americans may have left *Datura* as a useful weed.

Another plant with great allelopathic effect is the Barley. It is integrated with other crops to inhibit weed growth which is mediated by alkaloids released from barley plant components and/or exuded from living roots. High allelopathic effectiveness of barley has resulted in its wide adoption as a cover crop in sustainable agricultural systems for weed management. The allelopathic effectiveness varies among the barley cultivars, hence, selection programs might improve the allelopathic potential of new cultivars used for weed management [28]. In barley, alkaloids gramine (*N*,*N*-Dimethyl-3-aminomethylindole) and hordenine (*N*,*N*-dimethyl-4-hydroxyphenylethylamine) confer heritable self-defense capabilities against other plants [29]. In a study, effects of gramine and hordenine on white mustard were studied. The release of alkaloids by barley was quantified by HPLC. The alkaloids had a negative effect on white mustard which included reduction of radicle length and apparent reduction in health and vigor of radicle tips. The evidence of the morphological and primary effects of barley allelochemicals at the levels released by living plants indicates that the biologically active secondary metabolites of barley may lead to a significant role in self-defense by the crop [30]. In an experiment by Ovesi and coworkers [31] 17 Iranian barley cultivars of different developmental stages were undertaken to determine their allelopathic potential, and it was observed that barley leachates and residues decreased emergence and Germination Rate Index (GRI) of wild mustard (*Sinapis arvensis*) as the test plant. Although there were some fluctuations in the GRI value with time, the germination inhibitory effect has decreased as new, higher-yielding cultivars have been released. Taking into account of various laboratory studies on alkaloids, they have proved to play an important role in influencing neighboring plants. But in some cases there are discrepancies between the field and laboratory experiments. For example, in a study, the germination of *Ocimum* (a tropical herb), *Spermacoce* and *Catharanthus* (two tropical, alkaloid-producing plants), and *Cinchona* itself was strongly inhibited by the quinoline alkaloids of *Cinchona*, when applied at concentrations higher than about 0.3 mM. Although the roots of the plants contain high concentrations of these alkaloids (ca. 10 mM), only very low concentrations were found (ca. 0.02 mM) in the soil. Further, upon germination of seeds sown close by the plants, no toxic effects were observed. So, although several studies have reported inhibition of seed germination by *Cinchona* alkaloids under laboratory conditions, such results indicate that this property does not play a role under natural circumstances at realistic concentrations [32].

Similariły, *Lupinus albus* L. was mentioned to have allelopathic effects, limiting the growth of *Spergula arvensis* L., *Rumex obtusifolius* L., and *Chenopodium album* L. Such effect was attributed to the presence of quinolizidine alkaloids in *L. albus*. Some crops such as barley (*Hordeum vulgare* L.) [33] and pearl millet (*Pennisetum glaucum* (L.) R.Br.) [34] were reported to have self-toxicity effects and that type of effect was expected in *Lupinus* species because alkaloids are present in all plant components. However, in a work by Nava and coworkers [35] the occurrence of plants of *L. campestris* in the experimental areas before seed sowing did not affect seed germination. This result suggested that *L. campestris* does not present self-toxicity despite having alkaloids. There are many experiments which showed allelopathic effect of different plants, e.g., alkaloid content of mesquite (*Prosopis juliflora*) leaves showed allelopathic activity against root growth of lettuce (*Lactuca sativa*) and barnyard grass (*Echinochloa crus-galli*) [36]. Caffeine (1,3,7-trimethylxanthine), a purine alkaloid found naturally in over 100 plant species showed to possess phytotoxicity against plant species. A study proved the effect of caffeine on the rooting of hypocotyl cuttings of mung bean (*Phaseolus aureus*) and the associated biochemical changes. At 1,000 μM caffeine concentration, there was a 68 % decrease in the number of roots/primordia per cutting and 80 % decrease in root length. However, no root formation occurred at 2,000 μM caffeine [37]. Research on wheat allelopathy has progressed rapidly from the initial evaluation of allelopathic potential to the identification of allelochemicals and genetic markers associated with wheat allelopathy. Benzoxazinones, a novel class of alkaloids, along with other allelochemicals were identified as biologically active agents conferring weed suppression. Recent advances in metabolomics, transcriptomics, and proteomics will greatly assist in the identification of novel allelopathy genes. Ultimately, the allelopathy genes could be manipulated to regulate the biosynthesis of allelochemicals, thereby resulting in better weed suppression via elevated levels of allelopathic potential in commercial wheat cultivars [38].

3 Ecological Role of Alkaloids on Herbivores and Insects

Plant allelochemicals are nonnutritional compounds, synthesized by a plant species that are able to affect growth, health, and behavior or population biology of another species either as a stimulator or an inhibitor [39]. Most progress in the past decade in understanding how secondary metabolites influence herbivores has come from systems where the plant chemistry is well characterized through partnerships between chemists and ecologists [40, 41]. Up to 90 % of the energy flow through feeding in terrestrial ecosystems occurs at the interface between plant primary producers and animal primary consumers, thus this sector of the system includes many alkaloids produced by plants to repel, reduce, regulate, or redirect the pressure put on them by herbivores (consumers). Most known alkaloids in almost all classes were first isolated from edible parts of photosynthetic plants and have notable effects on the physiology and behavior of animals that normally or potentially feed on these plant parts (leaves, stems, sap, bark, roots, flowers, shoots, fruits, seeds). Among plant secondary metabolites, pyrrolizidine alkaloids (PAs), mainly those with a 1,2-double bond in the necine base moiety (1,2-dehydropyrrolizidines), are one of the best-studied examples of the flux of chemical compounds through trophic levels (In ecology, the trophic level is the position that an organism occupies in a food chain). In natural contexts, PAs are important elements of ecosystem and plant–animal relationships [42]. PAs flow through different trophic levels, suffering metabolic modifications and mediating many ecological interactions. Herbivorous species that are associated with PA-plants may have developed different ways to deal with these potentially toxic compounds. Some species like *Tyria jacobaeae, Zonocerus variegatus, Oreina* leaf beetles, *Mitopus morio*, and many more became PA-specialists and overcame this chemical barrier by detoxifying and incorporating PAs in their tissues [43]. Moreover, they use these toxic compounds for their own benefit as odor signal in intraspecific communication, and as a taste signal in interspecific interactions [44]. The reason behind the tolerance to toxic PAs by the organisms can be that the PAs are almost always stored as their *N*-oxides and not as a free base. Because PA *N*-oxides cannot diffuse freely across biomembranes, they can be easily stored and retained in specific organs or tissues, in general the integuments (as seen in different species of arctiid moths: *Spilosoma lubricipeda, Arctia caja, Phragmatobia fuliginosa, Callimorpha dominula, Diacrisia sannio*, and *T. jacobaeae, Creatonotos transiens*) [45]. In plants, PAs and other toxins occur in low concentrations (usually less than 1 % of the dry weight), so they are assumed to be a cheap defense. Digestibility reducers (e.g., trypsin protease inhibitor) occur in higher concentrations and are more expensive for the plant to produce [46] as the costs of secondary metabolites increase with their concentration [47]. Besides being beneficial traits, defense products can also lead to so-called ecological costs, such as increased susceptibility to other types of herbivores and pathogens, and deleterious effects on pollinators and herbivore predators and parasitoids [48].

The ragwort species, common or Tansy ragwort (*Jacobaea vulgaris*, formerly *Senecio jacobaea*), Marsh ragwort (*S. aquaticus*), Oxford ragwort (*S. squalidus*), and Hoary ragwort (*S. erucifolius*), members of Asteraceae family, are native to Europe, but invaded North America, Australia, and New Zealand as weeds. The abundance of ragwort species is increasing in west-and central Europe. Ragwort species contain different groups of secondary plant compounds defending them against generalist herbivores, contributing to their success as weeds [49]. They are mainly known for containing pyrrolizidine alkaloids, which are toxic to grazing cattle and other livestock causing considerable losses to agricultural revenue. Consequently, control of ragwort is obligatory by law in the United Kingdom, Ireland, and Australia. Cattle confront alkaloid poisoning primarily through the consumption of hay poisoned with *Senecio* material. The livers of mammals can be severely damaged by toxic pyrrols, the breakdown products of PAs, which are formed in the gut. Ragwort is one of the most frequent causes of plant poisoning of livestock. It is responsible for over 90 % of the complaints on injurious weeds in the United Kingdom [50, 51]. PAs also occur in other families, e.g., Boraginaceae and Leguminosae. Besides herbivores and insects, microorganisms are also affected by PAs [52].

For controlling *Senecio* different factors affecting its growth were explored. A study showed that nutrients level in soil can determine the outcome of competition between plants. For example, *Chenopodium album* competes most successfully with the *S. vulgaris* when potassium levels are high [53]. In agriculture, one might want to reduce weed pressure of *Senecio* species by adapting nutrient supply. Nutrients affect both the variety and the levels of PAs in the plant. Nutrient-poor pastures were found to be much more invaded by the weeds *S. jacobaea* and *S. aquaticus* than nutrient-rich pastures [54]. Apparently, the low amount of nutrients caused gaps in the vegetation which enabled propagules to invade the pastures. The effect of nutrients on competitiveness of *Senecio* even depends on its species, e.g., *S. madagascariensis* actually has increased competitive advantage over oats with increasing nitrogen and phosphorus levels [55]. Moreover, improved nutrient status in the field lowers the PA concentration in the *Senecio* plants and thereby pathogen did respond positively to the plants, and such plants are more susceptible to pathogens, though it remains to be seen whether this can improve biocontrol of *Senecio* weeds [52].

The most prominent insect for control of common ragwort is the cinnabar moth (*Tyria jacobaeae*, Arctiidae). The larva of cinnabar moth is specialized on feeding on common ragwort. It uses the PAs contained for host detection, sequestering, and detoxifying them through N-oxidation [43]. The cinnabar moth can have a major impact on the population dynamics of *S. jacobaea*. The caterpillars periodically completely defoliate their host plant and in certain years cause extinction of *S. jacobaea* on a local scale [56]. If the cinnabar moth has been a selective force in the evolution of different PAs of its host plant *S. jacobaea*, we expect that structurally related PAs differ in their effects on the moth. Other insects capable of deterring PA effect are Ragwort stem and crown boring moth (*Cochylis atricapitana*) and Ragwort plume moth (*Platyptilia isodactyla*) [51]. Toxins are rare within natural communities, but they exert profound effects on species interactions at multiple trophic levels and thus could function in keystone roles [57].

In a study, induced direct defenses in wild populations of the alpine plant *Adenostyles alliariae*, a species that possesses constitutive chemical defense (pyrrolizidine alkaloids mainly seneciphylline and senecionine) and specialist natural enemies (two species of leaf beetle, *Oreina elongata* and *Oreina cacaliae*, and the phytopathogenic rust *Uromyces cacaliae*), were tested. The host plant suffers a high proportion of leaves consumed by leaf beetles, and infection by the phytopathogenic rust. *O. cacaliae* spends the entire reproductive season on *A. alliariae*, whereas *O. elongata* also feeds on *Cirsium spinosissimum*. It was observed that the beetles were not deterred by the PAs in their host, and in fact sequestered them for their own defense. Such plants possesses inducible resistance involving the jasmonic acid and salicylic acid pathways that is capable of reducing the rate of beetle and rust attack that were undeterred by the pyrrolizidine alkaloids produced by the plant [11].

During the study on other specialist organisms, Van Dam and coworkers [58] observed adults of the monophagous weevil, *Mogulones cruciger* (Curculionidae) feeding preferentially on young leaves of *Cynoglossum officinale* (Boraginaceae) with a high PA (heliosupine, echinatine, and 30-acetylechinatine) content. Concerning the adult stage, PAs were demonstrated to be an oviposition stimulant to females of *T. jacobaeae* and the danaine butterfly, *Idea leuconoe*. In 1982, Eisner demonstrated for the first time that PAs were involved in the chemical defense of warning colored insects. He showed that the orb-weaver spiders *Nephila clavipes* (Nephilidae) and the domesticated scrub jays *Aphelocoma coerulescens* (Corvidae) rejected adults of the arctiid moth *Utetheisa ornatrix* as a food source, which contain PAs sequestered from their larval host plants, *Crotalaria* spp. When the larvae of *U. ornatrix* fed on artificial diet without PAs, the adults were predated by the orb-weaving spiders [59]. Cardoso [60] showed that mealworms treated with monocrotaline were tasted and afterward visually rejected by the pileated finch, *Coryphospingus pileatus* (Emberizidae). Thus, the unpalatability was learned, since in subsequent trials the birds started to reject visually palatable prey with the same coloration as the unpalatable ones. The insectivorous bat *Eptesicus fuscus* (Vespertilionidae) is also deterred by PAs present in arctiids. This bat species learned to associate the sounds emitted by PA-containing moths with unpalatability, thus using auditory cues signaling aposematism [61]. The protection conferred by these alkaloids is not totally effective. Tanager birds (*Pipraeidea melanota*, Thraupidae) have been found preying heavily on distasteful species of ithomiine butterflies, by discarding the cuticle and ingesting the abdominal contents, which is suggested to have a low PA content. In the overwintering sites of the danaine butterfly, *Danaus plexippus* in Mexico, these butterflies were preyed upon by the mouse *Peromyscus melanotis* (Cricetidae) that presented the same predatory behavior as the tanager birds [62]. The PA-derivatives, dihydropyrrolizines and b- and c-lactones have a sexual function as pheromones in males of ithomiines, danaines and arctiids. In these butterflies, females choose mate depending upon the amount of PAs on their bodies. This is ecologically significant, since males transfer PAs to females during mating. In turn females transfer PAs to the eggs, conferring protection for both [63]. These examples depict the flow of PAs through different trophic levels. Further example of tritrophic interaction can be seen in Ladybirds (*Coccinella*). These insects sequestered PA from *Aphis jacobaeae* feeding on PA rich *S. jacobaea*. It is likely that in ladybirds, besides the endogenously produced coccinellines the PA also serve as chemical protectants.

Some plants contain a diverse array of alkaloids that deter vertebrate herbivores. However, mammalian folivores have evolved a complex of physiological and behavioral strategies to counter these compounds, leading to the development of an "evolutionary arms race." Many alkaloids exhibit a bitter or pungent taste for vertebrates and a bitter or pungent diet is normally instinctively avoided [64]. Unlike most mammals, insect herbivores are more selective feeders. The majority of insects are specialized feeders of some sort, specializing on particular plant species, genera, or families or on particular plant organs [65]. As for example, lupins occur in two varieties, one which is alkaloid free (sweet lupins) and the other which is alkaloid rich (bitter lupins). They produce quinolizidine alkaloids (QA) in leaf chloroplasts and export them via the phloem all over the plant where they accumulate in epidermal tissues and especially in reproductive organs. Aphids passively feed on sap of phloem vessels in plants. Aphid like, *Myzus persicae* only suck sap from phloem of "sweet" lupins but not from alkaloid-rich varieties with high alkaloid contents in the phloem. Also many other animals, from leaf miner flies (Agromyzidae) to rabbits (*Oryctolagus cuniculus*) showed a similar discrimination. Sweet lupins were always highly susceptible and selected as favorable food [64]. The defense potential of bitter lupin is due to quinolizidine alkaloids which interfere with the nervous system of animals, affecting mainly nicotinic and muscarinic acetylcholine receptors and inhibiting Na^+ and K^+ channels.

Bitter lupins are not used as a diet because of their toxicity. However, they may have alternative uses as potential sources of natural insecticides. In a study, quinolizidine alkaloids of three Mexican *Lupinus* species: *L. montanus* (HBK), *L. stipulatus* (Agardh), and *L. aschenbornii* (Schauer), were analyzed by capillary

Gas chromatography-mass spectrometry. Sparteine was found in high amounts in both *L. montanus* and *L. aschenbornii* while aphylline and an epiaphylline-like compound in *L. stipulatus*. When extracts of these plants were tested for their insecticidal activity using larvae of the Fall Armyworm, *Spodoptera frugiperda* (Smith); (Lepidoptera, Noctuidae) as a model pest; it was found that extract of *L. stipulatus* was most toxic against the larvae of *S. frugiperda* in comparison to two others. This suggests that the various QA act differently on caterpillars, and could be used to control *Spodoptera* populations [66].

Besides being toxic, alkaloids of lupins are helpful to some plants and animals in direct or indirect ways. For example, specialized aphid, *Macrosiphum albifrons*, which lives on lupins and sequesters the dietary alkaloids and uses them for defense against predators [67]. Another beneficial effect of bitter lupin in maintaining the ecology of an area can be anticipated by studies on *Castilleja indivisa*, a hemiparasitic annual plant. Herbivory can result in decreased pollination by reducing resources available for floral displays, or by damaging attractive tissues. Preference of pollinators for less damaged plants has been found in systems with floral herbivory and with leaf herbivory [68]. Decreased pollination indirectly results in low seed set and disturb the normal seed number and thereby ecology of the particular area. In a study by Adler [69] alkaloids were manipulated experimentally in the *Castilleja indivisa* by growing these parasites with bitter or sweet alkaloid isogenic lines of the host *Lupinus albus* in the field. Over the flowering season, pollinators visited a greater percentage of *C. indivisa* with bitter (high-alkaloid) hosts than sweet (low alkaloid) hosts, and also visited more *C. indivisa* that had been sprayed with insecticide compared to natural herbivory controls, regardless of alkaloid content. Analysis indicated that the benefit of alkaloids was due to their effect on herbivory. Herbivory and pollination had both direct and indirect effects on lifetime seed set of a parasitic plant, *Castilleja indivisa*. Similar host plant effects on herbivores of a hemiparasite were seen in generalist herbivore cabbage looper (*Trichoplusia ni*) and the specialist Nymphalidae butterfly (*Euphydryas anicia)*. Both type of herbivores showed decreased performance on *Castilleja sulphurea* containing alkaloids from the host *Delphinium occidentale* compared to *Castilleja* parasitizing hosts without alkaloids [68].

Besides avoiding, some insects can tolerate the defense chemistry of their host. The potato beetle (*Leptinotarsa decemlineata*) lives on *Solanum* species containing steroidal alkaloids, which are tolerated but not stored by this species. The bruchid beetle, *Bruchidius villosus*, predates seeds of QA rich plants, such as *Laburnum anagyroides*. This beetle eliminates most of the dietary cystisine with the faces. Certain aphids can store the dietary alkaloids, for example, QA in *Aphis cytisorum*, *A. genistae*, and *Macrosiphum albifrons* and PA in *Aphis jacobaeae* and *A. cacaliaster* [70].

There are many examples which show that besides pyrrolizidine and quinolizidine alkaloids other classes of alkaloids are also quite toxic to herbivores and insects. In North America, reed canarygrass (*Phalaris arundinacea*) is important livestock forage on wet and poorly drained soils. It is often unpalatable and supports lower animal performance than would be predicted from its nutrient content. It contains at least eight different alkaloids, including five indole alkaloids and three *p*-carbolines. These alkaloids all contain an indole nucleus. Reed canarygrass also contains hordenine. Marten and coworkers [71] concluded that the threshold level for total indole alkaloid concentration in reed canarygrass at or above which diarrhea and reduced growth rate occurs is about 0.2 % of the dry weight. The low palatability of reed canarygrass is associated with the hordenine content. *Phalaris* spp. has caused animal mortalities in various parts of the world by affecting the animals in different ways. Low larkspurs (*Delphinium nuttallianum* Pritz., *Delphinium andersonii* Gray) and plains larkspur (*Delphinium geyeri* Greene) often poison cattle grazing on western North American rangelands. The dominant toxic alkaloid in larkspur is methyllycaconitine (MLA); other very toxic alkaloids in low and plains larkspurs are nudicauline and geyerline. Concentrations of toxic alkaloids in *D. nuttallianum*, *D. andersonii*, and *D. geyeri* often did not significantly decline during the growing season as typically found in tall larkspurs. Thus, risk to grazing cattle may remain high until these plants are dormant [72].

Despite such toxic effects, alkaloids are sequestered by herbivores and insects for their own benefit which in a way is important in maintaining the ecosystem of the habitat. Pitohuis, a passerine bird, is thought to sequester the alkaloid, homobatrachotoxin, from beetles in their diet into the feathers and skin. The utilized batrachotoxin helps the bird in deterring its predators and avoidance of this bird as food by natives of New Guinea. It is predicted by a group of workers that the rarity of examples of secondary metabolites sequestration in birds and mammals demonstrates that other predator-avoidance strategies such as cryptic behavior and physical “fight or flight” mechanisms are less costly than ingesting and sequestering these toxic compounds in these mobile animals [73]. Another example of alkaloid driven benefit was seen in wild tobacco. As assumed short visitation (time period of visits) times by legitimate pollinators due to increased level of alkaloid and phenols, do not necessarily represent a fitness disadvantage for plants. For example, nicotine in floral nectar of wild tobacco was found to decrease consumption rates by certain pollinators but consequently increased the number of individual visits and, thus, the number of successful pollen transfers. Likewise, nectar odors play a role in the attraction of mutualists to both floral nectar and extrafloral nectar, but repellent functions have to protect nectar from unintended consumption. Nectar chemistry serves both, the attraction of nectar visitors that exert a positive effect on plant fitness and the repellence or putative intoxification of exploiting organisms [74].

Numerous plant alkaloids with antiherbivore properties are classified according to their mode of action (e.g., toxins, antifeedants, antidigestive proteins, etc.) and have been used in agriculture to control insect pests. The pyridine alkaloid nicotine is one of the best-studied putative plant resistance traits. Because it can interact with the acetylcholine receptors in the nervous systems of animals, nicotine is extremely toxic to most herbivores and, consequently, was one of the first insecticides used to control pests in agriculture. Evidence for the resistance value of nicotine arises from the agricultural practice of using nicotine sprays and genotypes of cultivated tobacco differing in nicotine levels [75]. Similarly, experiments indicate a statistically significant dose dependent decrease in the survival rate and an increase in the percentage mortality of ants and aphids in the presence of *Datura alba*. This property of *Datura* suggests that alkaloid-rich extract of *Datura alba* at suitable concentration could potentially be useful to local farmers [76]. Another alkaloid, caffeine has recently been viewed as a safe chemical for management of pests including molluscs, slugs, snails, bacteria, and as a bird deterrent.

Work by Chaniago and coworkers [77] showed that barley has the potential for self-defense against broiler chickens and other species through its biologically active secondary metabolites, gramine and hordenine. Similar type of defense was also seen against aphid *Rhopalosiphum padi* (L.). The population growth rate of *R. padi* was negatively correlated with gramine content in leaves of barley seedlings. In wheat indole alkaloids and phenolics are important in the resistance to insects. The indole alkaloid (i.e., gramine) can be toxic to *Schizaphis graminum*, *R. padi*, and *Sitobion avenae*. Compared to total phenolics, the indole alkaloid content is highly correlated with the number of *S. avenae* infesting wheat ears [78]. Cotton bollworm is one of the most important pest insects in many countries of world and is responsible for destroying a lot of crop plants. In an experiment, when secondary compound gramine was incorporated into artificial diets of cotton bollworm (*Helicoverpa armigera*) larvae. The results showed that it decreased the mean larval and pupal weights of cotton bollworm [79]. Such types of studies make the background to attempt to breed crop varieties with increased level of allelochemicals that would be resistant to pests.

4 Ecological Role of Alkaloids on Microorganisms

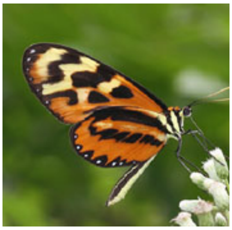
The existing evidence on the role of alkaloids against microorganisms is scarce especially in comparison to ecological studies on insects. However, there are many classes of alkaloids known to show antimicrobial activities, one of such classes is benzylisoquinoline alkaloids (BIAs). It is a large and diverse alkaloid group with more than 2,500 defined structures. The BIAs berberine, sanguinarine, and palmatine were specifically shown to confer protection against herbivores and inhibit the multiplication of bacteria, fungi, and viruses [80]. Other class with potential antibacterial activity is quinolizidine alkaloids. Recently, Erdemoglu and coworkers [81] reported that quinolizidine alkaloid extracts from the aerial parts of *Lupinus angustifolius* and *Genista vuralii* showed significant activity against several different bacterial species and moderate activity against the fungi; *Candida albicans* and *C. krusei*. In a work, alkaloid extracts of aerial parts and seeds of *Sophora alopecuroides* showed promising antimicrobial activities against different bacteria tested [82]. Tyski and coworkers [83] tested lupin alkaloids against a number of Gram-positive and Gram-negative bacteria, including one strain of *Bacillus thuringiensis* isolated from soil and concluded that the alkaloids present in lupins may have an allelopathic function against bacteria. Sepulveds and

Table 6.1 Selected examples of alkaloid containing plants and animals and their role in ecology

Alkaloid	Plants/animals	Ecological roles
Gramine, Hordenine	 *Hordeum vulgare* L. (Barley)	Allelopathic potential; Weed management
Quinolizidine alkaloids	 *Lupinus* sp. (Bitter lupins)	Allelopathic potential, natural insecticides
3-oxo-juliprosine, secojuliprosopinal, etc.	 *Prosopis juliflora*	Allelopathic potential
Benzoxazinones	 *Triticum* sp.	Allelopathic potential
Pyrrolizidine alkaloids	 *Jacobea vulgaris*	Toxic to cattles and other livestock

(*continued*)

Table 6.1 (continued)

Alkaloid	Plants/animals	Ecological roles
Indole alkaloids	 *Phalaris arundinacea* (Reed canarygrass)	Animal mortalities
Methyllycaconitine, nudicauline, geyerline	 *Delphinium* sp. (Low Larkspurs)	Cattle poisoning
PA-derivatives (dihydropyrrolizines, b- and c-lactones)	 Ithomiines, danaines, arctiids males	Phermones
Pyrrolizidine alkaloids	 *Coccinella* (ladybirds)	Sequester PA
Homobatrachotoxin	 Pitohuis (a passerine bird)	Sequester from beetles and deter its predators

Corcuera [84] identified gramine as being inhibitory to the growth of the bacterium *Pseudomonas syringae*, and Krischik and group [85] found nicotine to be toxic to five species of *Pseudomonas*: *P. syringae*, *P. syringae* pv. *angulata*, *P. syringae* pv. tabaci, *P. fluorescens*, and *P. solanacearum*, all plant pathogens. Wink [86] listed 183 alkaloids with antibacterial properties but indicated that many of these may have been investigated for possible pharmaceutical use rather than to elucidate their ecological roles.

Alkaloids also exhibit antifungal effects. There are many bioassay experiments which proved their efficacy. Ma and coworkers [87] showed that the isoquinoline alkaloids, corynoline and acetylcorynoline, inhibited the fungal growth of *Cladosporium herbarum*. Zhou and group [88] reported that steroidal alkaloids from the rhizomes and roots of *Veratrum taliense* inhibited the growth of the phytopathogenic fungi, *Phytophthora capisis* and *Rhizoctonia cerealis*. The alkaloid extract of the seeds of the plant *Sophora alopecuroides* showed significant activity against *Candida krusei* (MIC = 62.5 μg/mL) [89]. PAs produced by *Heliotropium subulatum* showed to be active against *Aspergillus fumigatus, Aspergillus niger, Rhizoctonia phaseoli*, and *Penicillium chrysogenum* [90].

Alkaloidal plant extracts from *J. vulgaris*, when tested never stopped fungal growth totally. After initial growth-delay the fungi were able to adapt and grow normally within 30 days. Hol and van Veen [91] hypothesized that this temporary growth-delay may have serious ecological significances. This delay may impact the competition between microorganisms in the rhizosphere and will give plant roots extra time to raise its defenses or escape. Experimental studies have proved that high-PA-containing plants (1.13–3.92 mg/g dw) with jacobine as the major root PA suppressed the development of some microorganisms in the rhizosphere and thereby play a role in the selection processes. This selection might favor those microorganisms that are tolerant or resistant to these defense compounds or in some cases even can degrade or utilize them. Such selections shape the soil-borne microbial community present in the rhizosphere. Despite all such findings the exact role that alkaloids play in plant protection against root-infecting bacteria and fungi is still not fully understood. Measuring low levels of chemical compounds that occur in the rhizosphere of the plant is still a challenge study area because of inadequate methods for analysis. Some examples of alkaloid containing plants and animals have been shown in (Table 6.1).

5 Ecological Role of Endophytic Alkaloids

Symbiotic interactions of green plants with bacteria and fungi are widespread. Enhanced nutrient uptake, greater stress tolerance, and protection from pests are among many benefits obtained by infected hosts. Most endophytes (organism that lives inside of a plant) produce one or more alkaloid classes that likely play some role in defending the host plant against pests. The bioprotective alkaloids in grass/endophyte symbiota are generally grouped as pyrrolizidines, ergot alkaloids, indole diterpenes, and pyrrolopyrazines. Such a production of alkaloid toxins by endophytes provides a clear mechanism for altered host physiology and ecology. Controlled and natural experiments suggest that endophyte symbiosis can have large effects on plant communities and their associated consumers [92]. An interesting example can be seen in ergot alkaloids. These alkaloids are mainly produced by saprophytic fungi: *Claviceps purpurea* and related species (Clavicipitaceae) which are symbionts on grasses. Rye is especially affected among cereals. *Claviceps* is a symbiotic organism that takes nutrients from its host but provides chemical defense against herbivores as compensation. Field experiments have shown that such a fungal infection is an ecological advantage for grasses in the wild.

The endophyte that has sparked the greatest research interest is *Neotyphodium coenophialum* (formerly *Acremonium coenophialum*), which is a ubiquitous symbiont of tall fescue (*Festuca arundinacea* = *Lolium arundinaceum*; a long-lived perennial bunchgrass species). It provides a wide range of host benefits and produces three of the four known classes of endophyte associated alkaloids which provide them protection against various pathogens. For example, infected tall fescue is more resistant to seedling blight caused by *Rhizoctonia* and crown rust caused by *Puccinia coronata* relative to uninfected plants [93]. The widespread occurrence of all four alkaloid classes among asexual endophyte species, and the hybrid origins of many such endophytes, it is reasonable to speculate that hybridization may have led to the proliferation of alkaloid-production genes among asexual endophytes, favoring hybrids. The ergot alkaloids ergovaline, lolitrems, and lolines are produced by only a single sexual species, *Epichloe festucae*, but they are common in seed-transmitted endophytes, suggesting that *E. festucae* contributed genes for their synthesis [94]. Alternatively, asexual hybrids may also be favored by the counteracting of the accumulation of deleterious mutations.

Other symbiotic fungi such as *Balansia* and *Myriogenspora* (Clavicipitaceae) also infect grasses and produce alkaloids. Ergot alkaloids (such as agroclavine, chanoclavine, ergine, ergosine, and ergometrine) are also common in some genera of the Convolvulaceae (including *Argyreia*, *Ipomoea*, *Turbina corymbosa*, *Stictocardia tiliafolia*). This is due to the ergot alkaloid formation by endophytic clavicipitaceous fungus, which is in symbiotic relationship with certain species in this plant family [95]. Loline, another alkaloid found in a grass *Lolium pratense*, e.g., *Festuca pratensis*, which has symbiotic relation with endophytic fungus *Neotyphodium uncinatum*. A study proved that endophyte infection increased persistency and competitive ability of *F. pratensis* [96] thus; help the grass and its hemiparasite to ward off herbivores. Besides endophyte/grass symbiota, loline alkaloids have also been identified in some other plant species; namely, *Adenocarpus* species (Fabaceae) and *Argyreia mollis* (Convolvulaceae). Loline alkaloids are less widely distributed among endophyte-infected grasses than ergot alkaloids but are generally found in higher concentrations than other alkaloids (exceeding 1 % of plant dry weight biomass. Further, Peramine, the only pyrrolopyrazine alkaloid found in endophyte-infected grasses, is more widely distributed in *Epichloe* and *Neotyphodium* host grasses than other alkaloids. Peramine has a relatively even distribution in the plant and over the growing season, and there is evidence of host genotype control of peramine concentrations [97]. Recently, a fungal endophyte, *Embellisia* spp., was isolated from locoweeds (*Astragalus* and *Oxytropis* spp.) and shown to produce toxic alkaloid swainsonine [98].

Endophytic symbionts need to cope with the secondary metabolites of their host plant. Usually fungi inactivate the toxins of its host by the ability to utilize the compounds as substrate for energy and biomass production or storage for their own defense against attackers. Werner and group [99] showed that endophytic fungi were able to metabolize the polyamine alkaloid aphelandrine. Several fungi were capable to use part of the polyamine alkaloid as nitrogen source. Nearly all fast-growing endophytes of the family of Nectriaceae were able to degrade and to grow on this alkaloid while slow-growing endophytes could only partially degrade aphelandrine.

Endophyte infection affects the host plants and these changes may negatively affect co-occurring plant species and their consumers as seen in some greenhouse experiments. White clover (*Trifolium repens*) was competitively suppressed by perennial ryegrass in infected pastures but not in uninfected pastures, making endophyte-infected grass/clover mixtures difficult to sustain [100]. Grasses and grasslands are dominant features of our landscape and some endophyte symbioses potentially have large-scale consequences. Clay and Holah [101] showed how infected tall fescue in successional fields increased in dominance over a 4-year period, resulting in a decline in plant species richness compared to plots with uninfected tall fescue. Large tall fescue grasslands in the eastern United States present a good example of endophyte-induced dominance.

Much attention has been focused on endophyte alkaloids during the last 25 years as these alkaloids play direct or indirect role in changing the ecology of the environment in which it exists. Toxicity in cattle grazing tall fescue and of sheep grazing perennial rye grass by endophytic infection is quite common. Chinese grass *Achnatherum inebrians* (drunken horse grass), infected by a *Neotyphodium* endophyte, is rapidly increasing its geographic range and frequency of afflicted livestock. Indigenous livestock rarely eat the grass because they have learned to avoid it, but naive animals are frequent victims of intoxication or death. Endophyte-infected *Echinopogon* species native to Australia are reported to cause stock poisonings much like those seen with perennial ryegrass [102]. Besides them, wild herbivores such as voles and rabbits, and Canada geese are also negatively affected by endophyte-infected tall fescue. In an experimental study, sex ratio and the reproductive status of female prairie voles was significantly affected by endophyte infection in field plots of tall fescue [103]. Laboratory studies with several passerine birds found strong avoidance of endophyte-infected tall fescue seeds in choice tests, and reduced survival and weight gain in nonchoice tests [104].

Community-wide effects on endophytes may be extended beyond herbivores to their associated parasites and predators in other trophic levels. For example, *Neotyphodium* endophytes of *Lolium multiflorum*, lower plant quality, reduce the densities of aphid herbivores, and indirectly reduce the rate of parasitism on aphids by parasitoids [105]. The potential for alteration of the interactions of grasses with herbivores and their natural enemies will clearly depend on many factors, including the frequency of infected hosts within the community, the types and relative levels of bioprotective alkaloids, and the spatial distribution of grasses and herbivores.

However, little is currently known about the impact of endophytes on food web dynamics in any natural ecosystem. There is a potential for the grass/endophyte symbiosis to impact more than the associated animal and plant communities. The ecosystem level consequences of endophytes have begun to be considered by a few researchers [106]. These consequences include carbon flow into soil food webs, such as the endophyte effects on litter decomposition and soil microbial activity and changes in soil nutrients availability and secondary compounds that may feedback to the plant community. The two agronomic species *L. arundinaceum* and *L. perenne* investigated the most are not native to places where they have been studied (United States and New Zealand) [106]. Further studies in natural ecosystem remain to be done before realistic ecological impact of endophytes can be studied.

6 Conclusion

Although the biological activities of many alkaloids have not yet been studied and their ecological functions remain to be discovered or proven, alkaloids have proved to play an important role in the ecosystem. Recognizing the potential of alkaloids, studies on the ecological effects of alkaloids have received considerable attention over the last few decades. Some alkaloid-rich plants, such as *Senecio* spp., *Crotalaria* spp., *Lupinus* spp., wheat, and barley, have provided a concise background to understand how alkaloids mediate ecological interactions. Most of the structural types of alkaloids can exhibit allelopathic effects. Numerous examples of allelopathic effects can be seen in members of the Solanaceae plant family. In crop plants, allelopathy can help in warding off weeds. Allelochemicals in barley make it a potential candidate as a natural herbicide. Integrating barley cover cropping with other cultural practices can possibly improve the sustainable agricultural systems for weed management. Taking into account various laboratory studies on alkaloids, they have proved to play an important role in influencing neighboring plants. But in some cases there are discrepancies between the field and laboratory experiments, as in the case of *Cinchona* and *Lupinus campestris*. However, recent advances in metabolomics, transcriptomics, and proteomics will greatly assist in the identification of novel allelopathy genes. Ultimately, the allelopathy genes could be manipulated to regulate the biosynthesis of allelochemicals, thereby resulting in better weed suppression via elevated levels of allelopathic potential. Among plant–animal relationships, pyrrolizidine alkaloids and quinolizidine alkaloids are the most studied examples of the flux of chemical compounds through trophic levels. Herbivorous species that are associated with alkaloid-rich plants may have developed different ways to deal with these toxic compounds. Some species became alkaloid specialists and overcame this chemical barrier by detoxifying and incorporating these toxins in their tissues. Regardless of this, there are numerous plant alkaloids with antiherbivore, insecticidal, and antimicrobial properties and are classified according to their mode of action (e.g., toxins, antifeedants, antidigestive proteins, etc.) and have been used in agriculture as safe chemicals for management of pests including molluscs, slugs, snails, bacteria, and as a bird deterrent. Thus, the field of alkaloids in relation to ecology presents a great potential for research and development of environmental friendly weeds/herbivores/insects/microbes management practices, without disturbing the ecosystem.

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Plant In Vitro Systems as Sources of Tropane Alkaloids

7

Vasil Georgiev, Andrey Marchev, Strahil Berkov, and Atanas Pavlov

Contents

1 Introduction 175
2 Distribution of Tropane Alkaloids 175
3 Qualification and Quantification of Tropane Alkaloids 177
3.1 Sample Preparation Procedures 177
3.2 Chromatography of Tropane Alkaloids 178
4 Biosynthetic Pathway of Tropane Alkaloids 181
5 Biotechnological Approaches for Tropane Alkaloids Production 185
5.1 Plant Cell Suspensions 186
5.2 Soot Cultures 186
5.3 Adventitious Root Cultures 188
5.4 Hairy Roots 189
5.5 Artificial Polyploidy as a Useful Tool for Tropane Alkaloids Production 197
6 Conclusion 204
References 204

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Abstract

The number of known tropane alkaloids exceeds 200. They are tertiary or quaternary (*N*-oxides) bases having in common the tropane nucleus as the key structural element. The tropane alkaloids are mainly distributed in the genera of Solanaceae family and they are frequently used in medicine because of their valuable pharmacological activities. The main representatives of this group of bioactive substances are hyoscyamine (its racemate atropine), scopolamine, and cocaine. These alkaloids have been investigated in details during the last two decades. In this chapter we summarize the knowledge about distribution of tropane alkaloids in plant kingdom and their biosynthetic pathway that has already been elucidated at enzyme and gene level, as well as the methods for their qualification and quantification. Biotechnological approaches for tropane alkaloids production are also discussed in details.

Keywords

Artificial polyploidy • bioreactor • hairy roots • hyoscyamine • plant cell cultures • scopolamine • tropane alkaloids biosynthesis

Abbreviations

ADC	Arginine decarboxylase
BAP	Benzylaminopurin
cDNA	Complemantary deoxyribonucleic acid
CE	Capillary electrophoresis
DESI	Desorption electrospray ionization
GC–MS	Gas chromatography–mass spectrometry
GUS	β-glucuronidase
H6H	Hyoscyamine 6β-hydroxylase
HPLC	High-performance liquid chromatography
IAA	Indoleacetic acid
IBA	Indolebutyric acid
LLE	Liquid–liquid extraction
LS	Linsmaier and Skoog nutrient medium
m/z	Mass-to-charge ratio
MPO	*N*-methylputrescine oxidase
MS	Murashige and Skoog nutrient medium
NAA	α-naphthylacetic acid
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance spectroscopy
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser effect spectroscopy
ODC	Ornithine decarboxylase
PTM	Putrescine *N*-methyltransferase
SAM	*S*-adenosyl-L-methionine

SPE	Solid-phase extraction
T-DNA	Transfer-deoxyribonucleic acid
TLC	Thin-layer chromatography
TR	Tropinone reductase
UV	Ultraviolet

1 Introduction

Tropane alkaloids are frequently used in medicine because of their valuable pharmacological activities [1]. To this group belong hyoscyamine **1**, its racemate atropine **5**, scopolamine **6**, and cocaine **2** [2, 3]. Hyoscyamine **1** and scopolamine **6** are widely applied as parasympatholytic agents who antagonize acetylcholine binding in a competitive manner [1, 4, 5]. Therapeutic doses of atropine **5** possess antispasmodic properties and are used in treatment of cardiac and gastrointestinal diseases [1, 4]. It is used to treat acute bronchitis, to control the symptoms of Parkinson's disease, and as an antidote in cases of poisoning by organophosphates [1, 6]. The better activity on the central nervous system at lower therapeutic doses of scopolamine **6** supported with less side-effect than atropine **5** [1, 4] determines its greater demand on the market than hyoscyamine **1** and atropine **5** [1, 7].

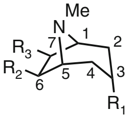
Tropane alkaloids are exclusively distributed in many Solanaceae species – *Mandragora, Brugmansia, Duboisia, Hyoscyamus, Datura, Atropa*, and *Scopolia* [4, 7, 8]. Cocaine **2** is produced by *Erythroxylum coca* Lam.[8, 9]. Tropane alkaloid content in plants differs according to the species and varies about 0.01–3% [1]. However, climate changes and other ecological problems lead to disruption and even to disappearance of natural habitats of medicinal plants, which has resulted in difficulties to obtain plant-derived bioactive substances. Promising alternative for commercial scale production of tropane alkaloids appears to be cultivation of plant in vitro systems in controlled conditions [7, 10]. To improve the production of secondary metabolites, different strategies like high productive line selection or culture condition optimizations are performed [11]. Another successful way to increase the productivity in plants or their in vitro systems is the application of genetic engineering. The detailed elucidation of the intermediates and enzymes that influence the biosynthetic pathway, and increasing the number of the regulatory genes, provides efficient approach to increase or decrease the amount of the target metabolites by enhancing the rate-limiting steps or by blocking competitive pathways [1, 5, 11, 12]. Changes in the plant ploidy are also useful approaches for improving the yields of valuable secondary metabolites [13].

2 Distribution of Tropane Alkaloids

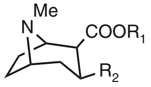
The number of tropane alkaloids known from natural sources exceeds 200. They are tertiary or quaternary (*N*-oxides) bases having in common the tropane nucleus (8-azabicyclo[3.2.1]octane) as the key structural element [14].

Their distribution is among plants of the families Solanaceae, Erythroxylaceae, Convolvulaceae, Proteaceae, Rhizophoraceae, Brassicaceae, and Euphorbiaceae [15].

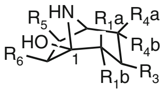
The greatest range of tropane alkaloids has been found in the Solanaceae family. Characteristic alkaloids for this family are mono-, di-, or tri-esters of tropine **27** or pseudotropine **25** (the OH group at C3 is in *β*-position). A number of organic acids, such as formic, acetic, hydroxyacetic, propionic, tiglic, methylbutyric, phenyllactic, tropic, senecic, angelic, mesaconic, cinnamic, etc., form esters with the alkamines [14, 16, 17]. Many solanaceous species from genera *Datura*, *Brugmansia*, *Hyoscyamus*, *Scopolia, Atropa*, and *Duboisia* accumulate the pharmacologically active hyoscyamine **1** and scopolamine **6** as main compounds in their alkaloid profiles and therefore, they have been used as industrial sources for their production [15]. As many tropane alkaloids, hyoscyamine **1** is a chiral molecule wherein chirality is introduced by the tropic acid. Only the *R* form of tropic acid occurs in natural tropane alkaloids. Racemization, however, readily occurs during isolation procedure. The racemic mixture of (*R*,*S*)-hyoscyamine is known as atropine **5**. Dimeric tropane alkaloids (belladonnine) and polyhydroxy nortropane alkaloids (calystegines) have been found in *Atropa belladonna.* Calystegines have been found in other species from the family such as *Physalis, Hyoscyamus, Scopolia*, and *Solanum* [15, 18]. Often, monocyclic analogues such as the pyrrolidinyl alkaloids hygrine **16**, cuscohygrine **4**, and their derivates have been detected as minor compounds in the alkaloid profiles of plants and in vitro cultures from genera *Datura*, *Hyoscyamus*, *Brugmansia*, etc. [16, 17, 19]. The main structures and some representatives of the tropane alkaloids (**1–7**) are given below.



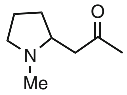
Derivatives of tropine



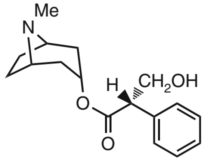
Derivatives of ecgonine



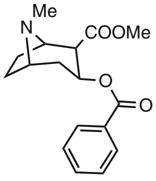
Polyhydroxynortropanes



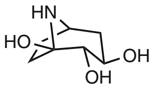
Monocyclic analogues



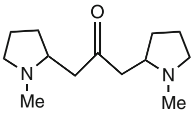
(–) Hyoscyamine
1



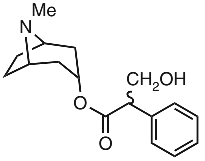
Cocaine
2



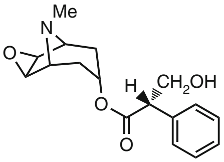
Calystegine A_3
3



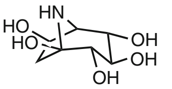
Cuscohygrine
4



(–/+) Hyoscyamine
(Atropine)
5



Scopolamine
6



Calystegine C_1
7

Along with 3α-tropine derivatives, characteristic for the plants of the family Erythroxylaceae are the esters of 3*β*-tropine (pseudotropine) and ecgonine with benzoic, phenylacetic, 3,4,5-trimethoxybenzoic, 3,4,5-trimethoxycinnamic acids, etc. Dimeric esters of methylecgonine (truxillines) and tropine (mooniines) have been also reported [15].

Unusual pyronotropane tropane alkaloids have been found in the family Proteaceae. Tropine and pseudotropine esters of benzoic acid have been isolated from the family Euphorbiaceae. Novel dithiolane esters, along with tropine esters of acetic, propionic, *n*-butyric, isobutyric, isovaleric, and benzoic acids have been found in the Rhizophoraceae. Calystegines, as well as a 3-hydroxybenzoate ester, have been found in Brassicaceae. The calystegines have been used as chemotaxonomic markers in the Convolvulaceae family [20]. Tropine esters with methoxy-substituted benzoic acids are also characteristic for the later family.

3 Qualification and Quantification of Tropane Alkaloids

3.1 Sample Preparation Procedures

The tertiary tropane alkaloids can be extracted from the plant material in the form of salt with acidic aqueous solutions or as bases at pH $>$ 8–9 with organic solvents such as methanol, ethanol, chloroform, etc. Sample purification procedures for tropane alkaloids before chromatographic analyses are based usually on liquid-liquid extraction (LLE) or solid-phase extraction (SPE). The choice of solvents depends on the solubility (p*Ka* and p*Kb*) in water of the target alkaloids. For quantitative analyses, exhaustive extraction or the use of suitable internal standard should be ensured.

Columns packed with diatomaceous earth (Extrelut®) are often used for the preparation of a large number of samples from various biological materials [21, 22]. In this case, the alkaloids are extracted with acidic aqueous solution which has to be basified before application on the column. The alkaloids are eluted with chloroform and chloroform:methanol (3:1).

N-oxide forms of the tropane alkaloids, found in many plants, are analyzed after reduction with dithionite. For that reason, two aliquots of the acid alkaloid extract are taken, and the first one is treated with dithionite. After basification of the extracts and partition of the alkaloids (as tertiary bases), the amount of the *N*-oxides is calculated after instrumental analysis by subtracting the yield of total alkaloids (first aliquot) minus yield of tertiary alkaloids (second aliquot). The method is proved to be quantitative [21].

The hydrophilic calystegines are extracted with water or methanol-water solutions. After centrifugation, the volume of the supernatant is reduced, treated with skin powder, filtered, and then applied to columns of strongly acidic cation exchange resin for purification. The nonbinding compounds are removed with water and alkaloids are eluted with 3.5% aqueous NH_3. After drying, the alkaloid fraction has to be silylated for further GC-MS analysis [20].

3.2 Chromatography of Tropane Alkaloids

Quantitative and qualitative analyses of the tropane alkaloids are based on their efficient separation. The pure compounds, obtained after preparative chromatography (column or thin-layer), are subjected to spectroscopic analysis for identification. NMR (1 and 2D mono- and hetero-atom experiments) is applied for structure elucidation. While relative configuration of chiral centers can usually be assigned by NMR (NOESY and/or NOE) experiments, the determination of the absolute configuration is much more difficult due to the identical NMR spectra of enantiomers. It can be determined by measuring the specific rotation [α] and circular dichroism or using chiral auxiliary reagents. Only X-ray crystallography provides direct access to the chirality of compounds but obviously relies on the ability to form crystals [23].

GC-MS is the method of choice for identification of the components in complex mixtures of tropane alkaloids combining the high resolution power of capillary columns with the specificity of the mass detectors. It allows identification of tropane alkaloids without using of standard compounds. Isomers (at positions C3 and C6/7) are tentatively identified by Kovats retention indexes and cochromatography with reference compounds [16, 17]. In some cases, tentative identification of new tropane alkaloids has been possible by GC-MS [16, 24–26]. In fact, this is the only way to assess the minor or trace compounds, which are difficult or impossible to isolate. More than 30–40 tropane alkaloids can be detected in a single species by GC-MS [19]. GC-MS chromatogram of alkaloid extracts of *D. stramonium* L. hairy roots and representative MS spectra are presented in Fig. 7.1.

The major MS characteristics of the tropane alkaloids and related compounds are as follows:

- The monocyclic (pyrrolidinyl) alkaloids (hygrine **16**, hygroline, cuscohygrine **4**, etc.) have M^+ with low intensity. The most characteristic ion fragments of these alkaloids are the base ion at *m*/*z* 84 and ions at *m*/*z* 85, 83, 82, 70, 55, and 42.
- The main fragments, characteristic for the tropane ring system, are at *m*/*z* 124, 113, 112, 96, 95, 94, 83, 82, and 42.
- The type of substitutions could be determined according to the base ion. For 3-monosubstituted tropanes, the base peak is at *m*/*z* 124; for 3,6-disubstituted, 3,6,7-trisubstituted, and 3-monosubstituted-6,7-epoxytropanes, at *m*/*z* 94; for nortropanes, at *m*/*z* 110; and for 6,7-epoxynortropanes, at *m*/*z* 122. For alkaloids without ester substitution at C-3, the base peak is at *m*/*z* 82. Each of these subgroups of alkaloids has characteristic fragmentation as well as ion fragments indicating the substituents (Fig. 7.1).
- Many tropane esters appear as double peaks in the gas chromatograms and show identical mass spectra. They are isomeric tropine and pseudotropine esters. The stereochemistry of substituents at C-3 of the tropane alkaloids could be determined by GC comparison with reference material only.

It should be mentioned that hyoscyamine **1** and littorine **26** are poorly separated by GC, and they have very similar mass spectral fragmentation. They could be distinguished by the characteristic ion fragments at *m*/*z* 91 and 142 for littorine **26**

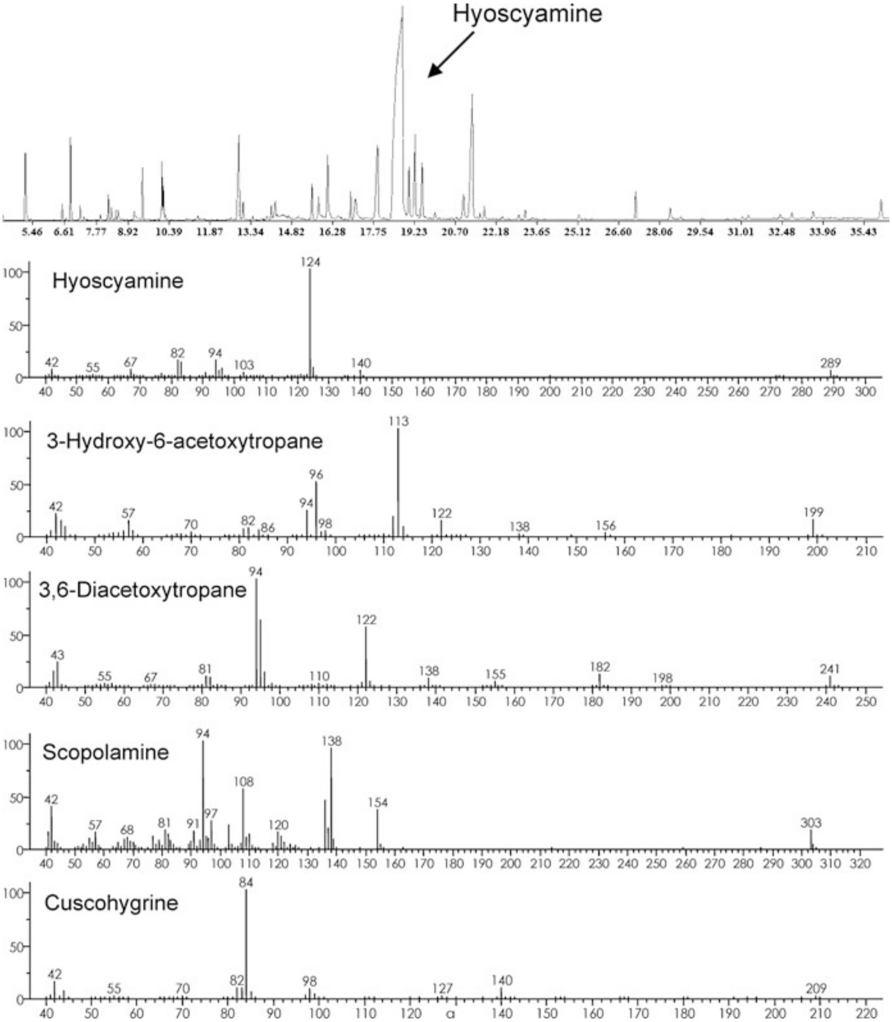


Fig. 7.1 GC-MS chromatogram of alkaloid extract of *Datura stramonium* L. hairy roots and representative MS spectra of monosubstituted (hyoscyamine **1**), disubstituted (3-hydroxy-6-acetoxytropane, 3,6-diacetoxytropane), epoxytropanes (scopolamine **6**) tropanes, and their monocyclic analogues (cuscohygrine **4**). The GC-MS analyses were performed with a Hewlett Packard 6,890+/MSD 5975 instrument operating in EI mode at 70 eV. An HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was: 100–180 °C at 15 °C/min, 180–300 at 5 °C/min, and 10 min hold at 300 °C. Injector temperature was 250 °C. The flow rate of carrier gas (helium) was 0.8 mL/min. 1 μL of the solution was injected

and at *m*/*z* 271 (the lost of water) for hyoscyamine **1** [16]. Good separation of hyoscyamine **1** and littorine **26** can be obtained after silylation [27].

GC-MS has been applied for quantitative and qualitative analysis of tropane alkaloids in various plant species and in vitro cultures [17, 24]. The MS detector,

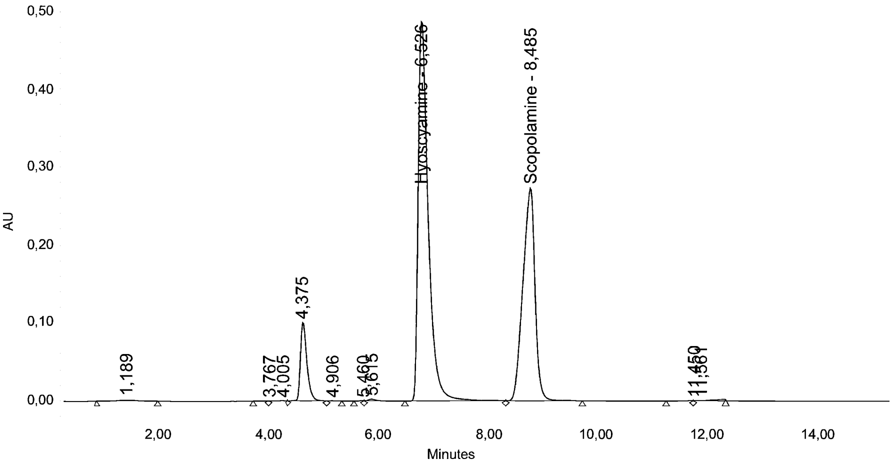


Fig. 7.2 HPLC separation of hyoscyamine **1** and scopolamine **6**. The analysis was performed by using Waters HPLC system, equipped with Waters 1,525 binary pump, a Dual λ Waters 2,487 absorbance detector, and Symmetry® C18 reversed phase chromatographic column (250 mm × 4.6 mm, 5 μm). The operation temperature was 26 °C. The mobile phase consisted of acetonitrile:methanol:0.05 mol ammonium acetate (20.9:27.9:51.2), and elution speed was 0.6 mL min^{-1} in isocratic regime

especially in selected ion monitoring (SIM), provides a high sensitivity allowing quantification of target compounds at ppb levels [28]. The main disadvantages of GC-MS, however, are the degradation of some thermally unstable compounds (hyoscyamine may lose water and form apoatropine under GC conditions), derivatization of nonvolatile compounds such as the calystegines, and impossibility for resolution of enantiomers.

In contrast to GC-MS, a wide range of tropane alkaloids, including *N*-oxides and calystegines, can be analyzed by HPLC without prior derivatization. Enantiomeric separation of atropine **5** and scopolamine **6** racemic mixtures has been also achieved [22]. A number of HPLC methods have been published for routine quantification of the major tropane alkaloids hyoscyamine **1** and scopolamine **6** in plant samples. The use of UV detectors however is limited to compounds with UV adsorbing (aromatic or other) functionality. This disadvantage, however, is easily overcome by using HPLC coupled with MS detectors [22]. A typical HPLC separation of hyoscyamine **1** and scopolamine **6** is presented on Fig. 7.2.

In the recent years, capillary electrophoresis (CE) is widely used in alkaloid analysis because the nitrogen is easily charged. In the analysis of tropane alkaloids, it is applied for enantiomeric separation of atropine **5** and for analysis of pharmaceutical substances [22].

Despite that thin-layer chromatography (TLC) is less informative as compared to the above-mentioned methods, it is frequently used in the laboratories for screening of plant samples and alkaloid fractions. TLC is a low-cost, simple, fast, and with high-throughput approach. It is less sensitive and less reproducible than other

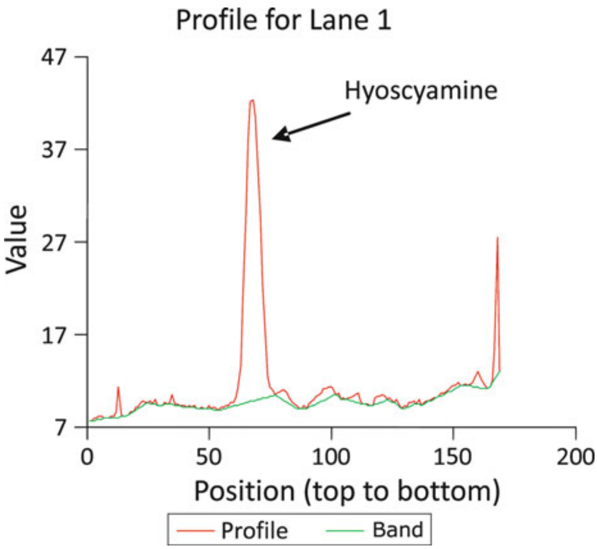


Fig. 7.3 TLC chromatogram of *Atropa belladonna* L. root extract. 20 μL of extracts were spotted on Silicagel G60 TLC plate. Hyoscyamine and scopolamine standards were spotted in concentrations of 2, 5, 10, and 20 μg on the same plate as well. The chromatogram was developed by using chloroform: methanol:26% ammonia (in proportion of 85:15:0.7) as mobile phase, and Dragendorff's reagent was used for spots visualization. The alkaloid contents were quantified densitometry by QuantiScan® software

chromatographic methods, and it has been considered as semiquantitative method for a long time. Usually, Dragendorff's reagent is used for visualization of the tropane alkaloids. The levels of alkaloids in the solanaceous intact plants and in vitro cultures, however, are high enough, and recently quantitative TLC methods for tropane alkaloids have been developed for analyses of plant tissues [29–31]. A TLC chromatogram of alkaloid extracts of *A. belladonna* L. roots is presented on Fig. 7.3.

It is worth mentioning that the recent work of Talaty et al. [32] uses desorption electrospray ionization (DESI), a nonchromatographic method, for detection of various alkaloids from *D. stramonium* L. and *A. belladonna* L. Using DESI, no sample preparation is required because native fresh tissues can be directly analyzed. The mass spectra are recorded under ambient conditions, in times of a few seconds.

4 Biosynthetic Pathway of Tropane Alkaloids

Tropane alkaloids are biosynthesized in the roots and transported to the aerial parts of the plant through xylem [1, 6, 7]. They comprise of pyrrolidine and piperidine ring with shared nitrogen and two carbon atoms [1]. The ring skeleton is considered to be an assembly of the amino acid ornithine **8**, acetate, and methionine. The C-1, C-5, C-6, C-7, N-8 of the five-membered ring derive from ornithine **8**, the *N*-methyl group from methionine, and C-2, C-3, C-4 come from acetate-derived C3 fragment [33, 34]. Over the last years, a lot of evidences have been gained providing data that 4-(L-methyl-2-pyrrolidinyl)-3-oxobutanoate **19** is the direct precursor on the pathway to tropine **27** but not hygrine **16** as considered before [7, 34, 35].

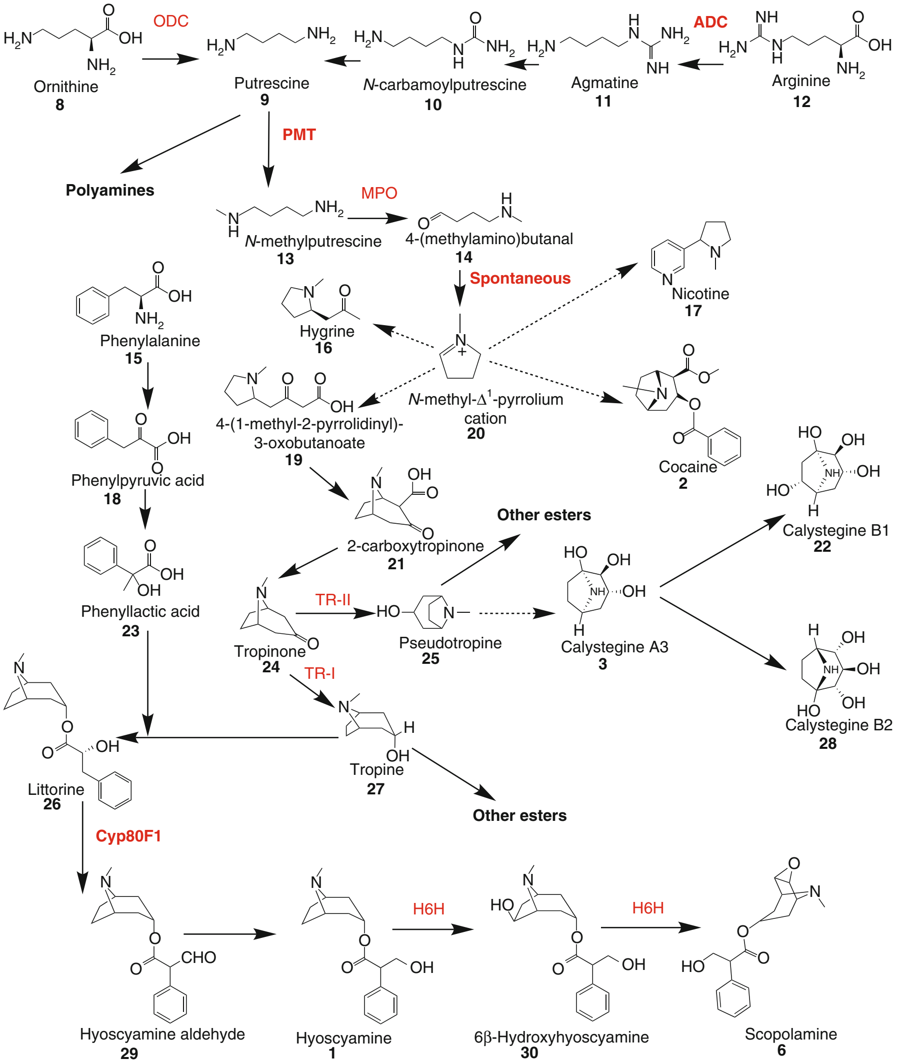
Tropane alkaloids share a similar biosynthetic pathway with nicotine although it is not part of them (Scheme 7.1) [36]. The pyrrolidine part of tropane alkaloids is formed from ornithine **8** or arginine **12** [1]. Ornithine decarboxylase (ODC; EC 4.1.1.17) is responsible for the direct formation of putrescine **9** via ornithine decarboxylation, while arginine **12** is firstly transformed to agmatine **11** and then to *N*-carbamoylputrescine **10** by arginine decarboxylase (ADC; EC 4.1.1.19) [1, 2, 7]. They are flux-limiting enzymes [1], since putrescine **9** formation is a common step with polyamine metabolism [2]. Using specific inhibitors, it has been demonstrated that in *Datura* both alternative routes for putrescine **9** formation are possible. However, the ADC activity is more important than ODC [1]. Arginine **12** seems to be the most preferred source for C_4N fragment C-1, C-5, C-6, C-7, *N*-8 than ornithine **8** [34].

While ODC is found in all living organisms, ADC has limited distribution to bacteria and plants [1]. Cloning of ODC cDNA from *D. stramonium* L. and its expression in *E. coli* revealed that the enzyme is highly similar with other prokaryotic and eukaryotic ODCs, and it is presented by more than one gene copy in the plant genome [1, 2].

The next step of the pathway is the *N*-methylation of putrescine **9** to *N*-methylputrescine **13** by putrescine *N*-methyltransferase (PMT; EC 2.1.1.53) [1, 5]. This is the key enzyme and the first committed step in the biosynthesis of tropane and nicotine alkaloids in which a methyl group from *S*-adenosyl-L-methionine (SAM) is transferred to an amino group of putrescine **9** [3, 7]. The PMT is a unique enzyme detected only in tropane and related to alkaloid-producing plants. The reaction is a flux-limiting step during which putrescine **9** is being removed from the polyamine pool and pointed toward alkaloid production [7] instead of polyamines such as spermidine and spermine [1]. Monoamines like *n*-butylamine or cyclohexylamine are competitive inhibitors of PMT [1, 4]. The PMT activity has been detected within branched roots of tropane alkaloid-producing *A. belladonna* L., *H. niger* L.*,* *D. stramonium* L., and *D. inoxia* L. and was absent in other plant organs [4, 6]. A cDNAs coding for this enzyme has been isolated from *H. niger* L., *A. belladonna* L., and *Solanum tuberosum* L. [37].

PMT expression occurs in the root pericycle of *A. belladonna* L. and *H. muticus* L. [2, 36, 37] and in endodermis, outer cortex, and xylem in *Nicotiana sylvestris* L. [36, 37]. The localization of PMT to the pericycle would facilitate the access to arginine **12** or ornithine **8** [2, 6]. In the same way, *N*-methylputrescine **13** or some biosynthetic intermediate could then be transported to the endodermis for further elaboration into tropine **27** [6].

Further step in the pathway is reaction catalyzed by the diamine oxidase enzyme, *N*-methylputrescine oxidase (MPO; EC 1.4.3.6). This enzyme leads to oxidatively deamination of *N*-methylputrescine **13** to 4-(methylamino)butanal **14**, which after spontaneous cyclization forms the reactive precursor of the tropane nucleus – *N*-methyl-Δ^1-pyrrolinium cation **20** [2, 7, 36]. This enzyme requires copper as a cofactor and belongs to a class of amine oxidases. cDNA was isolated from tobacco [37]. This enzyme has been isolated by root cultures of *H. niger* L.,


Ornithine
8
ODC
Putrescine
9
N-carbamoylputrescine
10
Agmatine
11
ADC
Arginine
12
PMT
Polyamines
N-methylputrescine
13
MPO
4-(methylamino)butanal
14
Spontaneous
Nicotine
17
Hygrine
16
N-methyl-Δ1-pyrrolium cation
20
Phenylalanine
15
4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate
19
Cocaine
2
Phenylpyruvic acid
18
2-carboxytropinone
21
Other esters
Calystegine B1
22
Phenyllactic acid
23
Tropinone
24
TR-II
Pseudotropine
25
Calystegine A3
3
Calystegine B2
28
TR-I
Tropine
27
Littorine
26
Other esters
Cyp80F1
H6H
H6H
Hyoscyamine aldehyde
29
Hyoscyamine
1
6β-Hydroxyhyoscyamine
30
Scopolamine
6

Scheme 7.1 Biosynthetic pathway of tropane alkaloids

D. stramonium L., and *N. tabacum* L. Its activity is suppressed to very low level by exogenous supply of plant hormones and subsequent cell dedifferentiation. The activity decreases also after feeding with tropine **27** [1].

No enzyme has been found so far which would catalyze the formation of tropinone **24** from *N*-methyl-Δ^1-pyrrolinium cation **20** [1]. It was demonstrated that the pathway most probably passed by formation of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate **19**, its cyclization to give 2-carboxytropinone **21**, was followed by decarboxylation step to form tropinone **24** [35].

The next step from the biosynthetic pathway is a branch point of the tropane alkaloid pathway [1, 2]. Tropinone **24** is the first intermediate with a tropane ring, and it is converted into intermediates that lead to hyoscyamine **1** or calystegines production depending on the stereochemistry of the reduction [37]. Two stereospecific tropinone reductases (TR; EC 1.1.1.236) – tropinone reductase I (TR-I) reduces the 3-carbonyl group of tropinone **24** to the 3α-hydroxy group of tropine **27** and tropinone reductase II (TR-II) to the 3β-hydroxy group of pseudotropine **25**. TR-I leads to hyoscyamine **1** and scopolamine **6** formation via tropine **27**, whereas pseudotropine **25** produced by TR-II is converted into calystegines and other nortropane alkaloids [1, 7, 36].

It should be noted that encoding TR-I and TR-II genes have been indentified in *A. belladonna* L. and *H. niger* L., but not in nicotine-synthesizing plants. cDNA clones for TR-I and TR-II isolated from *D. stramonium* L. and expressed in *E. coli* revealed that the proteins exhibit 64% amino acid identity and to be member of short-chain, nonmetal dehydrogenase family [34, 36]. The exchange of various domains of TR-I and TR-II revealed that the stereo- and substrate specificity are determined by the orientation of the tropinone at the substrate-binding site which consists mainly of carboxyl-terminal domain. The N-terminal region constitutes the NADPH-binding site [1, 2]. In the amino-terminal region there are glycine residues which form tight turns, called Rosmann folds, in the cofactor-binding domain. The carboxyl-terminal region contains reaction centers, with a tyrosine and a lysine residue [4]. The substrate-binding site consists of 120 amino acids, mostly hydrophobic which is present in both TRs [1–3] and 53 differ [4]. The presence of different charged residues gives different electrostatic environment on TR-I and TR-II [1].

TRs' activities are at very high levels in developing young branched roots [4]. TR-I is found in the endodermis and nearby cortical cells which determines the transport of the intermediates of tropane alkaloid metabolism between cell types. TR-II is localized to companion cells of sieve elements in the phloem of potato [36, 37]. TR-I is not considered to be a rate-limiting activity [1]. The TRs' activities differ with species which means that in this way, the flow at this branching point may be determined [4]. Demonstration of this has been done by Richter et al. [38] overexpressing TR-I and TR-II in *A. belladonna* L. Higher pseudotropine **25** concentration led to an increased accumulation of calystegines, while higher tropine **27** production resulted in several times higher tropane alkaloid levels and a 30–90% decrease in calystegine accumulation compared with controls [38]. Trihydroxylated calystegines are classified as the calystegine A-group, tetrahydroxylated calystegines as the B-group, and pentahydroxylated derivatives form the calystegine C-group [1].

In the next reaction, the tropate ester moiety of hyoscyamine is derived by condensation of tropine with the phenylalanine-derived (*R*)-phenyllactate to form littorine **26** [7, 37]. Patterson and O'Hagan [39] suggested that the rearrangement of littorine **26** into hyoscyamine **1** does not occur with a vicinal interchange process as previously considered. Recently, a multifunctional cytochrome P450 has been identified from *H. niger* L., classified as Cyp80F1 and catalyzes the oxidation of

(*R*)-littorine **26** with rearrangement to hyoscyamine aldehyde **29** [7, 37, 39]. In this way, the free tropic acid is not considered to be an intermediate in hyoscyamine biosynthesis [1].

The final step in the tropane alkaloid pathway is the formation of scopolamine **6**, which is the 6,7β-epoxide of hyoscyamine via two-step reaction. Both reactions are catalyzed by hyoscyamine 6β-hydroxylase (H6H; EC 1.14.11.11) which is a bifunctional enzyme. This dioxygenase first hydroxylates the tropane ring of hyoscyamine **1** by 6β-hydroxylation forming 6β-hydroxyhyoscyamine **30**, followed by intramolecular epoxide formation via removal of the 7β-hydrogen [2, 3, 7, 37]. The hydroxylase activity of H6H is 40 times higher than its epoxidase activity. H6H is a 2-oxoglutarate-dependent enzyme that requires alkaloid substrate, 2-oxoglutarate, Fe^{2+}, molecular oxygen, and ascorbate for catalysis [1]. H6H is a rate-limiting enzyme in scopolamine biosynthesis. Overexpression of H6H in low scopolamine *A. belladonna* L. increased the scopolamine **6** content [40]. In the same way, hyoscyamine **1** was favored to scopolamine **6** in *A. beatica* Wilk [41].

H6H is localized to the pericycle in the roots of *A. belladonna* L. and *H. muticus* L. and not found in stem, leaves, or cultured cells of the same species. Thus, the scopolamine **6** produced in the pericycle can be readily translocated to the leaves via the adjacent xylem [2, 3, 37].

5 Biotechnological Approaches for Tropane Alkaloids Production

Higher plants are valuable sources of diverse metabolites, many of which are used as pharmaceuticals. Further, in recent years, worldwide market has preferred natural products; thus, the market for natural plant products has expanded, and this trend will continue. However, the production of plant-derived substances is limited by environmental, ecological, and climatic conditions. These problems could be overcome using plant in vitro systems, which were introduced at the end of the 1960s as promising matrices for both producing and studying plant secondary metabolites [42]. At the moment, several problems still complicate industrial implementation of these systems. Instability of their secondary metabolite profiles and yields of the target molecules are the main problems. Nowadays, bioprocess engineering approach for development and optimization of plant in vitro system-based processes is well developed. Algorithms for media optimization, elicitation, bioreactor constructions adaptation, environmental conditions optimization, etc., have been described in details [43].

Hyoscyamine **1** and scopolamine **6** are the tropane alkaloids with the most important therapeutic value. The current commercially exploited sources for their production are different plants belonging to *Atropa* or *Datura* species in Europe and *Duboisia myoporoides* R.Br. in Australia [8]. Faced with the described above problems accompanying the production process based on plant material, many research groups focused their attention on development of alternative biotechnological process for tropane alkaloids production.

5.1 Plant Cell Suspensions

As the plant cell suspensions could be easily cultivated in common bioreactor systems, used in microbial biotechnology, several investigations were performed on initiation of callus and cell suspension cultures by different Solanaceae plants (Table 7.1) [44–49]. In general, once initiated, plant cell suspensions are easily adapted to submerged cultivation conditions and are amenable to subsequent scale-up in bioreactors [50]. Cell suspension of *H. muticus* L. showed fast growth and accumulates between 10 and 11 g dry biomass L^{-1} when cultured in both 5.0 L stirred tank and 9.0 L plastic-lined vessels bioreactors [51]. Following the scale-up process, a 40.0-L low-cost plastic air-lift bioreactor system, which produced 199 g dry weight of *H. muticus* L. cells in 13 days, was developed. This biomass yield was indistinguishable from the achieved in submerged cultivation of the same culture in shake flask [51]. However, as it is showed in Table 7.1, the overall content of produced alkaloids in Solanaceae callus and cell suspension cultures is rather low. Different strategies for obtaining of calli and cell suspension cultures with incensed production potential of target tropane alkaloids have been applied. It was demonstrated that during callogenesis, the different plant organs used as explants could generate calli with different potential to produce tropane alkaloids [49]. As example, the contents of scopolamine **6** in callus cultures, induced from leaf, stem, and root parts of *D. metel* L., varied between 14.15 and 0.06 mg g^{-1} dry biomass. At the same way, only the callus cultures induced from *D. metel* L. roots biosynthesized hyoscyamine **1** in concentration of 0.2 mg/g dry biomass [49]. Exposure of light could affect on tropane alkaloids accumulation as well. Transferring and cultivation of *H. niger* L. callus and cell suspension cultures under continuous illumination (3,000–5,000 lx) decreased almost three fold their scopolamine **6** contents, without any significant changes in hyoscyamine **1** levels (Table 7.1) [47]. The increased contact between the plant cells can also increase alkaloids production. Immobilization of *D. innoxia* L. cell suspension in Ca-alginate leads to almost six fold increase of both hyoscyamine **1** and scopolamine **6** levels, whereas the addition of pure alginate into the cultivation medium altered only scopolamine **6** content (almost by three fold higher than in control), probably due to increased viscosity and oxygen limitation [44]. Despite of all efforts made, in most of the cases, the undifferentiated Solanaceae callus and cell suspension cultures failed to biosynthesize considerable amounts of hyoscyamine **1** and scopolamine **6**.

5.2 Soot Cultures

Searching for other producing systems, differentiated shoot cultures of *H. albus* L., *Duboisia leichhardtii* F.Muell., *D. myoporoides* R.Br., *Datura metel* L., and *Scopolia parviflora* (Dunn.) Nakai. were initiated on different media (B5 or MS, supplied with 10 μM BAP alone, or in combination with 0.1 or 1.0 μM NAA) [45, 48, 49, 52, 53]. Analyses showed that hyoscyamine **1** and scopolamine **6** were not presented or were detected at extremely low concentrations in these plant

Table 7.1 Examples for undifferentiated cultures, producers of tropane alkaloids

Species	Cultures	Medium	Phytohormones (μmol)	Light conditions	Hyoscyamine (μg g^{-1} DW)	Scopolamine (μg g^{-1} DW)	References
Hyoscyamus niger L.	Callus	LS	10 NAA+5 BAP	Dark	150.00	11.90	[47]
Hyoscyamus niger L.	Callus	LS	10 NAA+5 BAP	Continuous light	199.80	4.30	[47]
Hyoscyamus niger L.	Suspension	LS	10 NAA+5 BAP	Dark	95.30	15.00	[47]
Hyoscyamus niger L.	Suspension	LS	10 NAA+5 BAP	Continuous light	98.60	4.80	[47]
Atropa belladonna L.	Callus	LS	10 NAA+5 BAP	Continuous light	0.00	0.00	[47]
Datura stramonium L.	Callus	LS	10 NAA+5 BAP	Continuous light	0.00	0.00	[47]
Duboisia leichhardtii F. Muell.	Callus	B5	10 NAA+1 BAP	Continuous light	tr[a]	tr	[48]
Hyoscyamus niger L.	Suspension	LS	10 NAA+5 BAP	Dark	0.00	0.00	[45]
Hyoscyamus albus L.	Suspension	LS	10 NAA+5 BAP	Dark	0.00	0.00	[45]
Datura innoxia L.	Suspension	S10	9 2,4-D	Light/dark	6.00	22.00	[44]
Duboisia myoporoides R. Br.	Callus	MS	1 NAA+10 BAP	Light/dark	0.41	0.23	[52]
Datura metel L.	Callus	MS	5 NAA+5 BAP	Light/dark	0.00	14.15[b]	[49]

[a] tr – Detected in traces
[b] Value is on mg g^{-1} DW

in vitro systems. This observation is not surprising, as it is well known that these alkaloids are synthesized in the roots [37]. When young roots were formed after transferring the *D. leichhardtii* F.Muell. shoots on root regeneration media (B5, supplied with 10 μM IBA), the appearance of tropane alkaloids in shoots biomass was recorded. With the increase of root biomass (2–3 weeks after root initiation), the hyoscyamine **1** and scopolamine **6** were accumulated at levels of 0.36% and 0.30% of dry shoots weight, respectively [48]. The biosynthesis of tropane alkaloid could occur in cultured shoots without root initiation, but in this case, the addition of appropriate phytohormone combinations, providing a suitable cell differentiation (development of xylem cells), is necessary [46, 52]. However, as the enzymes involved in tropane alkaloid biosynthesis are scattered in different specialized root tissues (PMT and H6H are localize to the pericycle in the roots, whereas TR-I resides in the endodermis and nearby cortical cells [37]), the mature roots remain the best system for their production.

5.3 Adventitious Root Cultures

Several adventitious root cultures have been obtained by direct cultivation of sterilized young root tips [45, 54–56], rhizomes [57, 58], calli [46, 52], or even single cells [59] of different Solanaceae plants in media (B5 or MS), supplied with higher auxin concentrations. In contrast with the case of plant cell suspensions, the growth of plant root cultures in bioreactors is accompanied by several difficulties. Thus, developments of new bioreactor designs or improvements of the existed apparatus constructions should be performed as an essential step for successful realization of such cultivation process. Adventitious root culture of *H. niger* L. was cultivated in 5.0 L stirred tank bioreactor, modified by addition of support mesh and in 5.0 L mist bioreactor [60]. In both cultivation systems, the levels of produced tropane alkaloids were not altered and remained to the similar levels, registered in shaking flasks (between 0.32% and 0.37% of dry weight for scopolamine **6** and 0.05–0.075% of dry weight for hyoscyamine **1**). However, significant decreases in obtained growth indexes in stirred tank (GI = 12.9) and mist reactor (GI = 4.76), compared to submerged cultivation in flasks (GI = 21.4), were observed [60]. The lower root growth in mist bioreactor was explained by the limited nutrient supply in these type cultivation systems. Small-scale (0.6 L) bubble column bioreactor has been developed for cultivation of *S. parviflora* (Dunn.) Nakai. adventitious roots [58]. It was found that the increased inoculum density from 3.0 g fresh weight to 15.0 g fresh weight stimulated scopolamine **6** production in the roots (from 1.44 to 1.82 mg g^{-1} dry weight) and negatively correlated with their hyoscyamine **1** content (from 3.29 to 2.66 mg g^{-1} dry weight) [58]. Large-scale stirred tank bioreactor (500 L with 300 L working volume), equipped with special inoculation and mixing systems, has been developed and applied for cultivation of *A. belladonna* L. adventitious roots as well [55]. After 60 days of cultivation (agitation for 30 min at 30 rpm in every 3 days was started after the 26th day), a total amount of 35.0 kg fresh roots, containing 334.0 μg g^{-1} dry weight scopolamine, were produced [55].

The long cultivation times and the low alkaloid yields make these in vitro systems inappropriate for commercial application. Moreover, the supplement of additional phytohormones, essential for support of the adventitious roots growth, could promote the appearance of culture instabilities, manifested by unpredictable alkaloid yields.

5.4 Hairy Roots

Hairy root cultures seem to be the most promising alternative to overcome the above- mentioned difficulties. These in vitro systems are obtained by genetic transformation of plant cells with the soilborne phytopathogenic bacteria *Agrobacterium rhizogenes* [61]. During this process, a small part of bacterial root-inducing plasmid (Ri plasmid), named transfer DNA (T-DNA), is introduced and permanently integrated into the random site of plant genome [62, 63]. Most of the *A. rhizogenes* strains contain only one fragment of T-DNA in their plasmids, whereas the plasmids of some super-virulent strains (as example, agropine strains) have two completely different T-DNA fragments (T_L-DNA and T_R-DNA), which could be transferred independently into the plant cell [61, 63]. However, only T_L-DNA encodes the genes, responsible for neoplastic root formations. The T_R-DNA encodes few genes (*aux1*, *aux2*, *RolB* TR, *mas1*, *mas2*, and *ags*) responsible for control of opine and auxin biosynthesis in plants; thus, it was considered that it could be required only to extend the host range in *A. rhizogenes* strains [64, 65]. For realization of T-DNA transfer, the agrobacteria should be in close contact with damaged plant tissues. When this happens, the release of plants phenolic compounds (mainly acetosyringone) and sugars acts as inducers for expression of several *chv* and *vir* genes, located in bacterial chromosome and Ri plasmid, respectively. These signals triggered the beginning of several processes including T-complex formation, its further transport into the plant cell, nuclei localization, and T-DNA integration. The molecular mechanisms involved in each steps of this complex interkingdom horizontal gene transfer are well studied and described elsewhere [63, 64]. In general, the T-DNA consists of left and right border sequences and several oncogenes flanked by eukaryotic regulatory sequences, encoded between them [66]. The availability of both left and right border sequences is crucial for successful implementation of T-DNA integration, no matter which genes were encoded between them [67]. This made T-DNA an attractive system for introducing and expression of foreign genes in plants [7, 68]. However, only the expression of *A. rhizogenes* oncogenes (the “root locus” *rolA*, *rolB*, *rolC*, *rolD*, as well as the ORF3n, ORF8, and ORF13) can perturb the signal transduction pathway involved in auxin perception in plant cells, increasing their sensitivity to endogenous phytohormone levels [61, 65, 66]. As results, a fast growing, laterally branched roots named “hairy roots” are formed by each transformed cell [62]. These roots are able to rapidly grow on phytohormones-free medium and accumulate similar or even higher amounts of secondary metabolites compared to nontransformed plant roots [62, 65, 69].

Most of the Solanaceae plants were susceptible to *Agrobacterium*-mediated transformation. Successful establishing of hairy root cultures has been reported for several genera including *Atropa*, *Anisodus*, *Brugmansia*, *Datura*, *Duboisia*, *Hyoscyamus*, and *Scopolia* [1, 24, 70–74]. The direct infection and cocultivation of sterile plant explants with *A. rhizogenes* seems to be the most effective procedure for hairy root induction by Solanaceae plants (Fig. 7.4). This method is relatively simple for implementation and does not require any special laboratory equipment. To induce hairy roots, sterilized explants or sterile in vitro plants are wounded and inoculated with *A. rhizogenes* suspension.

All known plant organs could be used as explants for transformation, but the young leaves and stems are preferred for Solanaceae plants [72, 75, 76]. Several *Agrobacterium* strains including *A. rhizogenes* ATCC 15834, ATCC13333, 8196, 1724, 2659, 1855, AR-10, TR-105, A41027, A4, LBA 9402, LBA 1334, R1601, and MAFF 03-01724 as well as *Agrobacterium tumefaciens* C58 C1 carrying the pRi 15834 plasmid have been reported to induce hairy roots formation in different members of Solanaceae family [69, 70, 72, 75, 77–80]. Double transformation by using two *A. rhizogenes* strains (ATCC 15834 and MAFF 03-01724) was reported as well. The obtained doubly transformed *A. belladonna* L. hairy roots have intermediate alkaloid content as compared to the singly transformed hairy root lines but showed improved growth [81]. However, the different *Agrobacterium* strains manifested varying capability to transform plant cells, depending on the current plant species. Moreover, different *Agrobacterium* strains have a great influence on the root morphology and degree of tropane alkaloids accumulation in obtained hairy root clones. Thus, the choice of appropriate *Agrobacterium* strain is very important and should be performed experimentally [1, 62, 70, 72, 75, 82]. Two or three days after infection, the explants should be transferred into solid media (B5 or MS) with antibiotic (usually cefotaxime in concentrations between 100 and 500 mg L^{-1}) to eliminate redundant bacteria (Fig. 7.4). The young hairy roots will be induced within a period of 1 week to over a month depending on different plant species. When their size reaches 2–3 cm, the individual root tips can be separated for detached growth on phytohormone-free media. After transformation, in many Solanaceae plants, an intensive callus formation could be observed simultaneously with hairy roots induction (Fig. 7.4). These calli (often referred as "rhizogenic" calli) could be formed directly on some separated hairy root tips as well. However, the hairy root lines carrying such callus formations often possess tendency toward dedifferentiation, which significantly increase their genetic instabilities and reduce the amounts of accumulated alkaloids [70, 71, 75, 82, 83]. Thus, when isolating single hairy root clones, it is essential to select those lines which do not form callus. It was reported that there were great differences in growth rate, morphology, and alkaloid production between individual hairy root clones, even isolated from the same explants [69, 70, 82]. As example, 500 hairy root lines of *D. stramonium* L. were isolated from the same mother plants and screened for growth, morphology, and total alkaloid content [69]. The levels of total alkaloids accumulated in their dry biomasses varied between 0.63% and 1.5%. In addition, only 8 lines showed high growth potential and stable alkaloid production for more

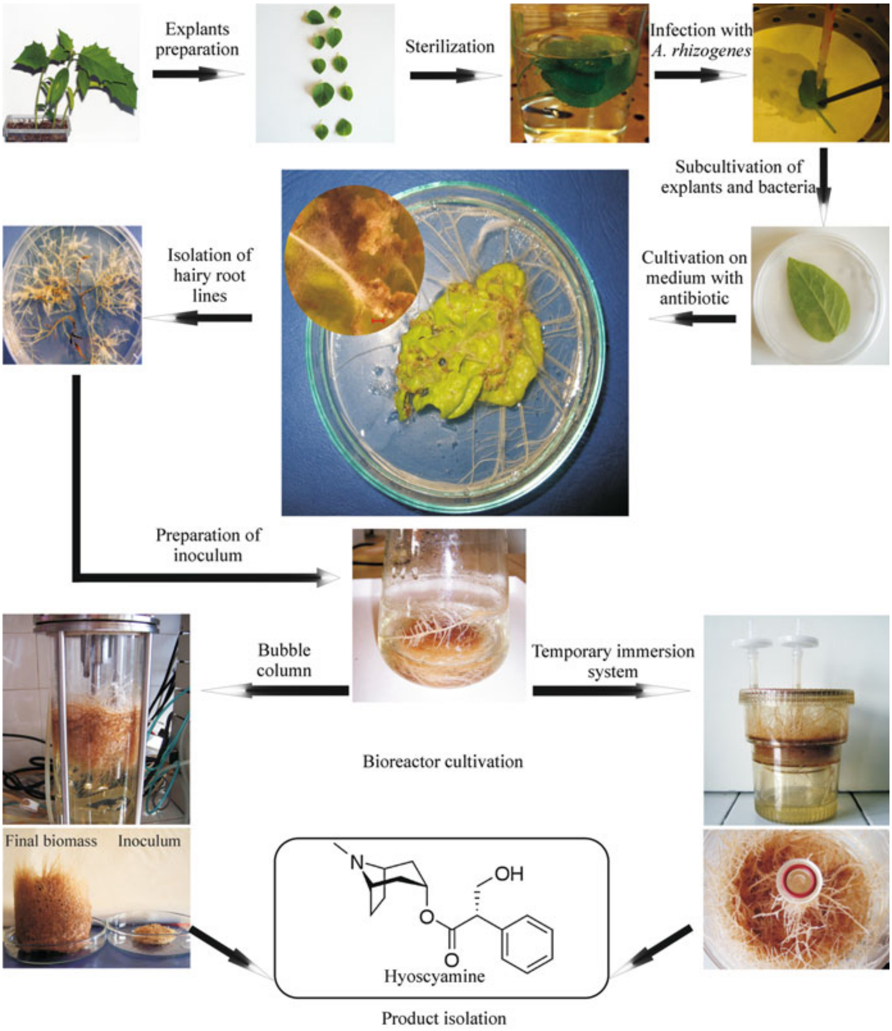
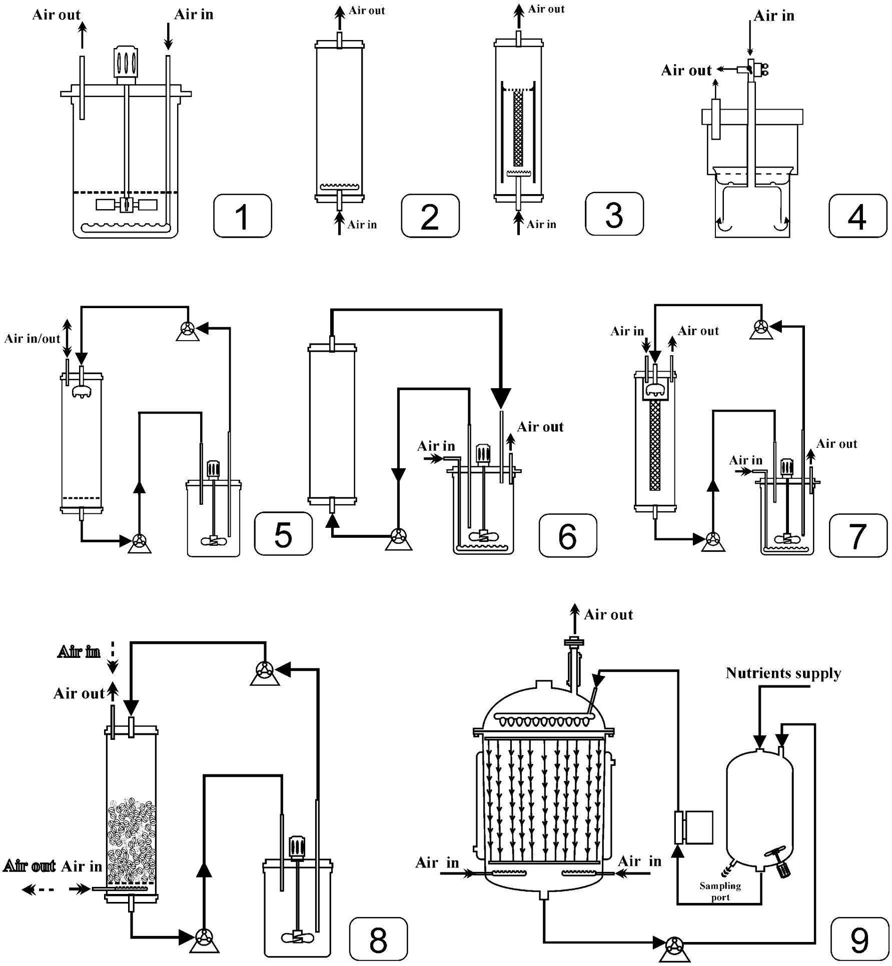

Explants preparation
Sterilization
Infection with A. rhizogenes
Subcultivation of explants and bacteria
Cultivation on medium with antibiotic
Isolation of hairy root lines
Preparation of inoculum
Bubble column
Temporary immersion system
Bioreactor cultivation
Final biomass
Inoculum
N
OH
O
O
Hyoscyamine
Product isolation

Fig. 7.4 *Datura stramonium* L. hairy roots induction, lines selection and their submerged cultivation in flasks and different bioreactor systems

than 5 years [69]. Substantial somaclonal variation was found to exist even in single-selected individual hairy root clones [82, 84]. This observation made possible the application of strategies of repeated selection or single protoplast selection for obtaining and screening of hairy root lines with high capabilities to accumulate biomass and tropane alkaloids. As example, after 8 repeated selections, the scopolamine **6** content in dry biomass of *D. myoporoides* R.Br. hairy root line was increased to 3.5% of dry weight, compared to a 0.15% at the parent line but decreased the growth from 7.0 to 2.2 g dry biomass L^{-1} [84]. Such negative correlation between tropane alkaloid contents and culture growth has been often reported and is thought to be the main reason for the observed low overall productivity [1, 70, 75, 84]. To overcome this substance, the growth and production conditions should be optimized individually for every selected hairy root line. Since each hairy root line is different, also its basic nutrient requirements could vary in some ranges. The growth and tropane alkaloids production of hairy root cultures could be differently affected by the type of basal culture media used for their cultivation [71, 83, 85–87]. Optimization of micro- and macronutrients of cultivation medium for selected hairy root line could have great effect on its overall tropane alkaloids productivity as well [86, 88, 89]. As example, after optimization of MS macronutrients composition for maximal biosynthesis of hyoscyamine by tetraploid and diploid *D. stramonium* L. hairy root cultures, the tetraploid hairy roots accumulated 24.7% more hyoscyamine **1** than the diploid culture, whereas before optimization, the last one has been found to produce 20.7% more hyoscyamine **1** than the former [89, 90]. The addition of exogenous growth regulators could have significant effects on both root morphology and tropane alkaloids content. In general, the addition of auxins stimulates disorganization of hairy root tissues and drastically decreases their tropane alkaloid productions [91, 92]. However, it has been reported that the exogenous supply of IAA or NAA at low concentrations (between 0.01 and 5.0 μmol L^{-1}) resulted in two fold increase of the alkaloids accumulation in hairy root cultures of *H. muticus* L., without affecting significantly the hairy root growth and morphology [1, 75]. On the other hand, the exogenous supply of GA was found to increase the roots lateral branches but to suppress significantly the tropane alkaloids production [93–95]. The elicitation with different biotic and abiotic elicitors has contradictory effects on tropane alkaloids accumulation in hairy root cultures of different Solanaceae species, but in all cases, they had increased the effects on alkaloids secretion in the culture medium [1, 62, 73, 77, 88, 96–98].

Introducing of genes encoding key enzymes of tropane alkaloids biosynthesis pathway in different Solanaceae hairy root cultures was applied as perspective strategy for increasing their scopolamine **6** and hyoscyamine **1** productions [1, 7, 68, 99]. Hairy root cultures of *Datura, Duboisia, Hyoscyamus*, and *Scopolia*, and overexpressing PMT have been obtained by introduction of *pmt* gene under the control of cauliflower mosaic virus 35S promoter (CaMV35-S) in their genomes [1, 7, 11, 100–102]. In general, the overexpression of *pmt* led to increase in both hyoscyamine **1** and scopolamine **6** levels in transformed hairy root lines, but in some cases, it was found to increase only the accumulation of the direct metabolite (*N*-methylputrescine **13**), while no effect on alkaloid production was observed [7, 101, 103]. The cDNA of TR-I and TR-II from *D. stramonium* L. was cloned into the binary plasmid pBI121 carrying a kanamycin resistance gene, a b-glucuronidase (GUS) reporter gene, and the CaMV 35-S-promotor. The constructs were introduced separately into *A. belladonna* L. and transformed hairy root cultures were obtained [38]. The hyoscyamine **1** and scopolamine **6** levels in hairy root lines overexpressing *trI* were increased three and five fold, respectively. On the other hand, the lines overexpressing the *trII* do not increase their hyoscyamine **1** or scopolamine **6** levels but elevated significantly the accumulation of pseudotropine-derived alkaloids (calystegines) [38]. In order to increase the scopolamine **6** levels in hyoscyamine **1** producing plants, the overexpression of H6H represents a promising target for obtaining transgenic hairy root cultures. The *h6h* gene under the control of CaMV 35-S-promotor was introduced in several transgenic hairy root lines of *Atropa*, *Duboisia*, *Hyoscyamus*, and *Scopolia* [41, 57, 102, 104, 105]. As general, the *h6h* overexpression led to a higher production of scopolamine **6**, whereas the hyoscyamine **1** content remained unaltered, which demonstrates that the *h6h* altered not only the last step of hyoscyamine **1** to scopolamine **6** conversion, but the overall alkaloid production of transgenic hairy root lines [7, 104, 105]. However, the most effective strategy for genetic engineering of tropane alkaloids producing hairy roots seems to be the simultaneous introduction and overexpression of couple genes, encoding different rate-limiting enzymes of scopolamine biosynthesis pathway in Solanaceae plants. Transgenic *H. niger* L. hairy root lines overexpressing both *pmt* and *h6h* were obtained after simultaneous introduction of genes encoding the PMT and H6H enzymes, both under control of CaMV 35-S promoter [12]. The best line produced over nine fold higher amount of scopolamine **6** (411 mg L^{-1}), compared to the wild-type hairy roots. This productivity was more than two fold higher than the scopolamine **6** content, registered in the *h6h* single-gene transgenic hairy root line as well [12]. On the other hand, the simultaneous overexpression of both *pmt* and *h6h* in transgenic *A. belladonna* L. hairy roots increased predominantly the hyoscyamine **1** production (11-fold, compared to control hairy roots), whereas the scopolamine **6** production was less improved (only 5 fold higher, compared to the control hairy root cultures) [10]. Recently, simultaneous introduction and overexpression of *pmt* and *trI* genes into *Anisodus acutangulus* C.Y. Wu & C. Chen hairy roots was reported [106]. The best producing transgenic line overexpressing both *pmt* and *trI* genes accumulated 8.104 mg g^{-1} DW tropane alkaloids, which was 8.66-, 4.04-, and 3.11-fold higher amount than the alkaloids in control hairy root line (0.935 mg g^{-1} DW), *pmt* single-gene transgenic hairy root line (2.004 mg g^{-1} DW), and *trI* single-gene transgenic hairy root line (2.604 mg g^{-1} DW), respectively [106].

The key point for successful realization of hairy root based-biotechnology for tropane alkaloid production is the large-scale cultivation in bioreactors. Since the hairy root cultures are sensitive to shear stress, which can cause disorganization of root tissue and decrease of tropane alkaloid productivity, the more complicated bioreactor systems should be used for their cultivations [107]. Several bioreactor systems were developed and applied for cultivation of hairy root cultures of different Solanaceae plants (Fig. 7.5, Table 7.2.) [107–114]. As can be seen on Table 7.2, the 14-L trickle-bed bioreactor (Fig. 7.5 position 8) operating in oxygen enrichment mode provides the highest biomass density (36.2 g L^{-1}) [111]. However, even the remarkably low energy costs for operation of this type bioreactor system, the presence of bed with support rings for root immobilization complicates the harvesting of accumulated biomass and confined the scale-up of the process. The problem with harvesting of immobilized root biomass was partially solved by development of detachable support matrix for large-scale (500 L) droplet


Air out
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Air out
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Air out
7
Air in
Air out
Air out
Air in
8
Air out
Nutrients supply
Air in
Air in
Sampling port
9

Fig. 7.5 Bioreactors used for cultivation of hairy root cultures of different Solanaceae plants: *1* stirred tank with isolated stirrer; *2* bubble column; *3* airlift with modified draft tube; *4* temporary immersion RITA system; *5* liquid-dispersed trickle reactor; *6* convective flow reactor; *7* modified liquid-dispersed reactor; *8* trickle-bed reactor; and *9* droplet reactor

bioreactor (Fig. 7.5 position 9) [107]. Hairy root culture of D. *stramonium* L. was successfully cultivated in this type bioreactor systems for 40 days with sucrose feeding. As a result, a 39.5-kg fresh hairy root biomass was produced [107]. Up to date, this is the largest bioreactor system used for cultivation of hairy root cultures reported in literature. During the last years, the low-cost bioreactors received increased attention for cultivation of hairy root cultures [115]. In our laboratory, we applied the temporary immersion RITA systems for cultivation of diploid and tetraploid hairy root cultures of D. *stramonium* L. [114]. The main advantages of these cultivation systems compared to conventional bioreactors are the simple

Table 7.2 Examples for different bioreactor systems applied for cultivation of hairy root cultures from different Solanaceae plants

Hairy roots	Bioreactor types	Vessel volume (effective volume) (L)	Final dry biomass density (g L^{-1})	Cultivation time (days)	Hyoscyamine (mg g^{-1} DW)	Scopolamine (mg g^{-1} DW)	References
Atropa belladonna L.	Stirred tank with isolated impeller	3.0 (2.5)	6.9	30	3.6	1.1	[109]
Atropa belladonna L.	Stirred tank with isolated impeller	30.0 (25.0)	6.0	30	5.4	0.9	[109]
Hyoscyamus muticus L.	Convective flow reactor	2.14 (1.92)	24.5	30	NR[a]	NR	[108]
Hyoscyamus muticus L.	Bubble column	2.14 (1.92)	15.3	30	NR	NR	[108]
Datura metel L.	Airlift with modified draft tube	4.0 (3.5)	2.6	28	NR	4.0	[110]
Hyoscyamus muticus L.	Bubble column/ trickle-bed	2.0 (1.6)	11.3	13	NR	NR	[111]
Hyoscyamus muticus L.	Bubble column/ trickle-bed	14.0 (14.0)	36.2	25	NR	NR	[111]
Datura stramonium L.	Bubble column/ droplet reactor	500.0 (500.0)	7.8	40	NR	NR	[107]
Atropa belladonna L.	Liquid-dispersed reactor	9.1 (4.4)	11.9	24.9	3.8	NR	[113]

(*continued*)

Table 7.2 (continued)

Hairy roots	Bioreactor types	Vessel volume (effective volume) (L)	Final dry biomass density (g L^{-1})	Cultivation time (days)	Hyoscyamine (mg g^{-1} DW)	Scopolamine (mg g^{-1} DW)	References
Datura stramonium L./diploid/	Temporary immersion systems	0.2 (0.2)	9.3	24	6.5	tr[b]	[114]
Datura stramonium L./tetraploid/	Temporary immersion systems	0.2 (0.2)	8.6	24	5.4	tr	[114]
Datura stramonium L./diploid/	Bubble column	3.0 (2.1)	3.3	21	2.9	tr	Unpublished data
Datura stramonium L./tetraploid/	Bubble column	3.0 (2.1)	4.5	21	2.7	tr	Unpublished data
Hyoscyamus muticus L.	Bubble column	1.0 (0.25)	3.2	18	NR	NR	[112]
Hyoscyamus muticus L.	Liquid-dispersed trickle reactor	1.0 (0.25)	4.4	18	NR	NR	[112]
Hyoscyamus muticus L.	Inclined reactor	1.0 (0.25)	5.0	18	NR	NR	[112]

[a] NR – Not reported
[b] tr – Detected in traces

construction, lack of complicated control system, and the relatively low price. When *D. stramonium* L. diploid and tetraploid hairy roots were cultivated in this temporary immersion system, they produced 2.8- and 1.9-fold more dry biomass, compared to when the same cultures were cultivated in standard bubble column bioreactor, respectively (Table 7.2) [114]. However, despite of all progress made with selection of high hyoscyamine **1** and scopolamine **6** producing hairy root lines, genetic engineering for improvements of alkaloid contents and design of bioreactor systems with specific constructions, the overall productivities of available hairy root cultures, is not sufficient for development of industrial process. Additional alternative strategies for improvement of hairy roots productivities should be applied.

5.5 Artificial Polyploidy as a Useful Tool for Tropane Alkaloids Production

While approaches for optimizing the yields of target metabolites are now well studied and appropriate strategies have been developed, the progress of modern science in understanding and controlling the profiles of produced metabolites is relatively small [50]. One way of changing the profiles of biosynthesized secondary metabolites is by artificial polyploidization [116]. Induced polyploidy makes possible both the increase in the yields of target metabolites and synthesis of new biologically active substances that are not normally synthesized in the source intact plants [13].

Polyploidy is a phenomenon that is part of the evolution of the plant kingdom [117, 118]. A polyploid is an organism that has more than two sets of chromosomes [119]. In nature, this phenomenon is rare in animal cells but widespread in plants. The term "polyploidy" or "polyploidy level" refers to the number of chromosome groups and is indicated by "x." An organism with two sets chromosomes is diploid (2x), with three sets of chromosomes – triploid (3x), with four sets of chromosomes – tetraploid (4x), etc. Polyploids may occur spontaneously (due to somatic mutation or abnormal mitosis, expressed in duplication of the chromosome number in the meristem cells) or may be induced in vivo or in vitro (with mitotic inhibitors or alteration of plant growth regulators) [120, 121]. It is important to note that induced polyploidy in plant cells does not introduce new genetic material but produces extra copies of existing chromosomes. This additional DNA replicates in subsequent cell divisions, and thus stable polyploids are obtained. Induced polyploidy has been intensively developed since the early 1930s (when the first mitotic inhibitors were discovered) in relation to improvement of agricultural crops [119].

The increase in the ploidy level often causes anatomical and structural changes, such as changes in the leaves, cell size, number of chloroplasts in cells, etc. [122, 123]. These effects of polyploidy may influence photosynthesis [124], the enzyme activities, the photosynthetic electron transport, and isoenzyme activity [13]. All these manifestations of polyploidy often lead to gigantism, i.e., to increased accumulation of plant biomass [116] and a change in secondary metabolism [13].

The general model to explain these changes is based on the assumption that the lower nuclear membrane: chromatin ratio is responsible for better contact between the chromatin and the nuclear envelope. Therefore, the gene activity increases, which improves the hormonal status and photosynthetic activity of the cell [125]. All these factors can affect the secondary metabolism and the yield of biologically active substances [13]. The data obtained by researchers over the years reveal that, with a few exceptions, polyploidy leads to a significant increase in the yields of biologically active substances. In addition, artificial polyploidy leads to a change in the chemical profile of secondary metabolites [126]. This fact itself is of particular importance both from practical (possibility to obtain new substances with high biological activity) and from fundamental points of view (an approach for studying the regulatory mechanisms of secondary metabolism of plant cells).

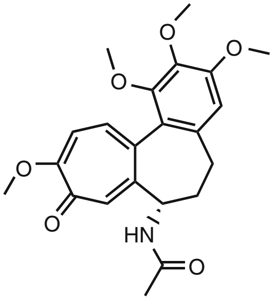
At present, plant in vitro systems are not used efficiently to study the relationship between the ploidy level and secondary metabolism. Research aimed at studying genetic stability suggests that most cells in a *Taxus* cell suspension are aneuploid within a 2-year period [127]. As far as ploidy variations in cell suspensions are cyclical, they can be related to the observed transient asymmetric cyclicity of the profiles of the biosynthesized secondary metabolites [128]. It is known that this cyclicity in the secondary metabolism subsides over time [129]. Plant cell cultures are not suitable matrices for exploiting the relationship between the ploidy level and secondary metabolism because they are inherently mixoploid systems and the achieved yields of target metabolites, and the analyzed metabolic profiles are a statistically average response of a heterogeneous cell association [50, 127]. Unlike plant cell suspensions, transformed root cultures are characterized by high genetic stability [130, 131], and it is adopted as a rule that their metabolic profile is identical to that of the intact plants from which they are derived [132]. This defines these cultures as promising systems both for fundamental research related to the regulation of secondary metabolism by means of artificial polyploidy and for applied research related to analysis of the relationships between ploidy levels and yields of target products and/or metabolic profiles of the studied in vitro systems.

5.5.1 Methods for Obtaining Polyploid-Transformed Root Cultures

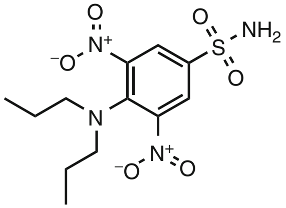
For polyploidization, both under in vitro and in vivo conditions, most frequently, the mitotic inhibitors colchicine **31** and oryzalin **32** are used. They inhibit the polymerization of the microtubules of the mitotic spindle, thereby preventing the duplicated genetic material from being divided between two daughter cells [133].

Colchicine **31** is an alkaloid synthesized by *Colchicum autumnale* L., which binds to tubulin (the polymer that makes up the microtubules of the mitotic spindle), thus preventing the formation of the mitotic spindle. This alkaloid is active in millimolar concentrations [133, 134].

Oryzalin **32** is a dinitrophenyl herbicide, which also exhibits high tubulin binding affinity, thereby inhibiting the polymerization of the microtubules of the mitotic spindle. Oryzalin **32** is active in micromolar concentrations [133].



Colchicine
31



Oryzalin
32

Two approaches are currently known for obtaining transformed root cultures of different ploidy level: transformation of autopolyploid intact plants and in vitro polyploidization of transformed root cultures obtained from diploid intact plants.

The methods of polyploidization of intact plants were developed in the second half of the last century and were well described in a number of articles [135]. The methods for obtaining transformed root cultures by transforming plant tissue with *Agrobacterium rhizogenes* have also been described in detail in numerous survey and scholarly articles [61, 136].

The approach of in vitro polyploidization of transformed root cultures obtained from diploid intact plants is presented schematically on Fig. 7.6. Most often, for in vitro polyploidization of transformed root cultures, colchicine **31** and oryzalin **32** are used in concentrations of 0.002–0.005% and 0.0002–0.0005%, respectively. Our experience shows that about 8–10% of the newly obtained lines are of different ploidy than the source line.

5.5.2 Biosynthetic Potential of Transformed Root Cultures with Different Ploidy Levels

Many years of research in our laboratory [24, 30, 89, 90, 114] on the impact of artificial polyploidy on the biosynthesis of tropane alkaloids from diploid- and tetraploid-transformed root cultures of *D. stramonium* L. have revealed that induced polyploidy makes it possible to obtain higher yields of target metabolites as well as to achieve biosynthesis of new biologically active substances that are normally not synthesized in the intact plants (Table 7.3). The comparative analysis of the alkaloid profile of diploid and tetraploid intact *D. stramonium* L. plants and transformed roots derived from them (Table 7.3) established 39 alkaloids, 33 of which were identified.

D. stramonium L. plants accumulate various alkaloids in different plant organs. However, no differences are detected in the alkaloid profiles of diploid and tetraploid plants. There are differences in the alkaloid spectra of plants of different ploidy levels in the minority alkaloids (less than 1% of the ion current).

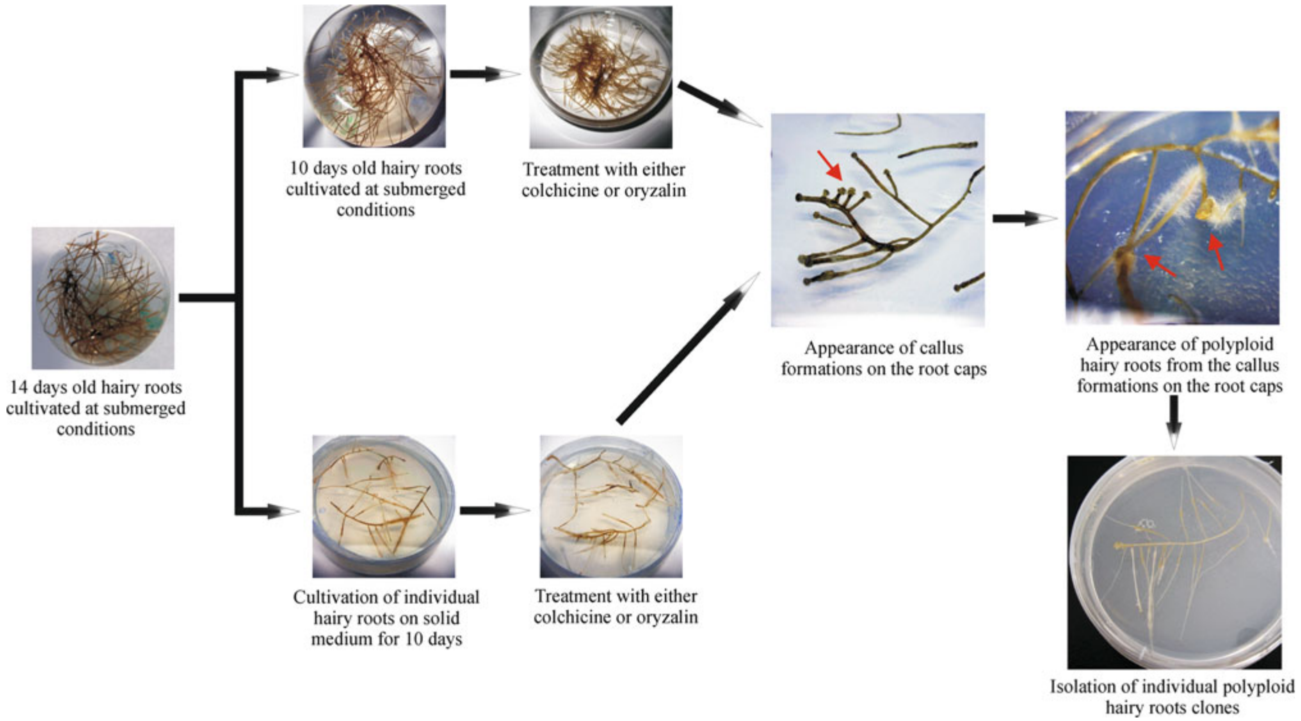

14 days old hairy roots cultivated at submerged conditions
10 days old hairy roots cultivated at submerged conditions
Treatment with either colchicine or oryzalin
Cultivation of individual hairy roots on solid medium for 10 days
Treatment with either colchicine or oryzalin
Appearance of callus formations on the root caps
Appearance of polyploid hairy roots from the callus formations on the root caps
Isolation of individual polyploid hairy roots clones

Fig. 7.6 Procedure for in vitro polyploidization of diploid hairy roots

Table 7.3 Alkaloids in different organs of diploid and tetraploid intact plants *Datura stramonium* L. and hairy roots obtained from them

			Roots		Leaves		Seeds		Hairy roots	
Entry	Alkaloids	M^+	2x	4x	2x	4x	2x	4x	2x	4x
1	Hygrine	141/84							+	+
2	Tropinone	139/82							+	+
3	3-Acetoxytropane	183/124							+	+
4	3-Tigloyloxytropane	223/124							+	+
5	3α-Phenylacetoxytropane	259/124					+	+	+	
6	3β-Phenylacetoxytropane	259/124					+			
7	3α-Apotropoyloxytropane	271/124		+	+	+	+	+	+	+
8	3β-Apotropoyloxytropane	271/124					+			
9	Hyoscyamine	289/124	+	+	+	+	+	+	+	+
10	3-(3′-Acetoxytropoyloxy)tropane	331/124	+	+					+	+
11	3-(2′-Hydroxytropoyloxy)tropane	305/124	+							
12	Alkaloid A	−/124	+	+						
13	Alkaloid C	−/124							+	+
14	Methylecgonine	199/82							+	
15	3-Acetoxy-6-hydroxytropane	199/94								+
16	3-Hydroxy-6-acetoxytropane	199/113							+	
17	3,6-Diacetoxytropane	241/94							+	+
18	3-Hydroxy-6-tigloyloxytropane	239/113							+	
19	3-Tigloyloxy-6-hydroxytropane	239/94	+	+					+	
20	3-Tigloyloxy-6-isobutyryloxytropane	309/94	+	+						
21	3-Tropoyloxy-6-hydroxytropane	305/94	+	+						
22	3-Tropoyloxy-6-methylbutyryloxytropane	323/94	+	+						
23	3-Tropoyloxy-6-tigloyloxytropane	387/94							+	
24	3-Tropoyloxy-6-acetoxytropane	347/94							+	
25	3,6-Ditigloyloxytropane	321/94	+	+					+	
26	3α-Tigloyloxy-6-isovaleroyloxy-7-hydroxytropane	339/94	+	+						
27	3β-Tigloyloxy-6-isovaleroyloxy-7-hydroxytropane	339/94	+	+						
28	3-Tigloyloxy-6-propionyloxy-7-hydroxytropane	341/94							+	

(*continued*)

Table 7.3 (continued)

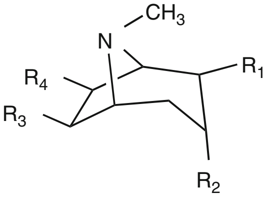
			Roots		Leaves		Seeds		Hairy roots	
Entry	Alkaloids	M^+	2x	4x	2x	4x	2x	4x	2x	4x
29	3-α,6b-Ditigloyloxy-7β-hydroxytropane	337/943	+	+						
30	3-β,6b-Ditigloyloxy-7b-hydroxytropane	337/94	+	+						
31	3-tigloyloxy-6,7-dihydroxytropane	255/94	+	+						
32	Aposcopolamine	285/94					+			
33	Methylscopolamine	317/94				+				
34	Norscopolamine	289/122	+							
35	Scopolamine	303/94	+	+	+	+	+	+		
36	Alkaloid B	−/94	+	+						
37	Alkaloid D	−/94							+	
38	Alkaloid E	319/94							+	
39	Alkaloid 325	325/94	+	+						

In transformed root cultures, the influence of the ploidy level on the qualitative composition of the biosynthesized alkaloids is clearly defined (Table 7.3). There are certain differences in the alkaloid profiles of intact roots and in the corresponding transformed root cultures of *D. stramonium* L., which contradicts the widespread assertion about the identity of the secondary metabolism of transformed root cultures and the respective intact roots [132, 134, 137]. Intact roots produce 3-monosubstituted, 2,3-disubstituted, 3,6-disubstituted, 3,6,7-trisubstituted alkaloids, and 3-substituted-6,7-epoxytropanes, while transformed roots biosynthesize mainly 3-monosubstituted and 3,6-disubstituted alkaloids (Table 7.3).

These results clearly indicate that induced polyploidy is a powerful approach to establish selection algorithms for obtaining highly productive lines of transformed root cultures as well as for biosynthesis de novo of unknown and/or secondary metabolites not synthesized in the respective intact plants (entries 1, 2, 3, 4, 13, 14, 15, 16, 17, 18, 23, 24, 28, 37 and 38, Table 7.3) that could carry new biological activities.

It should be noted that the two ploidy levels of the transformed root cultures of *D. stramonium* L. show significant differences as regards the spectrum of the biosynthesized alkaloids. Unlike the diploid, tetraploid-transformed roots biosynthesized ten alkaloids. One of them (entry 15, Table 7.3) was not detected in the diploid root culture of *D. stramonium* L. The pharmacologically active alkaloid hyoscyamine **1** is the principal alkaloid biosynthesized by both root cultures at 78.8% and 56.6% of the total ion current in the diploid and tetraploid-transformed roots of *D. stramonium* L., respectively (entry 9, Table 7.3). In contrast to diploid roots, which accumulate 7.12% apoatropine, the tetraploid-transformed root culture biosynthesize 3-acetoxytropane (28.4%) as a second alkaloid in terms of amount. C-3 monosubstituted tropanes dominate in the alkaloid mixture accumulated by the tetraploid culture, while disubstituted tropanes dominate in the alkaloid mixture biosynthesized by the diploid root culture of *D. stramonium* L. The significant differences established in the secondary metabolism of the diploid- and tetraploid-transformed root cultures of *D. stramonium* L. are probably due to differences in the genome organization of plant cells with different chromosome sets as well as to various physiological characteristics of both cultures that originate from their different ploidy level.

The subsequent detailed analysis of the alkaloid profiles of diploid- and tetraploid- transformed root cultures of *D. stramonium* L., cultivated in liquid medium, revealed that the studied cultures biosynthesized alkaloids that are untypical of intact plants: 3-tropoyloxy-6-acetoxytropane **33** (new for *Datura* genus), methylecgonine **34** (new for the Solanaceae family), and 3-tigloyloxy-6-propionyloxy-7-xydroxytropane **35** (a new tropane ester) [24].



		R_1	R_2	R_3	R_4
3-tropoyloxy-6-acetoxytropane	**33**	H	H O − COC −C_6H_5 I CH_2OH	O −$COCH_3$	H
methylecgonine	**34**	O −$COCH_3$	OH	H	H
3-tigloyloxy-6-propionyloxy-7-xydroxytropane	**34**	H	O − COC =CH I \\ CH_3 CH_3	O −COC_2H_5	OH

After optimization of the nutrient media for maximum yield of hyoscyamine **5** from diploid- and tetraploid-transformed root cultures of *D. stramonium* L. [90], the tetraploid root culture of *D. stramonium* L. accumulated 24.7% (2.01 g L^{-1}) more hyoscyamine **1** than the diploid culture (1.51 g L^{-1}), while in cultivation in standard MS nutrient medium [89], the diploid-transformed root culture synthesized 20.7% higher amounts. Therefore, it is not only the higher amount of nuclear DNA but also the optimal conditions for its expression that are essential for obtaining maximum yields of the target tropane alkaloids.

6 Conclusion

Despite of all progress made in the area of tropane alkaloids biotechnology, the overall productivities of available plant in vitro systems are not sufficient for development of industrial process. Hairy roots are the most promising plant in vitro culture that could be used as a technological matrix for development of an industrial process for hyoscyamine and scopolamine production. To realize this however, an integrated approach, based both on well- developed up to now strategies (selection of high-producing lines, genetic engineering for improvements of alkaloid contents, media optimization, and design of bioreactor systems with specific constructions) and on alternative strategies for improvement of hairy roots productivities (artificial polyploidy, cell cycle management, etc.), should be developed and applied.

As scopolamine is the constituent with a higher added value in the pharmaceutical market, more efforts on biotransformation of hyoscyamine to scopolamine should be done. This could happen either by genetically engineering cultures [138] or by using interspecies and/or intergenus organ cocultures [139]. Moreover, the genetic engineering of polyploidy hairy root cultures may prove an attractive source of new tropane alkaloids with better therapeutic value and higher biological activities.

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Biotechnology and Genetic Engineering for Alkaloid Production

8

Smita Srivastava and Ashok Kumar Srivastava

Contents

1 Introduction 215
  1.1 Chemical and Physical Characterization of Alkaloids 216
  1.2 Biosynthesis of Alkaloids in Plants 219
2 Production of Alkaloids Via Plant Cell Bioprocessing: Pregenomic Approach 224
  2.1 Screening and Selection of a High-Yielding Stable Cell Line 228
  2.2 Culture Condition Optimization 229
  2.3 Use of Differentiated Cultures for Enhanced Production of Alkaloids 231
3 Metabolic Engineering for Engineering Alkaloid Biosynthesis in Cellular Systems: A Genomic Approach 232
  3.1 Single-Step Engineering 233
  3.2 Multiple-Step Engineering 235
  3.3 Engineering Regulatory Genes 235
  3.4 Mutasynthesis of Novel Alkaloid Analogs 239
4 Engineering Alkaloid Biosynthesis in Microorganisms 240
5 Conclusion 243
References 245

Abstract

Alkaloids are a diverse group of complex organic molecules found in about 20% of plant species in small quantities. Their potent biological activity has led to their exploitation as pharmaceuticals, stimulants, narcotics, and poisons. Despite their

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importance, inefficiency of extracting alkaloids from a myriad of other metabolites remains a significant barrier toward inexpensive bioprospecting for drug development. Furthermore, the yield is inconsistent in the natural resource due to heavy dependence on genetic and geographical diversity and also on climatic conditions. Chemical synthesis has been successful for the class of indole alkaloids. However, it is still a challenge and also impractical on industrial scale to construct other commercially important class of alkaloids. In lieu of the given limitations, plant cell/tissue cultures serve as alternative production platforms in which the biosynthesis of alkaloids has been improved through various elicitation and culture manipulation strategies. In addition, recent advances made in metabolic engineering and systems biology now have the potential to more effectively maximize the alkaloid biosynthesis in such in vitro production systems. In this respect, the chapter provides information regarding recent advancements made in bioprocessing and genetic engineering of cellular systems for large-scale alkaloid production.

Keywords

Alkaloids • bioprocess optimization • genetic engineering • metabolic engineering • microbial culture • plant cell/tissue culture

Abbreviations

2, 4 D	2,4-Dichlorophenoxyacetic acid
4′-OMT	3′-hydroxy-*N*-methylcoclaurine-4′-*O*-Methyltransferase
6-OMT	6-*O*-methyltransferase
BBE	Berberine bridge enzyme
BIAs	Benzylisoquinoline alkaloids
CNMT	Coclaurine-*N*-methyltransferase
CPR	NADPH: cytochrome P450 reductase
CYP80B3	(*S*)-*N*-methylcoclaurine 3′-hydrolase
D4H	Desacetoxyvindoline 4-hydroxylase
DAT	Deacetylvindoline 4-*O*-acetyltransferase
EST	Enzyme tags
G10H	Geraniol-10-hydroxylase
H6H	Hyoscyamine-6-hydroxylase
MAO	Monoamine oxidase
MIAs	Monoterpenoid indole alkaloids
NAA	1-naphthaleneacetic acid
NCS	Norcoclaurine synthase
PMT	Putrescine *N*-methyltransferase
PUR-foam	Polyurethane foam
RNAi	RNA interference
SAM	*S*-adenosyl-L-methionine
SGD	Strictosidine b-D-glucosidase
SMT	(*S*)-scoulerine 9-*O*-methyltransferase
STR	Strictosidine synthase
TDC	Tryptophan decarboxylase

1 Introduction

Alkaloids are a diverse group of low-molecular-weight, complex organic molecules containing heterocyclic nitrogen ring. Alkaloids are produced by many different organisms, including plants, animals, and microbes, but a particularly diverse array of alkaloids is produced by plants. Approximately 10% of all plant species are thought to produce alkaloids as secondary metabolites. Many of the ≈12,000 alkaloids for which structures have been described function in the defense of plants against herbivores and pathogens [1–3]. The potent biological activity of alkaloids has led to their commercial exploitation as pharmaceuticals, stimulants, narcotics, and poisons (Table 8.1). Plant-derived alkaloids currently used as pharmaceuticals include morphine and codeine as analgesics, vinblastine and Taxol as anticancer agents, colchicines as gout suppressant, (C)-tubocurarine (muscle relaxant), ajmaline (antiarrythmic), sanguinarine as antibiotic, and scopolamine (a sedative). Other well-known alkaloids of plant origin include caffeine, nicotine, cocaine, and the synthetic *O,O*-acetylated morphine derivative heroin.

Alkaloids are mainly isolated from plants and produced commercially as fine chemicals. The complexity of these molecules means that it is in most cases impossible and impractical to produce them by total chemical synthesis, so extraction from the source plant remains the most economically viable strategy. However, plants often produce mixture of alkaloids with low yields; as a result, some commercially important alkaloids are highly expensive. With recent advances made in genetic engineering, research in manipulation of plants at genetic level has focused on the engineering of alkaloid biosynthesis to generate transgenic plants or cell lines that overproduce specific plant alkaloids. This has been proposed to be done via increasing the synthesis of a particular alkaloid and/or inhibiting the synthesis of related compounds for easier downstream processing. However, development of metabolic engineering strategies to enhance alkaloid biosynthesis in plants requires a thorough understanding of the endogenous biosynthetic pathways of alkaloids and its regulation mechanisms in plants. Plant cell and tissue cultures cannot only be used as model systems to elucidate the biosynthetic mechanisms of various secondary metabolites including alkaloids at cellular level but also as invaluable means of providing suitable biomass for large-scale production of these high-value alkaloids. Apart from this plant cell culture, investigations have also been done for biotransformation of alkaloids for enhanced bioactivity [4]. Despite the advantages, plant cell technology is not widely used as alternative production platforms for commercial production of alkaloids. The reason being that the level and manner of production of wide array of alkaloids in plants does not necessarily correlate with production in cell cultures. Moreover, issues related to slow growth and genetic and biochemical instability of plant cell cultures have been limiting their popular usage in industry. In this regard, the present chapter hence discusses some of the recent developments made in the bioprocessing and genetic engineering of plant cells/tissues for enhanced alkaloid production in cellular systems.

Table 8.1 Pharmacological applications of some important plant-derived alkaloids

Alkaloid	Plant source	Medicinal properties
Ajmaline	*Rauvolfia serpentina*	Antiarrhythmic, antihypertensive
Berberine	*Berberis vulgaris*	Antimicrobial
Caffeine	*Coffee arabica*	Stimulant, Insecticide
Camptothecin	*Camptotheca acuminate*	Antineoplastic
Cocaine	*Erythroxylon coca*	Analgesic, narcotic, local anesthetic
Codeine	*Papaver somniferum*	Analgesic, antitussive
Emetine	*Uragoga ipecacuanha*	Antiamoebic, expectorant, emetic
Hyoscyamine	*Atropa belladonna* and others	Anticholinergic
Irinotecan	Semisynthetic derivative of camptothecin	Chemotherapeutics
Morphine	*Papaver somniferum*	Analgesic, narcotic
Nicotine	*Nicotiana tabacum*	Stimulant
Noscapine	*Papaver somniferum*	Analgesic, antitussive
Oxycodone	Semisynthetic derivative	Analgesic
Oxymorphone	Semisynthetic derivative	Analgesic
Papaverine	*Papaver somniferum*	Vasodilator
Pilocarpine	*Pilocarpus jaborandi*	Cholinergic
Quinidine	*Cinchona* spp.	Antiarrhythmic
Quinine	*Cinchona* spp.	Antimalarial
Reserpine	*Rauvolfia serpentina*	Tranquilizer, antihypertensive
Sanguinarine	*Sanguinaria canadensis*	Antibacterial
Scopolamine	*Hyoscyamus niger* and others	Sedative, anticholinergic
Strychnine	*Strychnos nux-vomica*	Stimulant, poison
Taxol	*Taxus brevifolia*	Antineoplastic
Topotecan	Semisynthetic derivative	Chemotherapeutics
Vinblastine and vincristine	*Catharanthus roseus*	Antineoplastic, chemotherapeutics
Vindesine	Semisynthetic derivative	Chemotherapeutics
Vinflunine	Semisynthetic derivative	Chemotherapeutics
Vinorelbine	Semisynthetic derivative	Chemotherapeutics
Yohimbine	*Pausinystalia yohimbe*	Erectile dysfunction treatment

1.1 Chemical and Physical Characterization of Alkaloids

Alkaloids are a chemically heterogenous group of nitrogen containing substances found predominantly in higher plants. They occur in plants and are typically combined with sugars or simply as amides. They are also found in lower plants, animals, microorganisms, and marine organisms. Alkaloids exhibit dozens of different skeletal types. The structures of over 16,000 different alkaloids have been elucidated. Selected few important alkaloids are shown in Fig. 8.1 [5].

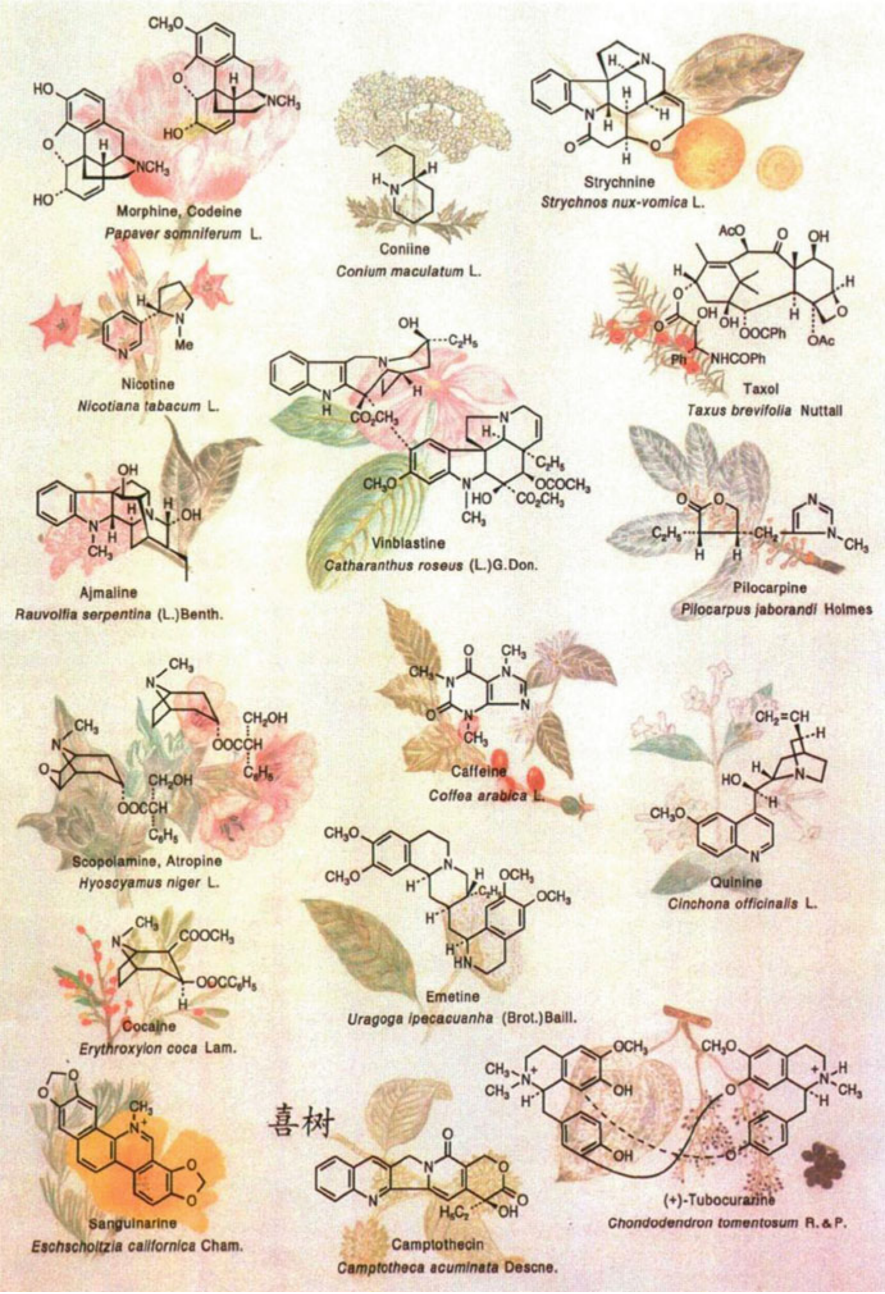

Morphine, Codeine
Papaver somniferum L.
Coniine
Conium maculatum L.
Strychnine
Strychnos nux-vomica L.
Nicotine
Nicotiana tabacum L.
Taxol
Taxus brevifolia Nuttall
Vinblastine
Catharanthus roseus (L.)G.Don.
Ajmaline
Rauvolfia serpentina (L.)Benth.
Pilocarpine
Pilocarpus jaborandi Holmes
Caffeine
Coffea arabica L.
Scopolamine, Atropine
Hyoscyamus niger L.
Quinine
Cinchona officinalis L.
Emetine
Uragoga ipecacuanha (Brot.)Baill.
Cocaine
Erythroxylon coca Lam.
喜树
Sanguinarine
Eschscholtzia californica Cham.
Camptothecin
Camptotheca acuminata Descne.
(+)-Tubocurarine
Chondodendron tomentosum R.&P.

Fig. 8.1 Structures of some commercially important alkaloids (Adopted from Ref. [5])

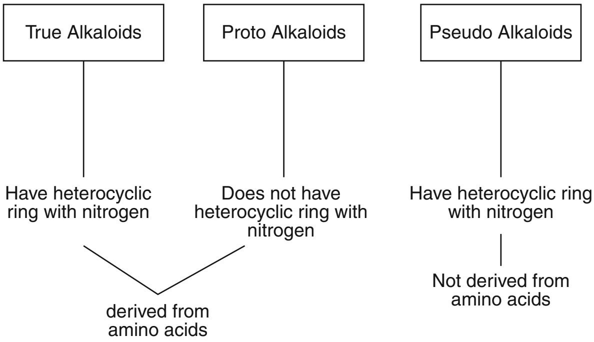

True Alkaloids
Proto Alkaloids
Pseudo Alkaloids
Have heterocyclic ring with nitrogen
Does not have heterocyclic ring with nitrogen
Have heterocyclic ring with nitrogen
derived from amino acids
Not derived from amino acids

Fig. 8.2 Classification of alkaloids on the basis of heterocyclic ring and amino acid (Adopted from Ref. [6])

No other class of natural products possesses such an enormous variety of structures. The term alkaloid was first coined in 1819 by a pharmacist, W. Meissner and meant simply, *alkalilike* (Middle English alcaly, from Medieval Latin *alkali*, from Arabic *alqaliy* = ashes of saltwort, from *qualey*, to fry). The first modern definition by Winterstein and Trier in 1910 [6] describes these substances in a broad sense as basic, nitrogen-containing compounds of either plant or animal origin [6]. Chemical, pharmacological, and botanical properties are usually considered when classifying these compounds as an alkaloid. "True alkaloids" were defined as compounds that fulfilled the additional four given criteria: (a) nitrogen atom being or not being a part of a heterocyclic system, (b) complex molecular structure, (c) manifesting significant pharmacological activity, and (d) restricted to plant kingdom. Compounds satisfying the definition of true alkaloids are restricted to certain families and genera of plant kingdom rarely being distributed in large group of plants. Though about 40% of all plant families contain at least one alkaloid-bearing species, true alkaloids have been reported in only 9% of over 10,000 plant genera. Other heterocyclic nitrogenous bases not classified as true alkaloids include the purines, of which caffeine, xanthine, and bromine are few examples.

Presently, alkaloids are classified into three main categories as shown in Fig. 8.2 [6]. This chemical classification of alkaloids is universally adapted and mainly depends on the type of heterocyclic ring structure present (Fig. 8.3). Alkaloids are further classified according to the amino acids (or their derivatives) from which they originate (Fig. 8.4) [6]. The most important classes are derived from the amino acids, ornithine and lysine, or from the aromatic amino acids, phenylalanine and tyrosine, or from tryptophan and a moiety of mavelonoid origin. A number of alkaloid-based compounds are also derived from anthranilic acid or from nicotinic acid.

Although majority of alkaloids and their derivatives are colorless, crystalline, nonvolatile solids, a few such as *cotinine* and *nicotine* are liquids, and few are even colored, for example, *berberine* is yellow. Alkaloids are insoluble in water but soluble in most organic solvents. Most of the alkaloids are levorotatory

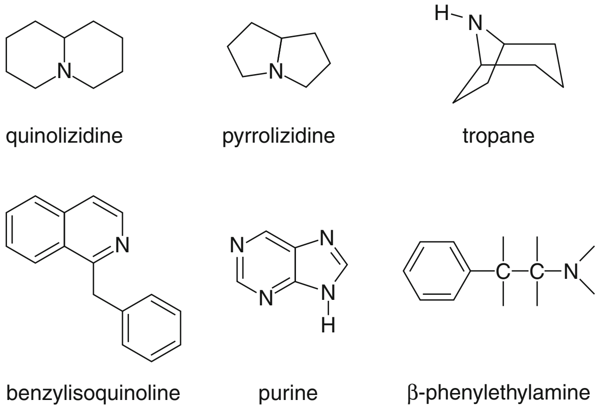

quinolizidine
pyrrolizidine
tropane
benzylisoquinoline
purine
β-phenylethylamine

Fig. 8.3 Heterocyclic ring systems present in different types of alkaloids

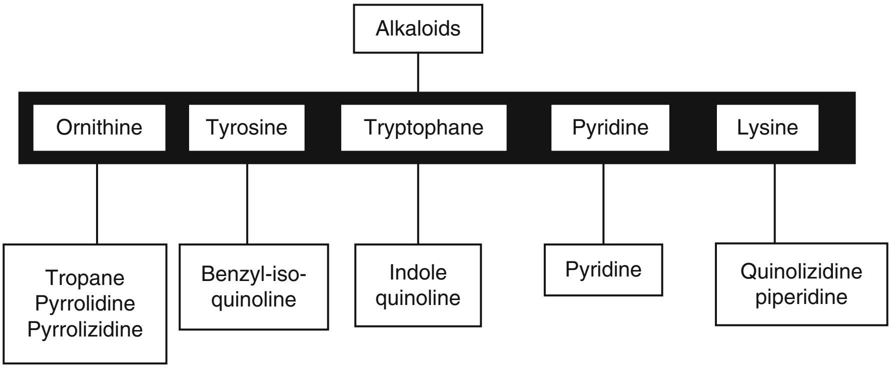

Alkaloids
Ornithine
Tyrosine
Tryptophane
Pyridine
Lysine
Tropane Pyrrolidine Pyrrolizidine
Benzyl-iso-quinoline
Indole quinoline
Pyridine
Quinolizidine piperidine

Fig. 8.4 Classification of alkaloids on the basis of amino acid from which it has been derived (Adopted from Ref. [6])

(optically active); however, a few are also known to be dextrorotatory (optically inactive), for example, *conine* and *papaverine*. Generally, the alkaloids are bitter in taste and have pronounced physiological activity.

1.2 Biosynthesis of Alkaloids in Plants

The pharmacological and commercial significance of alkaloids has motivated the characterization of their biosynthetic pathways. Also, before considering engineering strategies to exploit alkaloid biosynthesis, it is necessary to have a thorough understanding of its endogenous biosynthetic pathways. Research in the field of plant alkaloid biochemistry began with the isolation of morphine in 1806.

However, the structure of morphine was not elucidated until 1952 owing to the stereochemical complexity of the molecule. The technological advances made since then have significantly brought to light the underlying endogenous mechanisms of alkaloid formation in plants. The introduction of radiolabeled precursors has allowed the chemical elucidation of alkaloid biosynthetic pathways. The use of plant cell cultures as an abundant source of biosynthetic enzymes (which can be isolated, purified, and characterized) and widespread application of molecular techniques to the alkaloid field have facilitated the isolation of several genes involved in indole, tropane, and benzylisoquinoline alkaloid biosynthesis as shown in Table 8.2 [3].

Genomic and transcriptomic technologies have been used to rapidly identify biosynthetic steps. There are currently over 40,000 expressed enzyme tags (ESTs) generated from alkaloid-producing plants that have been used to isolate genes involved in the alkaloid pathway [7]. Some alkaloid biosynthetic steps occur as spontaneous chemical reactions without the use of enzymes, for example, conversion of the intermediate neopine into codeinone in the morphine biosynthetic pathway. Also, some enzymes may catalyze two or more separate reactions in the pathway, for example, hyoscyamine 6-hydroxylase, which carries out two consecutive steps in the scopolamine biosynthetic pathway. Alkaloid biosynthesis also involves compartmentalization. Tissue-specific localization studies have shown that sequential biosynthetic enzymes can occur in distinct cell types [8, 9]. During the biosynthesis of the indole alkaloids vinblastine and vincristine in *Catharanthus roseus*, different enzymatic steps are carried out in different cellular compartments (Fig. 8.5) [10]. Various steps in the pathway are carried out in different types of cell. This requires the intercellular transport of metabolic intermediates. Similarly, scopolamine biosynthesis also involves two different cell types.

To date, biosynthetic pathway for four classes of plant alkaloids has been characterized to some extent: the benzylisoquinoline, monoterpenoid indole, purine, and tropane alkaloids. Benzylisoquinoline alkaloids (BIAs) are derived from tyrosine and are comprised of ~2,500 defined structures found mainly in the *Papaveraceae, Ranunculaceae, Berberidaceae*, and *Menispermaceae* [11]. First step of BIA biosynthesis begins with the stereoselective Pictet-Spengler condensation of dopamine and 4-hydroxyphenylacetaldehyde to form (*S*)-norcoclaurine. Subsequently, through a series of methylations and hydroxylations, (*S*)-norcoclaurine gets converted into (*S*)-reticuline, which is the pivotal intermediate for many pharmaceutically important BIAs formed further down in the pathway (i.e., downstream pathways) (Fig. 8.6) [3].

Monoterpenoid indole alkaloids (MIAs) are derived from tryptophan metabolism. MIAs are one of the most structurally diverse class of compounds with over 2,000 structures. They are mostly found in the *Apocynaceae, Loganiaceae*, and *Rubiaceae* family of plants [12]. Similar to BIA biosynthesis, the committed step of MIA biosynthesis begins with the condensation of tryptamine (derived from tryptophan) and secologanin (derived from terpene biosynthesis) to form strictosidine

Table 8.2 Alkaloid biosynthetic enzymes for which the corresponding genes have been cloned (Adopted from Ref. [3])

Enzyme	Plant source
3′-Hydoxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT)	*Coptis japonica*
Acetyl COA: deacetylvindoline 17-o-acetyl transferase (DAT)	*Catharanthus roseus*
Anthranilate synthetase (AS)	
Berbamunine synthase (CYP80A1)	*Berberis stolonifera*
Berberine bridge enzyme (BBE)	*Papaver somniferum, Eschscholzia californica*
Caffeine synthase (CS)	*Camellia sinensis*
Codeinone reductase (COR)	*Papaver somniferum*
Desacetoxyvindoline 4-hydroxylase	*Catharanthus roseus*
Desacetoxyvindoline acetyltransferase	*Catharanthus roseus*
Desacetoxyvindoline-4-hydroxylase (OHT)	*Catharanthus roseus*
Geissoschizine	*Catharanthus roseus*
Geraniol 10-hydroxylase (GIOH)	
Glutamine synthetase (GS)	*Catharanthus roseus*
Hyoscyamine 6β – hydroxylase (HRH)	*Atropa belladonna, Hyoscyamus niger, Hyoscyamus muticus*
N-Methylcoclaurine 3′-hydroxylase (CYP80B1)	*Papaver somniferum*
	Eschscholzia californica
NADPH-cytochrome p-450 reductase	
Norcoclaurine 6-*O*-methyltransferase (6OMT)	*Coptis japonica*
Norcoclaurine 6-*O*-methyltransferase (OMT II; 1–4)	*Thalictrum tuberosum*
Ornithine decarboxylase (ODC)	*Datura stramonium*
Peroxidase	*Catharanthus roseus*
Putrescine *N*- methyltransferase (PMT)	*Atropa belladonna*
SAM:loganic acid methyl transferase (LAMT)	
SAM: Methoxy-2,16-dihydro-16-hydrotabersonine-*N*-methyltransferase (NMT)	*Catharanthus roseus*
Scoulerine 9-*o*-methyltransferase (SOMT)	*Coptis japonica*
Strictosidine (SG)	*Catharanthus roseus*
Strictosidine synthase (SS)	*Catharanthus roseus, Rauwolfia serpentina*
Tabersonine 16-hydroxylase	*Catharanthus roseus*
Tetrahydroalstonine synthase	*Catharanthus roseus*
Tropinone reductase 1 (TR-I)	*Datura stramonium*
Tropinone reductase II (TR-II)	*Datura stramonium*
Tryptophan decarboxylase (TDC)	*Camptotheca acuminata, Catharanthus roseus*
Tyrosine decarboxylase	*Papaver somniferum*
β- glucosidase	*Catharanthus roseus*

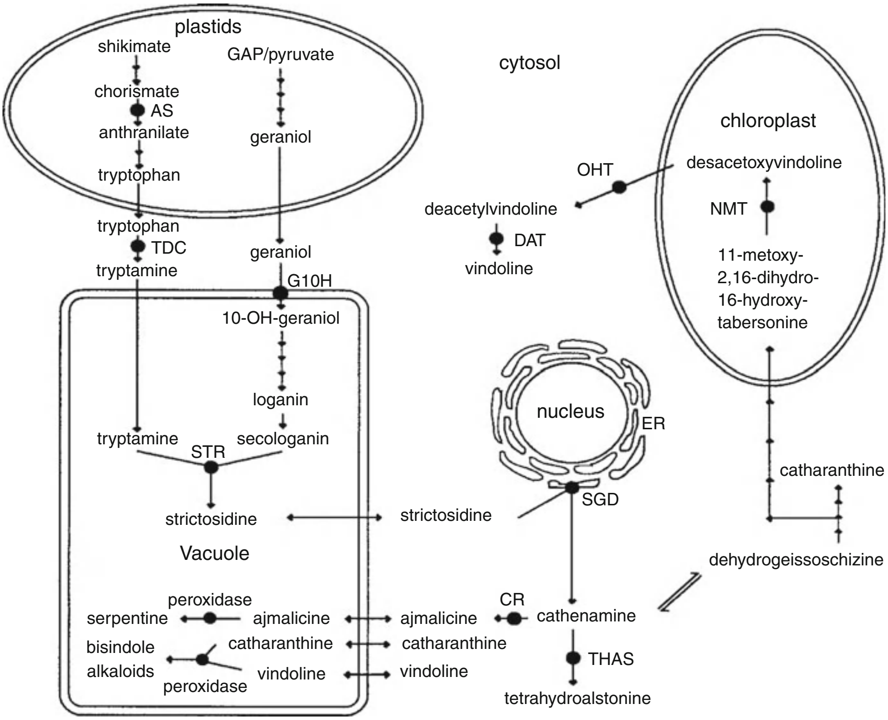

plastids
shikimate
chorismate
AS
anthranilate
tryptophan
GAP/pyruvate
geraniol
cytosol
tryptophan
TDC
tryptamine
geraniol
G10H
10-OH-geraniol
loganin
tryptamine
STR
secologanin
strictosidine
Vacuole
peroxidase
serpentine
ajmalicine
catharanthine
bisindole
alkaloids
vindoline
peroxidase
strictosidine
ajmalicine
catharanthine
vindoline
CR
cathenamine
THAS
tetrahydroalstonine
SGD
nucleus
ER
deacetylvindoline
DAT
vindoline
OHT
chloroplast
desacetoxyvindoline
NMT
11-metoxy-
2,16-dihydro-
16-hydroxy-
tabersonine
catharanthine
dehydrogeissoschizine

Fig. 8.5 Compartmentation of alkaloid biosynthesis in *Catharanthus roseus*. *AS* anthranilate synthase, *CR* NADPH: cathenamine reductase, *DAT* deacetylvindoline 17-O-acetyltransferase, *ER* endoplasmic reticulum, *G10H* geraniol 10-hydroxylase, *GAP* glyceraldehyde-3-phosphate, *NMT* S-adenosyl-L-methionine methoxy-2, 16-dihydro-16-hydroxytagersonine-*N*-methyltransferase, *OHT* desacetoxyvindoline-4-hydroxylase, *SGD* strictosidine _-glucosidase, *STR* strictosidine synthase; *TDC* tryptophan decarboxylase, *THAS* NADPH: tetrahydroalstonine reductase (Adopted from Ref. [10])

(Fig. 8.7) [3]. After the deglucosylation of strictosidine, equilibrium of the unstable aglycon intermediates leads to the formation of 4,21-dehydrogeissoschizine, the branchpoint precursor of MIAs [13].

Tropane alkaloids are found mainly in the *Solanaceae* [14] plant family. First biosynthetic step of tropane alkaloids starts with *N*-methylation of putrescine (derived from L-ornithine) to form *N*-methylputrescine. After the conversion to 1-methyl-Δ1 pyrrolinium cation, its condensation with nicotinic acid gives rise to nicotine synthesis, while other chemical conversions lead to the formation of tropinone, the precursor of many tropane alkaloids through branched pathways (Fig. 8.8a) [15].

The fourth class of alkaloids is derived from purine nucleotides instead of amino acids. A well-known example of a purine alkaloid is caffeine, whose biosynthetic

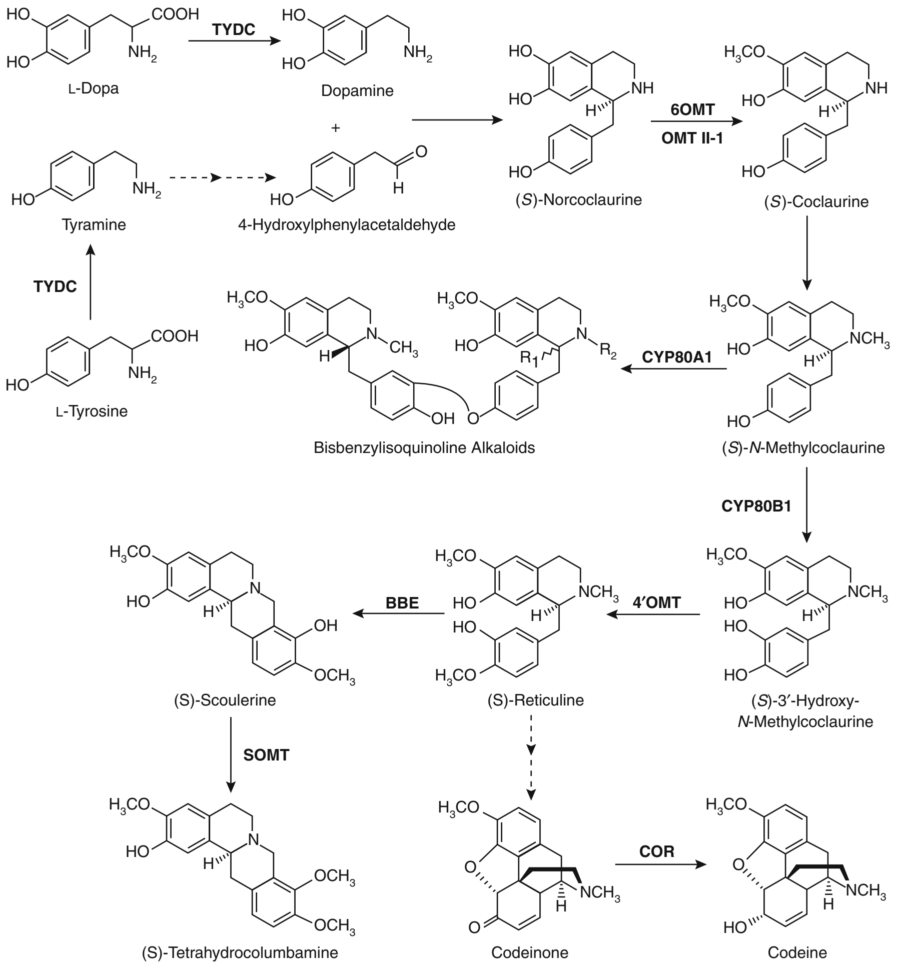
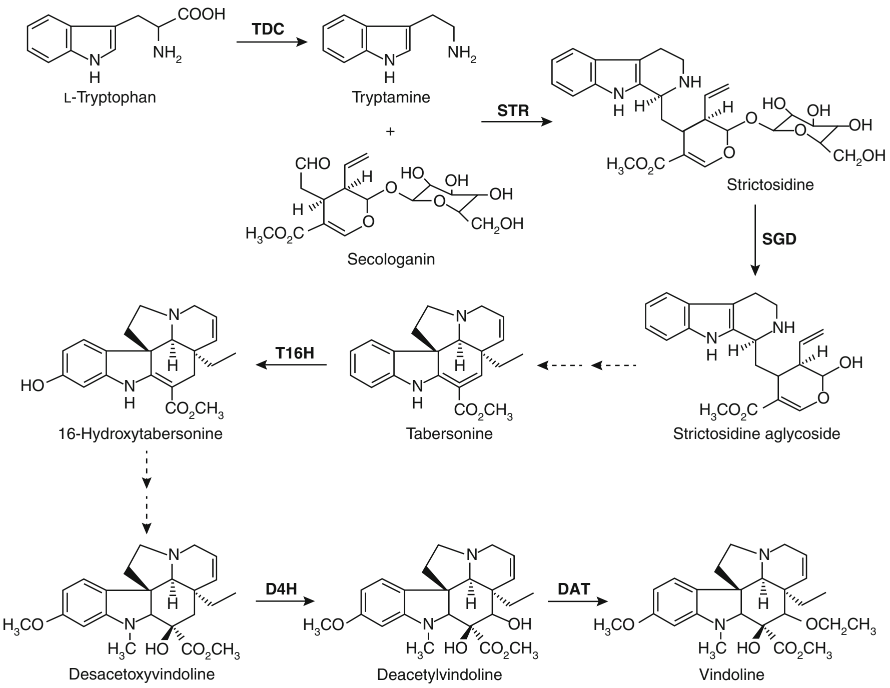

L-Dopa
TYDC
Dopamine
+
Tyramine
4-Hydroxylphenylacetaldehyde
(S)-Norcoclaurine
6OMT
OMT II-1
(S)-Coclaurine
TYDC
L-Tyrosine
Bisbenzylisoquinoline Alkaloids
CYP80A1
(S)-N-Methylcoclaurine
CYP80B1
(S)-Scoulerine
BBE
(S)-Reticuline
4′OMT
(S)-3′-Hydroxy-N-Methylcoclaurine
SOMT
(S)-Tetrahydrocolumbamine
Codeinone
COR
Codeine

Fig. 8.6 Biosynthetic pathway for benzylisoquinoline alkaloid (BIA) biosynthesis in plants. *TYDC* tyrosine/dopa decarboxylase, *6OMT* norcoclaurine 6-*O*-methyltransferase, *40OMT* 30-hydroxy-*N*-methylcoclaurine 40-*O*-methyltransferase, *OMT II-1* *O*-methyltransferase II-1, *CYP80A1* berbamunine synthase, *CYP80B1* (*S*)-*N*-methylcoclaurine 30-hydroxylase, *BBE* berberine bridge enzyme, *SOMT* scoulerine *N*-methyltransferase, *COR* codeinone reductase (Adopted from Ref. [3])

route has been extensively studied in *Camellia*, *Coffea*, *Theobroma*, and *Ilex* [16]. The biosynthetic pathway involves four enzymatic steps that consist of three *S*-adenosyl-L-methionine (SAM)-dependent methyl transfers and one nucleotide removal reaction from xanthosine (Fig. 8.8b) [15].



L-Tryptophan
TDC
Tryptamine
+
Secologanin
STR
Strictosidine
SGD
Strictosidine aglycoside
Tabersonine
T16H
16-Hydroxytabersonine
Desacetoxyvindoline
D4H
Deacetylvindoline
DAT
Vindoline

Fig. 8.7 Biosynthetic pathway for monoterpenoid indole alkaloid (MIA) biosynthesis in plants. *TDC* tryptophan decarboxylase, *STR* strictosidine synthase, *SGD* strictosidine -D-glucosidase, *T16H* tabersonine 16-hydroxylase, *D4H* desacetoxyvindoline 4-hydroxylase, *DAT* deacetylvindoline 4-*O*-acetyltransferase (Adopted from Ref. [3])

2 Production of Alkaloids Via Plant Cell Bioprocessing: Pregenomic Approach

The alkaloids are known as specialty chemicals, as their worldwide production volume is limited. Alkaloids such as quinine and quinidine have a yearly production of 300–500 metric tons, ajmalicine around 3,600 kg, and for the compounds like vinblastine and vincristine annual production is only in the range of few kilograms [17]. The market value of every known major alkaloid can be estimated to be in the range of several hundred million dollars. These specialty chemicals are conventionally produced by extraction from plant material that is cultivated or collected from the wild. There are several problems associated with this production method. Variable quantities and qualities of the plant material or plants that need to grow for several years before they are ready for harvesting (e.g., *Cinchona* bark), and overcollection of endangered species (e.g., *Taxus hevifolia)* are some of the few problems to mention. The amounts of plant material needed for the extraction of these compounds are also growing to fulfill the market demand which also calls for the need of alternative production methods. For example, it is in the

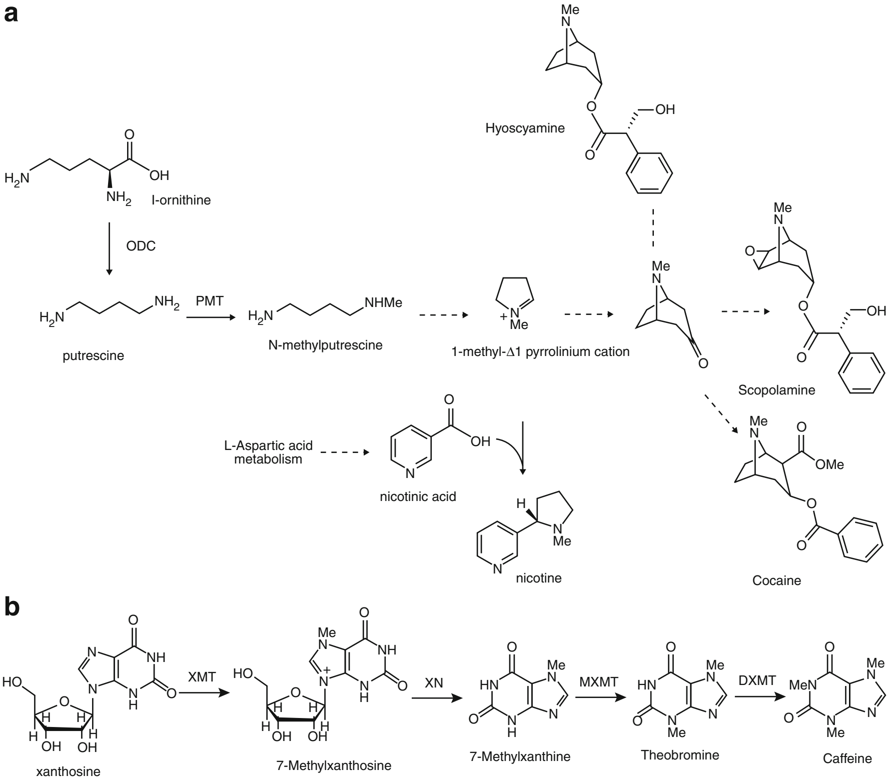

a
l-ornithine
ODC
putrescine
PMT
N-methylputrescine
1-methyl-Δ1 pyrrolinium cation
Hyoscyamine
Scopolamine
Cocaine
L-Aspartic acid metabolism
nicotinic acid
nicotine
b
xanthosine
XMT
7-Methylxanthosine
XN
7-Methylxanthine
MXMT
Theobromine
DXMT
Caffeine

Fig. 8.8 Biosynthetic pathway for tropane and purine alkaloids. (**a**) Tropane alkaloid (*ODC* ornithine decarboxylase, *PMT* putrescine *N*-methyltransferase). (**b**) Purine alkaloid (*XMT* xanthosine *N*-methyltransferase/7-methylxanthosine synthase, *XN* 7-methylxanthosine nucleotidase, *MXMT* 7-methylxanthine *N*-methyltransferase/theobromine synthase, *DXMT* dimethylxanthine *N*-methyltransferase/caffeine synthase) (Adopted from Ref. [15])

order of 5,000–10,000 metric tons of *Cinchona* bark for the extraction of quinine and quinidine and 200–300 t of *Catharanthus roseus* roots for the production of ajmalicine [18].

Looking into the increasing demand for high-value plant-derived products like alkaloids, alternative production methods are thus of great interest. As a consequence, a lot of investigations have been directed on the synthesis of natural products as an alternative to extraction. Although complete synthesis for most natural products including indole alkaloids has been shown to be possible, it is usually economically not feasible. Semi-synthesis of alkaloids from readily available precursors, has been successful in some cases, e.g., the coupling of monomers for the production of the dimeric alkaloid vinblastine [17, 19]. Also, novel useful compounds have also been developed in this way, for example, Taxotere as a result of the breakthrough on the chemical synthesis of Taxol [20]. Due to their complex structures, alkaloids are still most efficiently produced by plants. In the past few decades, the focus has shifted on plant cell biotechnology (cultured cells/tissues) as a possible alternative production method for alkaloids against conventional plant extraction (Table 8.3) [17]. As a promising alternative to produce plant secondary metabolites, plant cell culture technology has many advantages over traditional field cultivation and chemical synthesis. Large-scale plant cell culture is an attractive alternative as it offers following advantages: (1) controlled supply independent of plant availability, (2) a renewable and environmentally friendly resource, and (3) a biological factory for the production of high-quality products under strictly controlled conditions. Plant cell/tissue culture is also amenable to process optimization, scale-up and genetic engineering for yield and productivity enhancement of the desired metabolite. However, for industrial feasibility, plant cells should be grown in large fermenters and the price of the product from such a large-scale plant cell cultivation should be competitive with the existing production methods. Scaling up seemed to be a challenge when the first successful in vitro growth of plant cells and tissues was described almost 50 years ago [21]. However, many success stories on large-scale bioreactor cultivation of plant cells have been reported since then. The first successes were published in 1960 by Tulecke and Nickel [22], culturing cells of various plant species in a 134-l bioreactor. In 1977, Noguchi et al. [23] reported the culture of tobacco cells in a 20-m^3 tank mixed by aeration. In the past few decades, most work on large-scale culture has been directed toward the use of various types of low-shear bioreactors and configurations which facilitate effective mass transfer of nutrients/oxygen to the growing culture (e.g., airlift bioreactors). However, large investments in all sorts of new ingenious bioreactors can be a constraint in the commercialization of plant cell biotechnology. For the industrial applicability of plant cell cultures, it is of great importance that plant cells are tolerant to shear effects in stirred-tank reactors, which are currently the most exclusively used reactors in fermentation industry. Efforts have been made to overcome this issue by selecting more shear-tolerant cell lines which can be grown without any problem in stirred tanks [24–27]. Though shear sensitivity of most plant cell lines could limit the applicability in large-scale fermentations, Meijer and coworkers [26, 27] noted that "healthy" good-growing cell cultures were more shear resistant than apparently "stressed" (e.g., rapidly browning) cell cultures. As a matter of fact, the industrial production of shikonin is performed in 750-l stirred-tank bioreactors [28], and in Germany the Diversa company and Phyton Biotech GmbH. have successfully grown plant cell cultures in 60-m^3 and 75-m^3 working-volume stirred tanks, respectively [29].

Plant cells can be cultured on a large scale, and although the price of the products obtained is high, this price is still of the order of magnitude of that of many specialty chemicals. In order to have a commercially viable and reliable technology, it is important to obtain a stable, high-producing plant cell line. To further enhance the productivity of the culture, manipulation in the culture environment is recommended through exogenous additions (growth regulators, elicitors, precursors, permeability enhancers, etc.) and optimization of cultivation conditions like medium, pH, temperature, rpm, light, etc.

Table 8.3 In vitro plant cell/tissue cultivation for alkaloid production (Adopted from Ref. [17])

Plant source	Type of alkaloid	Alkaloid production by plant cell culture
Ailanthus altissima	Alkaloids	Cell suspension
Ailanthus altissima	Cathinone alkaloids	Cell suspension
Brucea javanica (L.) Merr.		
Catharanthus roseus	Indole alkaloids	Cell suspension
Choisya ternata	Furoquinoline alkaloids	Cell suspension
Cinchona L	Alkaloids	Cell suspension
Corydalis ophiocarpa	Isoquinoline alkaloids	Callus
Fumaria capreolata		
Duboisia leichhardtii	Tropane alkaloids	Callus
Hyoscyamus niger	Tropane alkaloids	Callus
Nandina domestica	Alkaloids	Callus
Nicotiana rustica	Alkaloids	Callus
Nothapodytes foetida	Camptothecin	Callus
Ophiorrhiza pumila	Camptothecin related alkaloids	Callus
Peganum harmala L.	β-Carboline alkaloids	Cell suspension
Ptelea trifoliata L.	Dihydrofuro [2,3-b] quinolinium alkaloids	Callus
Rauwolfia sellowii	Alkaloids	Cell suspension
Thalictrum minus	Berberine	Cell suspension
Catharanthus roseus	Catharanthine	Cell suspension
Ephedra spp.	L-Ephedrine	Cell suspension
	D-pseudoephedrine	
Cinchona ledgeriana	Quinoline alkaloids	Hairy root culture
Neotyphodium uncinatum	Loline alkaloids	Cell suspension
Catharanthus roseus	Ajmalicine	Cell suspension
Rauvolfia sp.		
Catharanthus roseus	Vinblastine	Cell suspension
Catharanthus roseus	Vincristine	Cell suspension
Rauvolfia sp.	Reserpine	Cell suspension
Vinca sp.	Vincamine	Cell suspension
Cinchona sp.	Quinine	Cell suspension
Cinchona sp.	Quinidine	Cell suspension
Ochrosia elliptica	Ellipticine	Cell suspension
Rauvolfia sp.	Rescinnamine	No data available
Camptotheca acuminala	Camptothecine	Cell suspension
Cephaelis ipecacuanha	Emetine	Root culture
Coffea, Thea	Caffeine	Cell suspension
Theobroma	Theobromine	Cell suspension

(*continued*)

Table 8.3 (continued)

Plant source	Type of alkaloid	Alkaloid production by plant cell culture
Atropa belladonna	Atropine	Hairy root culture
Atropa belladonna	Scopolamine	Hairy root culture
Duboisia leichhardtii	Scopolamine	Hairy root culture
Coptis japonica	Berberine	Cell suspension
Papaver somniferum	Morphine	Cell suspension
Papaver somniferum	Codeine	Cell suspension
Nicotiana sp.	Nicotine	Cell suspension
Colchicum autumnale	Colchicine	Callus culture
Dicentra peregrina	Alkaloids	Shoot culture
Calystegia sepium	Tropane alkaloids	Root culture
Hyoscyamus albus	Hyoscyamine	Root culture
Hyoscyamus muticus	Hyoscyamine	Hairy root culture
B. vulgaris	Betalains	Root culture
Papaver somniferum	Codeine	Biotransformation
Spirulina platensis	Morphine	Biotransformation

2.1 Screening and Selection of a High-Yielding Stable Cell Line

In a screening program a large number of cell lines are developed from high-producing plant variety. Subsequently, the yield of the desired compound in these cell lines is determined, and the cell line producing the maximum product is selected. However, the high production in plants does not always ensure high production in cell cultures. For example, highest berberine producing *Coptis japonica* cell culture was obtained from the cell line developed from a low berberine producing plant variety [30, 31]. Screening can also be done at the level of cell aggregates or even single cells or protoplasts. In case of the *C. japonica* cell cultures, it was found that at least three to four cloning steps were necessary before a stable, high-producing strain was obtained [32]. However, similar selection protocol for high-producing strains in *Catharanthus roseus* failed with respect to stability [33]. Stability is a major problem encountered in plant cell cultures. Hence, for selection, cultivation conditions are established in which only certain desired cell type can survive. For example, cells of *C. roseus* were grown on media containing the toxic compound 4-methyltryptophan to obtain cell lines that had a high alkaloid production capacity. Only cells that contained a high level of tryptophan decarboxylase, and thereby were able to convert the selection agent into the less toxic 4-methyltryptamine, survived. Although, the selected cell lines did have a higher tryptamine production, but only in one single case was terpenoid indole alkaloid production increased too [34].

2.2 Culture Condition Optimization

An important approach to increasing productivity is to improve the growth and production conditions. The production of alkaloids in cell suspension cultures is controlled by environmental conditions as well as by the genotype of the plant material. Medium optimization has also been shown to be an effective means to improve productivity. Single-factor and/or statistical optimization of medium composition with respect to major and minor nutrient level is carried out to model and optimize the independent and synergistic effect of various medium nutrients for maximum biomass density and product levels in the culture. Optimization of medium composition has been done for growth and production of indole alkaloids [35–38]. In many cases, production phase and growth phase are not correlated. Hence, a two-step cultivation process is often used. Initially, the biomass is maximized under optimal conditions, and then the medium is changed to a production-inducing composition. Developing a separate production medium is a useful and less cumbersome approach than manipulating a medium that is both suited for growth and production. The effect of any change in production medium is immediately observed after transferring the plant cells to the production medium, whereas in growth media, the effect of a change in medium composition is only observed after few cycles of subcultures. Use of production medium has been reported by Zenk et al. [39] and Knobloch et al. [40] for the induction of ajmalicine production in *C. roseus*. In *Tabernaemontana divaricata* cell cultures, replacement of auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) with 1-naphthaleneacetic acid (NAA) in the medium resulted in an increasing alkaloid production observed in five subculture cycles. This was followed by a decrease and eventually stabilization after 10 subcultures at a level that was not much higher than in the original medium [41]. However, manipulation in medium composition for high productivity is recommended only if a minimum basal level of the desired compound(s) is found in the cell culture.

The alkaloid productivity and the storage capacity in cultured plant cells can be influenced by the pH gradient between the medium and the accumulation sites inside the plant cell (vacuoles). A shift in the medium pH from low to high value was used to release the intracellularly stored alkaloids into the culture medium in cell suspension culture of *C. roseus* [42]. Similarly, transient modifications in the medium pH value led to the release of indole alkaloids in culture medium during immobilized cell cultivation of *C. roseus* [43].

Contradictory results like complete inhibition, stimulation, or no effects have been reported with respect to alkaloid production at lower temperatures in plant cell suspension cultures [44–46]. The effect of the temperature on alkaloid production happens to be cell line dependent. Use of open-loop temperature trajectories during the cultivation of plant cells in bioreactor can lead to an increase in alkaloid production and a shortening of the culture period [38]. It was shown that a single switch in the temperature increased the alkaloid accumulation by 22 % above that obtained at the optimum constant temperature [47].

Light is an important regulatory factor in the production of alkaloids in plant cell cultures. The light is known not only to affect the alkaloid production but also the accumulation site in cell culture of *C. roseus*. Through various studies, it is known that in *C. roseus* cell/tissue cultures, light influences the ajmalicine/serpentine accumulation ratio and also vindoline and catharanthine production [38]. However, in large-scale production in stirred-tank bioreactors, such conditions are difficult and costly to realize. Therefore, production conditions on small scale should preferably be optimized with dark-grown cultures.

The gaseous environment, mainly the availability of oxygen and carbon dioxide, significantly affects the production of secondary metabolites in plant cell cultures. It has been observed that increasing the dissolved oxygen concentration in high cell density cultivation of *C. roseus* stimulated the oxidative metabolism responsible for high production of ajmalicine [48]. Similarly, increasing the concentration of CO_2 up to 4 % of saturated air resulted in increased accumulation of indole alkaloids in cell suspension cultures of *C. roseus* [49]. In transferring results from batch-wise cultures in shake flasks to bioreactors, it has to be kept in mind that the gas phases in the two systems are completely different, thus possibly influencing the productivity. This may be illustrated by the recent finding that productivity of *C. roseus* cell cultures in bioreactors is drastically improved by recirculation of the air used for the aeration of the cells in the bioreactor [50].

Plant growth regulators, or phytohormones, like auxins, cytokinins, abscisic acid, gibberellins, and ethylene are known to affect growth and differentiation and thus affect secondary metabolism of cultured cells. Several studies have been carried out on the influence of the concentration of various growth regulators, especially auxins, on alkaloid production by *C. roseus* cultures [38]. Exogenous hormone addition like gibberellic acid in the growth medium results in higher accumulation of alkaloids in *C. roseus* plants [51].

Secondary metabolite yield in in vitro cultures can also be enhanced by exposing the cultures to stress factors like osmotic shock, addition of inorganic salts, heavy metal ions, fungal homogenates, and UV irradiation. As a response to the stress, the enzymes of secondary metabolism which is usually related to plant defense gets induced, as a result hyperaccumulation of secondary products is observed. These stress-inducing compounds are normally referred as elicitors. It is one of the most successful strategies employed to enhance secondary metabolite production in cultured plant cells/tissues. Several *C. roseus* cell lines upon elicitation have resulted in increased accumulation of indole alkaloids [35]. However, the response to elicitation is highly dependent on the cell line. It has been reported that by adding elicitors such as cell wall constituents of microorganisms, enzymes (e.g., cellulase and pectinase), or heavy metals, alkaloid biosynthetic pathways get induced (e.g., sanguinarine). Recently, Gundlach et al. [52] showed that jasmonic acid, functioning in signal transduction pathway, can be used to induce secondary metabolite pathways as well. The production of sanguinarine in *Eschscholtzia californica* and of raucaffricine in *Rauvolfia canescens* could be induced using the same. Addition of sodium chloride and sorbitol enhanced the production of catharanthine in cell suspension culture of *C. roseus* [53]. Similarly, addition of vanadyl sulfate could affect the ratio of catharanthine, serpentine, and tryptamine production in cell culture of *C. roseus* in a dose-dependent manner. Exposure to heavy metals has also been reported to enhance the production of alkaloids in *C. roseus* cultures [38]. Synergistic effect of combined addition of selected elicitors have also been studied using statistical tools for enhanced alkaloid production in plant cell suspension cultures [54].

Based on the knowledge of the regulation of alkaloid biosynthetic pathways, several organic compounds have been added to the culture medium in order to enhance the availability of alkaloid precursors. Two different approaches have been employed: directly feeding precursors into the culture medium or by addition of compounds interfering with precursor metabolism. For example, the alkaloid production was found to be significantly improved upon addition of secologanin to cell cultures of *C. roseus* [38]. Similarly, Moreno et al. [55] reported enhanced accumulation of ajmalicine and strictosidine in *C. roseus* cell culture upon addition of terpenoid precursor secologanin and its precursors loganin and loganic acid in the medium. Feeding of tryptophan to immobilized cultures of *C. roseus* increased tryptamine accumulation [56].

The immobilization of plant cells leads to larger cell aggregates in which cell-to-cell contact occurs. In some plant cell cultures, this results in higher productivity, probably due to a certain level of differentiation. Immobilization has been done in alginate, in small polyurethane (PUR)-foam cubes, or by “natural” immobilization by growing the cells as aggregates [57, 58]. The occurrence of differentiation was, for example, shown in the so-called compact-globular structures, which are large cellular aggregates formed by *Cinchona* cells. These aggregates do produce quinoline alkaloids such as quinine, but only in the outer layer, which has according to microscopic analysis similar morphology as the stem of the tree, that is the major source of these alkaloids in the plant [59]. However, limitations associated with immobilized cells are that they are expensive to produce on large scale and that for an economic continuous production process, excretion is needed into the medium [60].

2.3 Use of Differentiated Cultures for Enhanced Production of Alkaloids

By definition, secondary metabolism is a form of differentiation. In plants there is a clear correlation between cellular differentiation and secondary metabolism. The activation of many alkaloid biosynthetic pathways is tissue specific and a function of developmental stage [61]. Hence, plant tissue cultures apart from cell cultures have also been investigated as alternative production platforms [62–64]. In this context, the plant hairy root cultures are worth mentioning. These cultures are obtained by infection of the plant tissue with *Agrobacterium rhizogenes*, a soil bacterium that introduces root-inducing genes into the plant cells. These genes transform the cells into hormone-independent tumorous roots that can produce secondary metabolites similar to that of the natural plant.

The growth rate of hairy root culture for some species is comparable to their cell suspension culture. High productivity of tropane alkaloids has been reported in hairy root cultures [65]. An interesting aspect of the use of *A. rhizogenes* is that at the same time one can use these bacteria to introduce new genes into the cells. This approach was applied in *Atropa belladonna* hairy roots in which the enzyme that converts hyoscyamine into scopolamine was overexpressed [66, 67] and also in improving alkaloid productivity in *Cinchona* hairy roots [68]. A major disadvantage of differentiated organ cultures is that they are difficult to grow on a large scale. Hairy roots require specialized bioreactors, such as rolling drum fermenters or mist fermenters [69]. In these bioreactors the working volume is quite large as compared to the active biomass. Consequently, production costs are very high. Wilson et al. [70] and Rodriguez-Mendiola et al. [71] have proposed different solutions for the large-scale culture of hairy roots. A number of bioreactor configurations have been developed to facilitate mass cultivation of hairy roots for large-scale secondary metabolite production [72]. As a matter of fact, with very few exceptions like ROOTec bioactives Ltd. in Germany, the application of hairy roots is probably restricted to serve as models for in vitro studies like regulation of the biosynthesis.

3 Metabolic Engineering for Engineering Alkaloid Biosynthesis in Cellular Systems: A Genomic Approach

There are three basic goals of metabolic engineering: (1) producing more of a specific compound, (2) producing less of undesired compound, and (3) producing a novel compound (this can be a known compound that is heterologous in the expression system being used or a completely novel compound). Strategies for achieving these goals can involve the engineering of single steps in a pathway to increase or decrease metabolic flux to target compounds or block competitive pathways and modulation of multiple enzymatic steps. An alternative is to stimulate or inhibit the catabolism of the target compound as suggested by Kutchan [5].

As depicted in Fig. 8.9 [5], plant alkaloid genes can be functionally expressed in microorganisms to produce either single biotransformation steps or short biosynthetic pathways.

Similarly, using overexpression or antisense or cosuppression technologies, medicinal plants can also be tailored to produce important pharmaceutical alkaloids by introducing side pathways, eliminating side pathways, or by accumulating biosynthetic intermediates. For example, as shown in Fig. 8.9, suppose a known plant biosynthetic pathway contains an enzyme encoded by gene G3 that catalyzes a transformation step, of compound C to alkaloid D, which is difficult to achieve by chemical synthesis. The G3 gene can be heterologously expressed in a microorganism and the gene product used in a biomimetic synthesis of alkaloid D (i.e., the microorganism is supplied with compound C and produces alkaloid D). Likewise, a short pathway consisting of enzymes encoded by genes G1, G2, G3, and *G*4 could be expressed in a microorganism to produce alkaloid E directly from

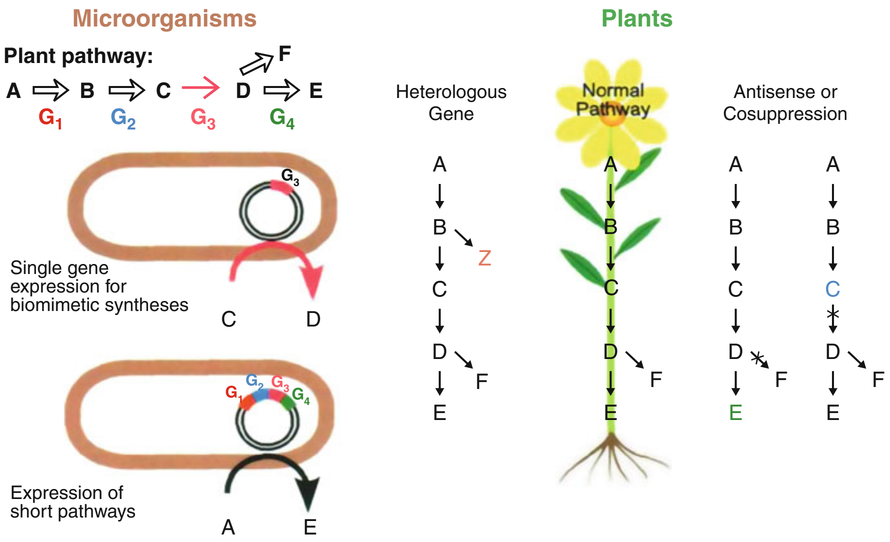

Microorganisms
Plant pathway:
A ⇒ B ⇒ C → D ⇒ E
F
G1 G2 G3 G4
G3
Single gene expression for biomimetic syntheses
C D
G1 G2 G3 G4
Expression of short pathways
A E
Plants
Heterologous Gene
Normal Pathway
Antisense or Cosuppression
A B Z C D F E
A B C D F E
A B C D F E
A B C D F E

Fig. 8.9 Genetic engineering for alkaloid biosynthesis (Adopted from Ref. [5])

precursor A. Many alkaloid biosynthetic pathways involve 20–30 enzymes. Our current knowledge of these pathways indicates that the genes encoding the biosynthetic enzymes are neither clustered nor coordinately controlled by one operon. Expression of an entire, long alkaloid pathway in a single microorganism is currently a technical challenge. However, it is possible to alter the pathway in the plant cell and produce the desired alkaloid either in culture or in the field. For example, to accumulate alkaloid Z, which is not normally produced in a particular plant species, a transgene (from another plant or a microorganism) can be introduced. To accumulate alkaloid E, a side pathway that also uses precursor D may have to be blocked. If intermediate alkaloid C is the target for accumulation, catabolism to D could be interrupted [5].

3.1 Single-Step Engineering

The simplest strategy to increase the levels of a specific target alkaloid is to increase the metabolic flux toward that compound. As discussed above, this requires a thorough understanding of the endogenous pathway because it is necessary to identify the rate-limiting step in the pathway and to use this as the primary target for engineering. For example, consider the biosynthesis of terpenoid indole alkaloids such as vinblastine and vincristine in *C. roseus*. Although, these valuable alkaloids are synthesized in whole plants, but they are not produced in cell culture due to the tissue-specific compartmentalization. The universal terpene indole alkaloid precursor strictosidine is produced in culture and is metabolized to form other alkaloids such as ajmalicine and catharanthine. For indole alkaloid biosynthesis,

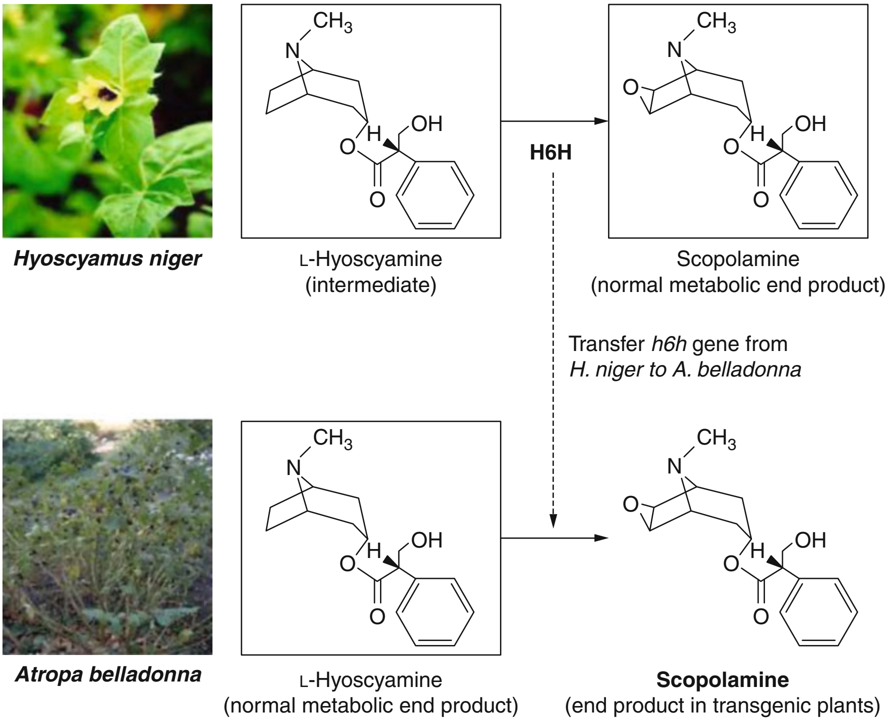

Hyoscyamus niger
CH3
N
OH
H
O
O
L-Hyoscyamine
(intermediate)
H6H
Scopolamine
(normal metabolic end product)
Transfer h6h gene from
H. niger to A. belladonna
Atropa belladonna
L-Hyoscyamine
(normal metabolic end product)
Scopolamine
(end product in transgenic plants)

Fig. 8.10 Single-step metabolic engineering in *Atropa belladonna* for scopolamine production (Adopted from Ref. [9])

a rate-limiting step is the conversion of the amino acid tryptophan to tryptamine, catalyzed by the enzyme tryptophan decarboxylase (TDC). Tryptamine is then condensed with the terpenoid molecule secologanin to produce strictosidine. To alleviate the regulation of TDC, the *C. roseus* TDC gene was overexpressed under the control of a strong and constitutive promoter. The resulting transgenic cultures produced high levels of tryptamine [9]. Single-step engineering has also been used to extend a metabolic pathway in a heterologous plant. The alkaloid scopolamine is produced in *Hyoscyamus niger* but not in *Atropa belladonna*, where the tropane alkaloid pathway stops at L-hyoscyamine. However, introduction of a cDNA from *H. niger* encoding hyoscyamine-6-hydroxylase into *A. belladonna* resulted in the production of scopolamine in this plant. As shown in Fig. 8.10, the enzyme hyoscyamine-6-hydroxylase (H6H) is responsible for two reactions that convert L-hyoscyamine into scopolamine. *A. belladonna* accumulates L-hyoscyamine because the enzyme H6H is not produced in this species. However, by transferring the enzyme from another plant such as *H. niger*, which produce scopolamine, the metabolic pathway in *A. belladonna* could be extended till scopolamine.

3.2 Multiple-Step Engineering

Recently it has become increasingly common to introduce multiple genes in plants either by crossing independent single-gene transgenics or by simultaneous multiple gene transfer. Such experiments use to mostly involve marker genes; however, simultaneous transfers of multiple genes conferring resistance to different pests and pathogens have now been attempted and reported [9]. One way to overcome the limitations of the single-gene approach is to simultaneously transform plants with genes encoding enzymes that act at different steps of a biosynthetic pathway. For example, transgenic tobacco (*Nicotiana tabacum*) plants and *C. roseus* cell cultures have been generated with simultaneous overexpression of tryptophan decarboxylase (TDC) and strictosidine synthase (STR) transgenes. The *C. roseus* cell culture demonstrated increased accumulation of strictosidine when fed with loganin [9]. Particle bombardment has been used to transform plants simultaneously with 13 different genes, so it is theoretically possible to transfer genes encoding the enzymes of an entire pathway into a suitable expression host. Leech et al. [73] used the particle gun to introduce the TDC and STR genes into tobacco plants. In the transgenic tobacco seedlings, a 24-fold variation and a 110-fold variation were found in TDC and STR activity, respectively.

3.3 Engineering Regulatory Genes

The intricate relationships among metabolic pathways and regulatory schemes in plant cells and tissues can hinder the metabolic engineering design used for the overproduction of the desired target compound. As an important feature of the regulatory system, a number of downstream genes are coordinately regulated, either in response to the organism's developmental process or in response to external inductive signals. A number of external stimuli are known to induce alkaloid biosynthesis, including UV light, fungal elicitors, auxin starvation, and the signaling molecules like jasmonic acid. It has been shown that the *C. roseus* genes for tryptophan decarboxylase (TDC), strictosidine synthase (STR), geraniol-10-hydroxylase (G10H), NADPH: cytochrome P450 reductase (CPR), strictosidine b-D-glucosidase (SGD), deacetylvindoline 4-*O*-acetyltransferase (DAT), and desacetoxyvindoline 4-hydroxylase (D4H) are all inducible by methyl jasmonate and/or fungal elicitor in cell culture [9].

In most organisms, transcription factors act as master regulators of complex pathways involved in development, signal transduction, and metabolism. The common regulation of many of the genes involved in terpenoid indole alkaloid biosynthesis in *C. roseus* suggests that a useful strategy for increasing the levels of alkaloids in this system would be to identify and manipulate transcription factors that control the expression of these genes [9]. Alkaloid metabolic regulatory machineries have been probed by using transcriptome analysis, leading to the identification of several transcription factors in monoterpenoid indole alkaloid (MIA) biosynthesis [74, 75]. This information can potentially be useful in determining metabolic engineering targets that can efficiently bring about the desired improvement in a specific alkaloid branch pathway. For instance, a metabolic engineering strategy to increase MIA production was devised to exploit the utility of the ORCA3 transcription factor to upregulate the expression of many MIA biosynthetic genes simultaneously [74]. However, initial ORCA3 overexpression in *C. roseus* cell cultures did not significantly improve MIA synthesis. It was discovered that even though ORCA3 positively regulates the expression of many genes that lead to the synthesis of strictosidine, it does not upregulate the expression of geraniol 10-hydroxylase (G10H), the enzyme in the terpenoid pathway that leads to the synthesis of secologanin [74–77]. Upon supplemental feeding of loganin, the precursor of secologanin, the overexpression of ORCA3 resulted in ~0.6% of dry weight of MIAs (~threefold increase). However, in a similar study in *C. roseus* hairy root cultures, it was discovered that although the MIA biosynthetic enzymes that were upregulated upon ORCA3 overexpression were similar to those upregulated upon ORCA3 over-expression in the cell lines, no significant improvement in MIA biosynthesis could be observed in hairy roots. The reason being that the transcriptional repressors ZCT1 and ZCT2 also got upregulated in the hairy root lines unlike in *C. roseus* cell lines [78]. The study reveals the complexity of MIA biosynthetic control by a variety of transcriptional regulators.

3.3.1 Metabolic Engineering for Indole Alkaloids

Most efforts in engineering of terpenoid indole alkaloid pathway have been concentrated on mapping the early part of the pathway and on overexpression of early genes, aiming to increase the metabolic flux into the alkaloid pathway [79]. In particular, the genes encoding tryptophan decarboxylase (TDC) and strictosidine synthase (STR) have been studied extensively in *Catharanthus roseus* cell cultures [4]. Overexpression of TDC resulted in higher levels of the immediate product tryptamine, but not in increased levels of alkaloids, while in the case of STR, higher levels of alkaloids were reported [80]. Feeding the transgenic cell lines with tryptophan and terpenoid intermediates showed high alkaloid production (up to 1,100 μmol/L) [81], indicating that the terpenoid branch of the pathway is limiting. Such studies indicate that there may be multiple rate-limiting steps. TDC and/or STR were also expressed in various other nonalkaloid-producing plants [82, 83]. Feeding transgenic tobacco cell cultures with secologanin [83, 84] led to the production of strictosidine, but this glucoalkaloid is excreted to the medium instead of being stored in the vacuole as in the indole-alkaloid-producing *C. roseus* cell cultures. This illustrates the importance of physiological aspects in secondary metabolism besides biosynthesis. Thus, not only genes that encode enzymes catalyzing biosynthetic steps are involved but also genes involved in, for example, pH regulation and transport. Cell cultures of *Weigelia*, capable of secologanin biosynthesis, upon overexpression of TDC and STR genes, also produced small amounts of ajmalicine and serpentine. This shows that indole alkaloid biosynthesis in otherwise nonalkaloid producing plants is feasible via expression of one or more heterologous pathway genes [83].

3.3.2 Metabolic Engineering for Isoquinoline Alkaloids

Yamada and coworkers [14] hypothesized that overexpression of an enzyme at a branch point in a pathway could lead to an increased flux through the affected branch. In the biosynthesis of berberine, the enzyme (*S*)-scoulerine 9-*O*-methyltransferase (SMT) is such an enzyme that might control the ratio of coptisine: berberine plus columbamine in *Coptis japonica* cells [14]. Overexpression of this gene resulted in a 20% increase in enzyme activity, with an increase of berberine and columbamine from 79% of the total alkaloid content in wild-type cells to 91% in transgenic cells. This observation proved that fluxes at a branch point can be changed by metabolic engineering. Overexpression of the *C. japonica* SMT gene in cell culture of *Eschscholzia californica*, a plant lacking this enzyme, resulted in the production of columbamine, which is normally not found in this species. Opening up a new pathway at the intermediate scoulerine apparently channeled the flux away from the sanguinarine branch, resulting in considerably lower levels of this alkaloid. An interesting approach for the production of new compounds in plants is to introduce enzymes with different substrate specificity (combinatorial biochemistry) [79]. For example, recombining *Thalictrum tuberosum O*-methyltransferase subunits to form heterodimeric enzymes with substrate specificity different from that of the homodimers was proposed as a possible way to generate new isoquinoline alkaloids [85].

3.3.3 Metabolic Engineering for Tropane Alkaloids and Pyrrolidine Alkaloids

Reasonable amount of research has been done on the genetic engineering of the pharmaceutically important tropane alkaloids [65]. The conversion of hyoscyamine to more valuable scopolamine has been the ultimate aim of these studies. The enzyme hyoscyamine-6β-hydroxylase (H6H) catalyzes this conversion. By the overexpression of the gene encoding H6H in *Hyoscyamus muticus* hairy root cultures, a 100-fold increase of scopolamine levels could be reached compared to control which produced hyoscyamine as the major alkaloid [86]. Recent efforts have been aimed at increasing the flux through the biosynthetic pathways [14, 79]. The tobacco putrescine *N*-methyltransferase (PMT) gene was overexpressed in *Atropa belladonna* and *Nicotiana sylvestris*, with the aim of increasing the production of tropane alkaloids and pyrrolidine alkaloids, respectively. For both types of alkaloids, the formation of methylputrescine is the first committed step. However, despite a modest increase in the PMT activity (up to 3.3-fold) in the transgenic *A. belladonna* plants, no increase in alkaloid levels was observed and only the level of the methylputrescine could be increased. In some transgenic *N. sylvestris* plants, the PMT activity was increased four- to eightfold, whereas in others cosuppression was noted. The transgenic lines showed a 40% increase in nicotine level, whereas in the case of cosuppression the nicotine level was only 2% of wild type. The latter plants had a clear increase in polyamine levels. A certain step in a pathway might appear to be rate limiting, but overexpression of the encoding gene in most cases immediately revealed new rate-limiting steps [79].

3.3.4 Metabolic Engineering for Eliminating Undesired Metabolites and its Unpredictability

Metabolic engineering strategies have also been used to eliminate undesired metabolite. For example, a transgenic coffee cultivar with 70% reduction of caffeine content was created by the introduction of RNA interference (RNAi) constructs in order to downregulate theobromine synthase (MXMT) [87]. A more pest-resistant tobacco cultivar has also been engineered by expressing three N-methyltransferases from coffee to divert flux from xanthosine to synthesize caffeine [88]. Although there are many examples of successful attempts to achieve a desired alkaloid production phenotype, the outcome of plant metabolic engineering strategies is often unpredictable. For instance, the downregulation of codeinone reductase with RNAi was a strategy which was expected to lead to suppression of morphine formation and the accumulation of codeinone and morphinone, the immediate precursors of codeinone reductase. Although the amount of the morphinan alkaloids was decreased, biosynthesis of (S)-reticuline, an early upstream metabolite in the pathway, was increased instead of codeinone and morphinone [89]. On the other hand, the overexpression of another enzyme in the pathway, the cytochrome P450 monooxygenase (*S*)-*N*-methylcoclaurine 3′-hydrolase (CYP80B3), resulted in hyperaccumulation of morphinan alkaloids [90]. This suggests that although there are multiple control points in the BIA pathway, CYP80B3 is an important target toward improving morphine biosynthesis. The suppression of the gene by an antisense construct led to reduced alkaloid content in transgenic opium poppy [90]. Similarly, by overexpressing the gene, *COR1*, the morphine and codeine content in transgenic opium poppy could be moderately increased by ∼22% and ∼58%, respectively [91]. However, thebaine, an upstream metabolite in the morphine branch pathway, was also unexpectedly and significantly amplified [15, 91].

3.3.5 Biotransformation of Alkaloids for Enhanced Bioactivity

The important pharmacological activity of many alkaloids has attracted and motivated chemists to make many derivatives of these natural compounds. The semisynthetic preparation alkaloids has resulted in the production of drugs with improved properties, such as the addition of a 14-hydroxy group to the morphine alkaloid structure which has been found to dramatically increase its potency [92]. However, the chemical synthesis of such compounds is often difficult to achieve on a commercial scale due to the chemical complexity of the starting material, cost, and environmental issues, in addition to the limited supply of the precursors required. Biotransformation can offer a number of advantages over conventional chemical processes. The specificity of enzyme-catalyzed reactions allows the stereospecific transformation of defined functional groups. The use of recombinant DNA technology has a substantial impact on biotransformation processes, resulting in the development of new approaches using biological systems. Recent advances made in the understanding of the genetic and biochemical basis of alkaloid biosynthetic pathways have made biotransformation of complex alkaloid molecules more feasible and credible.

3.4 Mutasynthesis of Novel Alkaloid Analogs

Functional group substitution in natural alkaloid structures have lead to the generation of compounds with improved pharmacological properties. For example, vinflunine (4′-deoxy-20′,20′-difluoro-C′-norvincaleukoblastine), a new compound was created by introducing two fluoro groups in vinblastine [93]. The current availability of novel alkaloids, however, remains limited because they are still semisynthetically derived from naturally isolated precursors. Precursor-directed biosynthesis, or a "mutasynthetic" approach, is a powerful strategy for increasing the availability of alkaloid derivatives. Several fluorinated tropane alkaloids could be produced by simply feeding fluorinated phenyllactic acid analogs to *Datura stramonium* root cultures [94]. Similarly, a wide variety of tryptamine and secologanin analogs could be introduced into *C. roseus* root cultures and seedlings in order to synthesize unnatural MIAs [95, 96]. The apparent flexibility of downstream alkaloid pathways opened the possibility of generating enzyme variants with increased selectivity toward unnatural substrate analogs, thereby improving the efficiency of precursor-directed biosynthesis and increasing the generation of novel alkaloids. Several STR variants with altered substrate specificity have been successfully engineered. For example, the structural elucidation of *Rauvolfia serpentina* STR led to the identification of several amino acid residues that form the binding pocket of *C. roseus* STR [97, 98]. An enzyme variant exhibiting increased selectivity toward an analog of secologanin (with a pentynyl group) was identified. Similarly, functional STR mutants were identified that can accept tryptamine analogs [99]. By applying a saturation mutagenesis strategy on several residues that form the binding pocket, two STR mutants (V214M and F232L) that turned over unnatural tryptamine compounds to synthesize β-carboline analogs were identified [98]. When the newly synthesized strictosidine analogs were fed to *C. roseus* hairy root cultures, a number of novel MIA analogs were obtained. As shown in Fig. 8.11 [15], STR mutants (identified from the development of colorimetric medium-throughput assay) that can efficiently turn over substrate analogs were used to synthesize unnatural strictosidine from unnatural tryptamine and secologanin. Unnatural complex alkaloids could be generated from feeding the unnatural strictosidine into *C. roseus* hairy root culture. Analogs of tryptamine could also be directly converted into complex alkaloids by metabolically reprogramming *C. roseus* hairy root [100].

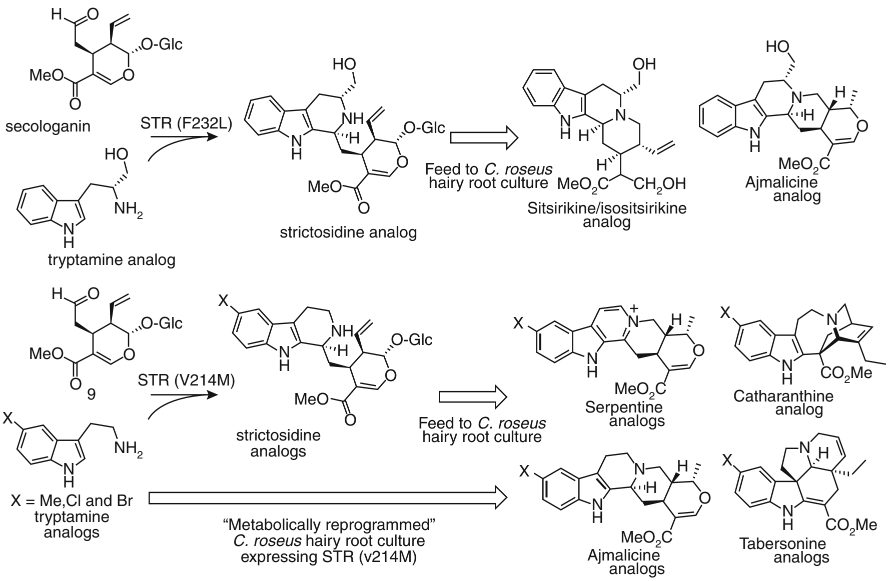

secologanin
STR (F232L)
tryptamine analog
strictosidine analog
Feed to *C. roseus* hairy root culture
Sitsirikine/isositsirikine analog
Ajmalicine analog
9
STR (V214M)
X = Me,Cl and Br tryptamine analogs
strictosidine analogs
Feed to *C. roseus* hairy root culture
Serpentine analogs
Catharanthine analog
"Metabolically reprogrammed" *C. roseus* hairy root culture expressing STR (v214M)
Ajmalicine analogs
Tabersonine analogs
O-Glc
MeO
HO
OH
NH_2
MeO_2C
CH_2OH
CO_2Me
X

Fig. 8.11 Mutasynthetic strategy for generating unnatural alkaloids in *Catharanthus roseus* hairy roots (Adopted from Ref. [5])

4 Engineering Alkaloid Biosynthesis in Microorganisms

Owing to the smaller genome size, the degree of complexity in microorganisms is significantly lower than that of plants. Moreover, microorganisms have fewer intracellular organelles compared to plant cells. Hence, metabolite transport between enzymatic steps can be negligible. With the ability to express alkaloid biosynthetic enzymes (difficult to achieve by chemical means), heterologously in microorganisms having better fermentation characteristics than plants, it is possible to achieve unlimited quantities of these enzymes required for synthesis of important drugs. The cDNAs for enzymes that catalyze the biosynthetic steps can be isolated from plants and heterologously expressed in microbes. Alternatively, instead of single transformation steps, microorganisms can be engineered to express short pathways, thus producing an end product alkaloid of interest.

The bacterium *E. coli* and yeast *S. cerevisiae* have been recently explored as production hosts of plant alkaloids [15, 101, 102]. In both cases, the metabolic engineering efforts in microorganisms entailed the reconstruction of the plant biosynthetic pathways (Fig. 8.12) [101]. To assemble an artificial pathway to achieve (S)-reticuline biosynthesis in *E. coli*, *Micrococcus luteus* monoamine oxidase (MAO) was introduced together with *Coptis japonica* norcoclaurine

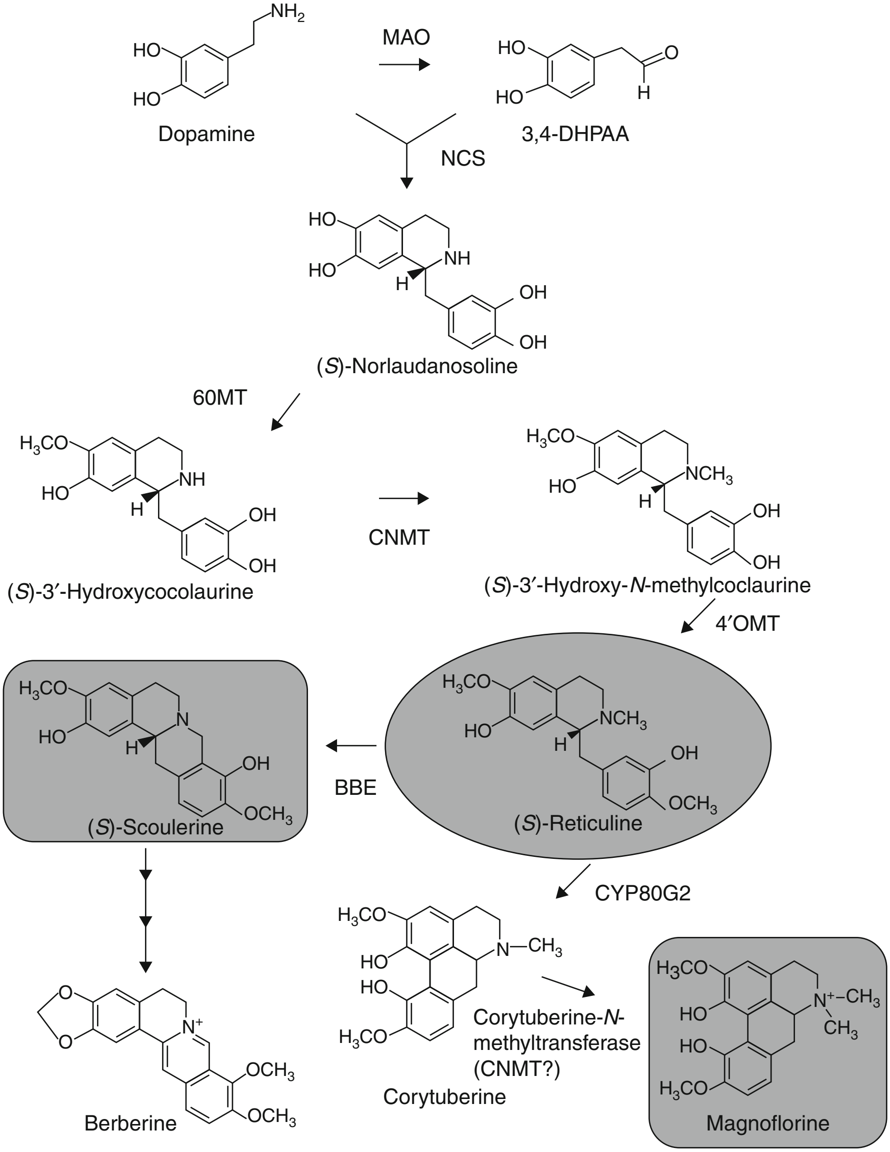

Dopamine
MAO
3,4-DHPAA
NCS
(S)-Norlaudanosoline
60MT
(S)-3′-Hydroxycocolaurine
CNMT
(S)-3′-Hydroxy-N-methylcoclaurine
4′OMT
BBE
(S)-Scoulerine
(S)-Reticuline
CYP80G2
Berberine
Corytuberine-N-methyltransferase (CNMT?)
Corytuberine
Magnoflorine

Fig. 8.12 Reconstruction of benzylisoquinoline alkaloid (BIA) pathway in microorganisms. *MAO* bacterial monoamine oxidase from *Micrococcus luteus*, *NCS* norcoclaurine synthase from *Coptis japonica*, *6-OMT* norcoclaurine 6-*O*-methyltransferase from *Coptis japonica*, *CNMT*, coclaurine-*N*-methyltransferase from *Coptis japonica*, *4-OMT* 3′-hydroxy-*N*-methylcoclaurine-4′-O-methyltransferase from *Coptis japonica*, *CYP80G2* plant cytochrome P450 enzymes from *Coptis japonica, BBE* berberine bridge enzyme from *Coptis japonica* (Adopted from Ref. [101])

synthase (NCS), 6-*O*-methyltransferase (6-OMT), coclaurine-*N*-methyltransferase (CNMT), and 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase (4′-OMT) in plasmid-based expression systems [101]. Upon induction of enzyme expression and supplementation of ~780 mg l^{-1} dopamine, nearly 11 mg l^{-1} of (*R*,*S*)-reticuline could be produced in the cell culture of the recombinant *E. coli*. Also, by coculturing the reticuline-producing *E. coli* with *S. cerevisiae* expressing the *C. japonica* berberine bridge enzyme (BBE) or plant cytochrome P450 CYP80G2, in the presence of dopamine, (*S*)-scoulerine and magnoflorine could be obtained in a short period of time (48–72 h). However, the use of two microbial systems for pathway construction relatively reduces the level of alkaloid production on account of metabolite transport required between the cells.

In another study, *S. cerevisiae* was used as a sole host organism for the assembly of artificial BIA pathways [103]. In this work, the biosynthesis of (*R*,*S*)-reticuline from (*R*,*S*)-norlaudanosoline was enabled by expressing 6-OMT, CNMT, and 4′-OMT derived from either *Thalictrum flavum* or *Papaver somniferum*. After stable insertion into the yeast genome under a reduced strength promoter variant [104], the heterologous gene expression resulted in the creation of an artificial plant pathway with reduced transcriptional activities while maintaining high catalytic activities. Furthermore, the plasmid-based expression of *P. somniferum* BBE together with *T. flavum* (*S*)-scoulerine 9-*O*-methyltransferase (SMT) in (*R*,*S*)-reticuline-producing yeasts resulted in the synthesis of ~60 mg l^{-1} (*S*)-tetrahydrocolumbamine from ~1 g l^{-1} of (*R*,*S*)-norlaudanosoline in 48 h. Additional plasmid-based expression of the *C. japonica* P450 enzyme CYP719A1 and the integration of the *A. thaliana* P450 redox partner protein in the genome gave rise to the accumulation of an estimated ~30 mg l^{-1} (*S*)-canadine, the direct precursor of the pharmaceutically important berberine. The synthesis of (*R*,*S*)-reticuline by the engineered yeast strain also enabled the synthesis of salutaridine, an intermediate in the branch pathway of morphine, through a shorter route. In plants, the synthesis of salutaridine from S-reticuline includes multiple enzymatic steps, many of which are not characterized. However, by expressing a human cytochrome P450 involved in morphine metabolism together with human CPR1 reductase in the reticuline-producing yeasts, ~20 mg l^{-1} salutaridine could be synthesized from (*R*,*S*)-norlaudanosoline. Yeast has also been engineered to accommodate the biosynthesis of high-value MIAs from feeding tryptamine and secologanin [105].

Transgenic yeast have also been created by expressing *C. roseus* STR and strictosidine β-glucoside (SGD) using a plasmid-based expression system. On feeding of the STR substrates, ~2 g l^{-1} of strictosidine could be detected in the medium on account of the presence of the heterologously expressed STR. Yeast has the ability to support the functionality of plant membrane-bound cytochrome P450 enzymes which is difficult in *E. coli* due to the absence of the endoplasmic reticulum required for anchorage. However, recently reported protein engineering strategies have allowed the functional expression of plant P450s in *E. coli* also [106, 107].

The recent demonstrations of engineering alkaloid pathways in microbes are promising but are limited by the need to provide expensive intermediate precursors exogenously. Thus, the complete elucidation of alkaloid biosynthesis is a necessary prerequisite to enable the synthesis of complex downstream alkaloids from simple precursors. However, this strategy will likely entail the implantation of numerous biosynthetic steps, which is a technical challenge. There are several other challenges that must be met before microorganisms can be used as an industrial alkaloid production platform. For example, many steps in alkaloid biosynthesis require methylation, and high-level production in microbial systems can be limited by the intracellular availability of *S*-adenosyl-L-methionine (SAM). Therefore, further metabolic engineering efforts are required to increase the SAM pool in the microbial hosts. Also, the cytotoxicity of alkaloids in yeast has also been reported [105] and is presumably present in other microbes as well. Therefore, practical and effective strategies need to be devised to mitigate cellular toxicity in order to generate alkaloid-overproducing microbes. A transcriptomic approach has been recently used to diagnose the effect of metabolite toxicity and resulted in a strategy to dampen the negative impact of the toxicity on growth [108]. Moreover, a new strategy to increase microbial tolerance toward toxic metabolites by engineering transcription factors can be applied in alkaloid-producing microbes [109]. In general, the current technology of supplying alkaloids from engineered microorganisms is not economical because it still relies on the supplementation of expensive intermediate metabolites. However, the complete elucidation of alkaloid biosynthetic pathways from the early amino acid precursors can lead to the generation of economically viable microbial production platforms. In fact, to some extent, the feasibility of plant metabolite biosynthesis from inexpensive precursors has been demonstrated in both *E. coli* and *S. cerevisiae* [110, 111].

5 Conclusion

The feasibility and success of industrial-scale production of scopolamine and berberine from cell culture by Sumitomo Chemical Industries and Mitsui Petrochemical Industries [112, 113] supports the applicability of plant cell/tissue culture bioprocesses for commercial production of alkaloids. However, one major limitation of plant tissues and cell lines is the inability to produce certain alkaloids owing to the lack of specialized cell types [114]. Metabolic reconstruction is feasible now for increasing the utility of plant cell lines and tissues for commercial production of alkaloids. Recent advances made in developing genetic tools for plant transformation can overcome the long-standing problems of slow growth, low yields, and instability in routine plant cell cultures. There have been some successful efforts on application of metabolic engineering strategies for enhanced alkaloid production (Table 8.4) [3]. However, pathway compartmentalization and the existence of multiple alkaloid biosynthetic pathways and regulatory control mechanisms can limit the robustness of the metabolic engineering strategies used for enhanced

Table 8.4 Metabolic engineering of plants for alkaloid production using genes involved in alkaloid biosynthesis (Modified and adopted from Ref. [3])

Transformation done in plant species	Source of gene	Enzymes involved	Result of metabolic modification
Nicotiana tabacum	*Catharanthus roseus*	Tryptophan decarboxylase (TDC)	Increased tryptamine
Solanum tuberosum	*Catharanthus roseus*	Tryptophan decarboxylase (TDC)	Redirection of shikimate metabolism; reduced phenylalanine; increased disease susceptibility
Catharanthus roseus	*Catharanthus roseus*	Tryptophan decarboxylase (TDC)	Increases tryptamine in crown gall tissue
Catharanthus roseus	*Catharanthus roseus*	Tryptophan decarboxylase (TDC), (STR)	Increases terpenoid indole alkaloids
Cinchona officinalis	*Catharanthus roseus*	Tryptophan decarboxylase (TDC), Strictosidine synthase (STR)	Increased quinoline alkaloids in root culture
Nicotiana tabacum	*Catharanthus roseus*	Tryptophan decarboxylase (TDC), Strictosidine synthase (STR)	Strictosidine production when supplied with exogenous secologanin
Brassica napus	*Catharanthus roseus*	Tryptophan decarboxylase (TDC)	Reduced indole glucosinolates
Brassica napus	*Papaver somniferum*	Tyrosine decarboxylase (TYDC)	Increased cell wall-bound tyramine; decreased cell wall digestibility
Nicotiana tabacum	*Saccharomyces cerevisiae*	Ornithine decarboxylase (ODC)	Increased putrescine and nicotine
Nicotiana tabacum	*Avena sativa*	Arginine decarboxylase (ADC)	Increased agmatine
Atropa belladonna	*Hyoscyamus niger*	Hyoscyamine 6 _-hydroxylase (H6H)	Increased scopolamine
Hyoscyamus muticus	*Hyoscyamus niger*	Hyoscyamine 6 _-hydroxylase (H6H)	Increased scopolamine
Peganum harmala	*Catharanthus roseus*	Tryptophan decarboxylase (TDC)	Increased serotonin
Nicotiana tabacum	*Hafnia alvei*	Lysine decarboxylase (LDC)	Increased cadaverine and anabasine
Peganum harmala	*Catharanthus roseus*	Tryptophan decarboxylase (TDC)	Increased serotonin and Camptothecin

alkaloid production in plant cell cultures. Complete elucidation of the complex alkaloid biosynthetic networks is hence required to design an effective metabolic engineering scheme without any unpredictability. The development of mathematical models of plant metabolism together with systems biology analyses can eventually be used to aid in determining effective metabolic engineering strategies [115, 116]. The inherent complexity of plant cellular systems often causes single-gene manipulations to be ineffective for altering a biosynthetic phenotype. Therefore, methods that are capable of effecting simultaneous changes in multiple metabolic points, such as the use of transcription factors, seem to be promising [117].

Microbes are even more scalable than plant tissue and cell cultures, with a long and successful history as chemical factories for the large-scale production of both bulk and specialized chemical products. The degree of complexity in microorganisms is significantly lower than that of plant systems, in terms of lack of preexisting branch pathways and transcription factors which simplifies the design of the metabolic engineering approach. Recent advances in synthetic biology and metabolic engineering have enabled tailored production of these secondary metabolites in microorganisms, but these methods often require the addition of expensive substrates.

In conclusion, both plant cell/tissue and microbial systems offer tremendous advantages as scalable alkaloid production platforms. Moreover, because the characteristics and metabolic capacities of plant cell/tissue and microbial systems are inherently different, they can serve as complementary unit operations in order to solve the long-standing problem of robust alkaloid production. However, better understanding of regulatory mechanisms and refinement for robust cellular and metabolic engineering of plant cells/tissues will further provide an impetus to popular use of plant cell technology in industry.

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Marine Pyrroloiminoquinone Alkaloids, Makaluvamines and Discorhabdins, and Marine Pyrrole-Imidazole Alkaloids

9

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Contents

1 Introduction 253
2 Marine Pyrroloiminoquinone Alkaloids, Makaluvamines, and Discorhabdins 253
2.1 Biosynthesis 253
2.2 Natural Makaluvamines and Discorhabdins 253
2.3 Approaches to Syntheses of Makaluvamines 257
2.4 Synthetic Approaches to the Discorhabdins 260
3 Marine Pyrrole-Imidazole Alkaloids 264
3.1 Biosynthesis 265
3.2 Natural Pyrrole-Imidazole Alkaloids 266
3.3 Synthetic Studies 270
4 Conclusion 278
References 279

Abstract

This chapter focuses on the biological origin and synthesis of makaluvamines and discorhabdins, and imidazole, pyrrole-imidazole alkaloids.

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Keywords

Discorhabdins • Marine metabolite • Makaluvamine • Pyrrole-imidazole alkaloid • Pyrroloiminoquinone alkaloid

Abbreviations

$(Boc)_2O$	di-*tert*-butyl dicarbonate
BocCl	*t*-butoxycarbonyl chloride
CAN	cerium(IV) ammonium nitrate
CbzN = C = S	benzyloxycarbonyl isothiocyanate
CCE	constant current electrolysis
CuTC	copper(I)-thiophene-2-carboxylate
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DIAD	diisopropyl azodicarboxylate
DIBAH	diisobutylaluminum hydride
DMAS	dimethylaminosulfonyl
DMT-MM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DTBMP	2,6-di-*tert*-butyl-4-methylpyridine
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDCl	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
Fremy's salt	potassium nitrosodisulfonate
IBX	*o*-iodobenzoic acid
KHMDS	potassium *bis*(trimethylsilyl)amide
m-CPBA	*meta* chloroperbenzoic acid
MsCl	methanesulfonyl chloride
NaHMDS	sodium *bis*(trimethylsilyl)amide
NBS	*n*-bromosuccinimide
Pd_2dba_3	tris(dibenzylideneacetone)dipalladium(0)
PEL	pig liver esterase
PIFA	phenyliodonium *bis*(trifluoroacetate)
PMBCl	*p*-methoxybenzyl chloride
PPTS	pyridinium *p*-toluenesulfonate
TBAF	tetra-*n*-butylammonium fluoride
TBAI	tetrabutylammonium iodide
TBSCl	*t*-butyldimethylsilyl chloride
TFA	trifluoroacetic acid
TFAA	trifluoroacetic anhydride
TMSCl	trimethylsilyl chloride
$TMSN_3$	trimethylsilyl azide
TMSOTf	trimethylsilyl trifluoromethanesulfonate
TrCl	triphenylmethyl chloride

1 Introduction

Natural products isolated from marine organisms, which contain various compounds that are used to sustain life and relationships with other organisms, have gained great attention because of their potential as new medicinally valuable agents, agricultural chemicals, cosmetics, and health foods [1–5]. Among these organisms, marine sponges of the genera *Latrunculia*, *Batzella*, *Prianos*, and *Zyzzya* are rich sources of alkaloid metabolites. This chapter focuses on the biological origin and synthesis of makaluvamines and discorhabdins, and imidazole, pyrrole-imidazole alkaloids.

2 Marine Pyrroloiminoquinone Alkaloids, Makaluvamines, and Discorhabdins

Several reviews have appeared describing the structures, biological activities, and synthesis of the pyrroloiminoquinone alkaloids and their analogues [6–10]. Because most substances in this family, including makaluvamines and discorhabdins, exhibit strong cytotoxicities toward human tumor cell lines, they serve as lead compounds for developing new anticancer drugs and have attracted the attention of several synthetic organic chemistry groups (Fig. 9.1).

2.1 Biosynthesis

The putative biosynthetic pathway linking the marine pyrroloiminoquinone and related metabolites, described by Munro et al., is shown in Scheme 9.1 [6, 11]. The route begins with oxidation of tryptamine to introduce hydroxyl groups at C-4 and C-7. Next, oxidation affords a quinone, which produces the iminoquinone intermediate by imine formation between the carbonyl group and the side chain amine. Further oxidation gives makaluvamines A-C, H, I, and N (Path A). In a branch of this pathway (Path B), thyrosine adds to the iminoquinone to generate makaluvamines D, E, G, J-M, and P. Furthermore, two additional routes exist to yield the discorhabdins, one of which produces makaluvamine F by early introduction of sulfur followed by phenol coupling to give the sulfur-containing fused hexacyclic discorhabdins A, B, I, Q, D, and L. The other route via discorhabdins C, E, F and O, which are generated by phenol coupling, involves sulfur introduction to produce discorhabdins S, T, U, A, B, I, Q, D, and L.

2.2 Natural Makaluvamines and Discorhabdins

Makaluvamine alkaloids, representative structures of which are shown in Fig. 9.2, were isolated from marine sponges collected in Fijian, Micronesia, Australia's

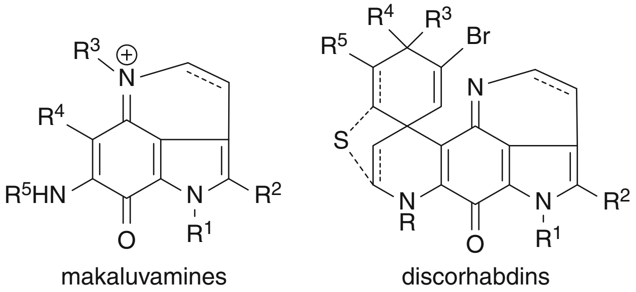
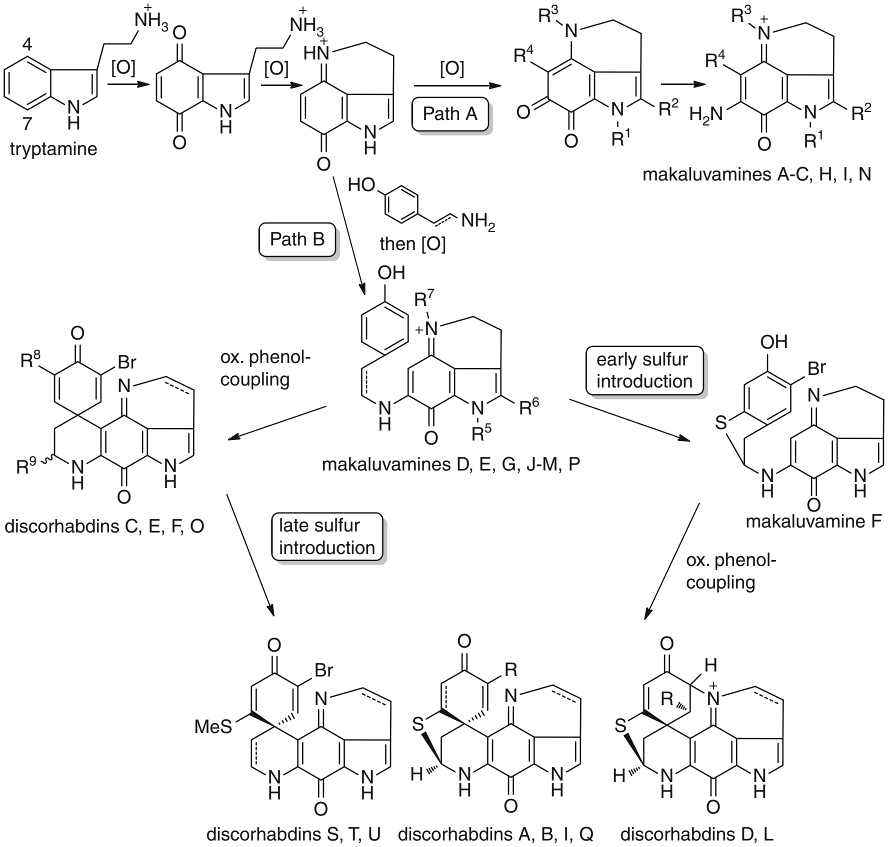
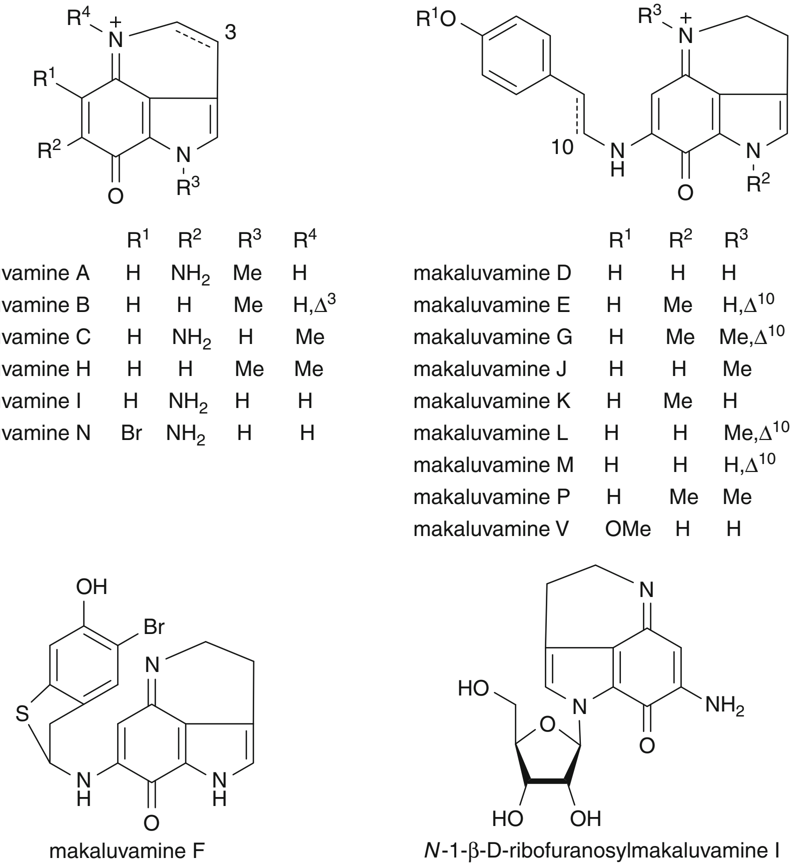

R3
R4
R5HN
O
R1
R2
makaluvamines
R4
R3
R5
Br
S
N
R
O
R1
R2
discorhabdins

Fig. 9.1 General skeletons of makaluvamines and discorhabdins


tryptamine
[O]
[O]
[O]
Path A
makaluvamines A-C, H, I, N
Path B
then [O]
makaluvamines D, E, G, J-M, P
ox. phenol-coupling
discorhabdins C, E, F, O
early sulfur introduction
makaluvamine F
late sulfur introduction
ox. phenol-coupling
discorhabdins S, T, U
discorhabdins A, B, I, Q
discorhabdins D, L

Scheme 9.1 Proposed biosynthesis of makaluvamines and discorhabdins



	R^1	R^2	R^3	R^4
makaluvamine A	H	NH_2	Me	H
makaluvamine B	H	H	Me	H,Δ^3
makaluvamine C	H	NH_2	H	Me
makaluvamine H	H	H	Me	Me
makaluvamine I	H	NH_2	H	H
makaluvamine N	Br	NH_2	H	H

	R^1	R^2	R^3
makaluvamine D	H	H	H
makaluvamine E	H	Me	H,Δ^{10}
makaluvamine G	H	Me	Me,Δ^{10}
makaluvamine J	H	H	Me
makaluvamine K	H	Me	H
makaluvamine L	H	H	Me,Δ^{10}
makaluvamine M	H	H	H,Δ^{10}
makaluvamine P	H	Me	Me
makaluvamine V	OMe	H	H

makaluvamine F

N-1-β-D-ribofuranosylmakaluvamine I

Fig. 9.2 Representative makaluvamine alkaloids

Great Barrier Reef, Indonesia, the Philippines, and Vanuatu areas [12–17]. The configuration of the stereogenic center in makaluvamine F has not yet been determined. *N*-1-*β*-D-ribofuranosylmakaluvamine I, which contains a carbohydrate moiety, was also isolated from a South African sponge [18].

The discorhabdin alkaloids, structures of whose representative members are given in Fig. 9.3, were isolated from marine sponges, including those from New Zealand of the genus *Latrunculia*, the Okinawan sponge *Prianos melanos*, the Fijian sponge *Zyzzya* cf. *Marsailis*. Discorhabdin Z was isolated from the Korean marine sponge *Sceptrella* sp. [19], and dihydrodiscorhabdin B and discorhabdin Y were isolated from a deep-water Alaskan sponge of the genus *Latrunculia* [20]. Among the various discorhabdins (A-X), discorhabdins A [21], B [21], D [22], H [23], I [24], J [6], L [24], M [6], N [6], Q [25], R [26], and X [27], and prianosins B and D [28] have sulfur-containing fused ring system. In contrast, discorhabdins

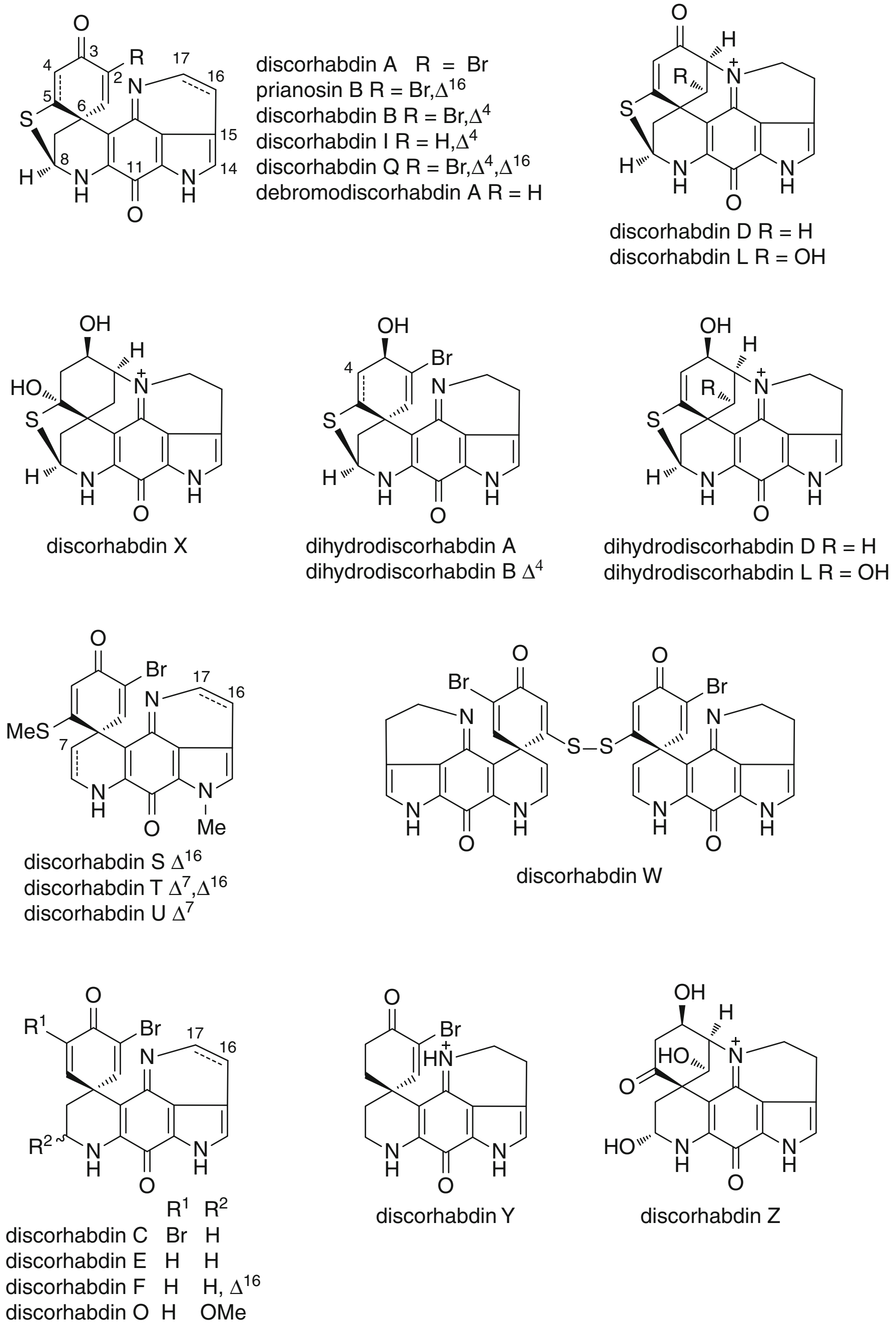

discorhabdin A R = Br
prianosin B R = Br,Δ^{16}
discorhabdin B R = Br,Δ^{4}
discorhabdin I R = H,Δ^{4}
discorhabdin Q R = Br,Δ^{4},Δ^{16}
debromodiscorhabdin A R = H
discorhabdin D R = H
discorhabdin L R = OH
discorhabdin X
dihydrodiscorhabdin A
dihydrodiscorhabdin B Δ^{4}
dihydrodiscorhabdin D R = H
dihydrodiscorhabdin L R = OH
discorhabdin S Δ^{16}
discorhabdin T Δ^{7},Δ^{16}
discorhabdin U Δ^{7}
discorhabdin W
R1 R2
discorhabdin C Br H
discorhabdin E H H
discorhabdin F H H, Δ^{16}
discorhabdin O H OMe
discorhabdin Y
discorhabdin Z

Fig. 9.3 Discorhabdin alkaloids

S, T, and U [29] possess methyl sulfide moieties, discorhabdin W [30] is a dimeric structure linked by a disulfide bond, while other members of this series do not contain sulfur. Furthermore, discorhabdins F [31], Q, S, and T and prianosin B contain a 16,17-dehydropyrroloiminoquinone moiety. The enantiomeric pairs of discorhabdins B, G*/I, L, and W were also isolated from *Latrunculia* species sponges [32]. A study focusing on the elucidation of the absolute stereochemistry of several discorhabdins was reported by Copp et al. [33].

2.3 Approaches to Syntheses of Makaluvamines

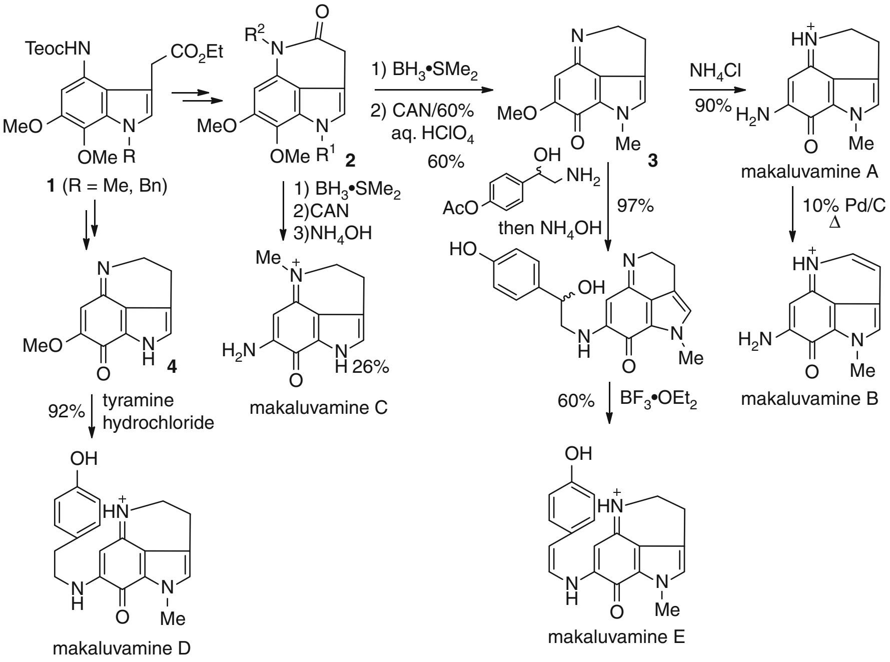
Makaluvamines A [34], B [35], C [36], D [37], E [38], I [34], and K [34], and *N*-1-*β*-D-ribofuranosyl-makaluvamine I [37] have been synthesized until now. Most of these syntheses were reported prior to 2000 and following that time, most attention in this area has been given to preparation of analogs and probing their biological activities.

For the most part, the routes developed for the synthesis of members of the makaluvamine family have mimicked the proposed biosynthesis route shown in Scheme 9.1. Specifically, pathways have been devised that involve construction of the pyrroloiminoquinone skeleton through initial formation of the quinone moiety followed by either imine formation, oxidation of the cyclic amine, or direct iminoquinone formation of substrates possessing an azide side chain.

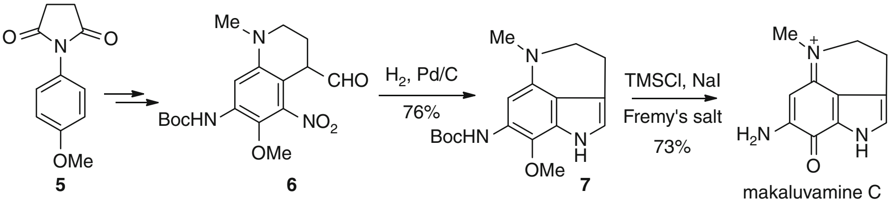
One example is found in the synthesis of makaluvamine A-E, reported by Yamamura and Nishiyama (Scheme 9.2). The route begins with conversion of indole **1** to lactam **2**. In the route to makaluvamines A, B, and E, **2** (R^1 = Me, R^2 = H) was transformed to pyrroloiminoquinone **3** by using a reduction and oxidation sequence. In a closely related fashion, makaluvamine C was prepared from **2** (R^1 = H, R^2 = Me) and makaluvamine D was synthesized from the iminoquinone **4** derived from **1** (R = Bn) [38].

Kraus et al. described a total synthesis of makaluvamine C that proceeds through initial formation of quinoline ring followed by formation of the indole moiety and Fremy's salt promoted oxidation of the 4-methoxyaniline group (Scheme 9.3) [36]. In this approach, the 6,6-bicyclic compound **6** was formed first.

In 1994, we discovered a process involving inter- and intramolecular nucleophilic addition of aromatic compounds that is mediated by a hypervalent iodine reagent in either CF_3CH_2OH or $(CF_3)_2CHOH$ [39]. In 2006, Nishiyama et al. used the hypervalent iodine reagent, prepared from iodobenzene under electrolytic conditions, as part of a new method for the synthesis of tetrahydroquinolines. This process was applied to syntheses of the pyrroloiminoquinones, makaluvamines D and I, and *N*-1-*β*-D-ribofuranosylmakaluvamine I (Scheme 9.4). In the synthetic pathways, arylpropionamide **10**, prepared from 3-benzyloxy-2-nitrotoluene (**8**), was converted to the quinolinone **11** when subjected to the hypervalent iodine prepared by anodic oxidation of PhI in CF_3CH_2OH. Quinolinone **11** was then transformed to aldehyde **12**, which gave **13** by reaction with Zn/AcOH, followed by treatment with TsCl–NaH and catalytic hydrogenolysis. Oxidation of the 2-hydroxyaniline moiety in **13** with


TeocHN
CO2Et
MeO
OMe R
1 (R = Me, Bn)
R2
O
MeO
OMe R1 2
1) BH3•SMe2
2) CAN/60% aq. HClO4
60%
MeO
O
Me
3
NH4Cl
90%
H2N
makaluvamine A
1) BH3•SMe2
2)CAN
3)NH4OH
OH
NH2
AcO
then NH4OH
97%
10% Pd/C
Δ
MeO
4
92%
tyramine hydrochloride
Me
H2N
H 26%
makaluvamine C
HO
OH
Me
60%
BF3•OEt2
H2N
makaluvamine B
OH
makaluvamine D
OH
makaluvamine E

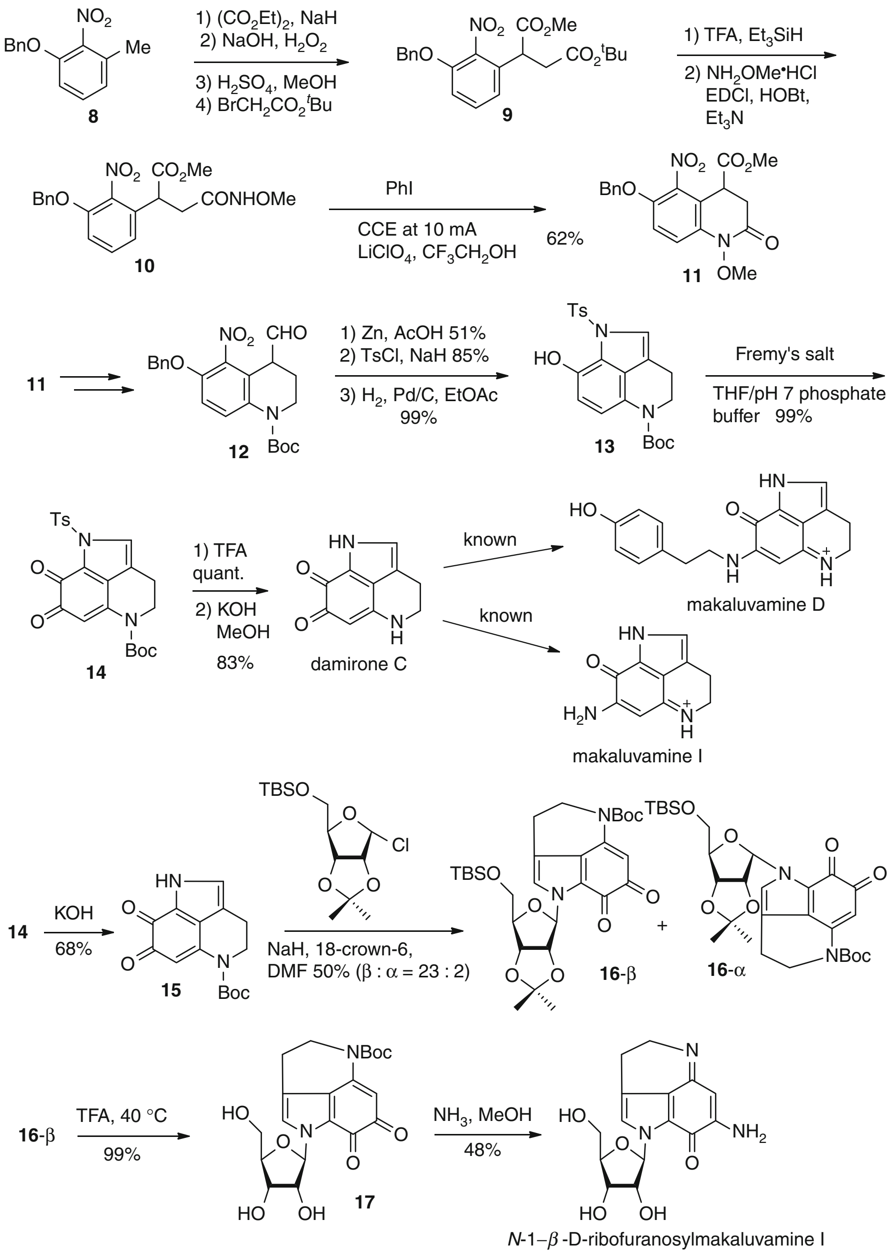
Scheme 9.2 Total synthesis of makaluvamines A-E


O
O
N
OMe
5
Me
CHO
BocHN
NO2
OMe
6
H2, Pd/C
76%
Me
BocHN
OMe
7
TMSCl, NaI
Fremy's salt
73%
Me
H2N
O
makaluvamine C

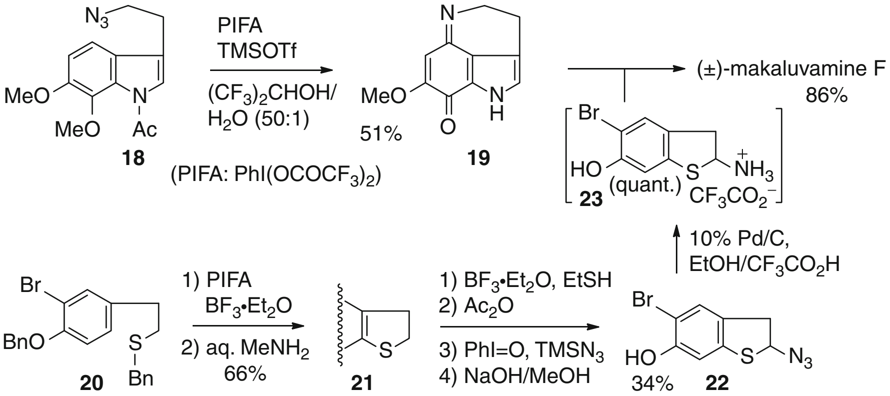
Scheme 9.3 Kraus's synthesis of makaluvamine C

Fermy's salt produced the α-diketone **14**, which was subjected to exhaustive deprotection to afford damirone C, a natural pyrroloiminoquinone alkaloid. Furthermore, amination of damirone C provided makaluvamine I and reaction of this substance with tyramine hydrochloride yielded makaluvamine D. In addition, reaction of **15**, obtained by deprotection of the amine functions in **14**, with a chloro-glycoside produced the desired glycosides **16-β** and **16-α** in 50 % yield as a 23:2 mixture. Removal of the TBS-protecting group in **16-β** afforded **17** and *N*-1-*β*-D-ribofuranosylmakaluvamine I was obtained by treatment of **17** with ammonia [37].

Among members of the makaluvamine family, makaluvamine F has been found to exhibit the most potent biological activity, exemplified by its cytotoxicity toward the human colon tumor cell line HCT-116 ($IC_{50} = 0.17$ mM) and inhibition of topoisomerase II.


NO2
BnO
Me
8
1) (CO2Et)2, NaH
2) NaOH, H2O2
3) H2SO4, MeOH
4) BrCH2CO2tBu
NO2 CO2Me
BnO
CO2tBu
9
1) TFA, Et3SiH
2) NH2OMe•HCl
EDCl, HOBt,
Et3N
NO2 CO2Me
BnO
CONHOMe
10
PhI
CCE at 10 mA
LiClO4, CF3CH2OH
62%
NO2 CO2Me
BnO
N
O
OMe
11
11
NO2 CHO
BnO
N
Boc
12
1) Zn, AcOH 51%
2) TsCl, NaH 85%
3) H2, Pd/C, EtOAc
99%
Ts
N
HO
N
Boc
13
Fremy's salt
THF/pH 7 phosphate
buffer 99%
Ts
N
O
O
N
Boc
14
1) TFA
quant.
2) KOH
MeOH
83%
HN
O
O
N
H
damirone C
known
known
HO
HN
O
N
H
N
H
makaluvamine D
HN
O
H2N
N
H
makaluvamine I
14
KOH
68%
HN
O
O
N
Boc
15
TBSO
O
Cl
O
O
NaH, 18-crown-6,
DMF 50% (β : α = 23 : 2)
NBoc
TBSO
O
N
O
O
O
O
16-β
+
TBSO
O
N
O
O
O
O
NBoc
16-α
16-β
TFA, 40 °C
99%
NBoc
HO
O
N
O
O
HO
OH
17
NH3, MeOH
48%
N
HO
O
N
NH2
O
HO
OH
N-1–β-D-ribofuranosylmakaluvamine I

Scheme 9.4 Syntheses of the pyrroloiminoquinones, makaluvamines D and I, and *N*-1-*β*-D-ribofuranosylmakaluvamine I

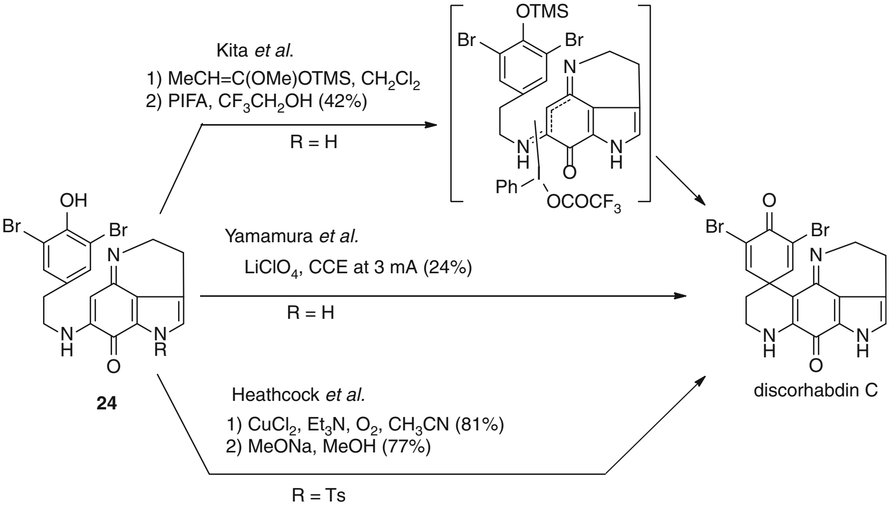


Scheme 9.5 Total synthesis of (±)-makaluvamine F

As a consequence of the presence of a labile *N,S*-acetal moiety, mild reactions are needed for the synthesis of makaluvamine F. We have developed two new methods that are valuable for the synthesis of complex pyrroloiminoquinone alkaloids. The processes involve hypervalent iodine-induced nucleophilic substitution of *p*-substituted phenol ethers via reactive cation radical intermediates. One involves reactions of quinone imine dimethylacetals or quinine imines from phenyl ethers with an alkylazido group [40, 41], and the other reactions of α-azidodihydrobenzothiophenes derived from dihydrobenzothiophenes [42]. The route devised for synthesis of makaluvamine F, utilizing the two new methods as key reactions, is shown in Scheme 9.5. The sequence begins with reaction of the 1-protected indole **18** with PIFA–TMSOTf in the presence of H_2O to afford the corresponding *N*-deprotected pyrroloiminoquinones **19**. In a parallel process, treatment of the phenol ether **20** bearing an alkyl sulfide side chain with PIFA–BF_3·Et_2O followed by the treatment with aq. $MeNH_2$ provided 5-bromo-6-benzyloxydihydrobenzothiophene **21**. Azidation of **21** gave only a trace amount of the expected α-azido compound. As a result, a new pathway was developed that involved debenzylation of **21** followed by acetylation of the resulting phenol to give a product that reacted with PhI = O and Me_3SiN_3 followed by hydrolytic deprotection to provide the desired 2-azido-5-bromo-6-hydroxydihydrobenzothiophene **22**. Catalytic hydrogenation of **22** using 10 % Pd-C in the presence of 4 equivalents of trifluoroacetic acid (TFA) resulted in complete reduction leading to the TFA salt **23** in quantitative yield. In the final step, coupling reaction in MeOH between **23** and **19** proceeded to give the TFA salt of makaluvamine F [43, 44].

2.4 Synthetic Approaches to the Discorhabdins

Discorhabdin alkaloids have the richest structure-diversity among the marine pyrroloiminoquinone alkaloids. Although many synthetic studies have been carried


Kita et al.
1) MeCH=C(OMe)OTMS, CH_2Cl_2
2) PIFA, CF_3CH_2OH (42%)
R = H
OTMS
Br
Br
N
N H
N H
O
Ph
I
$OCOCF_3$
Yamamura et al.
$LiClO_4$, CCE at 3 mA (24%)
R = H
Heathcock et al.
1) $CuCl_2$, Et_3N, O_2, CH_3CN (81%)
2) MeONa, MeOH (77%)
R = Ts
OH
Br
Br
N
N H
N R
O
24
O
Br
Br
N
N H
N H
O
discorhabdin C

Scheme 9.6 Final steps of the synthesis of discorhabdin C

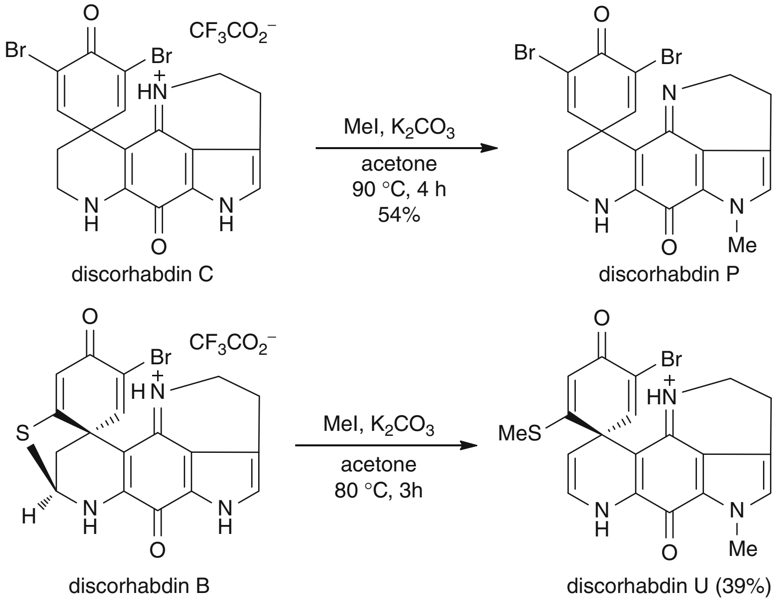
out, only a few total syntheses of the natural discorhabdins have been reported. Routes for the preparation of discorhabdin C have been developed by our group and Yamamura's group at almost the same time, and later by the group headed by Heathcock. Heathcock et al. also synthesized discorhabdin E, a 4-debromo derivative of discorhabdin C, using essentially the same procedure.

In Scheme 9.6 are shown the final steps of each of the completed synthetic pathways targeted at (±)-discorhabdin C. In our approach [45], the phenolic aminoindoloquinone imine **24** (R = H) was converted to its corresponding silyl ether, which subsequently underwent oxidative coupling using PIFA to form discorhabdin C in 42 % yield. In Yamamura's synthesis, discorhabdin C was generated in 24 % yield upon anodic oxidation of the bromophenol derivative **24** (R = H) [46]. Finally, in the route developed by Heathcock, discorhabdin C was produced by a phenolic coupling reaction of **24** (R = Ts) with $CuCl_2$ and Et_3N under O_2 followed by detosylation [47].

Copp et al. have also described the semi-synthesis of discorhabdins P and U starting with the natural discorhabdins C and B (Scheme 9.7) [48].

Sequences for the synthesis of the more complex discorhabdins, discorhabdin A and prianosin B, have been developed only in our laboratory. Discorhabdin A, which has a unique sulfur-containing fused ring structure, incorporating azacarbocyclic spirocyclohexanone and pyrroloiminoquinone systems, displays the most powerful cytotoxic activity among isolated members of the discorhabdin family.

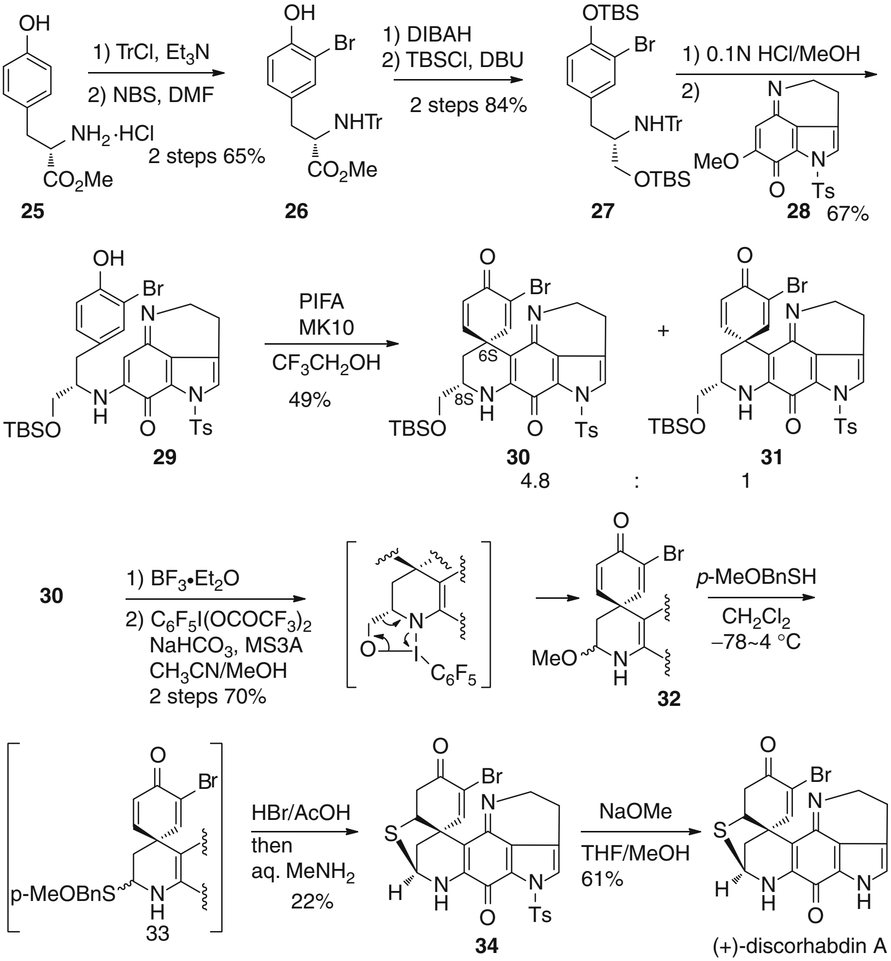
The synthetic approach we developed for the preparation of discorhabdin A involved a strategy in which the spirodienone system was preconstructed using a hypervalent iodine(III) reagent and final introduction of the sulfur to the cross-linked system (Scheme 9.8). The route began with tritylation of L-tyrosine methyl



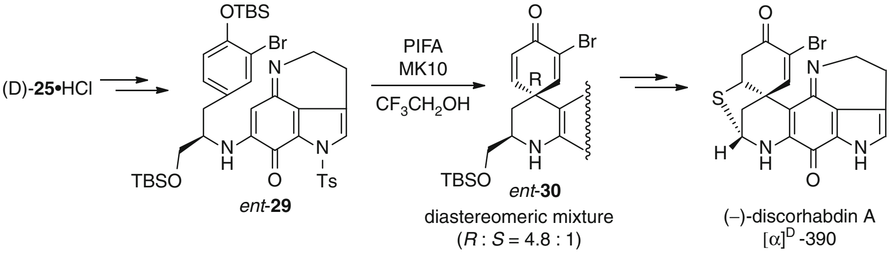
Scheme 9.7 Semi-synthesis of discorhabdin P and U

ester hydrochloride **25** followed by mono-bromination to yield **26**. Reduction of **26** with DIBAH followed by silylation gave the bis-silylated intermediate **27** that underwent coupling reaction with pyrroloiminoquinone **28**, prepared by using our previously developed method, to form **29**. Transformation of **29** to the spirodienone was promoted by treatment with PIFA in the presence of montmorillonite K10 (MK10) to give a diastereomeric mixture of **30** and **31**. The major isomer **30** has the same absolute stereochemistry (*S* configuration) at the C-6 spirocenter as that present in natural discorhabdin A. Oxidative fragmentation reaction of the α-amino alcohol, obtained from **30** by desilylation, with bis(trifluoroacetoxy)iodo (III) pentafluorobenzene ($C_6F_5I(OCOCF_3)_2$) in the presence of $NaHCO_3$, afforded the *N,O*-acetal **32** in 79 % yield. Introduction of the *p*-methoxybenzylthio group led to the unstable *N,S*-acetal **33** as a diastereomeric mixture, which was treated with 30 % HBr-AcOH followed by aqueous $MeNH_2$ work up to produce the *N*-tosyl derivative **34** of discorhabdin A. Removal of the tosyl group in **34** with NaOMe gave discorhabdin A in optically pure form (Scheme 9.8). In the same effort, we also completed a total synthesis of the unnatural discorhabdin A, (−)-discorhabdin A, starting from the D-tyrosine methyl ester by using the same general strategy (Scheme 9.9) [49, 50].

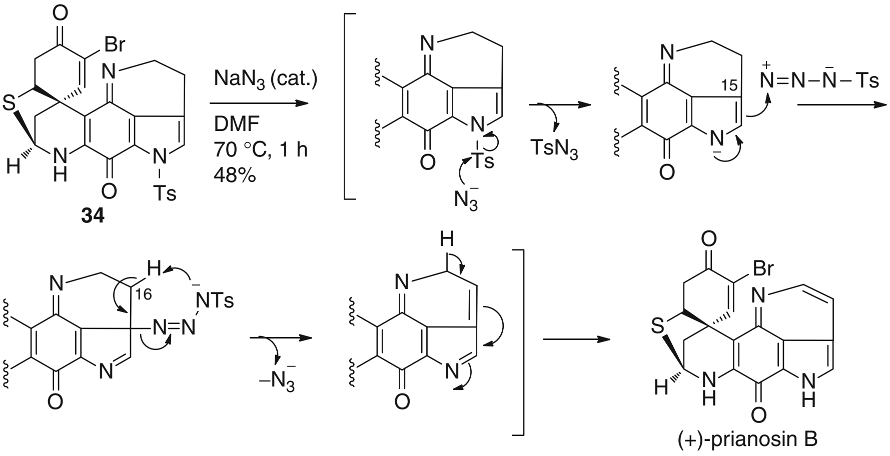
Prianosin B is a C16-17 dehydro-derivative of discorhabdin A. Thus, treatment of intermediate **34** in the discorhabdin A synthetic pathway (Scheme 9.9) with NaN_3 brought about simultaneous detosylation and dehydrogenation to produce prianosin B in 48 % yield. (Scheme 9.10) [51].


1) TrCl, Et$_3$N
2) NBS, DMF
2 steps 65%
1) DIBAH
2) TBSCl, DBU
2 steps 84%
1) 0.1N HCl/MeOH
2)
28 67%
25
26
27
29
PIFA
MK10
CF$_3$CH$_2$OH
49%
30
31
4.8 : 1
6S
8S
1) BF$_3$•Et$_2$O
2) C$_6$F$_5$I(OCOCF$_3$)$_2$
NaHCO$_3$, MS3A
CH$_3$CN/MeOH
2 steps 70%
32
p-MeOBnSH
CH$_2$Cl$_2$
–78~4 °C
33
HBr/AcOH
then
aq. MeNH$_2$
22%
34
NaOMe
THF/MeOH
61%
(+)-discorhabdin A

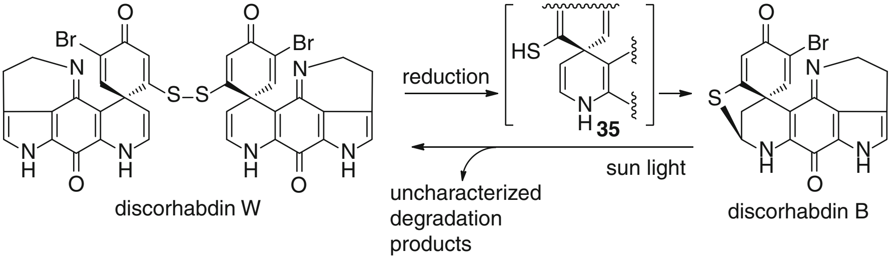
Scheme 9.8 Total synthesis of (+)-discorhabdin A


(D)-25•HCl
ent-29
PIFA
MK10
CF$_3$CH$_2$OH
R
ent-30
diastereomeric mixture
(R : S = 4.8 : 1)
(–)-discorhabdin A
[α]^D -390

Scheme 9.9 Total synthesis of (−)-discorhabdin A


34
NaN3 (cat.)
DMF
70 °C, 1 h
48%
TsN3
N3−
15
16
NTs
−N3−
(+)-prianosin B

Scheme 9.10 Total synthesis of (+)-prianosin B


discorhabdin W
reduction
35
sun light
uncharacterized degradation products
discorhabdin B

Scheme 9.11 Correlation of discorhabdin W and discorhabdin B

Discorhabdin B was also prepared in this effort by reduction of discorhabdin W, the dimer of discorhabdin B via the thiol **35**. Conversely, irradiation of discorhabdin B with sunlight afforded discorhabdin W (Scheme 9.11) [30].

3 Marine Pyrrole-Imidazole Alkaloids

The pyrrole-imidazole alkaloids, mainly isolated from various species of the *Agelasidae*, *Axinellidae*, *Dyctionellidae*, and *Hymeniacidonidae* sponge families, contain characteristic pyrrole-2-carboxamide and 2-aminoimidazoline moieties or derivatives thereof. Their architectural complexity ranges from simple, achiral, monomeric oroidin to the 16-stereocenter-containing tetrameric stylissadines A and B (Fig. 9.4). Because many reviews have appeared describing these alkaloids [52–56], we provide only a short discussion of some biosynthetically and synthetically pertinent information.

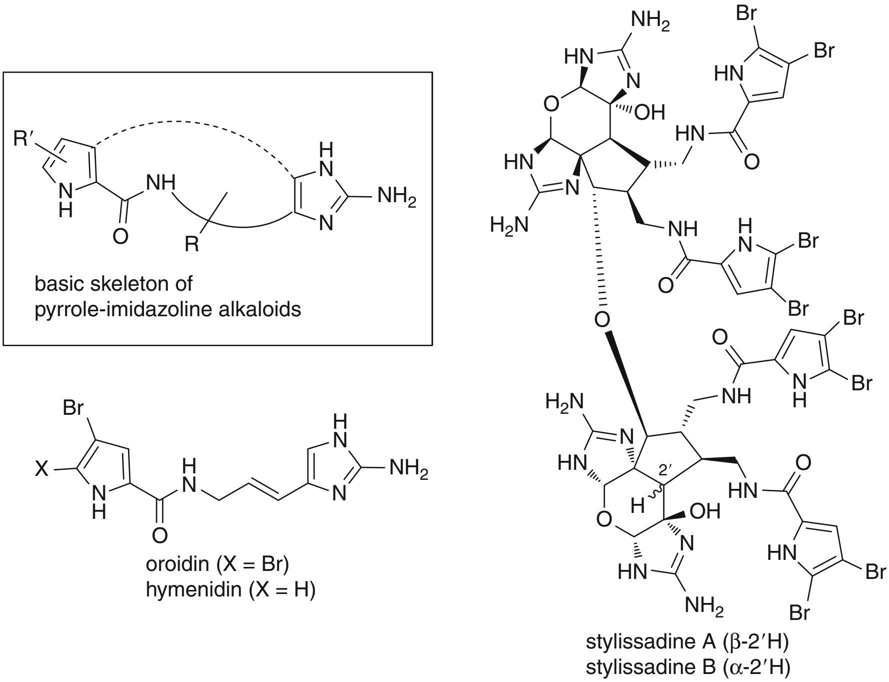


Fig. 9.4 General skeletons, monomeric and tetrameric pyrrole-imidazol alkaloids

3.1 Biosynthesis

The proposed biosynthetic route for formation of pyrrole-imidazole alkaloids is shown in Fig. 9.5. Amino acids, such as proline, histidine, ornithine, lysine, and arginine, are thought to be incorporated into simple monomeric intermediates in the route used to generate these substances in organisms [57, 58]. Indeed, feeding studies using ^{14}C-labeled amino acids by Kerr et al. demonstrated that histidine and ornithine (or proline) are the amino acid precursors of the oroidin alkaloids [59]. However, the radioactivity and specific incorporation levels were observed to be low. Based on the results of synthetic studies, Al-Mourabit et al. proposed that proline or arginine is the early precursor of both the pyrrole-carboxylic acid and the 2-aminoimidazole moieties [60]. In 2001, Al Mourabit and Potier reported that simple proposed monomeric intermediates were enzymatically converted to complex monomeric alkaloids and the and cyclized dimers [61]. Köck, Baran et al. also reported that oroidin is converted to the cyclized dimers, such as the palau'amines [62]. Furthermore, in 2010, Molinski and Romo et al. provided the first experimental evidence supporting the veracity of the common assumption that clathrodin/oroidin is the precursor to more complex pyrrole-imidazoline alkaloids, monomers, dimers, and tetramers [63].

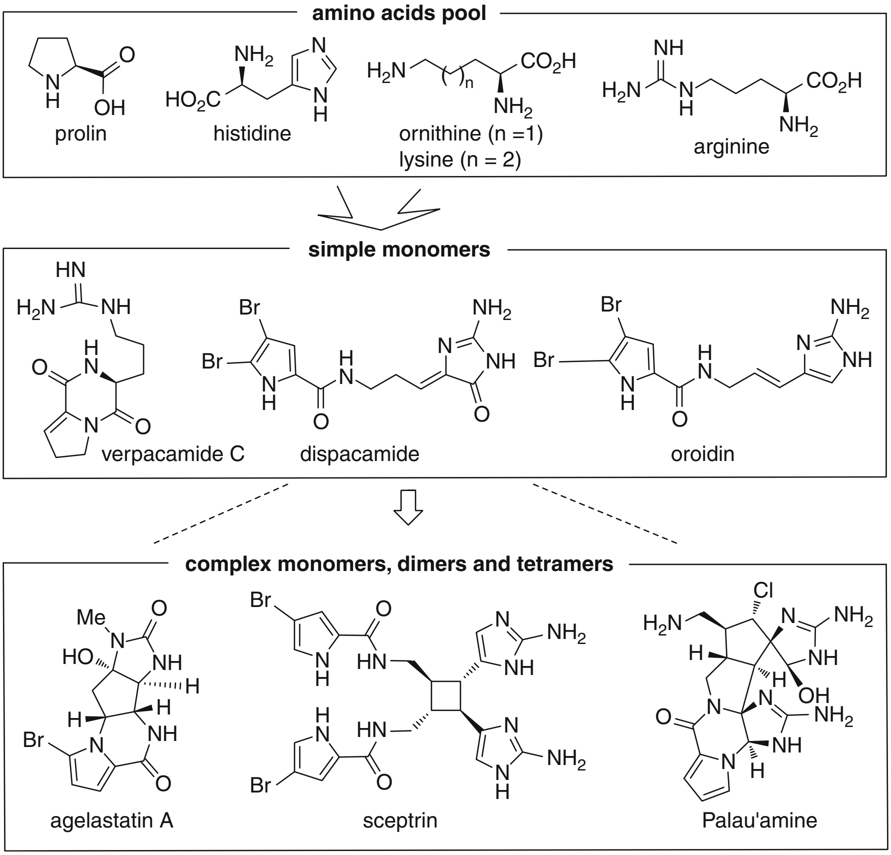

amino acids pool
prolin
histidine
ornithine (n =1)
lysine (n = 2)
arginine
simple monomers
verpacamide C
dispacamide
oroidin
complex monomers, dimers and tetramers
agelastatin A
sceptrin
Palau'amine

Fig. 9.5 Proposed biosynthesis of pyrrole-imidazole alkaloids

3.2 Natural Pyrrole-Imidazole Alkaloids

3.2.1 Monomers

Representative acyclic and cyclic monomers in the pyrrole-imidazole alkaloid family are shown in Fig. 9.6. Oroidin and hymenidin, from the sponge *Agelas oroides*, are acyclic while others members are cyclic [64]. Oroidin, the most abundant alkaloid in this group and considered to be the biogenetic precursor of the other alkaloids (Fig. 9.5), was found to inhibit larval metamorphosis of the barnacle *Balanus amphitrite*. Hymenidin is a mono-debromo derivative and clathrodine is the debromo analogue of oroidin [65, 66]. Dispacamides A and B, from the sponge *Agelas dispar*, are potent, noncompetitive, antihistaminic agents and dispacamide B was shown to display antihistaminic activity against the guinea pig ileum [67, 68]. (*Z*)-Hymenialdisine, from the sponges of the genera *Hymeniacidon, Acanthella, Axinella*, and *Pseudoaxinyssa*, has been observed to be potently active as a kinase inhibitor and to have utility for not only the treatment

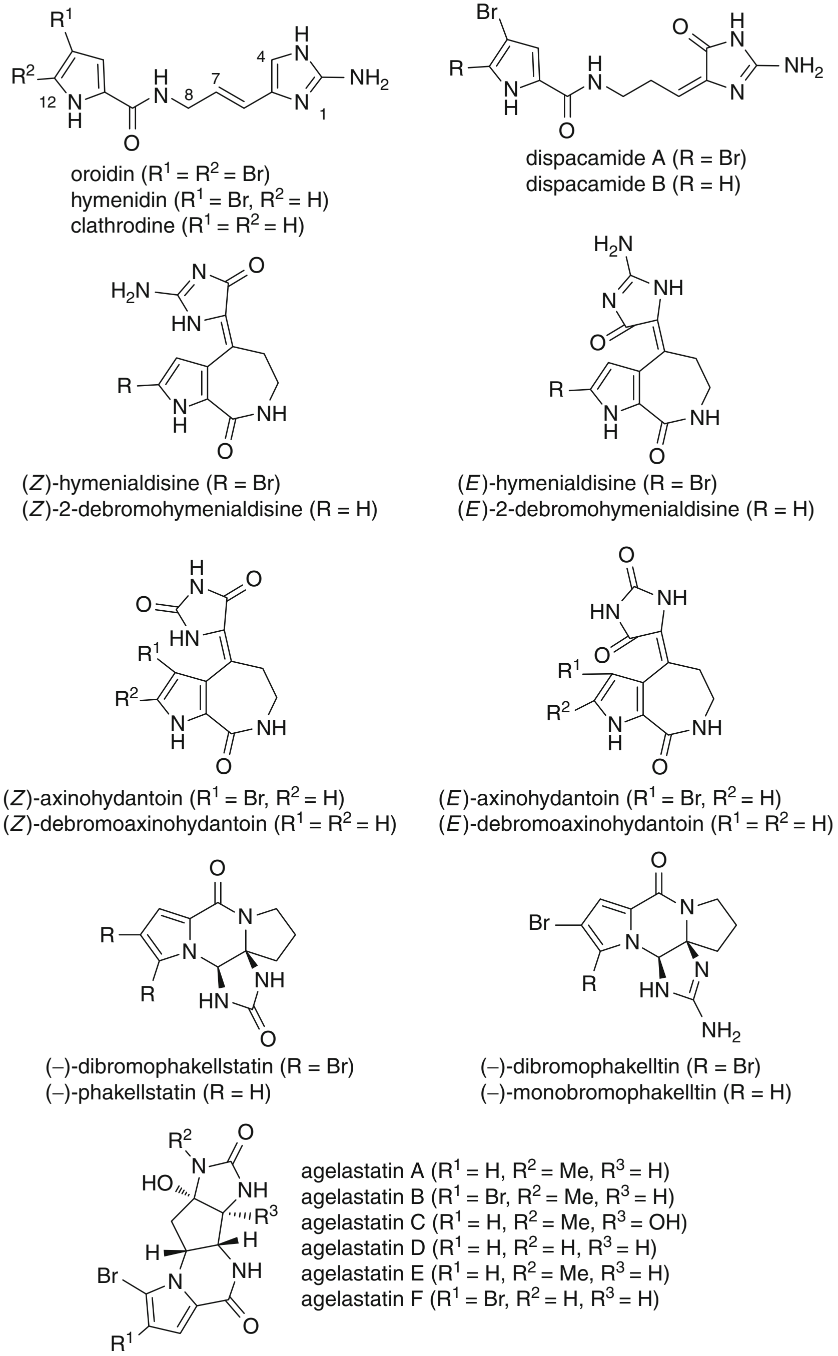

oroidin (R1 = R2 = Br)
hymenidin (R1 = Br, R2 = H)
clathrodine (R1 = R2 = H)
dispacamide A (R = Br)
dispacamide B (R = H)
(Z)-hymenialdisine (R = Br)
(Z)-2-debromohymenialdisine (R = H)
(E)-hymenialdisine (R = Br)
(E)-2-debromohymenialdisine (R = H)
(Z)-axinohydantoin (R1 = Br, R2 = H)
(Z)-debromoaxinohydantoin (R1 = R2 = H)
(E)-axinohydantoin (R1 = Br, R2 = H)
(E)-debromoaxinohydantoin (R1 = R2 = H)
(–)-dibromophakellstatin (R = Br)
(–)-phakellstatin (R = H)
(–)-dibromophakelltin (R = Br)
(–)-monobromophakelltin (R = H)
agelastatin A (R1 = H, R2 = Me, R3 = H)
agelastatin B (R1 = Br, R2 = Me, R3 = H)
agelastatin C (R1 = H, R2 = Me, R3 = OH)
agelastatin D (R1 = H, R2 = H, R3 = H)
agelastatin E (R1 = H, R2 = Me, R3 = H)
agelastatin F (R1 = Br, R2 = H, R3 = H)

Fig. 9.6 Representative acyclic and cyclic pyrrole-imidazole monomers

of cancer but also for illnesses such as Alzheimer's disease and type 2 diabetes [69]. (*E*)-Axinohydantoin and (*E*)-debromoaxinohydantoin have been isolated from the sponges *Axinella* sp and *Monanchora* [70, 71]. (Z)-Axinohydantoin and (*Z*)-debromoaxinohydantoin, isolated from the sponge *Stylotella aurantium*, are structurally similar to hymenialdisine [72, 73]. (−)-Dibromophakellstatin, isolated from the Indian Ocean sponge *Phakellia mauritiana*, is a tetracyclic monomeric pyrrole-imidazole alkaloid that showed interesting cell growth inhibitory activity against a minipanel of human cancer cell lines [74]. The structures of dibromophakellin and monobromophakellin are similar to that of dibromophakellstatin, and exist in nature as both enantiomers. The (−)-enantiomers were isolated from the marine sponge *Axinella vaceleti*, [75], and (+)-enantiomers were isolated from *Pseudoaxinyssa* cantharella [76]. Four closely related compounds, agelastatin A, its brominated congener agelastatin B isolated in 1993 from the marine sponge *Agelas Dendromorpha*, [77] agelastatin C, and agelastatin D isolated from the extracts of the sponge *Cymbastela* sp. [78], possess the same tetracyclic skeleton. Agelastatin E and F were isolated from the New Caledonian sponge *A. dendromorpha* [79]. In addition, (−)-agelastatin A was found to exhibit significant antitumor activity, toxicity toward arthropods, and selective inhibition of the glycogen synthase kinase-3b, which is a potential target in the treatment of Alzheimer's disease and bipolar disorder. The agelastatins are the only isolated pyrrole-imidazole alkaloids having C4–C8 and C7–N12 connectivities between oridin monomers.

3.2.2 Dimers

In Fig. 9.7 are displayed acyclic and cyclic dimeric members of the pyrrole-imidazole family. Nagelamide D, from Okinawan marine sponges of the genus *Agelas*, is a dimer comprised of oroidin monomer units connected between the C-10 and C-15′ positions [80]. However, the assigned structure of this substance is questionable (see below). Sceptrin, isolated in 1981 by Faulkner and Clardy from *Agelas sceptrum*, is the first reported symmetric dimer comprised of hymenidin subunits [81].

The ageliferins, isolated from the sponge *Agelas conifer*, are bicyclic, nonsymmetrical dimers that possess antibiotic and antiviral bioactivities [82]. Nagalamide E is a stereo-isomer of ageliferin [80]. Palau'amine, isolated in 1993 from the sponge *Stylotella agminata*, is a hexacyclic bisguanidine alkaloid that is the most well-known member of the pyrrole-imidazole alkaloid family. This substance, which has been observed to have exciting cytotoxic and immunosuppressive properties, was first assigned to be a diastereomer of the natural product [83]. This assignment was later questioned based on the known structure of carteramine A [84, 85], which is a structural component of palau'amine. The correct stereostructure of palau'amine was finally correctly assigned in 2007 [86]. Axinellamines A and B are the first reported tetracyclic pyrrole-imidazole alkaloids [87]. (−)-Massadine is a second tetracyclic pyrrole-imidazole alkaloid [88]. (−)-Massadine chloride is postulated to be a precursor of massadine [89].

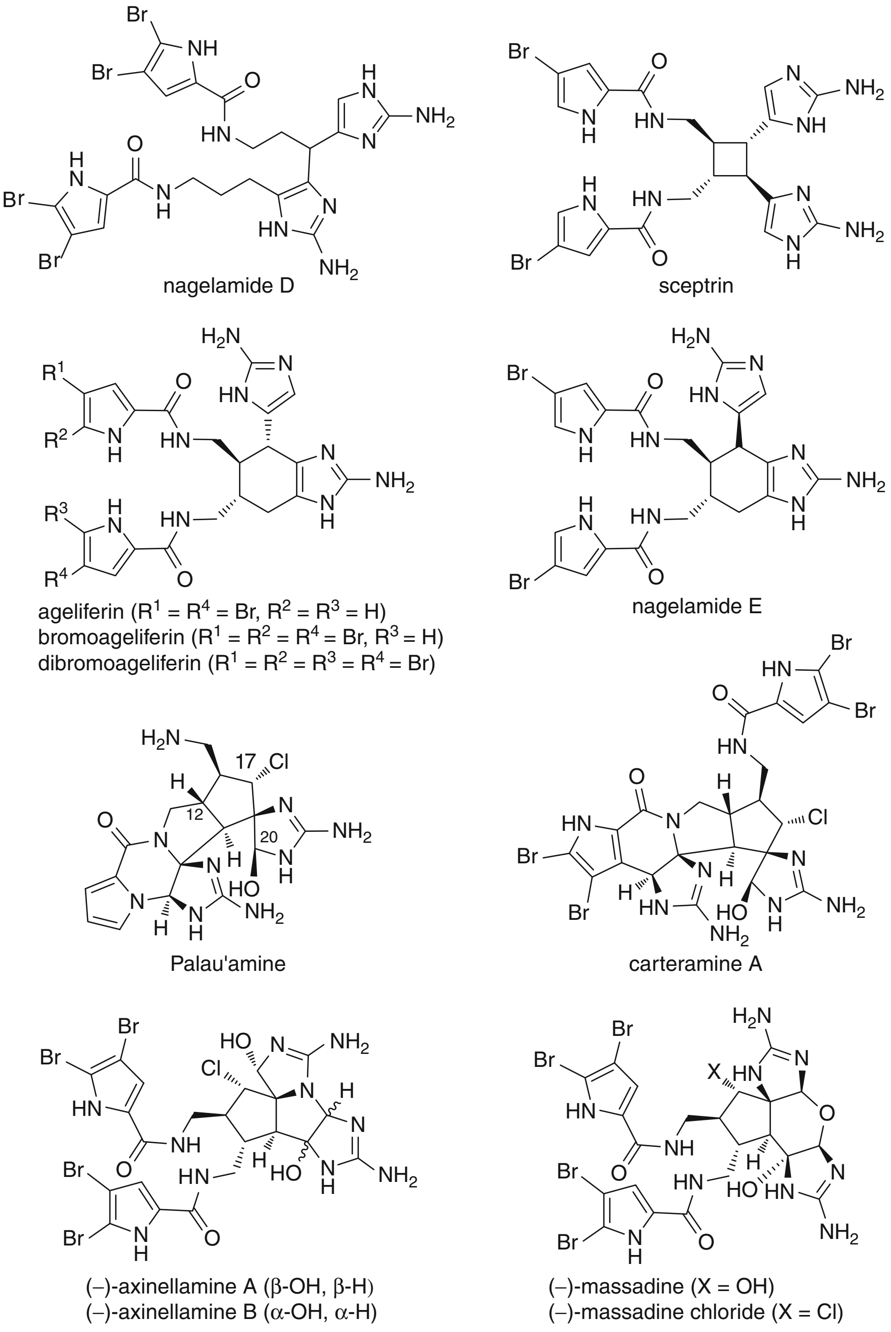

nagelamide D
sceptrin
ageliferin (R1 = R4 = Br, R2 = R3 = H)
bromoageliferin (R1 = R2 = R4 = Br, R3 = H)
dibromoageliferin (R1 = R2 = R3 = R4 = Br)
nagelamide E
Palau'amine
carteramine A
(−)-axinellamine A (β-OH, β-H)
(−)-axinellamine B (α-OH, α-H)
(−)-massadine (X = OH)
(−)-massadine chloride (X = Cl)

Fig. 9.7 Representative acyclic and cyclic pyrrole-imidazole dimers

3.2.3 Tetramers

The structures of two tetrameric alkaloids, stylissadine A and B that are ether-linked dimers of massadine, are shown in Fig. 9.4. Although these substances are antagonists of the P2X7 receptor involved in inflammatory diseases, no reports have appeared describing their syntheses, perhaps because they have the most complex structures of pyrrole-imidazole alkaloids discovered so far.

3.3 Synthetic Studies

Many studies have been targeted at the synthesis of marine pyrrole-imidazole alkaloids. As a result, in the chapter below, we will discuss only representative publications describing total and not partial syntheses of these alkaloids (Figs. 9.6 and 9.7). In addition, when several reports exist on the preparation of one of the alkaloids, we will cite only the most recent work.

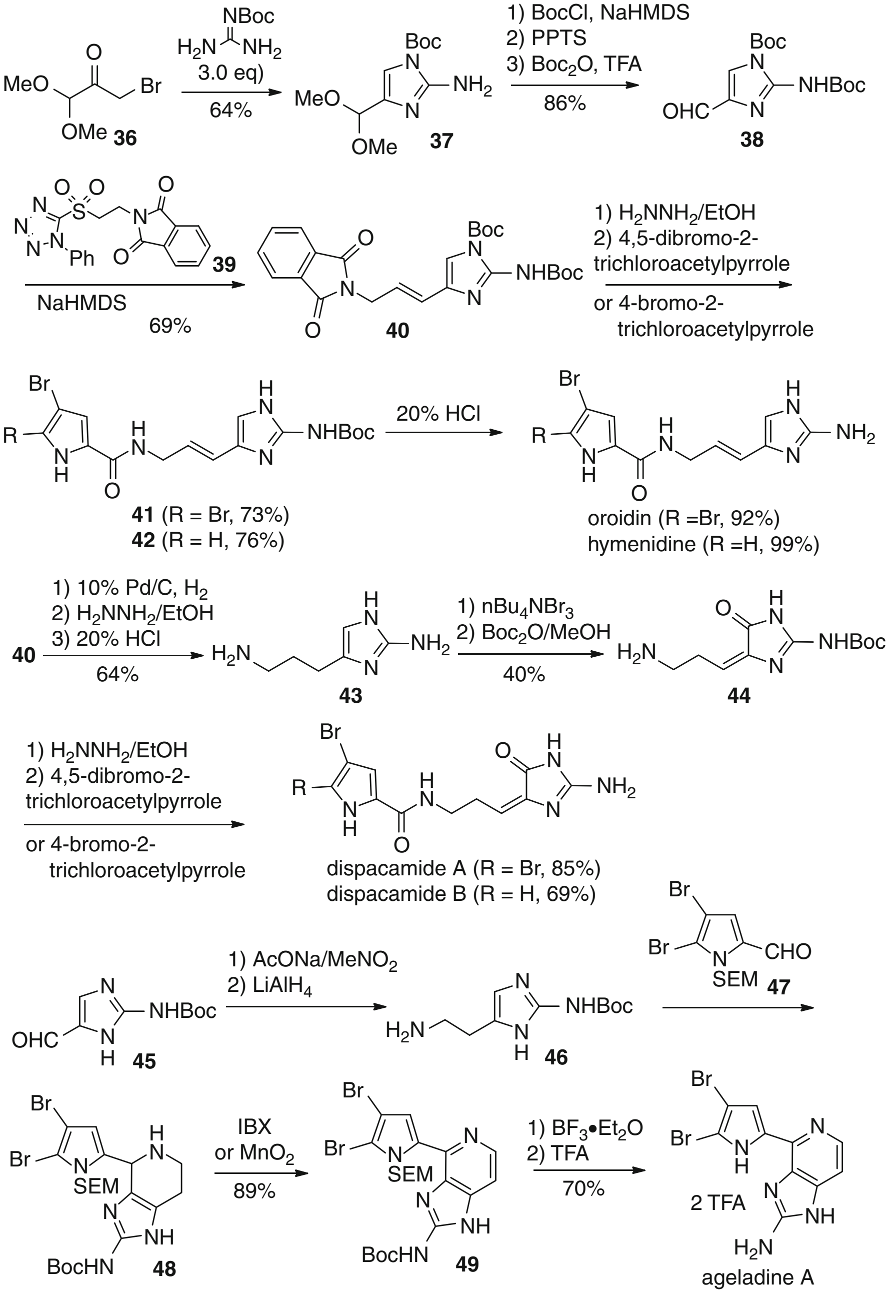
3.3.1 Synthetic Studies Focusing on the Monomers

Ando et al. developed a route for the total syntheses of the simple monomeric pyrrole-imidazole alkaloids, oroidin, hymenidin, dispacamide, monobromodispacamide, and ageladine A (Scheme 9.12) [90]. In the pathway, 3-bromo-1,1-dimethoxypropan-2-one (**36**) was converted to aldehyde **38**, which was then coupled with sulfone **39** by using the Julia/Kociensky olefination reaction to produce (*E*)-olefin **40**. Deprotection of **40** with hydrazine followed by introduction of pyrrole unit afforded **41** and **42**, which were acid-hydrolyzed to give oroidin and hymenidin, respectively. Catalytic reduction of **40** followed by removal of the phthaloyl group and the two imidazole Boc groups gave amine **43**, which was oxidized with tetrabutylammonium tribromide (nBu_4NBr_3) followed by Boc-protection of primary amine to afford the carbamate **44**. Sequential deprotection and acylation of the pyrrole units gave dispacamide and monobromodispacamide, respectively. Furthermore, **45**, generated from **37** by way of **40** prior to Boc-protection, was used for the synthesis of ageladine A. Thus, nitroaldol condensation of **45** followed by lithium aluminum hydride reduction gave the histamine derivative **46**, which was coupled with **47** through Pictet/Spengler reaction to give **48**. Dehydrogenation of **48** using IBX or activated MnO_2 gave the same results. Simultaneous removal of both the SEM and the Boc groups gave the TFA salt of ageladine A.

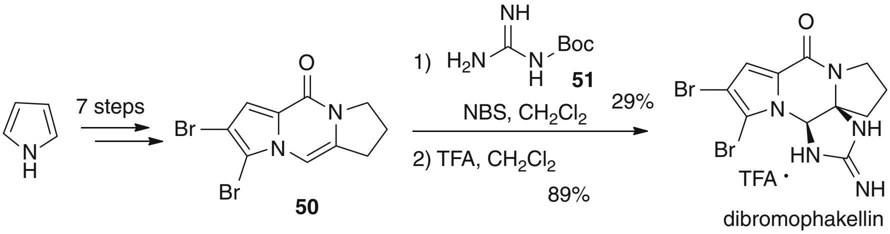
Although the syntheses of oroidin and hymenidin described above are the latest published, it would be remiss not to cite the preparation of these targets by Al-Mourabit's group, which is very short [91].

(±)-Dibromophakellin has been prepared using a two step sequence from the known alkene intermediate **50**, derived from pyrrole in seven steps (Scheme 9.13). Treatment of alkene **50** with NBS in the presence of Boc-protected guanidine **51**, followed by removal of the Boc group using trifluoroacetic acid afforded dibromophakellin as its TFA salt [92].

Nagasawa's group accomplished asymmetric total syntheses of (+)-phakellin, (+)-dibromophakellin, and (+)-dibromophakellstatin by utilizing a strategy that


MeO
OMe
O
Br
36
NBoc
H2N
NH2
3.0 eq)
64%
Boc
N
NH2
MeO
OMe
37
1) BocCl, NaHMDS
2) PPTS
3) Boc2O, TFA
86%
NHBoc
OHC
38
39
N
Ph
O
NaHMDS
69%
40
1) H2NNH2/EtOH
2) 4,5-dibromo-2-trichloroacetylpyrrole
or 4-bromo-2-trichloroacetylpyrrole
Br
R
H
41 (R = Br, 73%)
42 (R = H, 76%)
20% HCl
oroidin (R =Br, 92%)
hymenidine (R =H, 99%)
40
1) 10% Pd/C, H2
2) H2NNH2/EtOH
3) 20% HCl
64%
H2N
43
1) nBu4NBr3
2) Boc2O/MeOH
40%
44
1) H2NNH2/EtOH
2) 4,5-dibromo-2-trichloroacetylpyrrole
or 4-bromo-2-trichloroacetylpyrrole
dispacamide A (R = Br, 85%)
dispacamide B (R = H, 69%)
45
1) AcONa/MeNO2
2) LiAlH4
46
CHO
SEM
47
48
BocHN
IBX
or MnO2
89%
49
1) BF3•Et2O
2) TFA
70%
2 TFA
NH
ageladine A

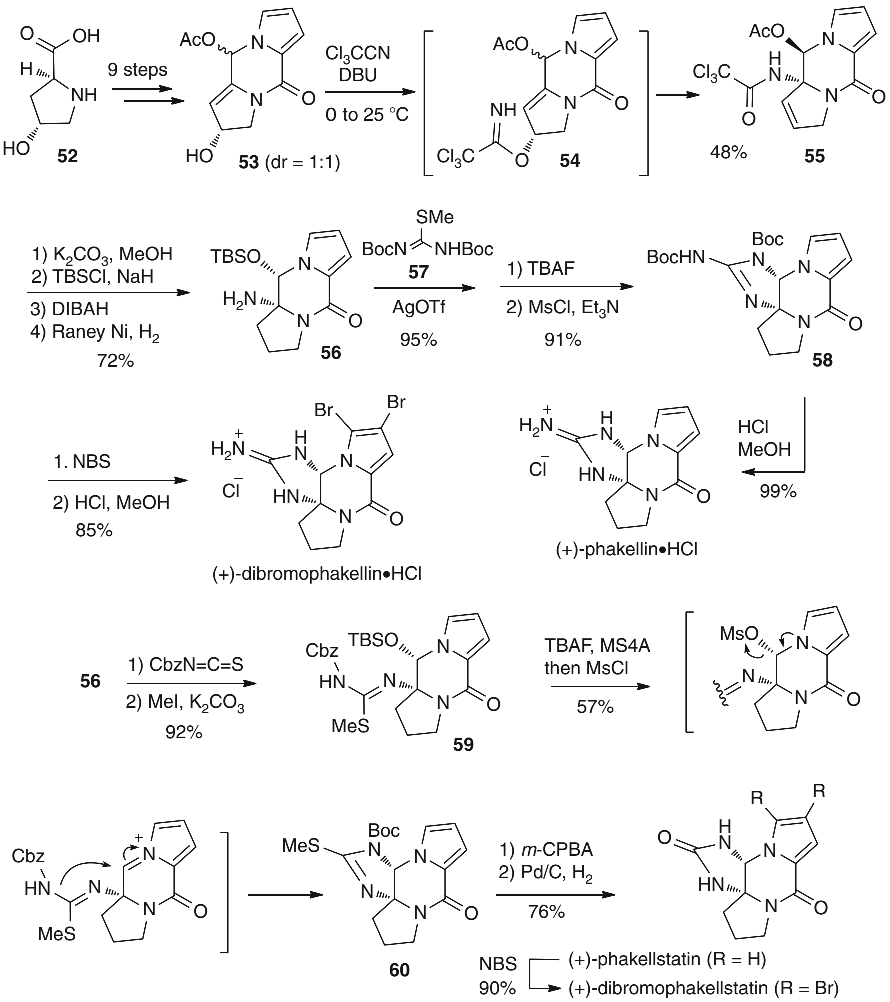
Scheme 9.12 Total syntheses of oroidin, hymenidin, dispacamide, monobromodispacamide, and ageladine A



Scheme 9.13 Synthesis of (±)-dibromophakellin

models the proposed biosynthetic pathway (Scheme 9.14). Accordingly, 4-hydroxy-L-proline (**52**) was converted to **53**, which then undergoes Overman rearrangement via **54** to give the aminal **55** as a single diastereomer. This aminal was then converted to **56** in four steps involving alkaline hydrolysis, TBS-protection, DIBAH reduction, and hydrogenation. Guanidination of **56** using **57**, followed by removal of TBS-ether group and treatment with MsCl in the presence of Et_3N, gave cyclic guanidine derivative **58**. Acid hydrolysis of **58** in methanolic HCl then gave (+)-phakellin, which upon NBS treatment followed by acid hydrolysis afforded (+)-dibromophakellin. Furthermore, **56** was converted to (+)-phakellstatin and (+)-dibromophakellstatin by suing a route that was initiated by treatment of amine **56** with benzyloxycarbonyl isothiocyanate followed by methylation to generate thiopseudourea **59**. This substance was converted to the cyclic thiopseudourea **60** by reaction with TBAF followed by addition of MsCl. m-CPBA oxidation of **60** and removal of the benzyloxycarbonyl group by hydrogenolysis furnished (+)-phakellstatin in 89 % yield. Bromine introduction at the C4 and C5 positions of (+)-phakellstatin was accomplished using two equivalents of NBS generated (+)-dibromophakellstatin in 90 % yield [93].

Although many efforts have succeeded in synthesizing the agelastatins, especially agelastatin A, Movassaghi's group recently described total syntheses of all known (−)-agelastatins, including the first total syntheses of agelastatins C, D, E, and F (Scheme 9.15) [94]. In the sequence, pyrrole (+)-**61** was brominated with NBS and the product was reacted with chlorosulfonyl isocyanate followed by desulfonylation to afford amide (+)-**62** in 75 % yield. Reaction of **62** with $NaBH_4$ followed by TsOH treatment and reaction with 4-methoxybenzenethiol and $AlMe_3$ led to the production of thioester **63** in 83 % yield. Reaction of thioester (+)-**63** and triazone **64** in the presence of copper(I)-thiophene-2-carboxylate (CuTC) followed by acid hydrolysis gave (+)-*O*-methyl-pre-agelastatin A in 85 % yield with 99 % ee. Acid hydrolysis of (+)-*O*-methyl-pre-agelastatin A afforded agelastatin A in 49 % along with *O*-methyl-di-*epi*-agelastatin A in 22 % yield. (−)-*O*-methyl-di-*epi*-agelastatin A was converted to agelastatin A in 66 % yield by treatment with acid. Bromination of (−)-agelastatin A afforded (−)-agelastatin B in 84 % yield. (−)-*O*-methyl-di-*epi*-agelastatin A was transformed to (−)-agelastatin C in three steps, involving heating in pyridine at 115 °C, oxidation with dimethyldioxorane, and heating in an aqueous solution containing a Brøonsted acid. Treatment of



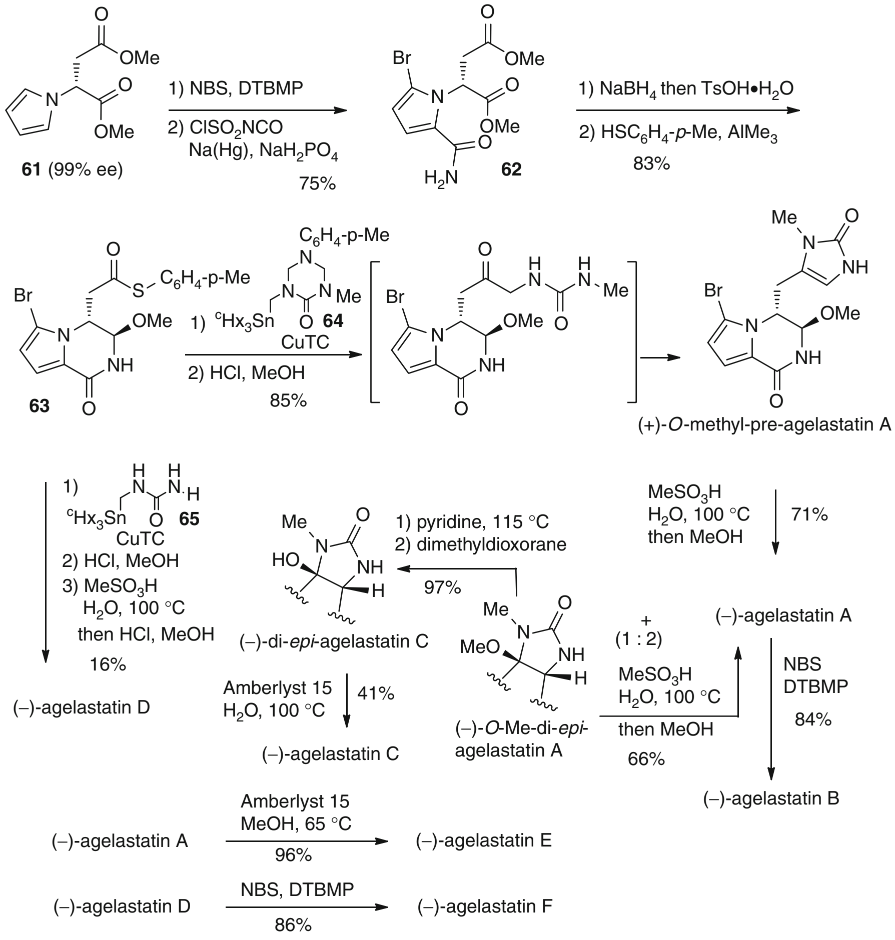
52
9 steps
53 (dr = 1:1)
Cl_3CCN
DBU
0 to 25 °C
54
48%
55
1) K_2CO_3, MeOH
2) TBSCl, NaH
3) DIBAH
4) Raney Ni, H_2
72%
56
57
AgOTf
95%
1) TBAF
2) MsCl, Et_3N
91%
58
HCl
MeOH
99%
(+)-phakellin•HCl
1. NBS
2) HCl, MeOH
85%
(+)-dibromophakellin•HCl
56
1) CbzN=C=S
2) MeI, K_2CO_3
92%
59
TBAF, MS4A
then MsCl
57%
60
1) m-CPBA
2) Pd/C, H_2
76%
NBS
90%
(+)-phakellstatin (R = H)
(+)-dibromophakellstatin (R = Br)

Scheme 9.14 Asymmetric synthesis of (+)-phakellin, (+)-dibromophakellin, and (+)-dibromophakellstatin

thioester (+)-**63** with stannylurea **65** and CuTC, followed by exposure of the product to methanolic hydrogen chloride and acid treatment, gave the desired (−)-agelastatin D in 16 % yield. Heating a methanolic solution of (−)-agelastatin A in the presence of a Bronsted acid afforded (−)-agelastatin E in 96 % yield, and bromination of (−)-agelastatin D gave (−)-agelastatin F in 86 % yield.

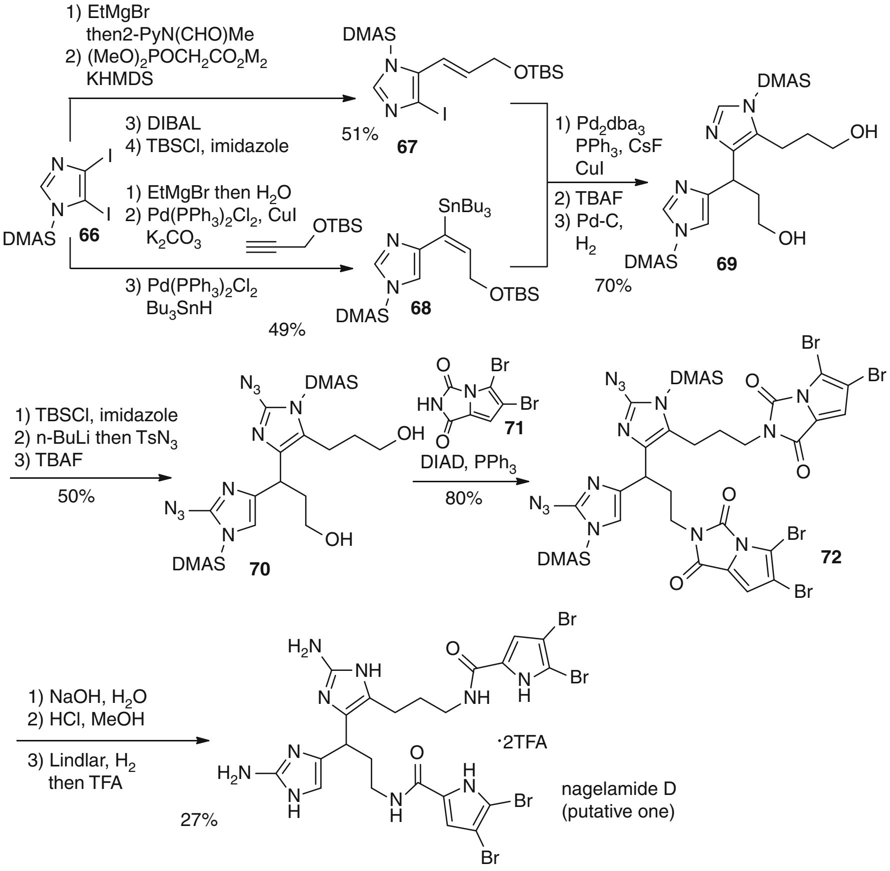
3.3.2 Synthetic Studies Targeted at the Dimers

The assigned structure of nagelamide D (Fig. 9.7), isolated from a marine sponge by Kobayashi et al., does not agree with data obtained from analysis of the synthesized natural product prepared by Lovely et al. (Scheme 9.16) [95]. The putative


61 (99% ee)
1) NBS, DTBMP
2) ClSO$_2$NCO
Na(Hg), NaH$_2$PO$_4$
75%
62
1) NaBH$_4$ then TsOH•H$_2$O
2) HSC$_6$H$_4$-p-Me, AlMe$_3$
83%
63
1) ^{c}Hx$_3$Sn 64
CuTC
2) HCl, MeOH
85%
(+)-O-methyl-pre-agelastatin A
1) ^{c}Hx$_3$Sn 65
CuTC
2) HCl, MeOH
3) MeSO$_3$H
H$_2$O, 100 °C
then HCl, MeOH
16%
(–)-agelastatin D
1) pyridine, 115 °C
2) dimethyldioxorane
97%
(–)-di-epi-agelastatin C
Amberlyst 15
H$_2$O, 100 °C
41%
(–)-agelastatin C
(–)-O-Me-di-epi-agelastatin A
MeSO$_3$H
H$_2$O, 100 °C
then MeOH
71%
(–)-agelastatin A
+
(1 : 2)
MeSO$_3$H
H$_2$O, 100 °C
then MeOH
66%
NBS
DTBMP
84%
(–)-agelastatin B
(–)-agelastatin A
Amberlyst 15
MeOH, 65 °C
96%
(–)-agelastatin E
(–)-agelastatin D
NBS, DTBMP
86%
(–)-agelastatin F

Scheme 9.15 Asymmetric syntheses of agelastatins

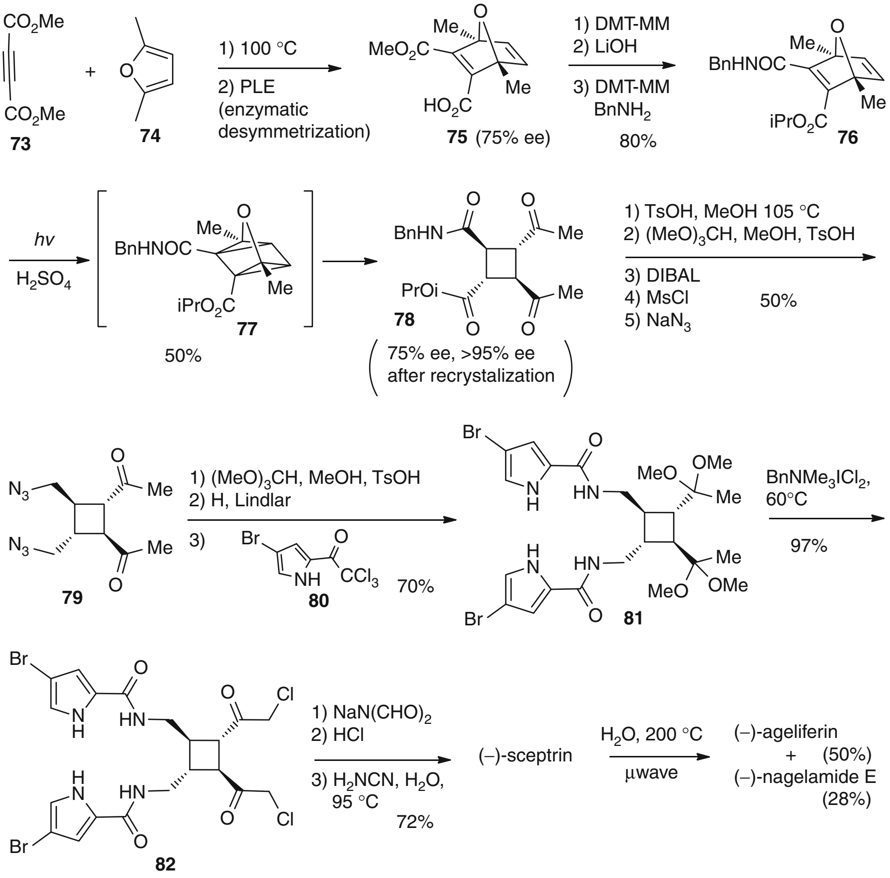
nagelamide D structure is a dimer comprised of oroidin monomeric units connected between C-10 and C-15′. The route employed by Lovely et al. to synthesize nagelamide D utilized coupling of two imidazoyl units. One fragment, **67**, was synthesized from diiodoimidazole **66** utilizing a four step-sequences, involving formylation, Horner-Wadsworth-Emmons reaction, DIBAL reduction, and protection as TBS-ether. The other fragment, **68**, was also produced from the same diiodoimidazole **66** through a three steps pathway, involving regioselective reduction of iodide, Sonogashira reaction using TBS-protected propargyl alcohol, and hydrostannylation. A Stille cross-coupling method was used to join the two fragments. The product generated in this manner was subjected to removal of the TBS-ethers, giving a substance that was hydrogenated to afford diol **69**. Protection of the diols as bis silyl ethers, followed by azidation at the C2 positions of imidazole rings


1) EtMgBr
then2-PyN(CHO)Me
2) (MeO)$_2$POCH$_2$CO$_2$M$_2$
KHMDS
3) DIBAL
4) TBSCl, imidazole
51%
DMAS
OTBS
67
66
DMAS
1) EtMgBr then H$_2$O
2) Pd(PPh$_3$)$_2$Cl$_2$, CuI
K$_2$CO$_3$
OTBS
3) Pd(PPh$_3$)$_2$Cl$_2$
Bu$_3$SnH
49%
SnBu$_3$
OTBS
68
1) Pd$_2$dba$_3$
PPh$_3$, CsF
CuI
2) TBAF
3) Pd-C,
H$_2$
70%
69
1) TBSCl, imidazole
2) n-BuLi then TsN$_3$
3) TBAF
50%
70
71
DIAD, PPh$_3$
80%
72
1) NaOH, H$_2$O
2) HCl, MeOH
3) Lindlar, H$_2$
then TFA
27%
·2TFA
nagelamide D
(putative one)

Scheme 9.16 Total synthesis of the putative structure of nagalamide D

and removal of the TBS-ethers, gave diol **70**. Double Mitsunobu reaction of **70** with the dibromopyrrolehydantoin derivative **71** gave **72**, which upon alkaline hydrolysis followed by treatment with methanolic HCl and then reduction of the azide groups generated the putative nagelamide D.

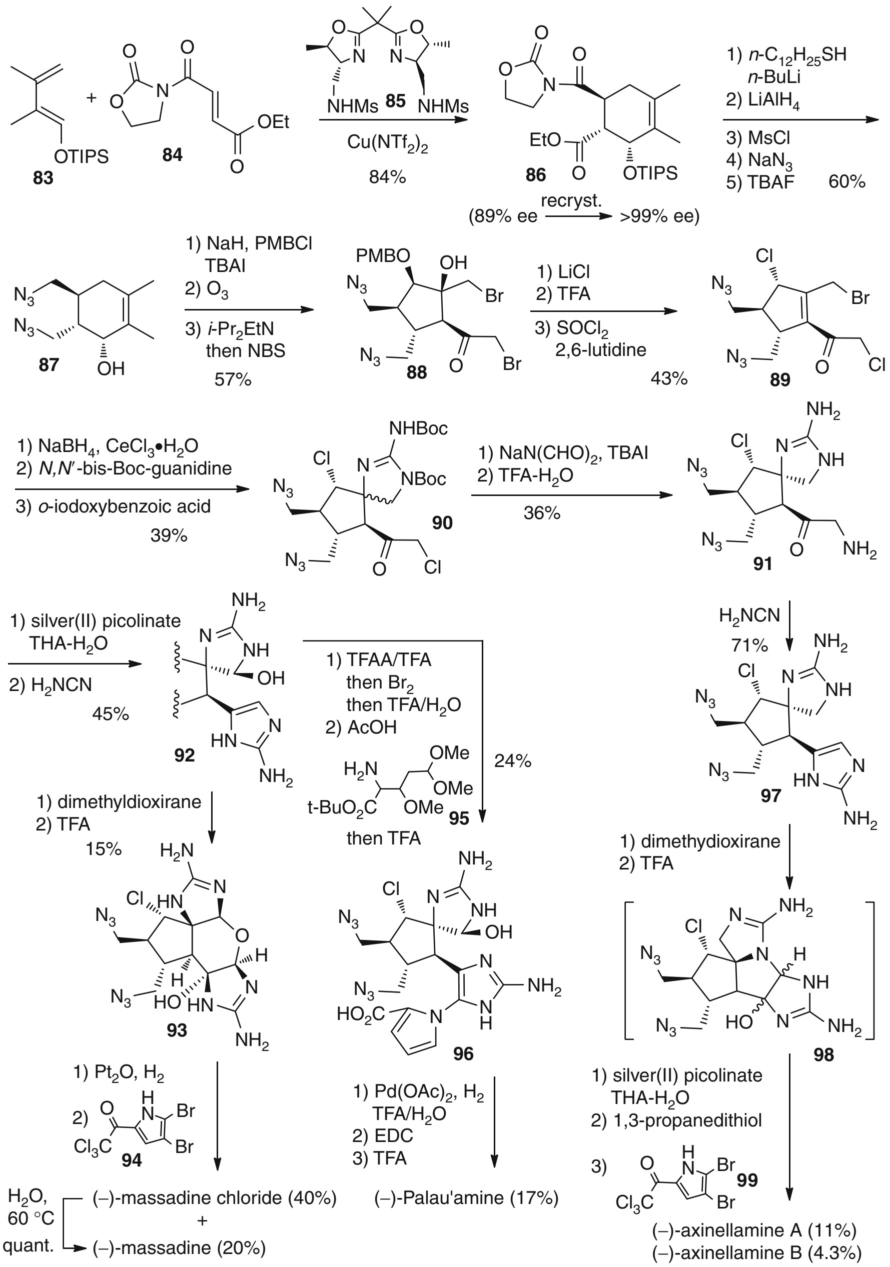
The first total syntheses of the dimeric pyrrole-imidazole alkaloids, including sceptrin, ageliferin, nagelamide E, nakamuric acid (and its methyl ester), and oxysceptrin, were reported by the group headed by Baran (Scheme 9.17). In addition, the first enantioselective total syntheses of sceptrin and ageliferin were accomplished by this group utilizing a strategy that relied on programmed fragmentation of an oxaquadricyclane [96]. A hallmark of the approach to these alkaloids is the minimal use of protecting groups. Diels-Alder reaction of dimethyl acetylenedicarboxylate **73** and 2,5-dimethylfuran **74** followed by enzymatic desymmetrization gave monoester **75** with 75 % ee. Esterification of **75** with 2-propanol, selective hydrolysis, and benzyl amide formation gave **76** in 80 %


CO2Me
CO2Me
73
+
74
1) 100 °C
2) PLE
(enzymatic
desymmetrization)
MeO2C
Me
O
HO2C
Me
75 (75% ee)
1) DMT-MM
2) LiOH
3) DMT-MM
BnNH2
80%
BnHNOC
iPrO2C
76
hν
H2SO4
77
50%
BnHN
PrOi
78
75% ee, >95% ee
after recrystalization
1) TsOH, MeOH 105 °C
2) (MeO)3CH, MeOH, TsOH
3) DIBAL
4) MsCl
5) NaN3
50%
N3
79
1) (MeO)3CH, MeOH, TsOH
2) H, Lindlar
3)
Br
NH
CCl3
80
70%
HN
MeO
OMe
81
BnNMe3ICl2,
60°C
97%
Cl
82
1) NaN(CHO)2
2) HCl
3) H2NCN, H2O,
95 °C
72%
(–)-sceptrin
H2O, 200 °C
μwave
(–)-ageliferin
+ (50%)
(–)-nagelamide E
(28%)

Scheme 9.17 Asymmetric syntheses of (−)-sceptrin, (−)-ageliferin, and (−)-nagelamide E

yield. UV irradiation of **76** gave rise to oxaquadricyclane **77**, which was immediately subjected to fragmentation to give cyclobutane **78** in 50 % yield and 75 % ee, which was recrystallized to produce a >95 % ee. Heating **78** with toluenesulfonic acid and methanol, followed by protection of the ketone moieties, DIBAL reduction, mesylation, and displacement with sodium azide, produced the key azide **79**. Ketalization of **79**, followed by reduction with Lindlar catalyst and acylation with pyrrole unit **80**, gave **81** in 70 % yield. Heating of **81** with benzyl trimethylammonium iododichloride gave chloroketone **82**, which underwent reaction with sodium diformimide followed by hydrolysis and the reaction with cyanamide to produce (−)-sceptrin. Microwave irradiation of (−)-sceptrin in water afforded (−)-ageliferin and (−)-nagelamide E.

Although (−)-palau'amine is an architecturally daunting natural product, its synthesis was finally accomplished by the Baran group in 2010. In the publication describing this work, Baran et al. also described the asymmetric syntheses of other


OTIPS
83
84
OEt
NHMs 85 NHMs
Cu(NTf2)2
84%
EtO
86
OTIPS
recryst.
(89% ee → >99% ee)
1) n-C12H25SH n-BuLi
2) LiAlH4
3) MsCl
4) NaN3
5) TBAF 60%
87
1) NaH, PMBCl TBAI
2) O3
3) i-Pr2EtN then NBS
57%
PMBO
88
1) LiCl
2) TFA
3) SOCl2 2,6-lutidine
43%
89
1) NaBH4, CeCl3•H2O
2) N,N′-bis-Boc-guanidine
3) o-iodoxybenzoic acid
39%
NHBoc
NBoc
90
1) NaN(CHO)2, TBAI
2) TFA-H2O
36%
91
H2NCN
71%
97
1) silver(II) picolinate THA-H2O
2) H2NCN
45%
92
1) TFAA/TFA then Br2 then TFA/H2O
2) AcOH
t-BuO2C
95
then TFA
24%
1) dimethyldioxirane
2) TFA
15%
93
96
1) dimethydioxirane
2) TFA
98
1) Pt2O, H2
2)
94
1) Pd(OAc)2, H2 TFA/H2O
2) EDC
3) TFA
1) silver(II) picolinate THA-H2O
2) 1,3-propanedithiol
3)
99
H2O, 60 °C quant.
(–)-massadine chloride (40%)
+
(–)-massadine (20%)
(–)-Palau'amine (17%)
(–)-axinellamine A (11%)
(–)-axinellamine B (4.3%)

Scheme 9.18 Asymmetric total syntheses of dimeric pyrrole-imidazole alkaloids, (−)-palau’amine, (−)-axinellamines, and (−)-massadines dimeric pyrrole-imidazole alkaloids, including (−)-axinellamines, and (−)-massadines, that relied on a bio-inspired approach (Scheme 9.18) [97]. In the route, highly enantioselective [4 + 2]-cycloaddition (95 % ee) of TIPS diene **83** and dienophile **84** was achieved by employing the Ishihara catalyst **85** to give the cyclohexene **86** in 89 % ee. This substance was recrystallized to give optically pure **86** prior to its conversion to a thioester, which was subjected to $LiAlH_4$ reduction, mesylation, displacement with azide, and TIPS deprotection to produce cyclohexenol **87** in 60 % yield. Protection of the alcohol moiety in **87**, followed by ozonolytic cleavage of the olefin, bromination, and cyclization, produced the fully substituted cyclopentane **88** in 57 %. Conversion of bromoketone **88** to chloroketone was followed by deprotection of PMB group, and exposure to sulfuryl chloride to afford the enone **89** in 43 % yield. Luche reduction of **89** followed by displacement of the allylic bromide with bis-Boc-guanidine and oxidation with IBX delivered the allylic guanidine **90** in 39 % yield. Treatment of **90** with $NaN(CHO)_2$ followed by removal of the Boc group furnished aminoketone **91** in 36 % yield. Reaction of **91** with silver(II) picolinate under acidic conditions, followed by treatment with H_2NCN, afforded **92** in 45 % yield. Oxidation of **92** with dimethyldioxirane and acid treatment gave **93** in 15 % yield, which was converted to (−)-massadine chloride and (−)-massadine in a 2:1 ratio by reduction of the azides and acylation with (trichloroacetyl)pyrrole **94**. Mild heating of (−)-massadine chloride in aqueous media afforded (−)-massadine quantitatively. Intermediate **92** was also converted to (−)-palau'amine, by a route involving initial bromination, attachment of the pyrrole surrogate **95**, and then pyrrole generation to afford **96** in 24 % yield. Hydrogenation of the azide moieties in **96**, condensation by using EDC, and then TFA exposure gave (−)-palau'amine in 17 % yield. Aminoketone **91** reacted with H_2NCN to afford **97** in 71 % yield, which upon oxidation with dimethyldioxirane followed by acid treatment gave the tetracyclic intermediate **98**. Treatment of **98** with silver(II) picolinate under acidic conditions followed by the reduction with propanedithiol and acylation with (trichloroacetyl)pyrrole (**99**) provided enantio-enriched axinellamine A and axinellamine B in 11 % and 4.3 % yields from **97**, respectively.

4 Conclusion

Marine organisms produce many types of biologically active compounds. Among them, the sponge-derived pyrroloiminoquinone alkaloids, makaluvamines and discorhabdins, and marine pyrrole-imidazole alkaloids are discussed in this chapter. Since these alkaloids have a wide variety of biological activities and unique structural architectures, they have been identified as lead compounds for developing new drugs. We hope that the studies focusing on structure-activity relationships and modes of action of these substances and their analogues will lead to the development of active drug candidates.

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Internet Natural Product Trade, Business, and Risk of Damaged and/or Contaminated Products

10

Maria Laura Colombo, Maria Teresa Pinorini, and Ario Conti

Contents

1 Introduction 286
2 Designer Drugs and Legal Highs 288
3 Athletes and Natural Products as Enhancing Substances 289
4 Natural Substances for Date Rape 291
5 Plant Toxins: Gender-Selective 293
6 Natural Toxins 294
7 Plants as Crime-Solvers: Forensic Botany 295
8 Natural Toxins and Bioterrorism 296
9 Conclusions 298
References 298

Abstract

The use of food or dietary supplements has increased enormously in the last years; among the different products present on the market, consumers show a special interest in those containing herbal ingredients. Some herbal products are emerging as popular drugs for recreational abuse. Plant and herbal

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supplements used recreationally can have a wide spectrum of clinical effects, ranging from euphoric and stimulant effects to hallucinogenic experiences. Despite the potential for abuse, addiction, and serious adverse effects, there may be a false perception that these products are all safe, legal, and organic. These perceptions and the ease of accessibility to herbal products could result in greater potential for recreational abuse and subsequent complications presenting to emergency departments. Health care professionals must be cognizant of this emerging problem as increased media coverage and marketing have made these products accessible and recognizable to many young adults and teenagers. Consumers may assume that because a label claims that something is good or right because it is natural, or that something is bad or wrong because it is "synthetic" or "industrial."

The use of food supplements containing herbal ingredients by athletes has become common, even though the scientific rationale is often insufficient. It is obvious that the uncontrolled use of food supplements can represent a risk for health, and this is particularly true when the herbal ingredients have known pharmacological effects. Moreover, the abuse of stimulant substances could represent an illicit way to improve performance during competitions, considering that herbal ingredients cannot always be detected by anti-doping tests. Other illicit uses of herbal ingredients are their inclusion among recreational drugs and, in some cases, among tools in criminal actions.

Keywords

Botanical food supplements • botanical dietary supplements • illicit drugs • plant toxins

1 Introduction

Foods play a critical role in the health of human beings and are often an important part of social, religious, and cultural activity. Several expressions illustrate the importance of foods in daily life, the most famous being "We are what we eat." Most traditions have a particular, easily recognizable cuisine, which is sometimes the focus of epidemiological studies (Mediterranean diet, vegetarian diet, etc.). More recently, foods have been identified as a means of decreasing crime and violence, since healthy food can reduce aggressive behavior. "We are what we eat" has become "we do what we eat." Thus, subjects on a vegetarian diet are often considered less aggressive than omnivorous ones. On the other hand, foods can pose a risk to human health if they contain unusual or unsuspected ingredients. Numerous cases of accidental food poisoning have been described in the scientific and popular literature, in particular the ingestion of poisonous mushrooms or plants harvested by inexpert collectors. Since ancient times, food and water have been used as tools for homicide, both because victims usually have confidence in their meals and because detecting the fatal agent can be difficult. Traditional poisons such as salts of arsenic have often been used in murder, but more recent investigations have hypothesized poisonous plants as the causative agent or identified them analytically. The most recent criminal approach involving foods is to use them to mask illicit substances, facilitating their transport across borders and wide distribution. Food supplements play an important role here, in particular those containing herbal ingredients. The use of food supplements has increased enormously in recent years since consumers believe that natural products are good or right for the body, whereas something of synthetic or industrial origin is bad or wrong. The use of food supplements containing herbal ingredients has become common among athletes even when the scientific rationale is non-existent. The uncontrolled use of food supplements can represent a health risk, particularly when the herbal ingredients have known pharmacological properties. Herbal ingredients are often undetectable by the usual anti-doping tests, which make those with stimulant properties particularly attractive to those seeking performance enhancement without penalty. The main hazards in the use of herbal supplements include effects due to the specific pharmacological properties (agitation, insomnia, tachycardia); the risk of exceeding the appropriate dose to obtain a pronounced enhancing effect; the risk of allergy and intolerances – several products specifically developed for sport activities actually contain proteins from animal and vegetable sources that are listed as major allergens; and finally, the possible presence of banned ingredients (hormones, etc.) that have been added to enhance the desired effects. Food supplements containing plants with known stimulant substances must be regarded as potential health hazards. The use of food supplements has increased enormously in recent years; among the different products present on the market, consumers show a special interest in those containing herbal ingredients.

In the twenty-first century, an ever-growing number of men and women are flocking to the Internet and World Wide Web to make purchases of products and to engage services from different providers. One type of product line that now is offered in abundance on the Internet is that of vitamins and dietary, food, and nutritional supplements. At the present time there are literally thousands of different website venues that cater to people who are interested in purchasing and utilizing vitamins and other nutritional supplements.

Unfortunately, there is also a segment of the Internet market consisting of scams and “flim-flam” artists. There are people who have created less than reputable sites in the vitamin and nutritional supplement business. Naturally, if one is new at seeking these types of products online, it is better to avoid these types of disreputable sites. Consumers may be misled by vendors’ claims that herbal products can treat, prevent, diagnose, or cure specific diseases, despite regulations prohibiting such statements. Physicians should be aware of this widespread and easily accessible information. More effective regulation is required to put this class of therapeutics on the same evidence-based footing as other medicinal products [1].

There are some tips and pointers that should be kept in mind when seeking reputable sites on the Internet that are involved in the vitamin and nutritional supplement trade. First, only visit websites that have been established in business for a reasonable amount of time. Of course, there are websites that deal in these products that crop up all of the time and many of these sites are very good.

However, until you get your feet wet and understand what to look for in regard to legitimate websites that market vitamins and nutritional supplements, you will be better served by relying on those sites that are well established.

Second, as you go about looking for a reliable website from which to make the purchase of vitamins and nutritional supplements, get references from other people. In many ways, referrals and recommendations are the best way to make reasonable and educated decisions about which websites to select when you are in the market for vitamins and nutritional supplements for your use and for use by your family. By educating yourself and by taking the time to research different websites, you will be able to find a reliable and reputable venue through which you can make the purchase of vitamins and nutritional supplements [2].

2 Designer Drugs and Legal Highs

The Internet has put the world at people's fingertips, but in some cases it has also allowed banned or controlled substances to slip within their grasp, British researchers warn. Their study, published online in *Drug Testing and Analysis*, looked at so-called "legal highs," where certain drugs sold online contain ingredients other than those claimed and can be hazardous. These products are quickly becoming big business for manufacturers and suppliers, who market them toward recreational drug users [3].

> "It is clear that consumers are buying products that they think contain specific substances, but that in reality the labels are unreliable indicators of the actual contents," Dr. Mark Baron, of the School of Natural and Applied Sciences at the University of Lincoln in England, said in a news release from the journal's publisher.

Although these "legal highs" are often readily available for purchase on the Internet, they are potentially dangerous for consumers, the study authors pointed out. The investigators found that these drugs, sometimes marketed as bath salts or plant food, commonly contain controlled substances and are being sold illegally. In investigating these drugs, Baron bought an array of pills from UK websites and analyzed their contents. Six out of seven products purchased did not contain the active ingredients advertised. In fact, in five out of seven cases, the products actually contained a controlled substance combined with caffeine. Among them was benzylpiperazine, a stimulant drug that is illegal in the United States and can cause anxiety, increased blood pressure, rapid heart rate, and blurred vision, among other side effects.

Although the United Kingdom and other governments have taken steps to crack down on these "legal highs," suppliers continue to market these drugs, sidestepping current regulations. As a result, Baron cautioned buyers to be wary of any products they buy online.

No guidelines exist as to what is sold and in what purity, and consumers are led to believe that purchased goods are entirely legal. The product name cannot be used as an indication of what it contains as there is variation in the content of the same product name between different Internet sites. The findings should serve as a red flag for consumers, as well as the lawmakers attempting to curb the illegal sale of controlled substances, the study authors noted. “As legislation deals with the current crop of products we can expect to see new products appearing that try to find a route of supplying previously banned substances,” Baron warned.

Many new drugs are produced in apartmentsand basements in large cities. Designer drugs are considered dangerous. They are difficult to detect and virtually unknown at the chemical level. This is a new phenomenon that frightens the authorities of all countries, which are seeing an increase in new pharmaceutical products based on substances that already exist but used for non-therapeutic purposes. Recent years have seen the appearance of a large number of synthetic substances on the Internet, including so-called legal highs. What are “legal highs”? This is huge number of new substances that circumvent legal controls and almost always are purchased on the internet. They are synthetic drugs used for fun, the so-called “party pills.” Sometimes they are called natural drugs to entice consumers who presume they cannot hurt.

For example, there is a whole series of drugs called “spice.” Spice represents herbs adulterated with many synthetic substances more potent than THC and very dangerous. These products may be sniffed, smoked, or inhaled [4].

3 Athletes and Natural Products as Enhancing Substances

Athletes use ergogenic products to face physical and emotional stress in a sport competition, but products need to be conscientiously evaluated in terms of the risk/benefit ratio. All athletes, whether beginners or professionals, must fully understand that the intake of some substances can have severe adverse effects, particularly products obtained in “alternative” markets that are only interested in profit. Continuous monitoring of the products present in these markets is the only effective tool to prevent the distribution of food supplements containing banned ingredients, and the availability of suitable analytical methods is critical if this goal is to be attained.

In fact, the use of food supplements containing herbal ingredients by athletes has become common even though the scientific rationale is often insufficient to support it. It is obvious that the uncontrolled use of food supplements can represent a risk for health, and this is particularly true when the herbal ingredients have known pharmacological effects. Moreover, the abuse of stimulant substances could represent an illicit way to improve performance during competitions, considering that herbal ingredients cannot always be detected in occasion of anti-doping tests. The main reasons to suggest caution in the use of herbal supplements include: (1) the side effects due to the specific pharmacological properties of a specific ingredient (agitation, insomnia, etc.); (2) the risk of exceeding the dose to obtain a certain effect (searching for improvement of performance); (3) allergy and intolerances; (4) the possible presence of illicit ingredients to enhance the effects (iodine, hormones, etc.).

Food supplements, containing plants or herbs with stimulant substances, must be regarded as potential health hazards, as stated in 2003–2004 by the FDA, and applied in Europe, Japan, Australia, and other countries to ban the sales of supplements containing ephedrine alkaloids [5].

The best-known case regarding dietary supplements is that of those containing *Ephedra* derivatives. Substantial evidence of harm emerged in 2003, when a major study reported more than 16,000 adverse events associated with the use of *Ephedra*-containing dietary supplements, including heart palpitations, tremors, and insomnia. The study also found little evidence of the claimed efficacy of *Ephedra* in boosting physical activities and weight loss. In 2004, the FDA banned sales of dietary supplements containing ephedrine alkaloids (*Ephedra* species) because such supplements presented an unreasonable risk of illness or injury for the consumers, and in the US the sale of products containing *Ephedra* alkaloids is now considered criminal unless they have been medically prescribed. Ephedrine is banned as an ingredient of food or food supplements in the European Union. This international ban, based as it is on data illustrating adverse effects of *Ephedra* alkaloids, stimulated a series of activities to monitor food supplements present on the market and developed for sport. There are thousands of products on the market containing herbal ingredients. The quality control of these products is often unsatisfactory and their market is wide and complex, with modern ways of distribution including athletes' training centers and direct delivery from purchase on the Internet.

Some of today's problems are without a solution. For example, in February of 2010, the Fédération Internationale de Football Association (FIFA) expressed concerns that some African herbs might enhance World Cup players' performance, thus providing them with an unfair advantage over other competitors. FIFA officials and team doctors have suggested that the use of some botanicals might go undetected by tests currently used in laboratories accredited by the World Anti-Doping Agency (WADA). They have also mentioned that some plants might produce byproducts that are not currently included on WADA's list of banned substances. FIFA has expressed its concerns to WADA, and WADA has been encouraging transnational programs that focus on traditional medicine usage. The chemistry and pharmacology of many of the functional foods and medicinal plants of the world are not at all well researched, and the net effect of complex polymolecular substances on sports performance is even less understood [6].

An actual problem without a solution (at this moment) are some food or dietary supplements that contain dimethylamylamine often list as rose geranium oil, geranium oil, or geranium stems on the label, putatively extracted from *Pelargonium graveolens* and from other *Pelargonium* species. Some supplement manufacturers claim that this is because rose geranium oil contains a small amount of dimethylamylamine. Dimethylamylamine is a drug originally synthesized by Eli Lilly in 1950s and used as a stimulant nasal decongestant. There is concern that it might be associated with adverse cardiovascular events, similar to other stimulants such as synephrine in bitter orange *Citrus aurantium* var. *amara* and ephedrine alkaloids from *Ephedra* species. Although some supplement manufacturers claim that dimethylamylamine is a natural compound found in geranium oil, this has not been verified by laboratory analysis. Some laboratories have not been able to detect dimethylamylamine in geranium oil. There is concern that some supplement manufacturers may be artificially spiking their supplements with this synthetic drug. The New Zealand government restricted sales of supplements containing dimethylamylamine to prevent the sale to people under 18 years of age due to concerns about safety [7]. Dimethylamylamine was added to the World Anti-Doping Agency's prohibited substances list for 2010, under the name methylhexaneamine [8].

4 Natural Substances for Date Rape

Belladonna, *Atropa belladonna* L., also known as deadly nightshade (Solanaceae family), is one of the most lethal, toxic plants known to man. The berries of the plant (which bear a slight resemblance to blueberries) have a low alkaloid concentration, generally requiring a minimum of three berries to poison a small child. However, the concentration of poison alkaloids (atropine, scopolamine, josciamine) in the leaves is much higher, with a single leaf capable of killing an adult human in a fairly short amount of time. The leaves feel like poison ivy to the touch, having an oily texture that is taken by some as a sign of being toxic. The roots have the highest concentration of alkaloids, though the berries are often seen as the most dangerous, as they can easily be mistaken for any number of non-poisonous berries by untrained eyes. The toxin works on the nervous system once ingested, targeting areas that control involuntary functions of the body, such as breathing and the heart. In essence, belladonna toxin shuts down the basic systems of the body. Scopolamine, also known as hyoscine, is a tropane alkaloid with muscarinic antagonist effects, obtained from plants of the same Solanaceae family (nightshades), such as *Hyoscyamus niger* (henbane) or *Datura stramonium* (jimson weed) and other Datura species. The drug can be highly toxic and should be used in minute doses. For example, in the treatment of motion sickness, the dose, gradually released from a transdermal patch, is only 0.33 mg of scopolamine per day. An overdose can cause delirium, delusions, paralysis, stupor, and death. Scopolamine, in common with the large percentage of anticholinergics that cross the blood-brain barrier such as diphenhydramine, dicyclomine, trihexyphenidyl, and related drugs, is said to produce euphoria at and around therapeutic doses as well as to potentiate this and other effects of morphine, methadone, hydromorphone, oxycodone, and other opioids. It is therefore occasionally seen as a recreational drug.

The use of medical scopolamine (most often in the form of tablets) for euphoria is uncommon but does exist and can be seen in conjunction with opioid use. The euphoria is the result of changes in dopamine and acetylcholine levels and ratios and appears to be related to some part of the chemical structure of the drug and other factors. Another separate group of users prefer dangerously high doses, especially in the form of Datura or Belladonna preparations, for the deliriant and hallucinogenic effects. The hallucinations produced by scopolamine, in common with other potent anticholinergics, are especially real-seeming and create a perception of a new world filled with frenzied, violent energy. The difference in realism of hallucinations caused by anticholinergics such as scopolamine and other hallucinogens such as the phenethylamines or dissociatives like PCP is quite large. Additionally, an overdose of scopolamine can often be fatal, unlike other more commonly used hallucinogens. For these reasons, naturally occurring anticholinergics are rarely used for recreational purposes. The use of scopolamine as a *truth drug* was investigated in the 1950s by various intelligence agencies, including the CIA.

In Colombia a plant admixture containing scopolamine called *Burundanga* has been used shamanically for decades. In recent years the criminal use of scopolamine has become an epidemic. Approximately 50% of emergency room admissions for poisoning in Bogotá have been attributed to scopolamine. Also in Caracas, Venezuela, crime related to *Burundanga* techniques has multiplied in the last years. Targets are easily approached and just with physical contact they administer the drug to the victim. Reports of techniques of administration include wafting the powder to the victim with a puff of air, drugged chewing gum, or even craftily dropping the powder into the collar of a shirt or the front of a woman's low-cut dress. Victims of this crime are often admitted to a hospital in police custody, under the assumption that the patient is experiencing a psychotic episode. A telltale sign is a fever accompanied by a lack of sweat. Scopolamine is used criminally as a date rape drug and as an aid to robbery, the most common act being the clandestine drugging of a victim's drink. It is preferred because it induces retrograde amnesia, or an inability to recall events prior to its administration or during the time of intoxication.

Scopolamine is being investigated for its possible usefulness alone or in conjunction with other drugs in assisting people in breaking the nicotine habit. The mechanism by which it mitigates withdrawal symptoms appears to be at least partially different from that of clonidine, meaning that the two drugs can be used together without duplicating or canceling out the effects of each other. Scopolamine (hyoscine) causes memory impairments to a similar degree as diazepam.

A date rape drug is a drug that can be used to assist in the execution of a sexual assault, such as date rape. Drugs used to facilitate rape may have sedative, hypnotic, dissociative, and/or amnesiac effects, and can be added to a food or drink without the victim's knowledge. The act of adding such substances to drinks is known as "drink spiking." The reasons for drink spiking range from personal amusement or maliciousness to theft or sexual assault.

The illicit uses of herbal ingredients are their inclusion among recreational drugs and, in some cases, among tools in criminal actions, such as date rape. Scopolamine, as such or as a herbal ingredient (*Datura* species), has been used in both illicit activities. In fact, scopolamine crosses the blood-brain barrier producing anticholinergic effects, such as euphoria and hallucinogenic effects; its use between young people is due to visual, auditory, and tactile hallucinations that may last for 24–48 h. The use of scopolamine and other similar alkaloids can also induce a victim to commit a crime they cannot remember committing.

In this context, food and food supplements can represent an “innocuous” carrier of criminal activities, both for international transport and for the administration in a specific crime. An example of this possibility is the case that occurred in 2005, when a herbal ingredient imported from India (*Coleus forskolii*), contaminated by scopolamine was then distributed on the Italian market. The chromatographic analysis of herbal ingredient, as usually performed in the quality control for forskolin, was not capable of easily detecting the presence of scopolamine, permitting its distribution and inclusion in different kinds of products (galenic preparations, food supplements, sport supplements, etc.). Even though this event in Italy was probably an accident, it is clear that the use of such a of vehicle could permit easier international distribution of illicit substances and a possible new way to criminally administer psychotic drugs [9].

5 Plant Toxins: Gender-Selective

Differences in exposure, anatomy, physiology, biochemistry, and behavior between males and females are a dominant theme in biology, transcending the plant and animal kingdoms. Information regarding gender-based differences in response to phytochemicals in both humans and animals are very important. Gender should be identified not only as a critical factor in human and animal responses to exogenous agents, but gender may be the most important factor regulating mammalian development. Gender-related differences and the dynamics of gender-based growth and development over the entire life cycle are topics of concerm. The potential interactions of dietary supplements and other exogenous agents that can act as drugs or modulate the potential effects of drugs differently in men, women, and developing children of both sexes must be taken into consideration. To this end, the health benefits of genistein or soy and the effects of dietary agents containing these plants mainly effect women.

Clivorine, a pyrrolizidine alkaloid (PA) from *Ligularia hodgsonii* Hook has been reported to cause hepatotoxicity and carcinogenicity in rodents and a positive mutagenic response in the Ames test in the presence of rat liver homogenates, suggesting the importance of hepatic metabolic activation in its intoxication. The toxicity of clivorine was revealed to be caused by cytochrome P450 mediated biotransformation to generate a chemically reactive “pyrrolic ester” (e.g., dehydroclivorine), which can further react with vital cellular macromolecules such as proteins and DNA to form bound pyrroles or DNA cross-linking, leading to toxicities. Female rats are reported to be less susceptible to clivorine intoxication than male rats. However, the biochemical mechanism causing such gender difference is largely unknown. The severity of PA intoxication depends upon an overall balance between the metabolic activation and detoxification pathways, which vary with enzyme systems and species. On the other hand, any factors, such as enzyme induction and/or inhibition caused by diets, dietary supplements, drug-drug and herb-drug interactions, may alter the metabolic balance in the body and thus have significant impacts on PA intoxication to human health.

6 Natural Toxins

Herbs, herbal products, food additives, and other dietary supplements derived from plants are widely consumed in many countries. The literature on intoxications from such behavior is increasing. Many and different are the factors predisposing to intoxication from the use of herbs, with examples drawn largely from pyrrolizidine and diterpenoid alkaloid-containing plants. Poisonings occur because of the misidentification of a plant, or the unknown or ignored toxicity of a correctly identified plant, or the poisoning is voluntary. A preliminary distinction must be drawn between "voluntary" and "involuntary" intoxication. If a person had an unexpected reaction to medicine or unwittingly consumed intoxicants, the accused is said to have been "involuntarily" intoxicated; such an accused could tender intoxication evidence to demonstrate that they lacked the fault for a crime, or that they could not have voluntarily performed the criminal act and so could not be liable. Factors contributing to problems include the difficulties of identifying chopped, processed herbs or plant mixtures, persistent use of a toxic plant, variability in the toxic constituents of a plant, problems of nomenclature, adulteration of a plant, and the difficulty in establishing the chronic toxic potential of a plant. Certain users of herbs are at high risk of intoxication. These include chronic users, those consuming large amounts or a great variety, the very young, the elderly, the sick, the malnourished or undernourished, and those on long-term medication.

Everything from herbal tea to natural muscle relaxants is touted as the new and better alternatives to more conventional medication. Everyone seems to have fallen into this new craze, even if most people don't quite understand what they're getting themselves into. However, nature can provide humanity with much more than natural muscle relaxants and stimulants, as anyone who has had experience with plant and animal toxins can likely attest to. Spiders, scorpions, and a variety of other animals all carry their own natural toxins, often with effects that rival the death rate and potency of even the most powerful manmade neurotoxins. The plant world also has an abundance of toxins, some of which are well known, some have played roles in history, and others have been adapted to serve medicinal roles in modern herbal lore, usually as sedatives and muscle relaxants.

One of the more famous herbs with potentially lethal toxic properties would be hemlock (*Conium maculatum* L.). Historically, doses of hemlock were used as a means of executing criminals in various ancient Greek city-states, with Socrates being the most notable victim of the practice. Plato, one of Socrates' contemporaries, wrote a detailed account of the effects that the hemlock serum had on the ancient philosopher, which were seen as being consistent with the effects of a suitable quantity of hemlock used as a poison. The herb has a powerful effect and, in small doses with low concentration, can serve as a substitute for most muscle relaxants. However, in the right mixture or in high enough concentrated dose, it makes for a very deadly drug. Lethal hemlock mixtures are relatively easy to prepare, provided one has the enough quantities of the said plant.

Curare is also known as a deadly plant, but can also act as a muscle relaxant. In high enough doses, curare can effectively paralyze the entire body.

The concentrations of poison in any given combination can vary: the concentration in the various organs of the plant can differ from specimen to specimen. Curare is made up of compounds that are not digestible by the body, however, so anyone who ingests the toxin is not going to feel the effects. The only way to be poisoned by curare is to have the toxin be mixed with the blood directly, either through an injection or through an open wound. Curare is usually used as a hunting poison in South America, both because it does not render meat toxic and because it is a quick, effective poison.

Other common plants associated with poisoning include oleander and foxglove. Both contain glucosides that exert a similar effect to *Digitalis purpurea* or *Digitalis lanata* and have been used in suicides for centuries. Ingestion of plant toxins may not only be for recreational purposes, as cases have been described where poisonings have occurred from toxins in homemade toothpaste, in wild vegetables, in herbal medicines, and in children experimenting.

A retrospective analysis of the plant poisonings in Northern Italy over a period of 11 years (1995–2006) was performed by the Poison Control Centre of Milan Niguarda Hospital. The registered cases of plant exposures were 10,959, about 1,000 per year. The more serious cases usually occurred in adolescents or adults who either mistook a plant as edible or deliberately ingested a toxic plant. Severe intoxications with plant materials occurred in adults with aconite (30 cases), meadow saffron (8 cases), and hemlock (21 cases) ingestions, some of which had lethal consequences. Atropa (108 cases), datura (93 cases), cherry laurel (133 cases), laurel (93 cases), autumn mandrake (41 cases), elder (127 cases), and mistletoe (238 cases) were the cause of severe reactions. The incidence of plant poisoning varies a great deal depending on local traditions, lifestyle, nutritional factors, climate, and the presence of wild plants. Notwithstanding plant species containing psychoactive toxins, the ingestion of a toxic plant is almost invariably accidental. The true problem is that many people currently believe that all natural substances are inherently safe and useful for human health, because they are not “true drugs.” Because of this, it is possible to observe an increasing number of poisoning cases, starting from the mid-1990s to the present time, thanks to the wide Internet diffusion of misleading information. Comparable results are reported in the Annual Report of the American Association of Poison Control Centers’ National Poison Data System (NPDS).

7 Plants as Crime-Solvers: Forensic Botany

Today, many criminal investigators are resorting to techniques used in the scientific world to accumulate evidence against the accused. The use of science to answer questions relevant to the legal system is termed *forensics*, and has branched into the many specialties seen in the scientific world. These include anthropology, ballistics, bloodstain analysis, botany, DNA fingerprinting, economic crime, entomology, and the list goes on. The area of forensic botany is a relatively new and untouched specialty. The first criminal investigation that included the analysis of plant material wasn't until 1935 and, today, few investigators rely on the science of plant material, although it is becoming more common. Forensic botanists examine plants and plant matter to determine their species and origin. In some cases, suspects may leave behind plant parts, spores, or seeds that had adhered to their clothing. If the plant species in question is found only in limited areas, its presence at the crime scene may indicate where suspects have been or where they live.

8 Natural Toxins and Bioterrorism

Toxin weapons are primarily illness-inducing chemicals formed from living creatures, such as bacteria, fungi, plants, and animals. Toxins range from disabling to acutely toxic. The most deadly compound currently known, botulinum toxin, is a bacterial toxin. Toxins are more potent than chemical weapons, requiring less material to produce equivalent casualties, but they are not self-reproducing, so more material is required than for a biological weapon. Symptoms from toxin exposure typically occur on a timescale intermediate between chemical and biological weapons, generally appearing over the course of several hours. Chemical, biological, and toxin weapons also differ in their medical treatment and the availability of effective prophylaxis. Chemical weapons, with their quick acting effects, must be treated as promptly as possible. Because of the large range of potential effects caused by chemical weapons, there is no universal treatment for chemical weapon exposure [10]. Exposure to nerve agents can be directly treated with medication to prevent or reduce symptoms. Exposure to vesicants, such as mustard gas, better known as Yprite, is generally untreatable; most people exposed will exhibit the agent's effects. The mustard gas is an irritant compound derived from the dried ripe seed of *Brassica (Sinapis) alba*, *B. nigra*, or *B. juncea*, and contains toxic allyl isothiocyanate in nontoxic glycoside form, though the plant also contains myrosinase, an enzyme that converts the glycoside into the toxic form. It was one of the most lethal of all the poisonous chemicals used during the World War I. It took 12 h to take effect. Yprite was so powerful that only small amounts had to be added to high-explosive shells to be effective. Once in the soil, mustard gas remained active for several weeks.

Ricin toxin is found in the beans of the castor plant, *Ricinus communis.* It is one of the most lethal and easily produced plant toxins. The toxin is present in the entire plant but is concentrated in its seeds. Ricin can be in the form of a powder, mist, or pellet, or dissolved in water or weak acid. It is a very stable substance and is not affected by extremes in temperature. Castor beans are processed throughout the world to make castor oil. Ricin is part of the waste "mash" produced when castor oil is made. Ricin irreversibly blocks protein synthesis. Ricin has some potential medical uses, such as bone marrow transplants and cancer treatment (to kill cancer cells).

Abrin and ricin are natural toxic protein toxins isolated from plant seeds, that is, they are phytotoxins. Both proteins are composed of two peptide chains, signified as A-chain and B-chain, which are linked by a disulphide bond. Both toxins have large scale molecular similarity, and the A-chains of abrin and ricin have a 102 conserved amino acid homology; it is possible to create hybrid toxins (in a reciprocal manner) between the A- and the B-chains of abrin and ricin. Also, the mechanism of their toxic action is the same. The A-chain inhibits protein synthesis, whereas the B-chain binds to cell surface receptors containing terminal galactose and acts as an immunotoxin. The A-chain contains the toxic activity and the B-chain gives the toxin a cell recognition and binding function to facilitate transport across the cell membrane. The A-chain is not active until it is internalized by the cell, where it halts protein synthesis.

Three routes of ricin exposure are known to exist for humans and animals: inhalation, natural infection by ingestion, and injection. Depending on the route of exposure (such as injection), as little as 500 μg of ricin could be enough to kill an adult. A 500-μg dose of ricin would be about the size of the head of a pin. A much greater amount would be required to kill a human being by either the inhalation or ingestion routes. Toxins are not usually transmitted person-to-person or animal-to-animal. Secondary aerosolization is not thought to be of concern.

The incubation period for ricin is dependent on the route of transmission. If the toxin is inhaled, the incubation is about 8 h. The incubation period for ingestion is a few hours to days. If ricin is injected under the skin, the incubation period could be immediate to several hours, depending on the location of injection and the dose of toxin injected.

The initial symptoms associated with inhalation of ricin toxin are cough, weakness, fever, nausea, muscle aches, chest pain, and cyanosis. Pulmonary edema occurs about 18–24 h after inhalation, and severe respiratory distress and death from hypoxemia ensues at 36–72 h.

The least toxic form of poisoning is by ingestion. Symptoms are also less severe if the seeds are swallowed whole, as they have a thick coating that limits absorption of the toxin. Less than a few hours after ingestion, severe gastrointestinal signs occur, including nausea, vomiting, intestinal cramps and headache. These signs are followed by diarrhea and hemorrhage from the anus and dilation of the pupils. This generally leads to vascular collapse and death in 3 days or more. The seeds of the castor bean are very attractive, which is why they are popular in certain costume jewelry. Unfortunately, they also look good enough to eat, which is why small children are the most common victims of castor poisoning.

There are no specific data on injection of ricin into humans, and likely human symptoms are based on those of animal models. Initially it would be expected that a person would experience local pain and necrosis at the site of injection, followed by systemic signs. Death would likely ensue at 3 or more days after exposure.

The diagnosis of ricin toxicosis is often based on clinical symptoms. Detection of the toxin in serum or respiratory secretions can be done by ELISA and immunohistochemistry on infected tissues. Ricin is very immunogenic, thus the toxin can be detected via serology.

There is no treatment or vaccine currently available for ricin poisoning. Supportive care is recommended based on the route of exposure, and includes respiratory support for inhalational exposure, gastric washing (cleaning), and cathartics to remove the toxin from the GI tract if ingestion has occurred.

Ricin can affect all domestic animals. Clinical signs were observed in experimental studies in laboratory animals. Inhalation of ricin would likely cause pulmonary edema, respiratory distress, and death within 36–72 h. Ingestion of ricin is the most common form of naturally occurring infection. If the seeds are eaten whole the severity of signs is decreased and seeds may pass with no ill consequences occurring. Severe vomiting and diarrhea are often seen, with depression, weakness and shortness of breath. Trembling and incoordination accompanied by sweating or shivering can be seen. The diagnosis and treatment of animals infected with ricin are similar to that of humans with ricin toxicosis, which is based on the clinical signs. Research is being conducted on vaccines that are immunogenic and can offer protection against aerosolized ricin.

Promising research is being conducted in animals for ricin antisera and vaccination. If exposure is suspected, decontamination of the area or exposed skin should be done with soap and water or with a 0.1% sodium hypochlorite bleach solution. A protective mask is effective against aerosol exposure. Standard safety precautions should be followed by all health care workers if exposure is suspected. Because of ricin's extreme ease of production, wide availability, and the fact that it is one of the most potent plant toxins known, it is considered to be a potential agent that could be used for bioterrorism.

9 Conclusions

The findings from this survey indicate that botanical food supplements or dietary supplements can be marketed for recreational purposes and as alternatives to illicit drugs. Some products presented in the web can be likened to marijuana or Ecstasy, and most claimed to have hallucinogenic or stimulating properties. Products containing stimulant compounds, performance-enhancing drugs, herbal drugs of abuse, and natural alternatives to Viagra were the most commonly encountered. Physicians, chemists, and healthcare professionals should be familiar with these products so that they are better able to identify products, ingredients, and causes of toxicity in persons utilizing food supplements for recreational purposes.

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Part II

Alkaloids: Classes - Occurrence, Biosynthesis, Structure and Chemistry, Distribution

Alkaloids Derived from Lysine: Piperidine Alkaloids 11

Éva Szőke, Éva Lemberkovics, and László Kursinszki

Contents

1 Introduction 304
2 Piperidine Alkaloids Derived from Lysine 305
2.1 Punica Alkaloids 305
2.2 Piper Alkaloids 309
2.3 Lobelia Alkaloids 318
3 Conclusion 337
References 338

Abstract

The biosynthesis, occurrence, and biological activities of piperidine alkaloids arising from lysine have been studied (*Punica granatum*, *Piper* species, and *Lobelia inflata*). The isolation process, chromatographic purification/analysis, and structure elucidation of the significant alkaloids are discussed.

Common characteristics in the biosynthesis of these alkaloids, that they are elaborated from the lysine derived Δ^1-piperideine, coupling either to an aliphatic- (from acetyl-CoA precursor, e.g., pelletierine and co-alkaloids), or an aromatic part (from cinnamoyl-CoA precursor, e.g., piperine and other amides; lobeline, lobelanine and related alkaloids).

The biological activities of piperidine alkaloids are summarized as follows:

– The pomegranate alkaloids, like isopelletierine, have anthelminthic activity and were used primarily as a taenicide.

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- Due to the pungency of the fruits of *Piper* species, among them the most frequently used black pepper, is one of the most ancient spices and also known as a preservative food additive. Piperine, the chief constituent, increases the bioavailability of numerous therapeutical drugs (e.g., propranolol, theophylline, curcumine, β-carotene) perhaps by increasing intestinal absorption or by exerting an antioxidant effect during the first pass through the liver. The piperine can reverse MDR by multiple mechanisms and may be a promising lead compound for future studies.
- Lobelin, the chief alkaloid of *Lobelia inflata*, is a respiratory stimulant, which enhances and accelerates the respiratory movements. It has been used in asthma, gas poisoning, and narcotic poisoning. Lobeline is currently the subject of renewed interest for the treatment of drug abuse and neurological disorders, like Alzheimer's or Parkinson's disease, which pose an important public health problem in industrial countries. Lobeline is a competitive nicotinic receptor antagonist and is still commercialized in antismoking preparations. Interest in Lobelia alkaloids, and in particular the most active (-)-lobeline, has increased in recent years due to their activity on the central nervous system (CNS) and the multidrug-resistance (MDR).

Keywords

Biological activities • biosynthesis of piperidine alkaloids • HPLC and LC-MS/MS • Lobelia inflata • lobeline and their derivates • pelletierine • Piper nigrum • piperine • Punica granatum

1 Introduction

The piperidine, quinolizidine, indolizidine alkaloids derived from lysine are belonging to so-called "true alkaloids" because their nitrogen atoms originate from an amino acid, and in general, the carbon skeleton of the particular amino acid precursor is also largely retained intact in the alkaloid structure. Relatively few amino acid precursors are actually involved in alkaloid biosynthesis, the principal ones being also lysine [1].

While for developing of simple piperidine alkaloids, e.g., pelletierine (*Punica granatum*), piperine (*Piper nigr*um et *longum*), and lobeline (*Lobelia inflata*), only one molecule of lysine is necessary, for quinolizidine alkaloids – e.g., lupinine (*Lupinus luteus*), sparteine of antiarrhythmic activity (*Sarothamnus scoparius*), and cytisine of respiratory stimulant effect (*Laburnum species*) – two molecules of lysines are indispensable. It was also proved that lycopodine (*Lycopodium tristachyum*, clubmoss) of quinolizidine structure has no polyketide origin, but it is a modified dimer of pelletierine, which, in turn, is derivable from lysine and acetate.

For completeness it could be mentioned that indolizidine alkaloids, e.g., castanospermine (*Castanospermum australe*) derived from pipecolic acid which is also a lysine derivate. Castanospermine, as a polyhydroxylated indolizidine alkaloid, displayed activity against the AIDS virus HIV, by its ability to inhibit glycosidase enzymes involved in glycoprotein biosynthesis.

Lysine has also a role in the biosynthesis of Nicotiana alkaloids, e.g., anabasine, in which beside nicotinic acid, the precursor of pyridine ring, lysine formed the piperidine core.

At the same time, there are alkaloids of piperidine skeleton which are derived from acetic acid or nicotinic acid pathways, e.g., coniine (*Conium maculatum*) or arecoline (*Areca catechu*) and anatabine (*Nicotiana tabacum, Lobelia inflata*) [1].

In this chapter, we intensively studied the Punica, Piper, and Lobelia piperidine alkaloids. These plants and their main alkaloids are undoubtedly known and used in therapy for ages, but at present, numerous scientific results were also born on their biological activities and pharmacological and clinical tests; further, new alkaloids were isolated and semisynthetic analogues prepared, which could be important in the modern phytotherapy.

2 Piperidine Alkaloids Derived from Lysine

L-lysine is α- ε-diaminocarbon acid, the homologue of L-ornithine, contains with one methylene group more than that.

While L-ornithine is the precursor of pyrrolidines, L-lysine is that of piperidine alkaloids (Fig. 11.1). Lysine is formed from aspartic acid, one of the members of Krebs cycle (Fig. 11.2). From lysine, the carboxyl group is lost; the ε-amino is retained in the ring. Lysine thus supplies C_5N-building block for alkaloids (Fig. 11.3) [1].

2.1 Punica Alkaloids

2.1.1 Biosynthesis of Pelletierine and its Co-alkaloids

The biosynthesis starts from Δ^1-piperideine-derived lysine. From lysine by decarboxylation cadaverine then 5-aminopentanal by oxidative deamination and finally Δ^1-piperideine are developed respectively (Fig. 11.4). To Δ^1-piperideine an acetoacetyl-CoA is connected providing an intermediate. From it, by hydrolysis and decarboxylation, pelletierine is formed, then *N*-methylpelletierin by methylation. This biosynthetic pathway was supported by C^{13}-NMR spectroscopic investigation already in 1999 [2]. *N*-methylpelletierine gave pseudopelletierine (homatropine) by *intramolecular* Mannich reaction. In the present of another Δ^1-piperideine molecule, pelletierine could give also anaferine by *intermolecular* Mannich reaction, but anaferine is firstly the characteristic alkaloid of *Withania somnifera* (Solanaceae) [1]. We have to mention that *N*-methylpelletierine, pseudopelletierine, and anaferine are the homologues of the pyrrolidine alkaloids derived from ornithine as the hygrine, tropinone, and cuscohygrine.

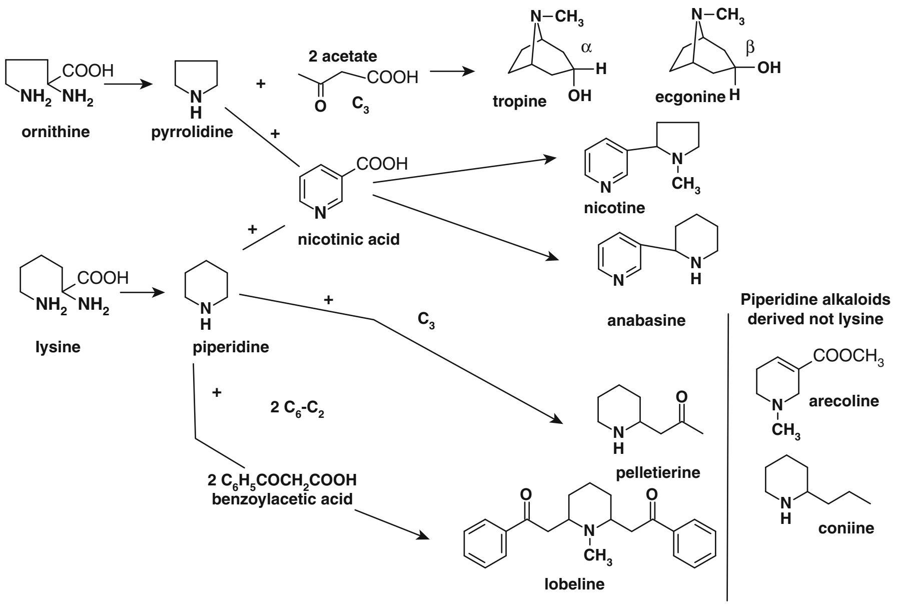

N–CH3
N–CH3
2 acetate
α
β
COOH
COOH
H
OH
NH2
NH2
N
H
+
O
C3
tropine
OH
ecgonine
H
ornithine
pyrrolidine
+
COOH
N
N
CH3
nicotine
+
N
nicotinic acid
COOH
N
N
H
NH2
NH2
N
H
+
Piperidine alkaloids
derived not lysine
C3
anabasine
lysine
piperidine
COOCH3
arecoline
N
CH3
+
2 C6-C2
O
N
H
pelletierine
N
H
coniine
2 C6H5COCH2COOH
benzoylacetic acid
O
O
N
CH3
lobeline

Fig. 11.1 Pyrrolidine and piperidine alkaloids derived from ornithine and lysine

The barks contain about 0.5–0.9 % of total alkaloids, which are volatile liquid. The alkaloids are bounded to tannins, in about 22 % [3].

In the barks of *Punica granatum which* originated from the region of Yugoslavia, Neuhofer et al. [4] found also other piperidine alkaloids beside pelletierine, *N*-methylpelletierine, and pseudopelletierine, namely, norpseudopelletierine, sedridine, and further hydroxypropyl and hydroxypropenyl Δ^1-piperideines (Fig. 11.5). *N*-methylsedridine and *N*-methylpelletierine were also detected in *Picea breweriana* [5]. For identifying of alkaloids, they use capillary GLC-MS method. It is interesting that pyrrolidine alkaloids – hygrine and norhygrine – were also detected by them but only in the root bark.

In 1963, Beyerman and Maat [6] already described the synthesis and absolute configuration of Tanret's (-)-pelletierine. The synthesis is described of (-)-pelletierine picrate as identical with *Tanret's* pelletierine picrate from *Punica granatum* L. and its antipode by oxidation, respectively, of (-)- and of (+)-sedridine. The sedridines are alkaloids with known absolute configuration of the piperidine asymmetric carbon atom. From this follows the D_S-configuration of (-)-pelletierine.

2.1.2 Occurrence of Punica Alkaloids: *Punica granatum* L. (Pomegranate)

The pomegranate (*Punica granatum* L., Punicaceae) is a shrub widespread in North Africa and West Asia but it is cultivated in South Europe, region of the Mediterranean Sea. Its flowers, which have five to seven bright red petals, and its

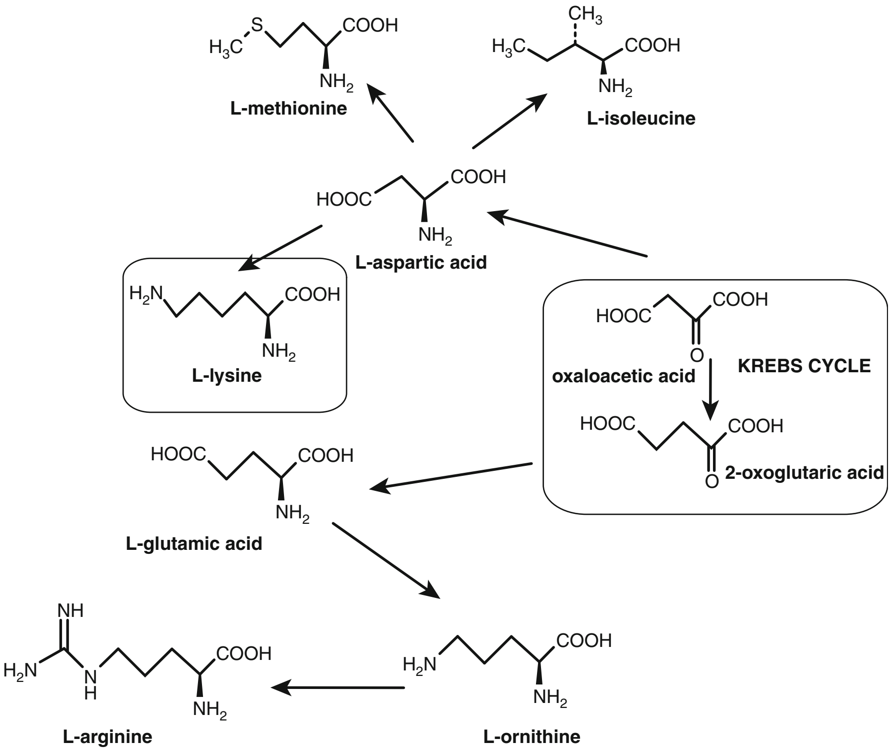

L-methionine
L-isoleucine
L-aspartic acid
L-lysine
oxaloacetic acid
KREBS CYCLE
2-oxoglutaric acid
L-glutamic acid
L-arginine
L-ornithine

Fig. 11.2 Biosynthesis of lysine amino acid

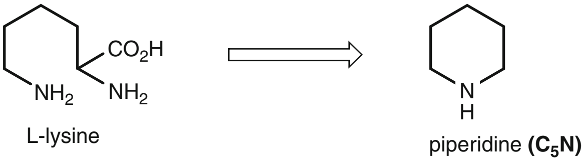

L-lysine
piperidine (C_5N)

Fig. 11.3 Lysine, the precursor of piperidine skeleton

fruit – a rounded corticos berry, with the teeth from the calix remaining on top – make it easy to identify. The fruit (Fig. 11.6) pulp is eatable; it is the starting material for authentic grenadine syrup [7]. The stem and root barks and the fruit rind and seeds all are found in medicinal use. Both barks are used and occur in curved or channeled pieces about 5–10 cm long and 1–3 cm wide. The outer surface of the stem bark shows longitudinal corky furrows, a few shallow depressions, and bark apothecia of lichens, while that of the root bark shows depressions where the outer layers have exfoliated. The barks are smooth and yellowish on their inner surfaces and break with a short granular fracture.

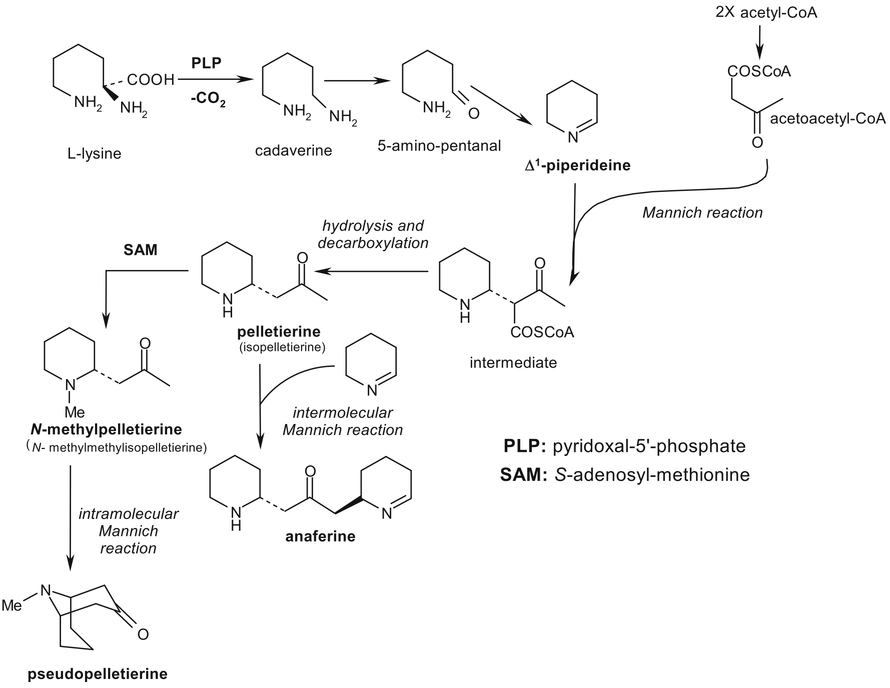

2X acetyl-CoA
COOH
NH2
NH2
PLP
-CO2
NH2
NH2
NH2
O
COSCoA
O
acetoacetyl-CoA
L-lysine
cadaverine
5-amino-pentanal
N
Δ1-piperideine
Mannich reaction
hydrolysis and decarboxylation
SAM
O
N H
O
N H
COSCoA
pelletierine
(isopelletierine)
intermediate
O
N
Me
N-methylpelletierine
(N- methylmethylisopelletierine)
N
intermolecular Mannich reaction
PLP: pyridoxal-5'-phosphate
SAM: S-adenosyl-methionine
intramolecular Mannich reaction
O
N H
N
anaferine
Me
N
O
pseudopelletierine

Fig. 11.4 Biosynthesis of pelletierine and co-alkaloids

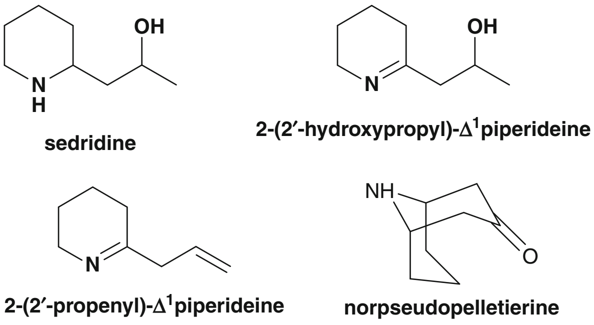

OH
N H
sedridine
OH
N
2-(2′-hydroxypropyl)-Δ1piperideine
N
2-(2′-propenyl)-Δ1piperideine
NH
O
norpseudopelletierine

Fig. 11.5 Other piperidine alkaloids in Punica barks

2.1.3 Biological Activity and Therapeutical Use of Punica Alkaloids

The *root bark* was already recommended in the Ebers Papyrus and later in more Pharmacopoeias (e.g., pelletierine tannate in BP 1948) to treat worm infestations and was used as an anthelmintic, primarily against tapeworm, until the first half of the twentieth century. The greatest anthelmintic activity of Punica extracts was chiefly due to isopelletierine [8]. Our daily harmful effects due to the fraction absorbed in the intestine have led to full abandonment of the drug and also its alkaloids [7].

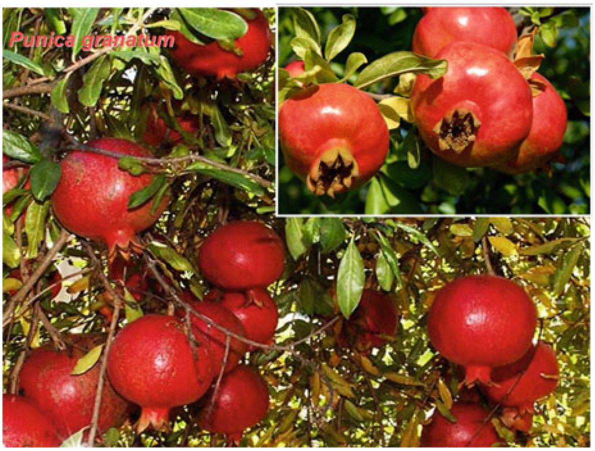


Fig. 11.6 *Punica granatum* L.

The *dried pericarp* of the fruit occurs in thin, curved pieces about 1,5 mm thick, some of which bear the remains of the woody calyx or a scar left by the stalk. The outer surface is brownish-yellow or reddish and the inner surfaces bear impressions left by the seeds. The height tannin content (28 %) affords its use as an astringent in the treatment of diarrhea.

The *seeds* have been studied for their glyceride content, and the validity of a seed extract for use in the treatment of diarrhea, as practiced in traditional Indian and Bangladesh medicine, has been experimentally verified [3].

2.2 Piper Alkaloids

2.2.1 Biosynthesis of Piperidine Amide: Piperine

Piperine consists from a piperidine part (tertiary amide) and a phenolic part: the piperic acid. During the biosynthesis, the piperidine formed from Δ^1-piperideine is connected to piperic acid. Piperic acid is developed from methylenedioxy cinnamoyl-CoA (Fig. 11.7), [1].

2.2.2 Occurrence of Pepper: *Piper nigrum* L. (Pepper)

Pepper (*Piper nigrum* L., Piperaceae) is one of the most ancient spices. Used since time immemorial in India, it was known in the Greece and Rome of antiquity. It is the fruit of *Piper nigrum*, a perennial plant originally from the south-west of India (Malabar Coast) and now cultivated in India (Kerala), Indonesia, Malaysia (Sarawak), Sri Lanka, and also South America (Brazil) (Fig. 11.8).

The pepper vines have a ligneous voluble stem affixed to their support by secondary branches. The leaves have an oval acute blade and are alternate.

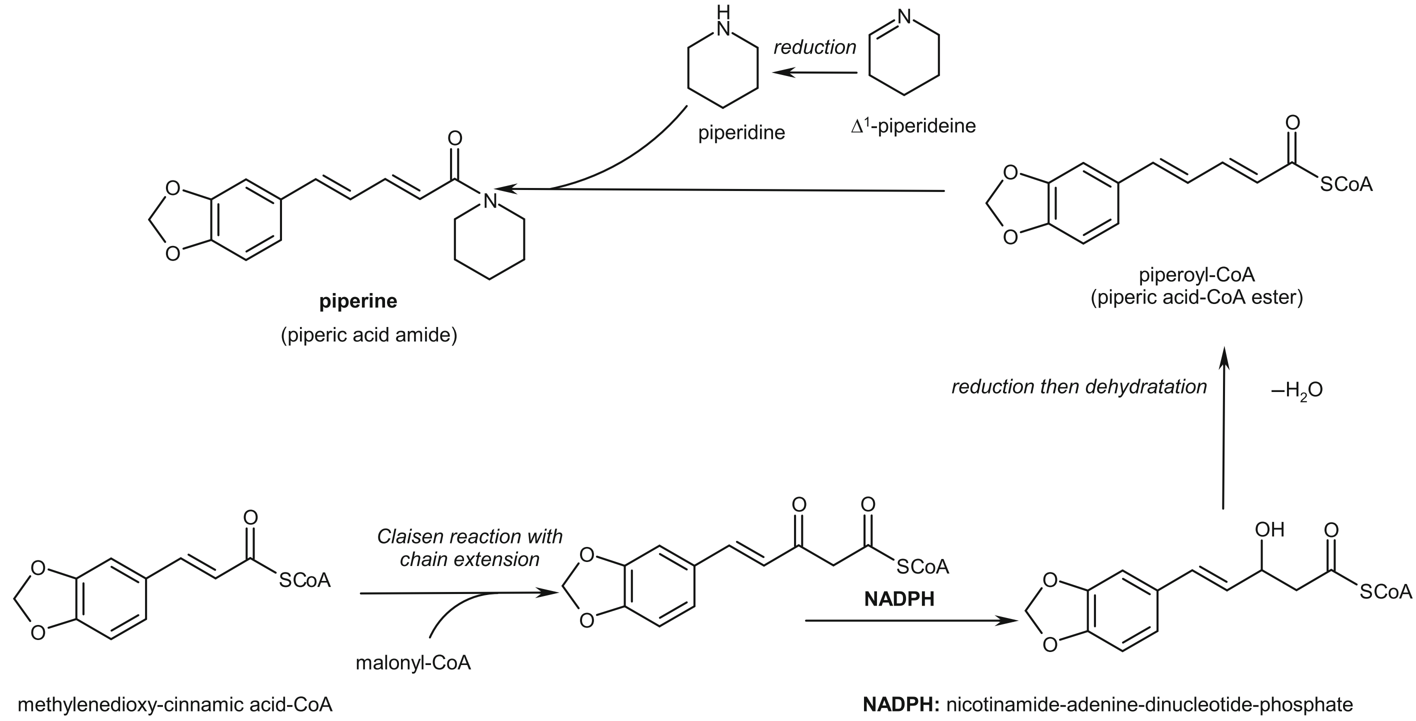

reduction
piperidine
Δ¹-piperideine
piperine
(piperic acid amide)
piperoyl-CoA
(piperic acid-CoA ester)
reduction then dehydratation
−H₂O
SCoA
Claisen reaction with chain extension
malonyl-CoA
NADPH
methylenedioxy-cinnamic acid-CoA
NADPH: nicotinamide-adenine-dinucleotide-phosphate

Fig. 11.7 Biosynthesis of piperine

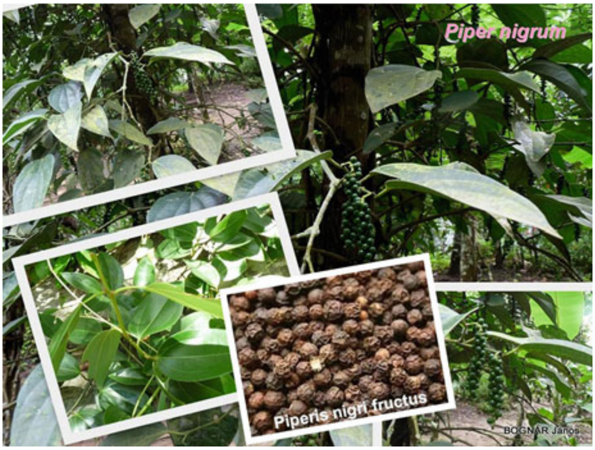


Fig. 11.8 *Piper nigrum* L.

The sessile flowers have no perianth and are grouped by 20–30 units into dangling spikes. The fruit is a berry of 4–8 mm in diameter, which turns from green to red as it ripens.

The different kinds of pepper (fruit of plant) are well recognized:

– Green pepper consists of whole fresh berries. Generally conserved in acidic aqueous solution (or frozen, or pasteurized), it is highly aromatic.
– White pepper consists of the fruits collected at full maturity. After being soaked in water for several days, the fruits' pericarp and the external layers of mesocarp are removed and the fruits are dried.
– Black pepper is prepared from the spikes collected immediately after the first berries turn red. After drying, the fruits are separated from the stalks. The dried fruits are spherical (3–6 mm) and particularly hard. Their surface is brownish-black and extremely wrinkled [7].

2.2.3 Ingredients of Pepper

The peppery odor is due to 1–3.5 % of an essential oil rich in terpenoid hydrocarbons, firstly β-caryophyllene sesquiterpene (22–27 %), then numerous monoterpenes: limonene (21–22 %), sabinene (8.5–17.5 %) β-pinene (9–11 %), α-pinene (5–6 %), myrcene (2.2–2.3 %), p-cymene (0–0.2 %), and oxygenated constituents (3–5 %). Recently, about 40 compounds were identified in oil [3].

The pungency of pepper is ascribed to piperidine amides (5–10 %).

The chief constituent is piperine (amide of piperidine and piperic acid) and its geometric isomer chavicine (amide of piperidine and chavicic acid). The other piperidine amides are piperanine (instead of two double bond, only one is in the side chain) and piperettine, a piperine homologue (the side is longer with two methylene group); further, a pyrrolidine amide, the piperylline, is also findable in piper (Fig. 11.9). In *Piper longum* beside piperidine amides, also not amide alkaloids, as the cepharadine A and B of 4,5-dioxoaporphins skeleton were found [9].

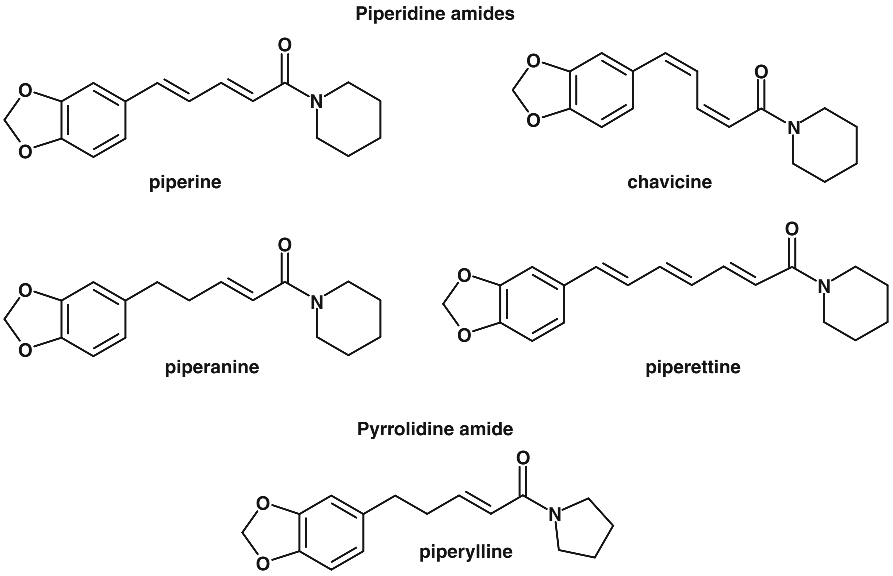


Fig. 11.9 Characteristic alkaloids of pepper

2.2.4 Isolation and Structure Elucidation of Piperine from *Piper nigrum, P. longum*, and *P. officinarum*

Piperine was firstly isolated in 1819 from *P. nigrum*, but it was later found in *P. longum* (long pepper) and Ashanti pepper, the fruits of *P. guineense* [3].

Regarding the therapeutical (digestive, anodyne) and antioxidant antibacterial activities of piperidine amides – and that the piperine and piperic acid could be used as natural agents in both food preservation and human health – more isolation methods were used for them from *Piper* species [10]. From *P. longum* fruits, Shihua et al. [11] carried out the preparative isolation and purification of amides by upright countercurrent chromatography (CCC) and reversed-phase liquid chromatography (RPLC) (Fig. 11.10). They isolated nine target amides with over 95 % purity (Fig. 11.11). Structures of all compounds were identified by electrospray ionization MS, electron impact ionization MS, and one- and two-dimensional NMR spectra.

Kanaki et al. [12] worked out a rapid, economical method for isolation of piperine from *Piper nigrum* L. The method involves extraction of the fruit powder with glacial acetic acid from which piperine was partitioned into chloroform and subsequently crystallized.

Pedalkar et al. [13] extracted piperine from *P. nigrum* by aqueous solutions of surfactant plus hydrotrope mixtures. They established that the combination of butyl benzene sulfonate as hydrotrope and sodium dodecyl sulfate (SDS) gave increased percentage extraction of piperine as compared to the hydrotrope alone. The piperine crystallized from aqueous solutions of surfactants and hydrotrope also showed cleaner structures with sharp edges, unlike the particles crystallized from organic solvents. Chen et al. [14] studied a preparative high-performance centrifugal

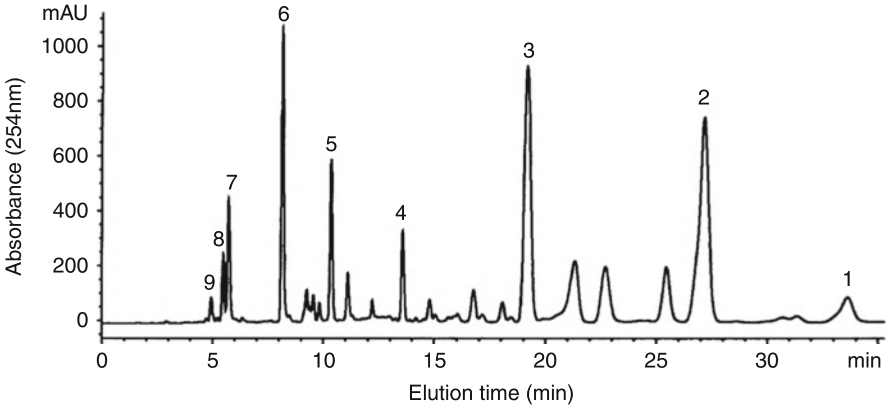


Fig. 11.10 HPLC of red oil from *Piper longum* containing the mixture of the nine isolated amides (Legends see in Fig. 10.11)

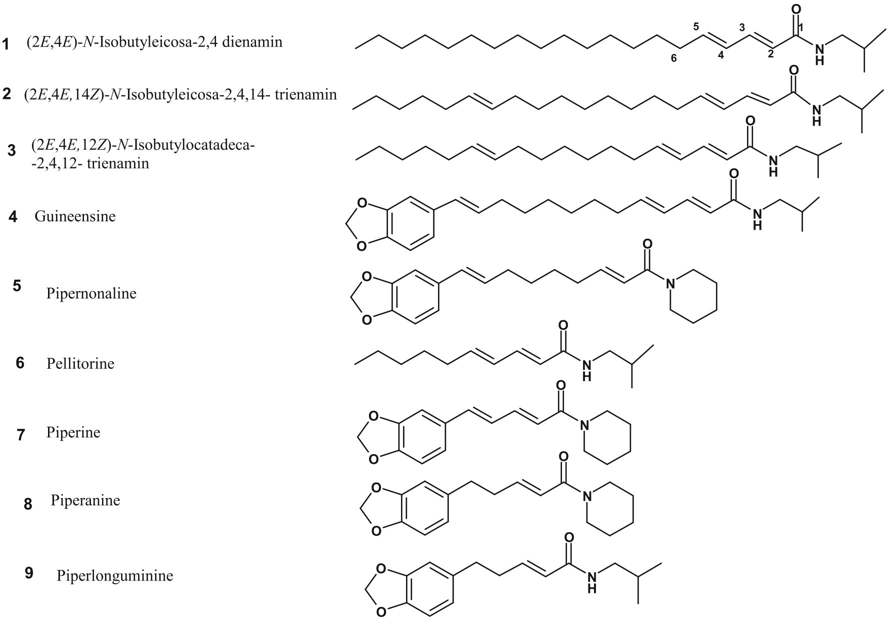

1 (2*E*,4*E*)-*N*-Isobutyleicosa-2,4 dienamin
2 (2*E*,4*E*,14*Z*)-*N*-Isobutyleicosa-2,4,14- trienamin
3 (2*E*,4*E*,12*Z*)-*N*-Isobutylocatadeca- -2,4,12- trienamin
4 Guineensine
5 Pipernonaline
6 Pellitorine
7 Piperine
8 Piperanine
9 Piperlonguminine
O
N
H
1
2
3
4
5
6

Fig. 11.11 Piperidine amides isolated from *Piper longum* [11]

partition chromatography (HPCPC) method for isolation and purification of the bioactive component piperine directly from the ethanol extract of *Piper nigrum* L. was successfully established by using n-hexane-ethyl acetate-methanol-water as the two-phase solvent system. The upper phase of n-hexane-ethyl acetate-methanol-water (6:5:6:5 *v*/*v*) was used as the stationary phase of CPC. Under the optimum

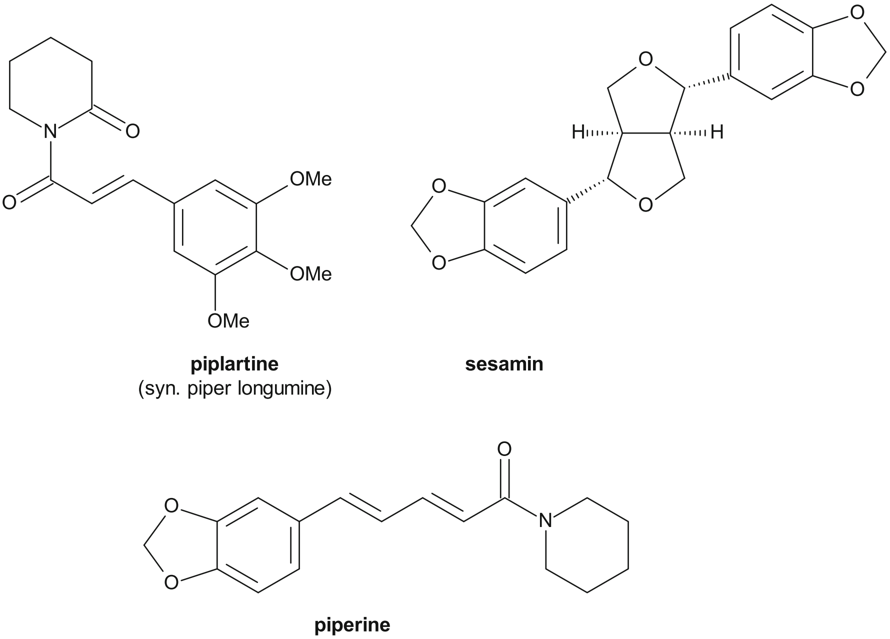


Fig. 11.12 Isolated compounds from *Piper officinarum*

conditions, 40 mg of piperine at 98.5 % purity, as determined by HPLC, was yielded from 300 mg of the crude extract in a single CPC separation. The peak fraction of CPC was identified by (1) H NMR and (13) C NMR.

Mrutyunjaga and Venkateswartu [15], from the dichlormethanic root extract of *Piper officinarum*, isolated three compounds after column chromatographic fractionation. Of the three compounds, piplartine and piperine were alkaloids and sesamin a lignin derivate (Fig. 11.12).

In the last decades, several reports were found about the potential of piperine to increase the bioavailability of drugs in humans. For example, Bano et al. [16] reported an increase in oral bioavailability of propranolol and theophylline by piperine in healthy volunteers. Shoba et al. [17] showed that piperine increases oral bioavailability of curcumin (*Curcuma longa*) also in rats. Increased serum β-carotene levels by piperine were also reported by Badmaev et al. [18].

In an attempt to further study, Bajad et al. [19] searched the differences of urinary metabolites of piperine – as omnipresent food component – between human and animal (rat) organisms. They detected a new major urinary metabolite in rat urine and plasma using HPLC. The metabolite was partially purified using reversed-phase column chromatography on Sephadex-LH 20 and characterized as 5-(3,4-methylenedioxyphenyl)-2 *E*, 4 *E*-pentadienoic acid-*N*-(3-yl propionic acid)-amide with the help of LC/NMR/positive ESI-MS studies (Figs. 11.13–11.15]. Complete mass fragmentation pattern could be assigned with MS/MS studies (Fig. 11.16).

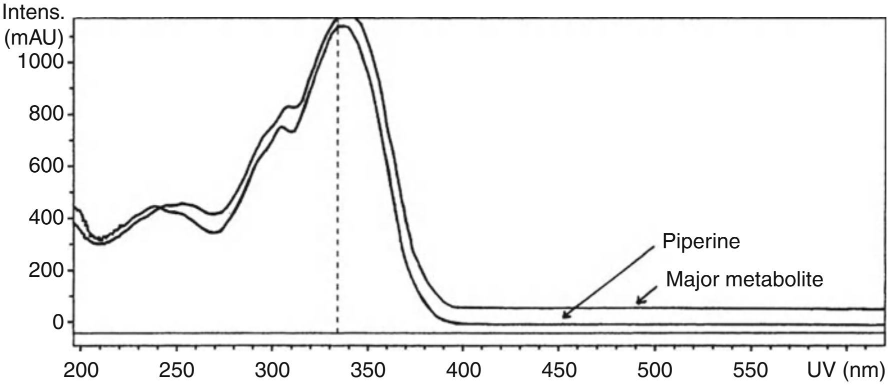


Fig. 11.13 UV spectrum of piperine and its major metabolite [19]

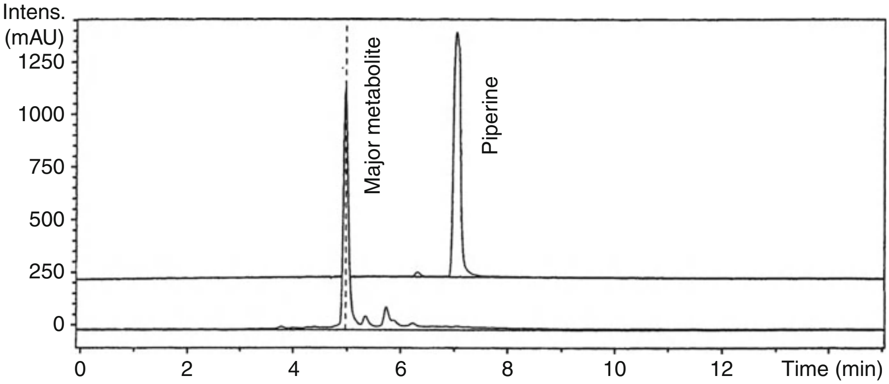


Fig. 11.14 LC chromatogram of piperine and partially purified metabolites at 300 nm obtained using LC/NMR/MS system. Separation was performed on ODS-AQ column [19]

2.2.5 Pharmacological and Therapeutical Activities of Piperine and its Derivates

Lee et al. [20] established that piperine and its related compounds, methylpiperate, (piperic acid methyl ester) guineensine, and piperlonguminine (Fig. 11.11), showed significant MAO inhibitory activities. Piperlonguminine is a novel therapeutic agent for Alzheimer's disease. The mix of piperlonguminine and dihydropiperlonguminine significantly inhibit the expression of amyloid precursor protein (APP). The decreased production of the peptide amyloid β (Aβ) in SK-N-SH cells is a beneficial effect to Alzheimer's disease [21].

During the newest researches, the inhibition of lipopolysaccharide (LPS)-induced inflammatory responses by piperine was intensively studied. The results suggested that piperine inhibited LPS-induced endotoxin shock through inhibition of type 1 IFN (interferon) production [22].

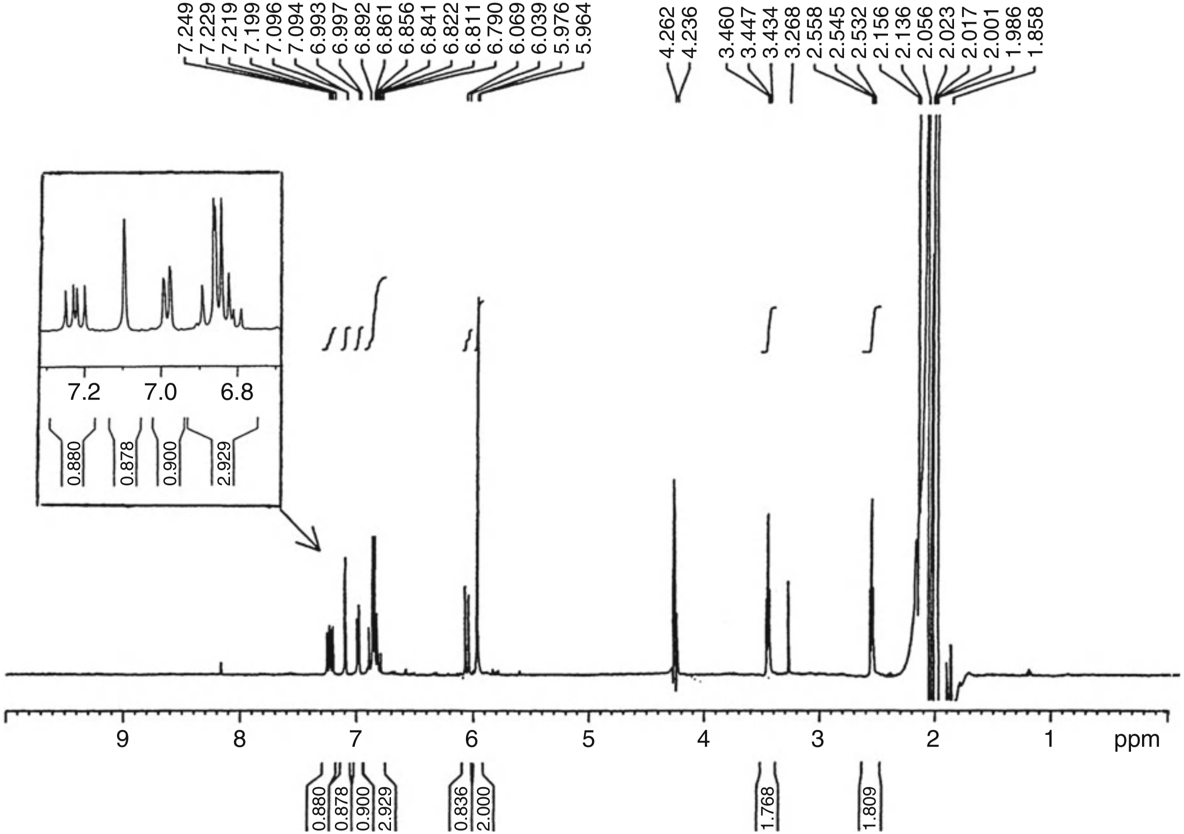


Fig. 11.15 H NMR spectrum (500 MHz) of major metabolite obtained by using LC/NMR/MS system [19]

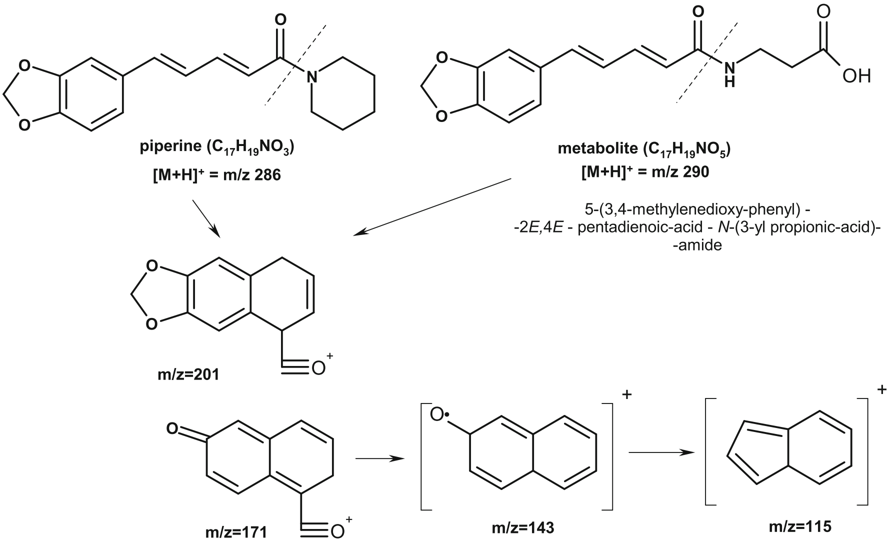

piperine ($C_{17}H_{19}NO_3$)
$[M+H]^+$ = m/z 286
metabolite ($C_{17}H_{19}NO_5$)
$[M+H]^+$ = m/z 290
5-(3,4-methylenedioxy-phenyl) -
-2E,4E - pentadienoic-acid - N-(3-yl propionic-acid)-
-amide
m/z=201
m/z=171
m/z=143
m/z=115

Fig. 11.16 Comparative mass fragmentation pattern of piperine its major metabolite in rat urine obtained using MS/MS studies [19]

Piperine is known to modify the biotransformation of drugs. Piperine-treated rat liver microsomes demonstrated a tendency to enhance [3H]-aflatoxin B1 binding to calf thymus DNA in vivo. The effect of piperine on aflatoxin B1 metabolism thus closely resembles the mode of action of SKF 525-A on biotransformation of foreign compounds [23].

Pradeep and Kuttan [24] established that piperine is a potent inhibitor of nuclear factor – κB (NF- κB), c-Fos, CREB, ATF-2, and proinflammatory cytokine gene expression in B16F-10 melanoma cells.

The antidepressant-like effects of piperine was proved by pharmacological test in chronic mild stress-treated mice [25].

Piperine significantly enhanced accumulation and decreased the efflux of ethidium bromide in *Mycobacterium smegmatis*, which suggests that it has the ability to inhibit mycobacterial efflux pumps [26].

It was also established that piperine has paralyze effect for CNS (central neurosystem) and has also spasmolytic activity. Its semisynthetic compounds are used as antiepilepsy remedy in China.

Recently was studied the multidrug-resistant (MDR) activity of piperine and established it can reverse MDR by multiple mechanisms.

Summary: Black pepper is used not only in human dietaries but also for a variety of other purposes such as medicinal, as a preservative, and in perfumery. Many physiological effects of black pepper, its extracts, or its major active principle, piperine, have been reported in recent decades. Dietary piperine, by favorably stimulating the digestive enzymes of pancreas, enhances the digestive capacity and significantly reduces the gastrointestinal food transit time. Piperine has been demonstrated in in vitro studies to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. Black pepper or piperine treatment has also been evidenced to lower lipid peroxidation in vivo and beneficially influence cellular thiol status, antioxidant molecules, and antioxidant enzymes in a number of experimental situations of oxidative stress. The most far-reaching attribute of piperine has been its inhibitory influence on enzymatic drug biotransforming reactions in the liver. It strongly inhibits hepatic and intestinal aryl hydrocarbon hydroxylase and UDP-glucuronyl transferase. Piperine has been documented to enhance the bioavailability of a number of therapeutic drugs as well as phytochemicals by this very property. Piperine's bioavailability-enhancing property is also partly attributed to increased absorption as a result of its effect on the ultrastructure of intestinal brush border. Although initially, there were a few controversial reports regarding its safety as a food additive, such evidence has been questionable, and later studies have established the safety of black pepper or its active principle, piperine, in several animal studies. Piperine, while it is non-genotoxic, has in fact been found to possess antimutagenic and antitumor influences [27].

2.3 Lobelia Alkaloids

The piperidine ring – the core of Lobelia alkaloids – is derived generally from lysine; but their biosynthetic pathways are depentent on the type (phenolic or aliphatic) of side chains, although the piperidine core seldom could be derived from nicotinic acid (similarly to pyridine alkaloids), e.g., in case of anatabine.

2.3.1 Biosynthetic Pathways of Lobelia Alkaloids

In biosynthesis of Lobelia alkaloids, the two basic compounds (direct precursors) are Δ^1-piperideine and benzoylacetic acid. Δ^1-piperidine is derived from lysine via two pathways: direct from the cadaverine or the 5-aminopentanal, a cadaverine derivate (Fig. 11.17).

The possible pathway for the biosynthesis of benzoylacetic acid as building block of phenolic side chain is the following (Fig. 11.18). Phenylalanine is converted into trans-Cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL). Hydroxylation of Cinnamic acid gave 3-hydroxy-3-phenylpropanoic acid which is transformed to benzoylacetic acid by β-oxidation (chain degradation).

Biosynthesis of Lobelia Alkaloids of Phenolic Side Chains

Δ^1-piperideine and benzoylacetic acid is condensated by Mannich reaction, hydrolysis, and decarboxylation. Intermediate-1 (phenacylpiperidine) furnished sedamin alkaloid by reduction, lobinaline by dimerization, and intermediate-2 by oxidation. From the latter, compound norlobenanine developed with another molecule of benzoylacetic acid by Mannich reaction, hydrolysis, and decarboxylation too. From norlobelanine, lobelanine was formed by methylation, and from it lobeline by reduction (Fig. 11.19), [1, 28].

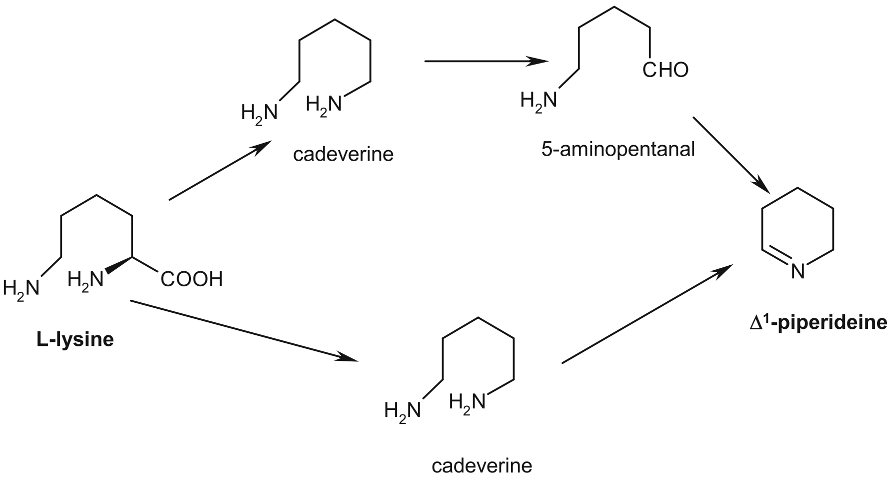

H2N H2N COOH
L-lysine
H2N H2N
cadeverine
H2N CHO
5-aminopentanal
N
Δ1-piperideine
H2N H2N
cadeverine

Fig. 11.17 Two possible biosynthetic pathways of Δ^1-piperideine from lysine in Lobelia

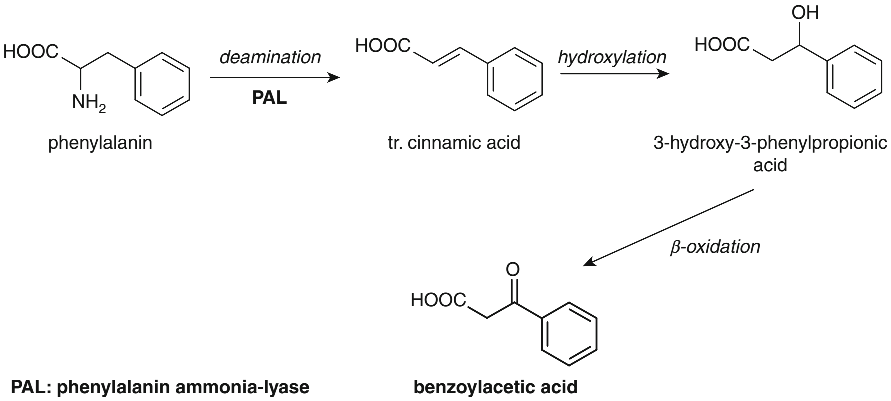

HOOC
NH2
phenylalanin
deamination
PAL
HOOC
tr. cinnamic acid
hydroxylation
OH
HOOC
3-hydroxy-3-phenylpropionic acid
β-oxidation
O
HOOC
benzoylacetic acid
PAL: phenylalanin ammonia-lyase

Fig. 11.18 Biosynthesis of benzoylacetic acid

Biosynthesis of Lobelia Alkaloids of Aliphatic Side Chains

The aliphatic side chains of Lobelia alkaloids derived from acetate. The 3-oxohexanoic acid formed from three units of acetate reacted with Δ^1-piperideine to yield intermediate-1 and its imine, in which subsequent condensation of another molecule of 3-oxohexanoic acid generated the dipenthyldione derivate. Demethylation of the side chains, then *N*-methylation, and reduction of the carbonyl functions provided 8,10-diethyllobelidiol (Fig. 11.20).

Similarly is the biosynthesis of 8-methyl-10-ethyllobelidiol, but one of its side chain is formed from 3-oxobutanoic acid instead of 3-oxohexanoic acid. For biosynthesis of 3-oxobutanoic acid, two acetate units are enough [28].

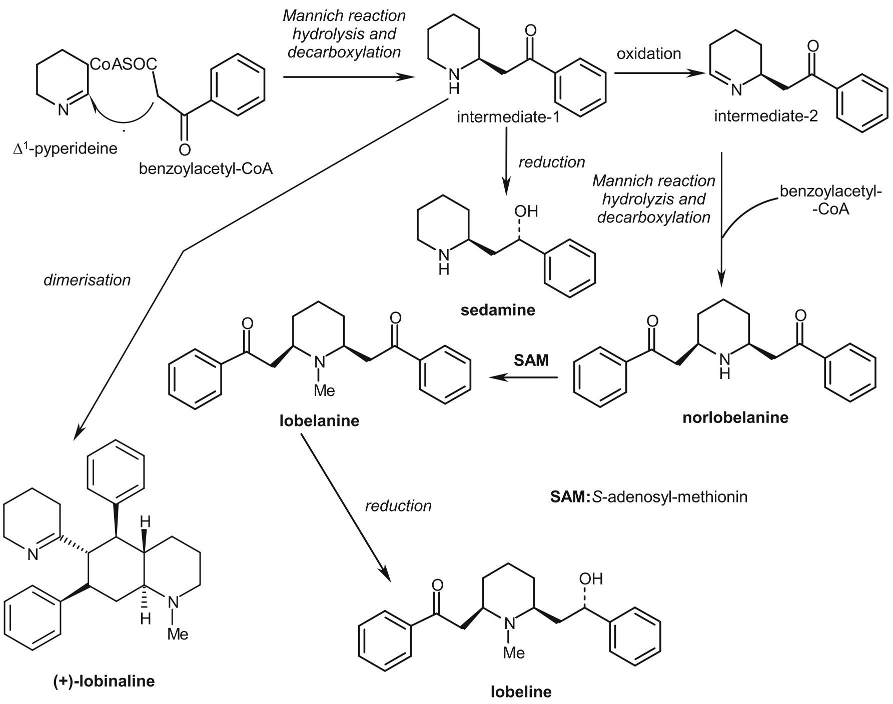

Mannich reaction
hydrolysis and
decarboxylation
oxidation
CoASOC
Δ1-pyperideine
benzoylacetyl-CoA
intermediate-1
intermediate-2
reduction
Mannich reaction
hydrolyzis and
decarboxylation
benzoylacetyl-
-CoA
sedamine
dimerisation
SAM
lobelanine
norlobelanine
SAM: S-adenosyl-methionin
reduction
(+)-lobinaline
lobeline

Fig. 11.19 Biosynthesis of Lobelia alkaloids containing phenolic side chains

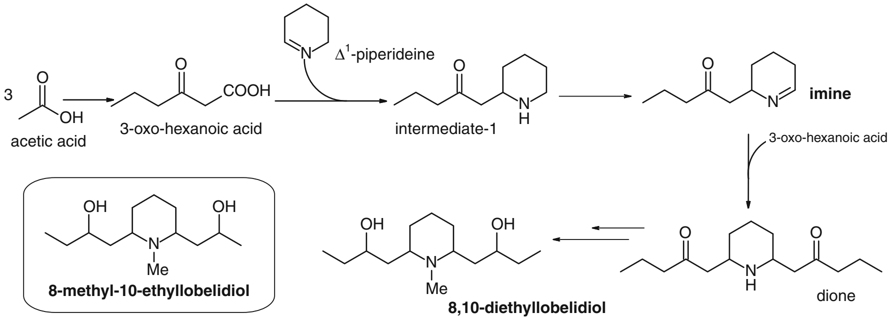

3
acetic acid
3-oxo-hexanoic acid
Δ1-piperideine
intermediate-1
imine
3-oxo-hexanoic acid
8-methyl-10-ethyllobelidiol
8,10-diethyllobelidiol
dione

Fig. 11.20 Biosynthesis of Lobelia alkaloids containing aliphatic side chains

Biosynthesis of Lobelia Alkaloids with Core of Δ^3-Dehydropiperidine

The biosynthetic pathways of Lobelia alkaloids in which the core is Δ^3-dehydropiperidine (tetrahydropyridine) ring instead of piperidine have not been studied, except of the anatabine.

It is interesting that anatabine was not derived from lysine, but nicotinic acid. (This alkaloid is characteristic for *Nicotiana* plants but was also detected in *Lobelia inflata*) (Fig. 11.21), [1].

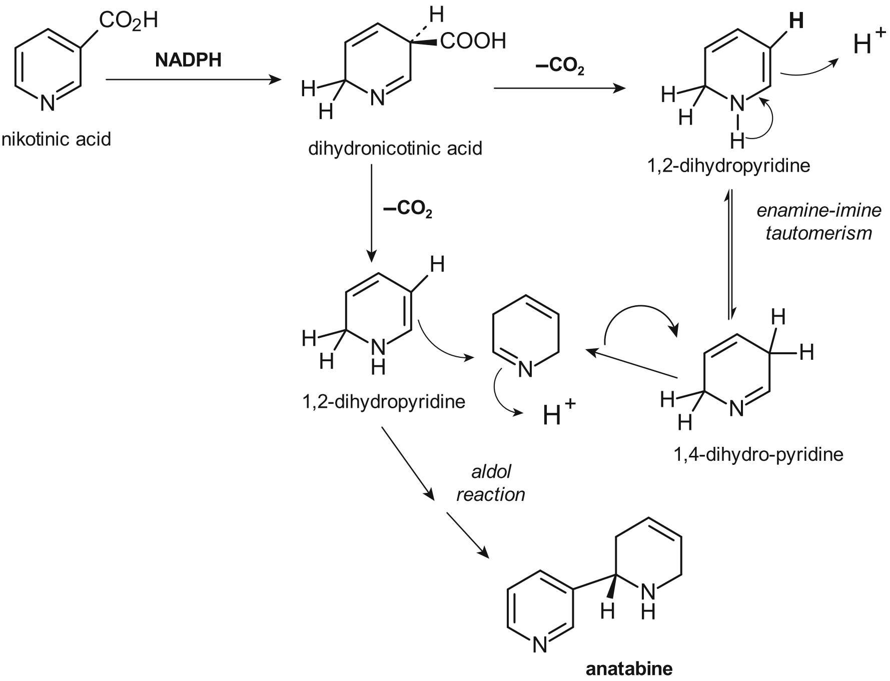

CO2H
NADPH
nikotinic acid
COOH
dihydronicotinic acid
−CO2
1,2-dihydropyridine
H+
enamine-imine tautomerism
−CO2
1,2-dihydropyridine
H+
1,4-dihydro-pyridine
aldol reaction
anatabine

Fig. 11.21 Biosynthesis of anatabine

2.3.2 Occurrence of Lobelia Alkaloids: *Lobelia inflata* L. (Indian Tobacco)

Lobelia inflata L. (Fig. 11.22) belongs to the order *Campanulales* and to the family *Lobeliaceae*. There are 400 species in Lobelia genus and 2 of them are native in Europe (*Lobelia dortmanna* L. and *Lobelia urens* L.) [29]. The Lobelia can grow as a biennial but usually grows as an annual [30, 31].

The habitat of *L. inflata* L. is North America, where it grows wild in forest, pastures, and at roadsides, though in many countries (the USA and Holland) it is also cultivated [3, 32] because of its active ingredients.

L. inflata is grown from seed which is shown either in the autumn or in March and April. It is a 40–60 cm high annual plant flowering from June to August. It bears alternate leaves 3–8 cm long and pale blue and about 7 mm long bilabiate flowers. The inferior ovary develops into an inflated capsule. The drug is supplied by the flowering shoot. After drying, the drug is exported in bales or compress packets. The seeds are sometimes separated by thrashing.

The plant was given its Latin name after Mathias von L'Obel, Flemish physician and botanist (1538–1616), who was the first to describe it. The term "inflata" refers to the bladder-like inflated capsule [33].

Lobelia has long been used by the North American Indians. It was recommended for use in asthma by Cutler in 1813 and was introduced to the English medical

Lobelia inflata

Fig. 11.22 *Lobelia inflata* L.

profession by Reece in 1829. The drug is known under several designations: e.g., in America the leaves of the plant are used in pipe by name of "Indian tobacco" (they recall the smell of tobacco), or are put into circulation as "*emetic herba*" owing to their nauseating effect [34].

2.3.3 Analysis of Lobelia Alkaloids

The Lobelia alkaloids were first detected by Procter in 1850. At the beginning of the twentieth century, Wieland and Scheuing isolated the first alkaloids from *L. inflata*. The most important alkaloid of the family Lobeliaceae is lobeline, which is an α, α_1-disubstituted piperidine. Further, other alkaloids of *L. inflata* have been isolated, with their structures described [29, 34, 35].

The typical alkaloids of the family Lobeliaceae have a piperidine skeleton. There are alkaloid bases disubstituted in the α, α_1-position (i.e., in C_2 and C_6) and those monosubstituted in the α-position (C_2) (Figs. 11.23, 11.24] [36].

Group I. Alkaloids, Substituted in Positions C_2 and C_6

1. Lobelidione and norlobelidione analogues
2. Lobelionol and norlobelionol analogues
3. Lobelidiol and norlobelidiol analogues

Group II. Alkaloids, Substituted in Positions C_2

1. Lobelol and norlobelol analogues
2. Lobelone and norlobelone analogues

Alkaloid Extraction

The efficiency of the extraction methods in acid-aqueous media (0.1 M HCl, or 0.1 M HCl-methanol) or in chloroform solution (for pre-alkaline samples) was investigated. Various devices (ultrasound device, shaking machine, Soxhlet-apparatus) were used for the extraction. The best method was the extraction with 0.1 M HCl- methanol (1:1, *v*/*v*) in ultrasound device.

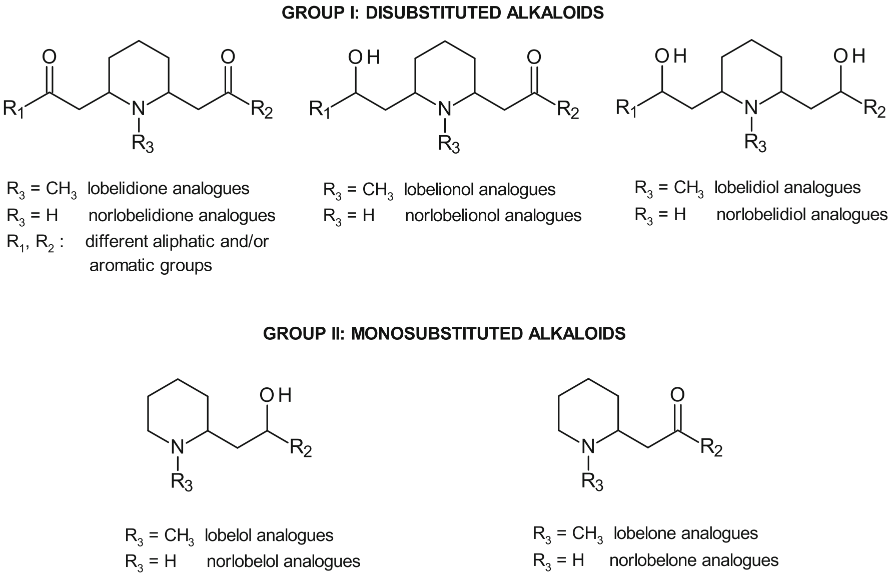

GROUP I: DISUBSTITUTED ALKALOIDS
O
O
R1
N
R2
R3
R3 = CH3 lobelidione analogues
R3 = H norlobelidione analogues
R1, R2 : different aliphatic and/or aromatic groups
OH
O
R1
N
R2
R3
R3 = CH3 lobelionol analogues
R3 = H norlobelionol analogues
OH
OH
R1
N
R2
R3
R3 = CH3 lobelidiol analogues
R3 = H norlobelidiol analogues
GROUP II: MONOSUBSTITUTED ALKALOIDS
OH
N
R2
R3
R3 = CH3 lobelol analogues
R3 = H norlobelol analogues
O
N
R2
R3
R3 = CH3 lobelone analogues
R3 = H norlobelone analogues

Fig. 11.23 Alkaloids characteristic of the genus *Lobelia*

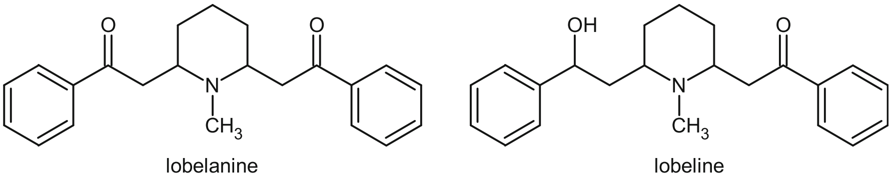

O
O
N
CH3
lobelanine
OH
O
N
CH3
lobeline

Fig. 11.24 Two characteristic representatives alkaloids of the genus *Lobelia*

The alkaloid extraction method was suitable for the extraction of polyacetylenes as well; in this way, several kinds of effective substances could be analyzed from one stock solution.

Samples of this solution were filtrated and purified by solid-phase extraction (SPE) before HPLC analysis.

Determination of Total Alkaloid Content by Spectrophotometric Method

The total alkaloid content was determined by spectrophotometry after Mahmud El-Masry [37] and Krajewska [38, 39]. 0.01 % methyl-orange solution was added to the neutralized extract and then it was transferred to the separating funnel and extracted with chloroform. The chloroform layer was removed and transferred to another separating funnel and extracted with 0.1 N HCl solution containing 5 % NaCl. The absorption of the red color solution was measured at 510 nm. Total alkaloids were calculated as lobeline base.

SPE Purification

Solid-phase extraction (SPE micro-columns) was efficient for the purification of alkaloidous solutions. Aliquots were passed through a pre-activated (by methanol than water) SPE cartridge (Supelclean LC-8 columns 500 mg, 3 mL, supplied by Supelco) which was then washed with water. Extraction was performed on a vacuum manifold processor (LiChrolut extraction unit; Merck).The alkaloid (lobeline)-containing fraction was eluted from the tube with methanol. On the basis of the HPLC determination, the recovery of lobeline from the SPE step was total [40].

HPLC-DAAD Analysis: Quantitative Determination of Lobeline

An HPLC-DAD method was developed for determination of lobeline from in vitro and in vivo cultures of *L. inflata*.

HPLC analysis was performed on a Surveyor (Thermo Finnigan) HPLC system consisting of a quaternary gradient pump with an integrated degasser, a PDA detector, and an autosampler. Compounds were separated on a Knauer Eurospher 100-C8 (5 μm) reversed-phase column (250 × 3 mm i.d.) integrated with a precolumn (5 × 3 mm i.d.). The column temperature was 25 °C and the injection volume 5 μL. The mobile phase was 30:70 (*v*/*v*) acetonitrile-0.1 % trifluoroacetic acid. The flow rate was 0.8 mLmin^{-1}. The lobeline peak was identified by the addition of authentic standard (lobeline base) and by diode-array detection [40].

Quantitative Determination of Lobeline

Quantitative determination of lobeline was performed by the external standard method. Standard solutions containing lobeline at 2.25, 5, 12.5, 20, 40, and 80 μg mL^{-1} were prepared in 0.1 N HCl. Each standard solution was injected (5 μL) in triplicate on to the HPLC column and the absorbance was recorded at 250 nm. The calibration graph for lobeline was constructed by plotting the peak areas against the corresponding concentrations. The concentration of lobeline in samples was calculated from its peak area by use of the calibration plot [41].

Validation studies proved that the repeatability of the method was good and the recovery was satisfactory [40].

HPLC-MS/MS Characterization of Piperidine Alkaloids of *Lobelia inflata*

For direct characterization of disubstituted piperidine alkaloids in extracts of *L. inflata*, tandem mass spectrometric method was developed using electrospray ionization. Analysis was performed in positive ion mode on a triple quadropole LC/MS system. The identification and structural elucidation of the alkaloids were performed by comparing their changes in molecular mass (Δ*M*), full-scan MS-MS spectra with those of lobeline, lobelanine, norlobelanine, and lobelanidine. These alkaloids and ten other derivatives were identified in the plant extracts.

Analysis was performed in positive ion mode on an Agilent 6410 Triple Quad LC/MS system (Wilmington, DE, USA) using electrospray ionization. Alkaloids were separated on a Knauer Eurospher 100-C8 (5 μm) reversed-phase column (250 mm x 3 mm I.D.), with precolumn (5 mm x 3 mm I.D.). The column temperature was set at 25 °C. The mobile phase was 30:70 (*v*/*v*) acetonitrile-30 mM ammonium formate, pH 2.80. The solvent flow rate was 0.8 mLmin^{-1}. The injection volume was 5 μL. By solvent splitting, 40 % eluent was allowed to flow into the mass spectrometer. The conditions of the LC-MS/MS analysis were as follows: nebulizer pressure 45.0 psi, drying gas flow rate 9 Lmin^{-1}, drying gas temperature 350 °C, capillary voltage 3500 V, and scan range from *m*/*z* 50–700 at collision energy of 15 or 20 eV, depending on the molecular structure [40].

LC-MS and LC-MS/MS Analysis of Lobeline and Norlobelanine Standards

At beginning of the investigations the chromatographic and mass spectrometric conditions were optimized by using alkaloid standards. The voltage for fragmentation and collision energy was optimized using dead volume in LC-MS and MS/MS experiments. Full-scan analysis provided information about the molecular ion of standards. Molecular ions were then used as precursor ions for product ion analysis. The MS/MS spectra obtained provided information about the characteristic fragment ions and neutral losses which were the bases for identification of analytes in the extract of *L. inflata*.

The mass spectra of lobeline and norlobelanine standards revealed a base peak at *m*/*z* 338 and 322, respectively, corresponding to the molecular ions $[M + H]^+$. The product ion mass spectra were obtained by choosing the molecular ions as the precursor ions (Fig. 11.25).

Fragmentation of the protonated molecular ion of lobeline in the instrument led to product ions of *m*/*z* 320, 218, 216, 200, 105, 98, and 96 (Fig. 11.25). The subordinate product ion at *m*/*z* 320 was formed by loss of H_2O from the molecular ion at *m*/*z* 338. The ion at *m*/*z* 218 was produced by the loss of a phenyl-2-ketoethyl side chain ($C_8H_8O_1$, 120 Da). The more abundant product ion at *m*/*z* 216 was formed by loss of a phenyl-2-hydroxyethyl unit ($C_8H_{10}O_1$, 122 Da). A loss of water can be observed at *m*/*z* 200 (218-18). The most abundant product ion at *m*/*z* 96 was formed by loss of both side chains and corresponds to *N*-methylated 1,3-dihydropyridine ion.

Fragmentation of the molecular ion at *m*/*z* 322 of norlobelanine led to product ions at *m*/*z* 202 and 82. These fragment ions and their characteristic neutral losses 120 Da (322 → 202) and 2x120 Da (322 → 82) indicated that two phenyl-2-ketoethyl side chains were present in the molecule. The daughter ion at *m*/*z* 82 was 14 Da less than the daughter ion at *m*/*z* 96 for lobeline, indicating that norlobelanine is a *N*-demethyl derivative of lobelia alkaloids.

These characteristic product ions and neutral losses were the sound bases to identify derivates of lobeline.

LC-MS/MS Identification of Alkaloids in the Herb of *L. inflata* (In Vivo)

The total ion chromatogram of an extract of *L. inflata* is presented by method of Kursinszki et al. [40] in Fig. 11.26. The LC–MS/MS spectrum of each analyte is presented in Fig. 11.27. The LC-ESI-MS data are shown in Table 11.1.

Among them, the retention time and the MS and MS-MS spectra of the molecular ions at *m*/*z* 338 (Peak **j**, Fig.11.27j), 340 (Peak **h**, Fig. 11.27h), and

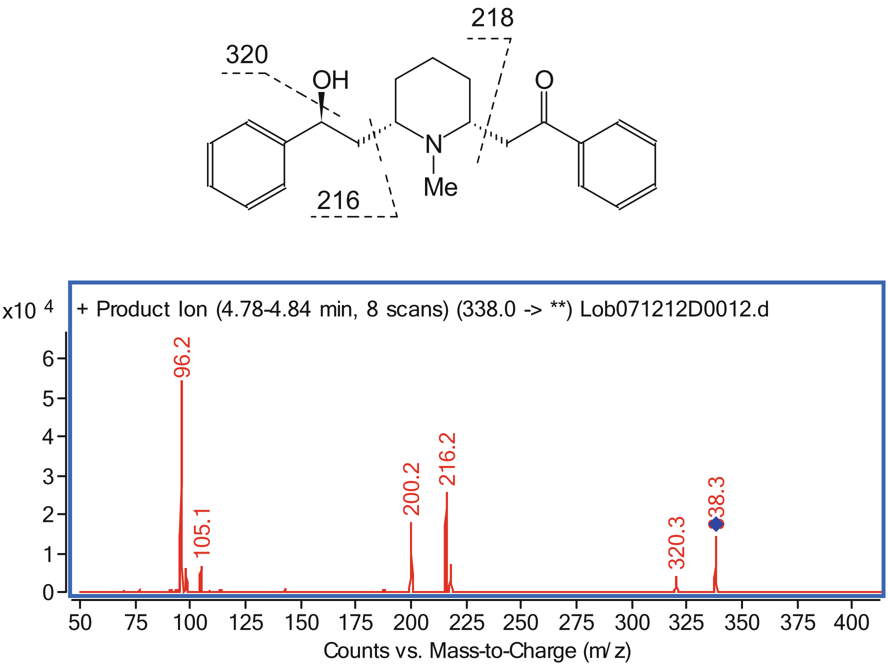


Fig. 11.25 Predominant fragmentation pattern and MS-MS product ion spectrum of (−) lobeline

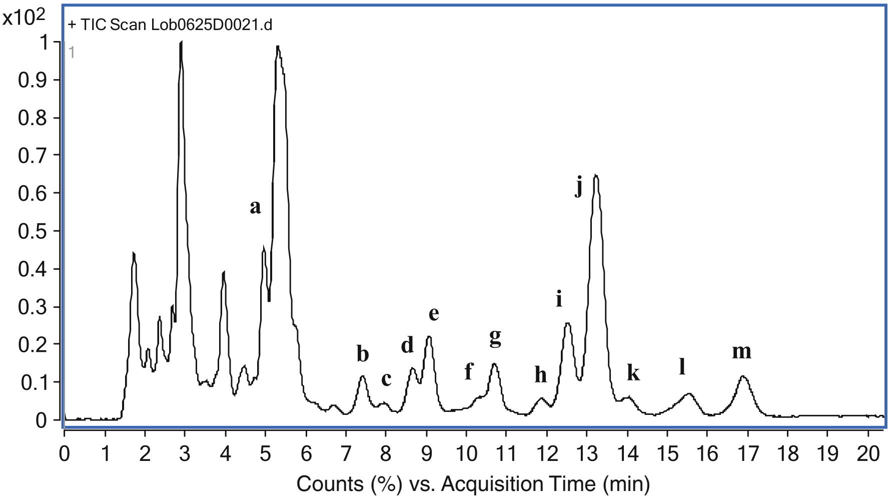


Fig. 11.26 HPLC-MS total ion chromatogram for the extract of the herb of *Lobelia inflata*

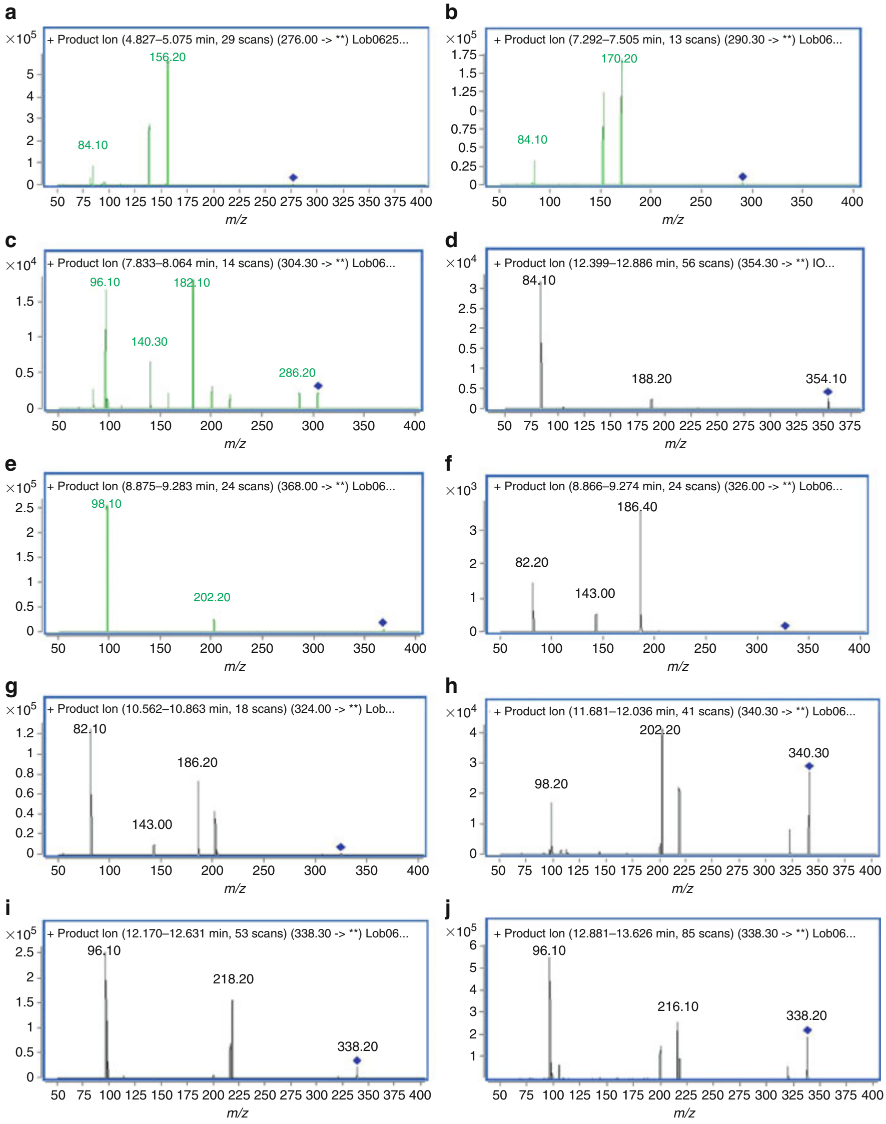


Fig. 11.27 (continued)

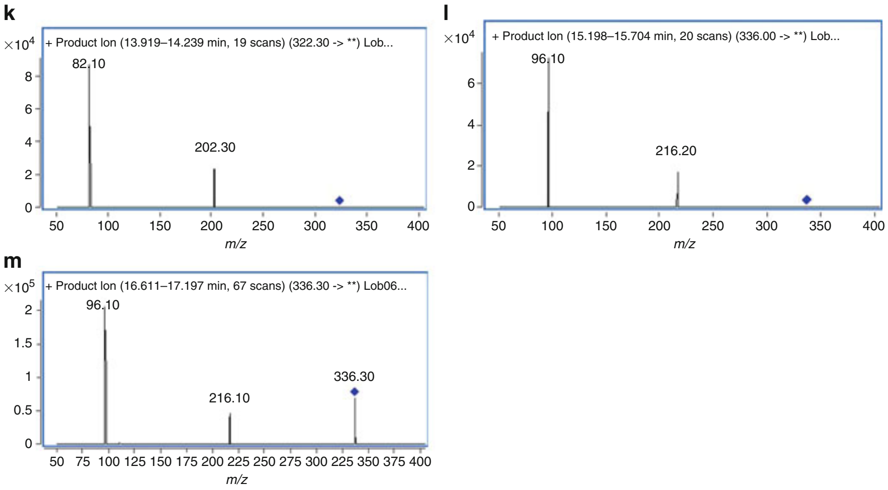


Fig. 11.27 (**a**–**m**) HPLC-MS/MS product ion spectra of alkaloids detected in the extract of the herb of *Lobelia inflata* cultivated in the field

Table 11.1 LC-ESI-MS data of the alkaloid extract of *Lobelia inflata*

Peak	t_R(min)	$[M + H]^+$	MS-MS (*m*/*z*)
a	4.95	276	82.1; 84.1; 138.2; 156.2
b	7.41	290	84.1; 152.2; 170.2
c	8.00	304	83.9; 84.3; 96.1; 98; 140.3; 158; 182.1; 200.3; 218.1; 286.2; 304.3
d	8.66	354	84.1; 188.2; 354.1
e	9.07	368	98.1; 202.2
f	10.29	326	82.2; 143; 185.9; 186.4
g	10.70	324	82.1; 143; 186.2; 202.1
h	11.86	340	98.2; 200.3; 202.2; 218.1; 322.3; 340.3
i	12.54	338	96.1; 98.1; 216.1; 218.2; 338.2
j	13.23	338	96.1; 98.1; 105.1; 200.2; 216.1; 218.1; 320.3; 338.2
k	14.06	322	82.1; 202.3
l	15.53	336	96.1; 216.2
m	16.87	336	96.1; 216.1; 336.3

322 (Peak **k**, Fig. 11.27k) were almost the same as those of lobeline, lobelanidine, and norlobelanine standards, respectively. Peak j can therefore be confirmed as lobeline, Peak **h** as lobelanidine, and Peak k as norlobelanine.

Above lobeline (Peak **j**), another chromatographic peak of *m*/*z* 338 was also detected in its LC-MS-MS chromatogram with retention time of 12.54 min (Peak **i**, Fig. 11.27i). The characteristic product ions at *m*/*z* 218, 216, and 96 were the same in both cases. Therefore, Peak **i** could be identified as an isomer of lobeline (lobelidine).

The molecular ion at 324 (Peak **g**, Fig. 11.27g) and its daughter ions at *m*/*z* 202, 186, and 82 were all 14 Da less than the molecular ion *m*/*z* 338 of lobeline standard and its daughter ions *m*/*z* 216, 200, 96, respectively. Therefore, Peak g could be identified as *N*-demethyl derivative of lobeline (norlobeline).

The molecular ion at *m*/*z* 326 (Peak **f**, Fig. 11.27f) was 14 Da less than the molecular ion at *m*/*z* 340 for lobelanidine. The fragment ions at *m*/*z* 186 and 82 could be formed by concomitant loss of two phenyl-2-hydroxyethyl units. Peak **f** can therefore be confirmed as norlobelanidine.

Two chromatographic peaks of *m*/*z* 336 were detected in its LC-MS-MS chromatogram with retention times of 15.53 min (Peak **l**, Fig. 11.27l) and 16.87 min (Peak **m**, Fig. 11.27m). Fragmentation of their molecular ions showed the same pattern. Their product ions at *m*/*z* 216 and 96 and their characteristic neutral losses 120Da and 2 × 120Da, respectively, indicated that two phenyl-2-ketoethyl side chains were present in the molecule. The product ion at *m*/*z* 96 can be *N*-methylated 1,3-dihydropyridine ion. Therefore, Peak **l** and Peak **m** should be lobelanine isomers.

Fragmentation of the molecular ion at *m*/*z* 276 (Peak **a**, Fig. 11.27a) led to product ions at *m*/*z* 156, 138, 84 and 82. The most abundant product ion at *m*/*z* 156 was formed by loss of a phenyl-2-ketoethyl part (120 Da, 276 → 156). A loss of water can be observed at *m*/*z* 138 (156-18). The ion at *m*/*z* 82 can be produced by loss of an ethyl-2-hydroxyethyl unit (74 Da, 156 → 82). Therefore Peak **a** can be identified as 8-ethyl-10-phenyl-norlobelionol.

The molecular ion at *m*/*z* 290 (Peak **b**, Fig. 11.27b) and the characteristic product ions at *m*/*z* 70 and 152 are all 14 Da more than *m*/*z* 276 and its daughter ions *m*/*z* 156 and 138, respectively. Therefore, Peak b could be identified as an *N*-methyl derivative of Peak **a** (8-ethyl-10-phenyllobelionol).

The molecular ion at *m*/*z* 304 (Peak **c**, Fig. 11.27c) can lead to six characteristic product ions at *m*/*z* 286, 218, 200, 182, and 96. The ion at *m*/*z* 286 was formed by loss of water from the molecular ion at *m*/*z* 304. The daughter ion at *m*/*z* 182 can be due to loss of a phenyl-2-hydroxyethyl unit (122 Da). Similar fragment loss 122 Da could be possible at *m*/*z* 96 (218-122). The *m*/*z* 182 ion could lose neutral fragment 86 Da to produce ion at *m*/*z* 96. This neutral fragment is 14 Da more than an ethyl substituted 2-ketoethyl unit characteristic to ethyl substituted lobelidion or lobelionol alkaloids of *L. inflata*. The results indicate that Peak **c** could be 8-propyl-10-phenyllobelionol. Our assumption is promoted by the fact that 8,10 dipropyllobelidione is described as a reaction product of 3-oxohexanoic acid and 2,3,4,5-tetrahydropiperidine and is considered as an intermediate in the biosynthesis of ethyl substituted lobelia alkaloids.

The fragmentation of the molecular ion at *m*/*z* 354 (Peak **d**, Fig. 11.27d) led to daughter ions, only two at *m*/*z* 188 and 84. The ion at *m*/*z* 188 might be explained by a neutral loss of 3-hydroxy-3-phenylpropanoic acid from its ester form resulting in dehydroxylated norallosedamine. This acid has been isolated from *L. inflata* and is considered as an intermediate in the biosynthesis of the phenyl-2-hydroxyethyl side chains of lobelia alkaloids. A possible fragment loss of 104 Da at *m*/*z* 84 (188–84) can correspond to the removal of dehydroxylated phenyl-2-hydroxyethyl unit. Further, the ion at *m*/*z* 84 can be an indicator of that the parent piperidine ion was monosubstituted. Peak **d** can therefore be an ester of 3-hydroxy-3-phenylpropanoic acid and norallosedamine (3-hydroxy-3-phenylpropanoic norallosedaminate). This proposed structure has not been described in *L. inflata*.

The molecular ion at *m*/*z* 368 (Peak **e**, Fig. 11.27e) and its daughter ions at *m*/*z* 202 and 98 were all 14 Da more than *m*/*z* 354 (Peak **d**) and its daughter ions *m*/*z* 188 and 84, respectively. Therefore, Peak **e** could be identified as *N*-methylated derivative of Peak **d** (3-hydroxy-3-phenylpropanoic allosedaminate).

Summarizing, the HPLC-DAD method of Kursinszki et al. [40] is reliable, simple, and sensitive. It can be used for determination of lobeline in the extracts of *L. inflata*. LC-MS-MS quadropole with electrospray ionization method was very suitable for the identification of lobeline and related piperidine alkaloids in *L. inflata*.

Piperidine Alkaloids in Culture of *Lobelia inflata* Under In Vitro, Greenhouse, and Field Conditions

Previously, the biosynthetic activity of cell suspension, callus, and genetically transformed hairy root cultures of *L. inflata* have been reported [34, 42–44]. The establishment of *L. inflata* in vitro cultures with high amount of active ingredients was an important precondition of investigating the in vivo cultures. With the aim of establishing the large-scale production of pharmaceutically important active ingredients, *L. inflata* was cultivated in greenhouse and in the field.

The secondary metabolite production of *L. inflata* was studied under in vitro, greenhouse, and field conditions. In vitro organized cultures with intensive growth and considerable secondary metabolite production were suitable objects for the cultivation of plants in greenhouse and in the field. The alkaloid content of greenhouse grown plants increased. The survival rate of the plants cultivated in the field was 100 %.

In Vitro Cultivation of *L. inflata*

The organized plants were cultured on solid Murashige-Skoog media [45] containing 3 % sucrose. Intensive growth could be observed for in vitro cultivation (Fig. 11.28) [44, 46, 47].

Cultivation in Greenhouse

In vitro cultures were transplanted to greenhouse after 2.5 weeks. The cultures were transplanted into a mixture of soil, perlite, and peat (2:1:1, *v*/*v*/*v*). Acclimatization of the plants was achieved by cultivation in greenhouse for 4 weeks (Fig. 11.28) [48].

Cultivation in the Field

After 4 weeks of greenhouse cultivation, *L. inflata* seedlings were transplanted to the field with a small soil ball, in early July, in Hungary. The distance between the plants was 5 cm (in every line) and between the lines it was 30 cm. After the first week of field cultivation, the plants were watered only rarely (Fig. 11.28) [48–51].

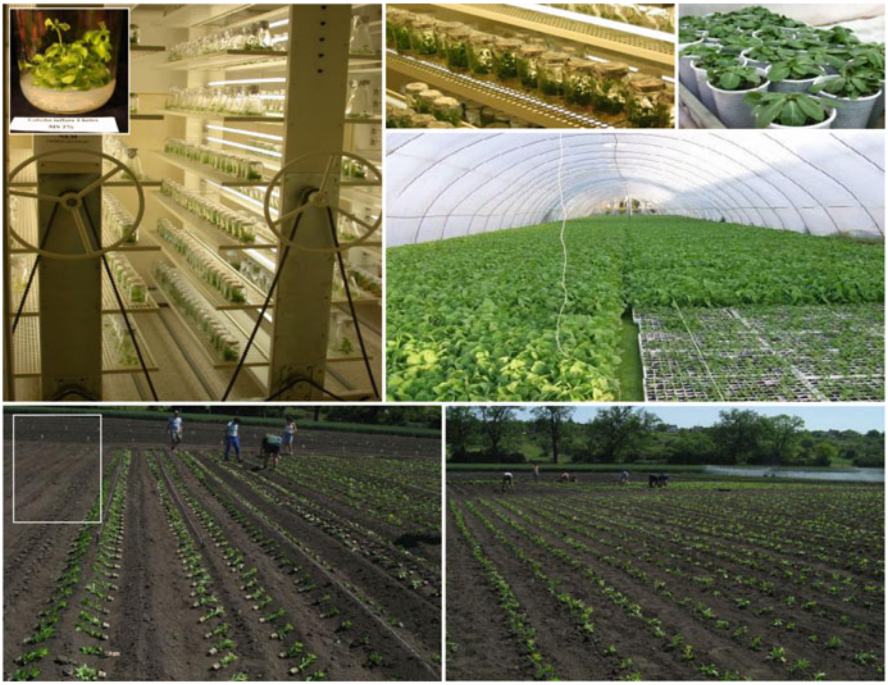


Fig. 11.28 Propagation of *Lobelia inflata* organized cultures in vitro and their acclimatization in greenhouse and cultivation in the field

The aim of experiments was the in vivo cultivation of *L. inflata*, in Hungary. In view of the good in vitro biosynthetic activity of cultures, it seems promising to start the experiments with in vivo cultures. The total alkaloid and lobeline content increased during the greenhouse cultivation. The total alkaloid production was three- to fourfold higher in both organs, and the lobeline production was 12-fold higher in the shoots of the plants cultivated in greenhouse than in the in vitro cultures. The total alkaloid and lobeline content was lower in plants cultivated in the field than in the seedlings cultivated in greenhouse.

For identification of alkaloids of plant obtained by vegetative micro propagation, the LC-MS/MS quadropole with electrospray ionization method was very suitable. In plant cultivated on field, 52 alkaloids (lobeline and related piperidine alkaloids) were found and identified. Of them, as main component, was identified the 8-ethyl-10-phenyl-lobelienonol isomer of molecular ion $[M + H]^+$ of m/z 288 (Fig. 11.29), [52].

It can be concluded that the in vitro organized cultures of *L. inflata* with intensive growth and considerable secondary metabolite production were suitable objects for the cultivation of plants in greenhouse and in the field. The highest alkaloid content was measured in the cultures cultivated in greenhouse.

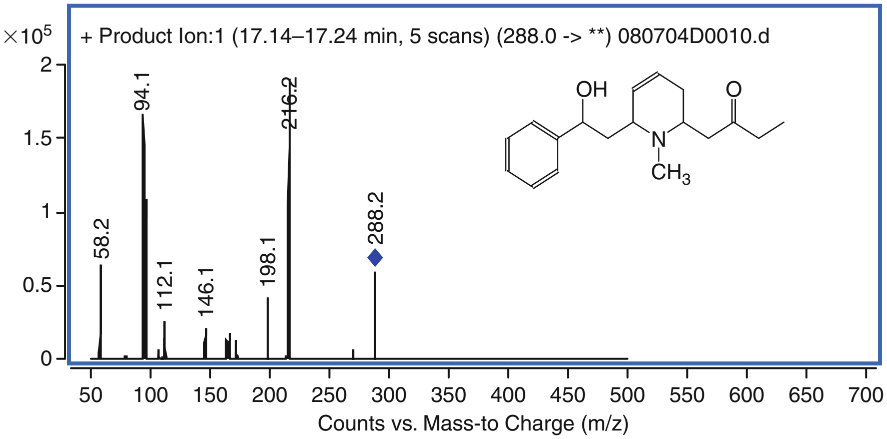


Fig. 11.29 HPLC-MS/MS product ion spectra of 8-ethyl-10-phenyl-lobelienonol isomer (*m*/*z* 288) detected in the extract of the herb of *Lobelia inflata* cultivated in the field

NMR Characterization of Lobeline

On the basis of qualitative and quantitative chromatographic results – isolation and spectroscopic (^{1}H-NMR, ^{13}C-NMR, MS/MS) identification of compounds, as well as comparison with an intact plant and authentic substances – it demonstrated that organized cultures have the ability to synthesize the characteristic alkaloids of the intact plant.

Identification of lobeline by ^{13}C-NMR spectroscopy is presented in Fig. 11.30 [53].

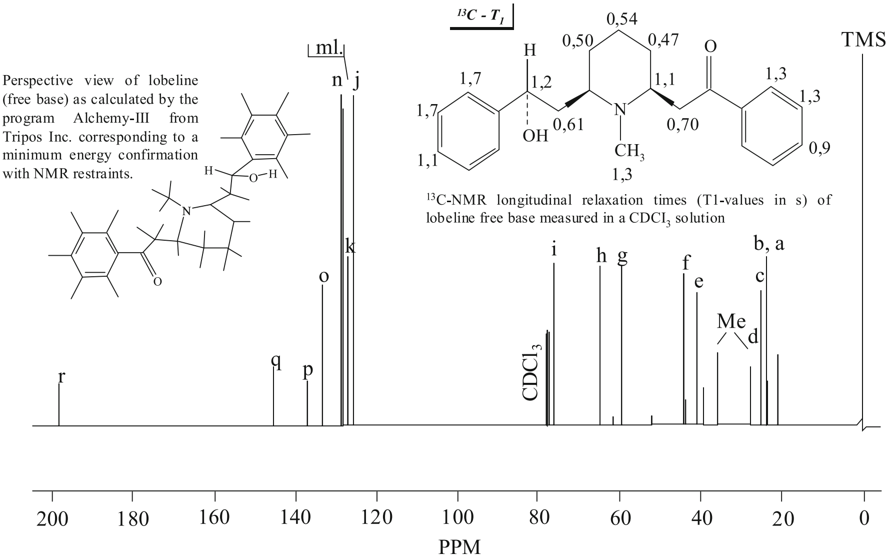
2.3.4 Biological Activity of Lobeline

L. inflata L. is a traditional medicinal plant native to North America. It contains several piperidine alkaloids. The main alkaloid is lobeline which has a stimulatory effect on the respiratory center and it is applied in the cases of asthma, gas- and narcotic-poisoning. Thus, lobeline is currently the subject of renewed interest for the treatment of drug abuse and neurological disorders, like Alzheimer’s or Parkinson’s disease, which pose an important public health problem in industrial countries. As lobeline can act as a competitive nicotinic receptor antagonist, it is frequently used in antismoking preparations. Interest in Lobelia alkaloids, and in particular (-)-lobeline, the most active of them, has increased in recent years due to their activity on the central nervous system (CNS).

Respiratory Stimulant

For a long time, lobeline was known as a powerful respiratory stimulant. This important property has been explained by the activation of the carotid and aortic body chemoreceptors at therapeutic doses.

This alkaloid on the respiratory system has therefore been used with success in numerous applications. Chronic pneumonia, asthma, bronchitis, and laryngitis are all conditions in which lobeline has been a great service.



Perspective view of lobeline (free base) as calculated by the program Alchemy-III from Tripos Inc. corresponding to a minimum energy confirmation with NMR restraints.

^{13}C-NMR longitudinal relaxation times (T1-values in s) of lobeline free base measured in a $CDCl_3$ solution

Fig. 11.30 NMR characterization of lobeline from *Lobelia inflata* in vitro cultures (^{13}C-NMR spectrum of lobeline at 75 MHz in $CDCl_3$ solution)

Lobeline has also been advantageous for the treatment of victims who have been electrocuted or asphyxiated by toxic gasses. Moreover, it was useful in the case of the paralysis of respiratory centers after drug poisoning with alcohol, soporifics, or morphine or after narcosis. Lastly, it has also been used to treat asphyxia in newborn infants.

In 2011, Anand et al. [54] investigated the influence of codeine on lobeline-induced respiratory reflexes and sensations and on ventilation with exercise in healthy subjects. In 15 healthy subjects, the effect of 60 mg oral codeine and placebo was examined on intravenously injected lobeline-elicited respiratory reflexes and sensations. Its influence was also studied on ventilation and appearance of distressful respiratory sensations with modest but incremental exercise. This is the first report of an attenuation of lobeline-elicited respiratory reflexes and sensations that are attributable to J receptors (pulmonary C fibers) by a pharmacological entity. It also suggests that codeine decreased these receptors' known contribution to respiratory augmentation and motor inhibition during exercise, which was seen as a delay in the onset of and a decrease in the magnitude of respiratory discomfort during treadmill walking and an increase in the duration walked by more than half the subjects.

In conclusion, the attenuation of respiratory influences that arose with i.v. lobeline and during treadmill walking by oral codeine suggests that an amelioration of respiratory distress as well as an increase in the duration of exercise undertaken by the latter could be achieved in situations or clinical conditions in which the J receptors would be naturally stimulated.

Drug Abuse

Lobeline has been reported as a useful agent to treat dependency on drugs such as cocaine, amphetamine, caffeine, phenylcycline, opiates, barbiturates, benzodiazepines, cannabinoids, hallucinogens, alcohol, and, especially, nicotine. Dwoskin [55] described a novel mechanism of action and potential use for lobeline as a treatment for psychostimulant abuse.

The most promising area in this field is the ability of lobeline to be a substitute of nicotine. Lobeline produces several physiological effects similar to those produced by nicotine. The use of it as a smoking deterrent was reported in 1936, but several later studies led to a dispute between positive and negative reports.

In 2010, Glover et al. [56] investigated the safety and efficacy of sublingual lobeline sulfate for smoking cessation. A multicenter (3 sites), double-blind, parallel, placebo-controlled, phase 3 smoking cessation trial of sublingual formulation of lobeline sulfate. A total of 750 smokers (250 per site) were randomized to either treatment (lobeline sulfate) or placebo with individual smoking cessation counseling lasting up to approximately 10 min. Results: Efficacy revealed no statistical significance ($P = 0.62$) for lobeline sulfate as a smoking cessation aid. Conclusion: Sublingual formulation of lobeline sulfate does not appear to be an effective smoking cessation aid.

Of late years, several results were born on methamphetamine abuse using pyrrolidine nor-lobelane analogues as lobeline derivates (Fig. 11.31), [57].

Both lobeline and lobelane attenuate methamphetamine self-administration in rats by decreasing methamphetamine-induced dopamine release via interaction with vesicular monoamine transporter-2 (VMAT2). A novel derivative of nor-lobelane, cis-2,5-di(2-phenethyl)-pyrrolidine hydrochloride (UKCP-110), and its transisomers, (2*R*, 5*R*)-trans-di-(2-phenethyl)-pyrrolidine hydrochloride (UKCP-111) and (2*S*, 5*S*)-trans-di-(2-phenethyl)-pyrrolidine hydrochloride (UKCP-112), were evaluated for inhibition of [(3)H]dihydrotetrabenazine binding and [(3)H]dopamine uptake by using a rat synaptic vesicle preparation to assess VMAT2 interaction. Compounds were evaluated for inhibition of [(3)H]nicotine and [(3)H]methyllycaconitine binding to assess interaction with the major nicotinic receptor subtypes.

The most promising compound, UKCP-110, was evaluated for its ability to decrease methamphetamine self-administration and methamphetamine discriminative stimulus cues and for its effect on food-maintained operant responding. It is noteworthy that UKCP-110 decreased the number of methamphetamine self-infusions, while having no effect on food-reinforced behavior or the methamphetamine stimulus cue. Thus, UKCP-110 represents a new lead in the development of novel pharmacotherapies for the treatment of methamphetamine abuse.

Central Nervous System (CNS) Disorders

The most promising bioactivity of lobeline concerns its use as an agent in the treatment of central nervous system (CNS) diseases and pathologies [28].

The effect of lobeline on the CNS was extensively exploited by the Native Americans, who smoked the dried leaves of Lobelia plants. Several studies have

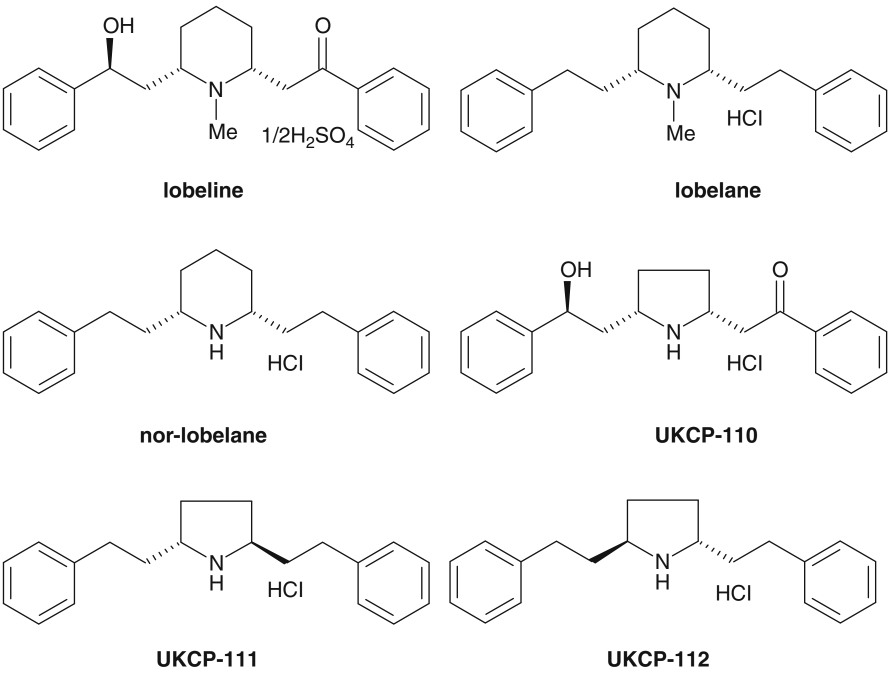


Fig. 11.31 Structures of pyrrolidine norlobelane analogues (UKCP110-112) as lobeline derivates

shown that lobeline improves memory in rodents, probably due to its involvement in cholinergic mechanisms of neurotransmission.

This pharmacological profile may be of great importance in the treatment of learning disorders like Alzheimer's disease (AD), the most common cause of dementia in the elderly.

Indeed, 2 years ago, the cholinergic hypothesis was claimed to explain the cognitive symptoms of AD. Thus, the development of new agents that selectively interact with cholinergic receptors could offer a new opportunity for AD therapy. AD is characterized by a gradual and progressive decline in intellectual function and behavioral abnormalities.

Multidrug-Resistant (MDR) Activity

The emergence of natural products as potential anti-MDR molecules is of particular significance. Many of these essential components may possibly represent a new generation of MDR modulators.

Cancer cells can develop resistance to the anticancer drugs used in cancer therapy. Furthermore, the cells become resistant to other chemotherapeutic drugs whose structures and functions are unrelated. This phenomenon is called multidrug resistance (MDR) [58, 59]. MDR is multifactorial, but P-gp, also known as P-glycoprotein, Pg-170 or P-170, plays an important role in MDR.

Recently, numerous publications were presented which were interested in the multidrug-resistant activity of piperidine like lobeline, piperine [60, 61], and other alkaloids, e.g., cinchonine and acridone [62, 63].

Ma and Wink [60] investigated Lobelia extracts, chiefly lobeline as a new multidrug resistance reversal agent. MDR can limit efficacy of chemotherapy. The best-studied mechanism involves P-gp-mediated drug efflux. This study focuses on MDR reversal agents from medicinal plants, which can interfere with P-gp. Lobeline, a piperidine alkaloid from *L. inflata* and several other *Lobelia* species, inhibited P-gp activity. MDR reversal potential of lobeline could be demonstrated in cells treated with doxorubicin in that lobeline can sensitize resistant tumor cells at nontoxic concentrations. However, lobeline cannot block BCRP (Breast Cancer Resistance Protein)-dependent mitoxantrone efflux. Lobeline could be a good candidate for the development of new MDR reversal agents.

Chemotactic Activity

Chemotactic activity of substances extracted from *L. inflata* was investigated in the frequently used eukaryotic ciliate model, *Tetrahymena pyriformis* GL [64, 65]. The chemotactic activity of cells was measured in a concentration course study ($v/v\% = 10^{-7}$–10^{-1}) by capillary assay [66]. The extracts of *L. inflata* (total extract in methanol, lobeline HCl, and polyacetylenes) proved to elicit diverse migratory responses: (1) a wide range chemoattractant character of the total extract was registered while (2) lobeline HCl and polyacetylene had strong chemorepellent effects (Fig. 11.32).

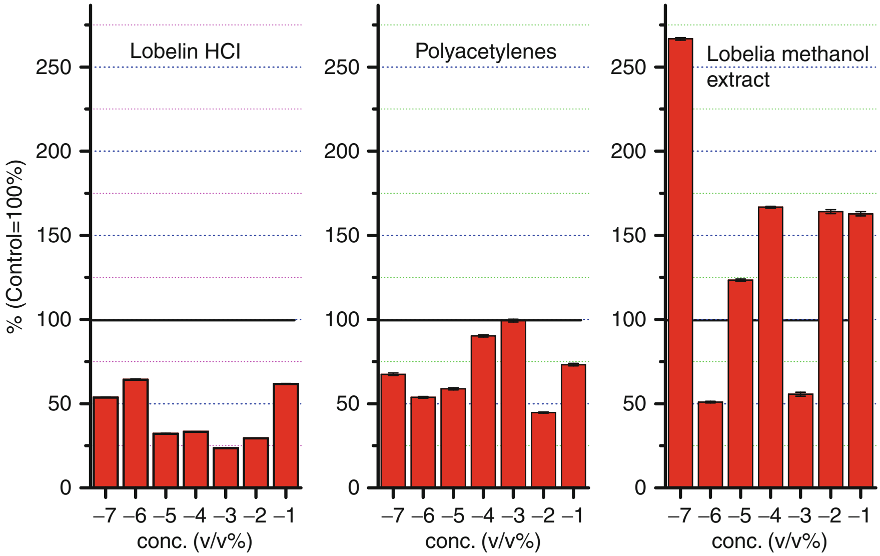


Fig. 11.32 Chemotactic activity of lobeline (repellent), polyacetylenes (repellent) and *Lobelia inflata* extract (attractant) in *Tetrahymena pyriformis*

However, the molecular backgrounds of the detected cell physiological responses and their diversities are still not known the presented data may explain the well known physiological/clinical effects of the extracts prepared from *L. inflata*. The registered chemorepellent characters of extracts have a good correlation with the presence of the generally chemorepellent aromatic R groups [67] in the more than 20 piperidine skeleton alkaloids of Lobelia.

3 Conclusion

In *Punica granatum* beside the long since known compounds as pelletierine, *N*-methylpelletierine, anaferine, and pseudopelletierine, other alkaloids – sedridine, norpseudopelletierine, hydroxypropyl – and propenyl-Δ^1-piperideine were also detected; further, the absolute configuration of (-)- and (+)-sedridine and (-)-pelletierine were determined.

The greatest anthelmintic activity of Punica extracts was chiefly due to isopelletierine but the harmful side-effects led to full abandon of the drug (root bark) and its alkaloids.

In *Piper nigrum* beside piperine were detected its geometric isomer chavicine, then other piperidine amides piperanine, piperettine, and also a pyrrolidine amide the piperylline. From *Piper longum*, nine piperidine amides were isolated, between guigeensine and piperlonguminine, which showed significant MAO inhibitory activities. Piperlonguminine is a novel therapeutic agent for Alzheimer's disease. From *Piper officina*rum, beside piperine, the piplartine piperidine amide and sesamin lignin derivate were detected. A new major urinary metabolite of piperine the 5-(3,4-methylenedioxyphenyl)-2*E*, 4*E*-pentadienoic(3-yl propionic acid)-amide was identified in rat urine and plasma and certified the similar mass fragmentation by MS/MS studies.

Of the numerous new pharmacological and therapeutical activities of piperine are summarized as follows:

– Piperine inhibited LPS-induced shock trough inhibition of type 1 IFN (interferon) production.
– Piperine-treated rat liver microsomes demonstrated a tendency to enhance [3H]-aflatoxin B1 binding to calf thymus DNA in vivo.
– Piperine is a potent inhibitor of nuclear factor κB.
– The later studies have established that black pepper could be safety used as a food additive, regarding the piperine is non-genotoxic and possess antimutagenic and antitumor influences.
– The piperine can reverse MDR by multiple mechanisms and may be a promising lead compound for future studies.

In *Lobelia inflata*, two types of piperidine alkaloids were detected: alkaloids of phenolic side chaines, as lobeline, lobelanine, norlobelanine, sedamine, and the dimer lobinaline; further, alkaloids of aliphatic side chains: diethyllobelidiol and methyl-ethyllobelidiol. It is important to mention that lobelane and nor-lobelane as reduced derivates of lobeline and the three pyrrolidine analogues of nor-lobelane are metamphetamine abuse agents, respectively.

Lobeline, the main alkaloid of *Lobelia inflata*, has the following important biological activities and therapeutical uses:

- Like respiratory stimulant was used success in chronic pneumonia, asthma, bronchitis, laryngitis
- Useful agent against drug abuse
- Promising agent in the treatment of central nervous system (CNS) disorders, like Alzheimer's disease, attention deficit, restlessness, impulsiveness, hyperactivity
- Positive effects in the treatment of multidrug-resistant tumor cells
- Strong chemorepellent effect of lobeline HCl; chemoattractant character of Lobelia alkaloid extract

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Alkaloids Derived from Ornithine: Tropane Alkaloids 12

Stefan Philipov and Tsvetelina Doncheva

Contents

1 Introduction 344
2 Tropane Alkaloid Classification 346
  2.1 Alkaloids Derived from Tropinone 346
  2.2 Alkaloids Derived from Ecgoninone 351
  2.3 Alkaloids Derived from 4-Benzyltropanone 352
  2.4 Pyronotropane Alkaloids 353
  2.5 Dimeric and Trimeric Tropane Alkaloids 354
3 Conclusion 356
References 356

Abstract

The tropane alkaloids are among the first ones detected in nature that have a wide application as various remedies. In this chapter, the tropane alkaloids are classified according to their subsequent biosynthetic pathway of formation. From this point of view, the alkaloids are described as derivatives of four main intermediates – tropinone, ecgoninone, 4-benzyltropanone, and pyronotropane. The derivatives of tropinone are the largest group of tropane alkaloids and are typical for family Solanaceae and Convolvulaceae. By the number of substituents in the tropane ring, they are divided into five subgroups: mono-, di-, and trisubstituted tropanes, epoxytropanes, and calystegines. Alkaloids derived from ecgoninone are mostly distributed in family Erythroxylaceae. 4-Benzyltropanes and pyronotropanes are small groups typical for family Proteaceae. Dimeric and

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trimeric tropane alkaloids found in families Solanaceae, Erythroxylaceae, and Convolvulaceae are also described.

Keywords

Alkaloids • dimeric tropanes • ecgoninone • tropanes • tropinone • L-ornithine • 4-benzyltropanone

Abbreviations

TR I	Tropinone reductase I
TR II	Tropinone reductase II

1 Introduction

The tropane alkaloids comprise a large well-defined structurally group of natural products, occurred mainly in family Solanaceae but also are found in the families Convolvulaceae, Erythroxylaceae, Proteaceae, Rhizophoraceae, etc. [1]. Common structural element of these alkaloids is the tropane skeleton, consisting of a pyrrolidine and piperidine ring sharing the nitrogen atom and two carbon atoms. The systematic name of the tropane skeleton is 8-methyl-8-azabicyclo[3.2.1.]octane (Fig. 12.1). Currently, the number of these alkaloids is more than 200 [2–4].

Precursor in the biosynthesis of tropane alkaloids is the amino acid L-ornithine (**1**) [5–8]. It was found that ornithine undergoes enzymatic decarboxylation to putrescine (**2**) which then is methylated to *N*-methylputrescine (**3**) [9–12]. The oxidation of *N*-methylputrescine (**3**) leads to 4-methylaminobutanal (**4**) that is further converted in the *N*-methyl-Δ^1-pyrrolinium cation (**5**). From the last compound, the four carbon atoms C-1, C-5, C-6, and C-7 and *N*-methyl group in tropane skeleton are originated [9, 13, 14] (Scheme 12.1).

The next steps of formation of the tropane skeleton require addition of two acetate units to *N*-methyl-Δ^1-pyrrolinium cation (**5**) to obtain tropinone (**6**) – dominating pathway for alkaloids in plants belonging to family Solanaceae [15, 16] and ecgoninone (**7**) dominating pathway for alkaloids in plants belonging to family Erythroxylaceae [11, 17, 18]. On the other hand, biogenetically tropinone (**6**) is

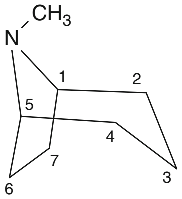
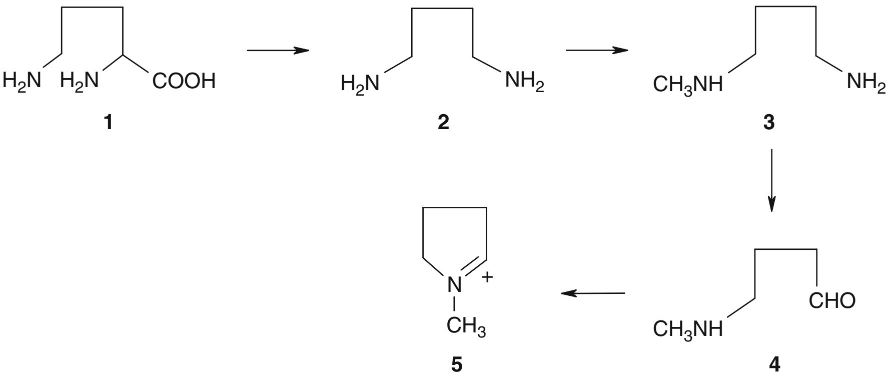
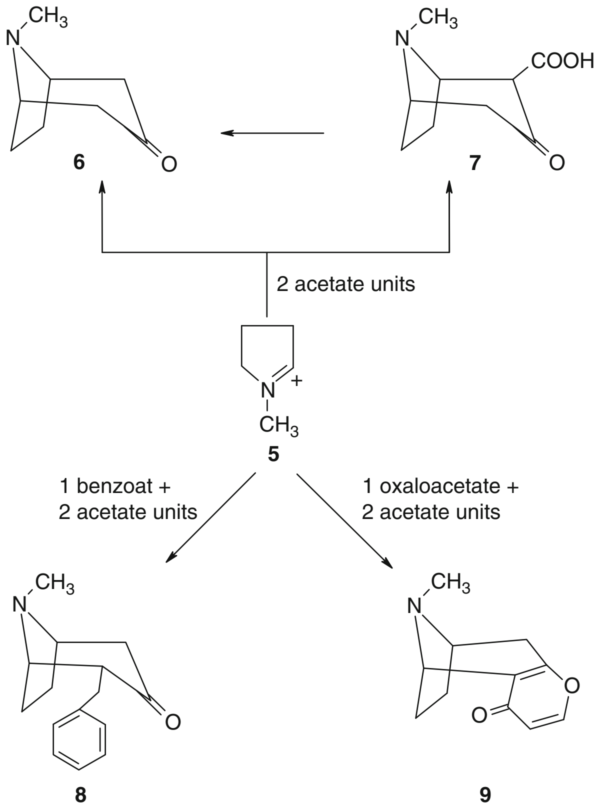


Fig. 12.1 Tropane skeleton


H2N
H2N
COOH
1
H2N
NH2
2
CH3NH
NH2
3
CH3NH
CHO
4
N
+
CH3
5

Scheme 12.1


N
CH3
6
O
N
CH3
COOH
7
O
2 acetate units
N
+
CH3
5
1 benzoat +
2 acetate units
1 oxaloacetate +
2 acetate units
N
CH3
O
8
N
CH3
O
O
9

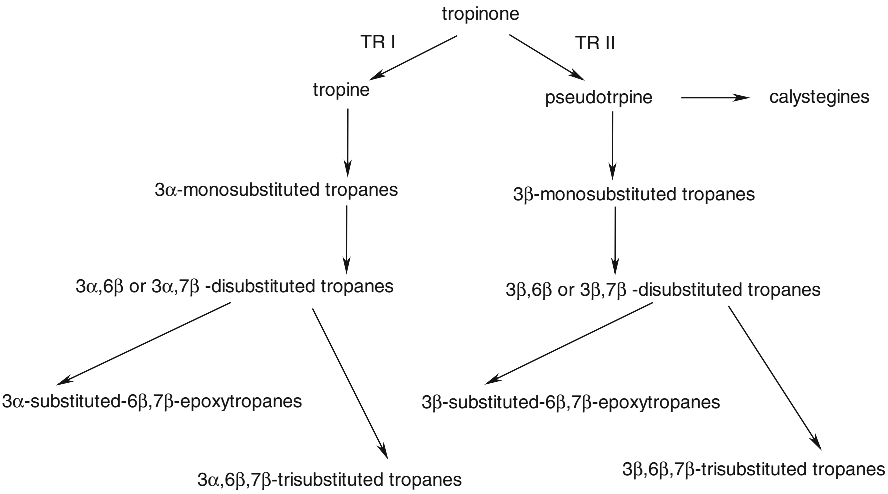
Scheme 12.2

formed and by decarboxylation of ecgoninone (**7**). (Scheme 12.2) [15]. The derivatives of 4-benzyltropanone (**8**) and pyronotropane (**9**) detected in plants belonging to family Proteaceae are assumed to be built up by addition of two acetate units and benzoate and oxaloacetate units to *N*-methyl-Δ^1-pyrrolinium cation (**5**) [19].

2 Tropane Alkaloid Classification

2.1 Alkaloids Derived from Tropinone

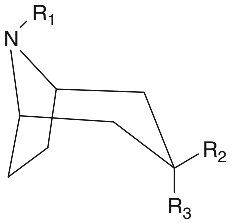
By the action of tropinone reductase I (TR I) and tropinone reductase II (TR II), tropinone (**6**) is reduced to tropine (**10**) and pseudotropine (**11**), respectively [20–23] (Scheme 12.3). The correlation between the two enzymes is essential for the diversity of the alkaloid spectrum of the plants producing 3-monosubstituted, 3,6- or 3,7-disubstituted, 3-substituted-6,7-epoxy- and 3,6,7-trisubstituted tropane alkaloids, respectively [24, 25]. It is determined that TR I is dominant for the species of family Solanaceae; therefore, the alkaloids of α-series, derived from tropine, are prevailing [26]. It is established the configuration of the substituents at C-6 and C-7 is always β- [27]. The obtained tropine (**10**) and pseudotropine (**11**) further is esterified with various aliphatic and aromatic carboxylic acids such as tropic, apotropic, tiglic, propionic, butyric, isobutyric, and other acids [19].


tropinone
TR I
TR II
tropine
pseudotrpine
calystegines
3α-monosubstituted tropanes
3β-monosubstituted tropanes
3α,6β or 3α,7β -disubstituted tropanes
3β,6β or 3β,7β -disubstituted tropanes
3α-substituted-6β,7β-epoxytropanes
3β-substituted-6β,7β-epoxytropanes
3α,6β,7β-trisubstituted tropanes
3β,6β,7β-trisubstituted tropanes

Scheme 12.3

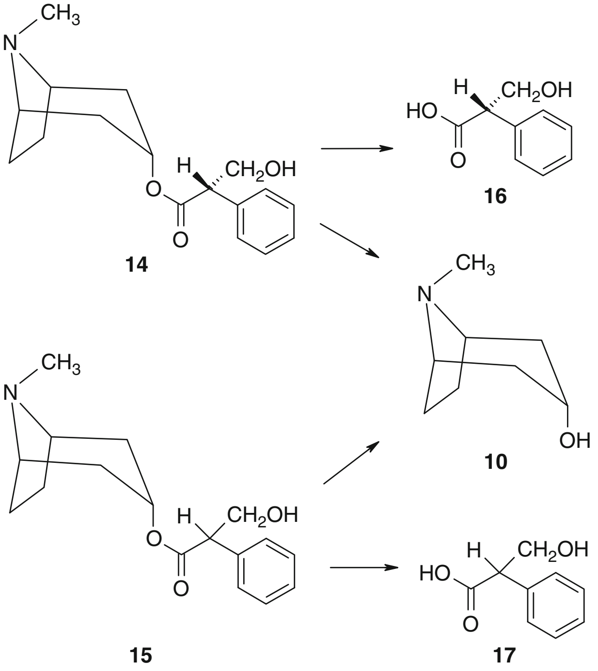
2.1.1 3-Monosubstituted Tropanes

All members of the monosubstituted tropane group are derived from tropine (**10**), pseudotropine (**11**), and their norderivatives nortropine (**12**) and pseudonortropine (**13**).



	R_1	R_2	R_3
10	CH_3	H	OH
11	CH_3	OH	H
12	H	H	OH
13	H	OH	H

The most important representative of this group is the alkaloid (*S*)(-)-hyoscyamine (**14**). The alkaloid is ester of tropine with tropic acid. The α-carbon atom of tropic acid is asymmetric, so that two stereoisomers are possible. The natural form, the one occurring in pharmacologically active hyoscyamine, is the (-)-form. The alkaloid is

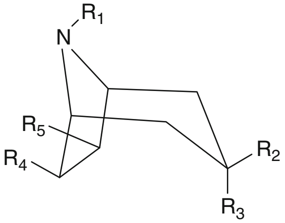


Scheme 12.4

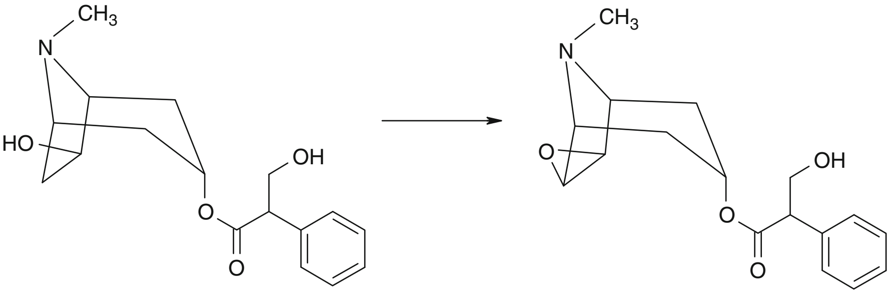
easily racemized, yielding atropine ((±)-hyoscyamine) (**15**), which has the same pharmacological effects as (-)-hyoscyamine (**14**), but with less pronounced effect. The hydrolysis of **14** results in **10** (optical inactive) and (±)-tropic acid (**16**), and **14** to **10** and (-)-tropic acid (**17**) (Scheme 12.4). Atropine (**15**) is one of the first isolated alkaloid from the tropane group as the name "tropane" comes from the species in which it is found – *Atropa belladonna* L. [28].

2.1.2 3,6- and 3,7-Disubstituted Tropanes

The main building unit of disubstituted tropane alkaloids are 3α,6β-, 3β,6β-dihydroxytropane (**18**, **19**) and 3α,7β-, 3β,7β-dihydroxytropane (**20**, **21**) and their corresponding norderivatives (**22**, **23**, **24**, **25**). The alkaloids belonging to this group are mono- or diester derivatives of these intermediate products.



	R_1	R_2	R_3	R_4	R_5
18	CH_3	H	OH	OH	H
19	CH_3	OH	H	OH	H
20	CH_3	H	OH	H	OH
21	CH_3	OH	H	H	OH
22	H	H	OH	OH	H
23	H	OH	H	OH	H
24	H	H	OH	H	OH
25	H	OH	H	H	OH



26 **27**

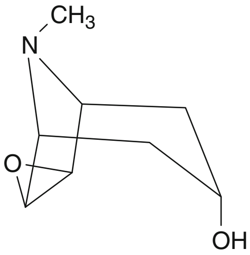
Scheme 12.5

One of the important representatives of this group is the alkaloid 3-tropoyloxy-6-hydroxytropane (**26**) which is the biosynthetic precursor of scopolamine (**27**) (Scheme 12.5).

2.1.3 3-Substituted-6,7-Epoxytropanes

A characteristic feature of this group of alkaloids is that they contain 6β,7β-epoxy ring.

Hydrolysis of esters of 6,7-epoxytropane alkaloids depending on the conditions of the process resulted in obtaining scopine (**28**) (by mild conditions with pancreatic lipase) and oscine (**29**) (by alkaline or acidic hydrolysis) [29].



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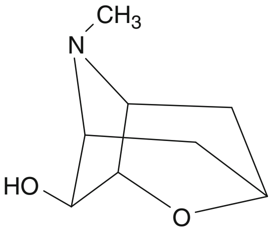

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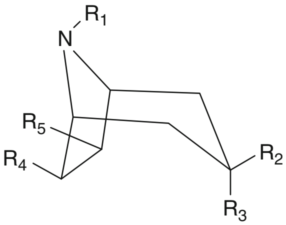
The most noted representative of this group is the alkaloid scopolamine (**27**), whose name comes from the species of the genus *Scopolia* from where it was originally isolated [30].

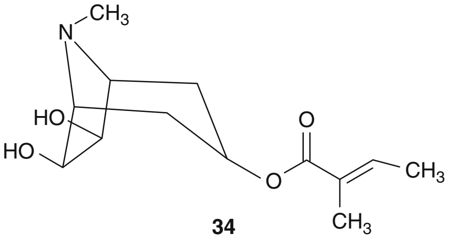
2.1.4 3,6,7-Trisubstituted Tropanes

Trisubstituted tropanes are formally derived from 3α,6β,7β- and 3β,6β,7β-trihydroxytropane (**30**, **31**) as well as their corresponding norderivatives (**32**, **33**).



	R_1	R_2	R_3	R_4	R_5
30	CH_3	H	OH	OH	OH
31	CH_3	OH	H	OH	OH
32	H	H	OH	OH	OH
33	H	OH	H	OH	OH

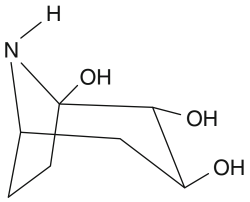
One of the early identified representative of this group is the alkaloid meteloidine-3-tigloyloxy-6,7-dihydroxytropane (**34**) whose name comes from the species *Datura meteloides* from where it was isolated for the first time [31].



34

2.1.5 Calystegines

Pseudotropine, product of TR II, is the specific precursor of calystegines, alkaloids which originally were isolated from different species of genus *Calystegia* (fam. Convolvulaceae) and later from several genera of families Solanaceae, Moraceae, etc. (Scheme 12.3) [32–35]. They are polyhydroxylated nortropane derivatives where the hydroxyl groups are situated on various positions. The trihydroxylated calystegines are summarized as calystegine A-group, the tetrahydroxy derivatives as the calystegine B-group, and the pentahydroxy respectively calystegine C-group [33, 36–38]. In contrast to the most tropane alkaloids, their hydroxyl groups are not esterified [39]. Widespread representatives of calystegine group are the alkaloids calystegine A$_3$ (**35**), calystegine B$_1$ (**36**), calystegine B$_2$ (**37**), and calystegine C$_1$ (**38**)



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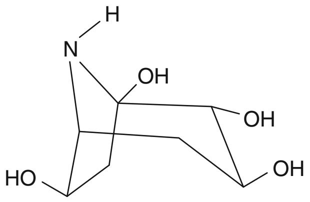

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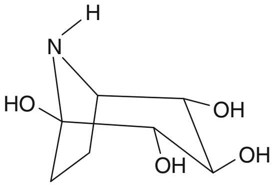

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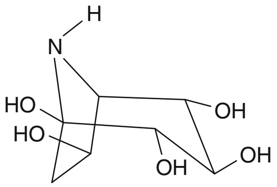

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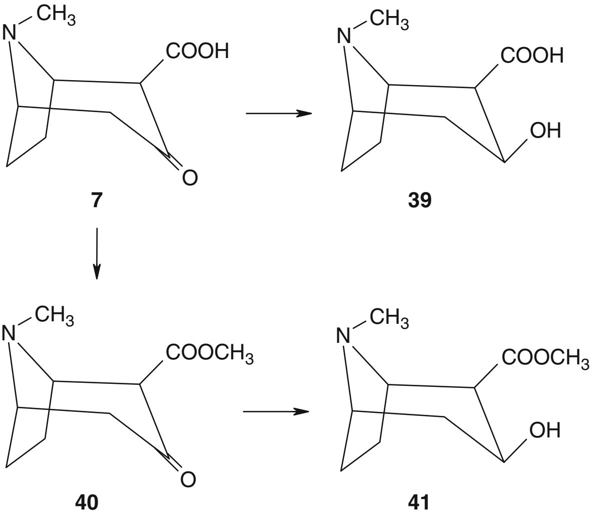
Scheme 12.6

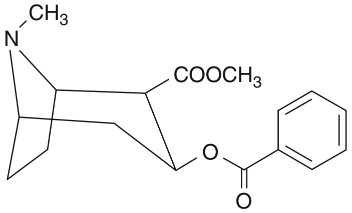


2.2 Alkaloids Derived from Ecgoninone

All members of this group of alkaloids are biosynthesized through the reduction of ecgoninone (**7**) to ecgonine (**39**) and subsequent esterification of the hydroxyl group at C-3 with various aliphatic and aromatic acids. In some cases, this stage is preceded by methylation of carboxylic group to methylecgoninone (**40**), which is then reduced to methylecgonine (**41**) (Scheme 12.6).

The alkaloids derived from ecgonine are typical for the plants of the family Erythroxylaceae. It is established the configuration of the substituents at C-2 and C-3 in all identified alkaloids of this type is always β- [27].

Well-known alkaloid of this group is cocaine (**42**), whose name comes from the species *Erythroxylum coca* from where it was originally isolated [40].



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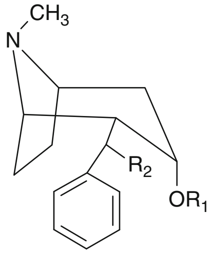
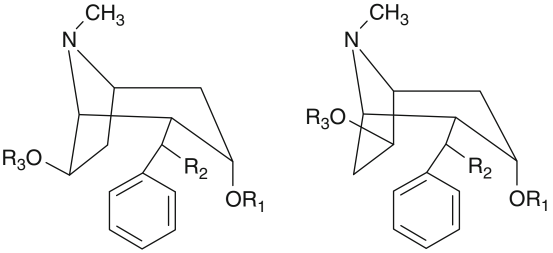


Fig. 12.2 3-Substituted-4-benzyltropanes

Fig. 12.3 3,6- and 3,7-Disubstituted-4-benzyltropanes



2.3 Alkaloids Derived from 4-Benzyltropanone

The members of this small group of tropane alkaloids according to the number of substituents are divided into two subgroups – monosubstituted- and disubstituted-4-benzyltropane alkaloids.

2.3.1 3-Substituted-4-Benzyltropane Alkaloids

The general formula of these alkaloids is presented in Fig. 12.2.

Most often the substituent at C-3 is acetyl group, but it also can be a benzoyl or cinnamoyl group. R_2 can be hydrogen, hydroxy group, or its acyclic derivatives. In all identified alkaloids of this group it is determined that the configuration of the substituent at C-3 can be α- or β-, and at C-4 it is always α- [27].

2.3.2 3,6- and 3,7-Disubstituted-4-Benzyltropanes

This group is derived from 3-substituted-4-benzoyltropane alkaloids with one additional functional group R_3 at C-6 or C-7, most often benzoyl, which is always in β-position (Fig. 12.3).

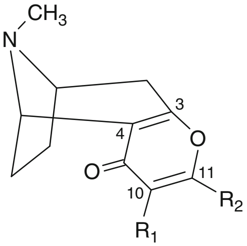


Fig. 12.4 Pyronotropanes

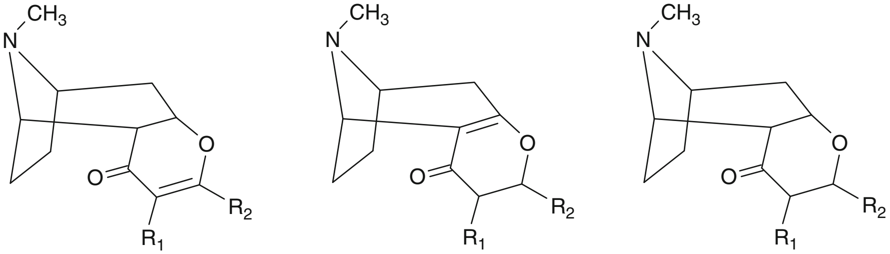


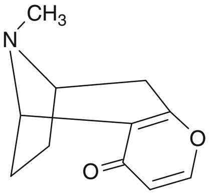
Fig. 12.5 Dihydro- and tetrahydropyronotropane

2.4 Pyronotropane Alkaloids

Pyronotropane alkaloids are natural compounds with γ-pyrone ring condensed with tropane ring at C-3 and C-4 (Fig. 12.4).

The substituents R_1 and R_2 at C-10 and C-11 most often are hydrogen atom or methyl group, and rarely phenyl or hydroxyl group [27].

One of the earliest found pyronotropane alkaloid is strobiline (**43**) isolated from *Knightia strobilina* (family Proteaceae) [19].

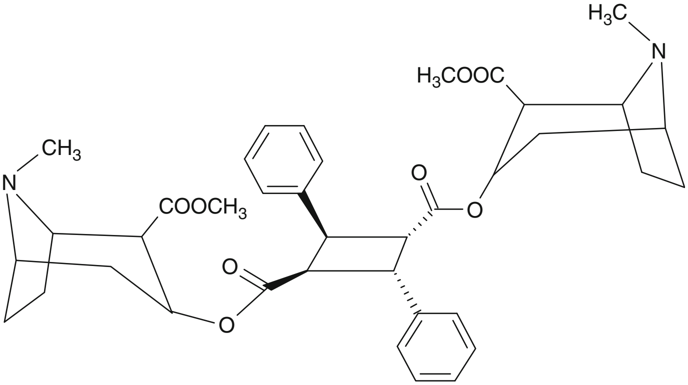


43

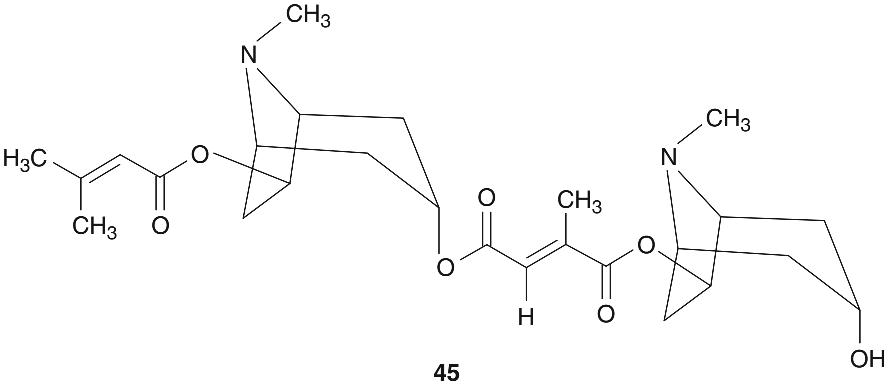
To the group of pyronotropanes also are included the alkaloids in which the γ-pyrone ring has hydrogenated double bond at C-3, C-4, and/or at C-10, C-11 (Fig. 12.5) [41].

2.5 Dimeric and Trimeric Tropane Alkaloids

The dimeric tropane alkaloids are a small group of natural compounds structurally composed by enzymatically catalyzed process of two tropane nuclei derived from tropinone or ecgoninone, linked by dicarboxylic acid, such as mesaconic, itaconic, truxillic, etc., at C-3, C-3' or C-3, C-6' or C-3, C-7' [27, 42]. Typical examples for these types of linkage are the alkaloids α-truxilline (**44**) and schizanthine D (**45**) [42]. Another type of linkage of the two tropane nuclei can take place between the nitrogen atoms via CH_2-CH_2 or > C=O bridge as in subhirsine (**46**) and convolvidine (**47**) [27, 42–45].



44



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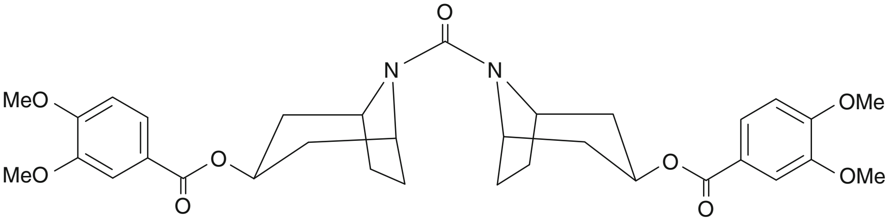
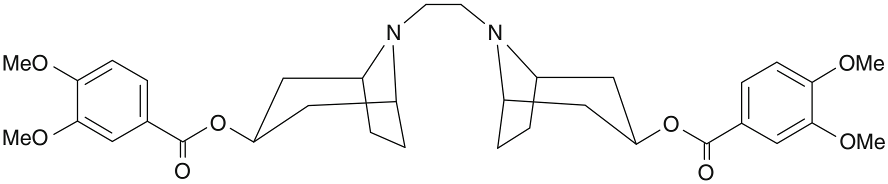
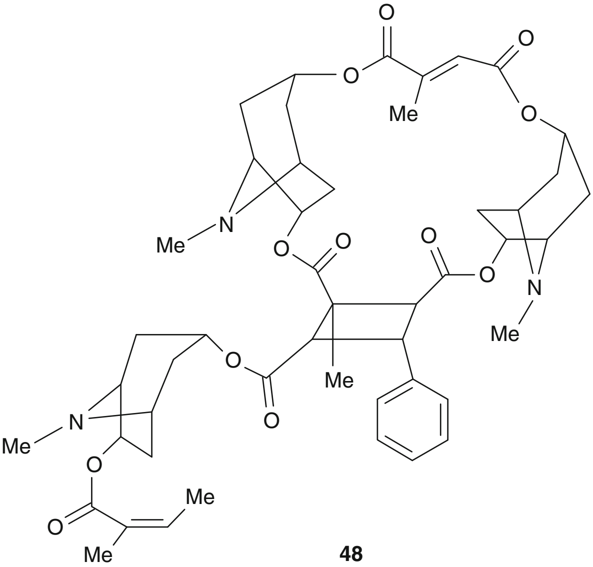

O
N
N
MeO
MeO
O
O
O
O
OMe
OMe

46


N
N
MeO
MeO
O
O
O
O
OMe
OMe

47

Till now the only known trimeric tropane alkaloid is grahamine (**48**) found in *Schizanthus grahamii* (Solanaceae) [43]. The structure of **48** is composed of three 3,7-disubstituted tropane rings and one unit truxillic acid.


O
O
O
Me
O
N
Me
O
O
O
O
N
Me
O
Me
O
N
Me
O
O
Me
Me

48

3 Conclusion

The tropane alkaloids are a large important class of natural products which have variety of chemical structures and interesting pharmacological activities. These alkaloids are characteristic secondary metabolites for the families Solanaceae, Convolvulaceae, Erythroxylaceae, Proteaceae, and Rhizophoraceae, and they have been used as chemotaxonomical markers. The intensive investigations in the branch of tropane alkaloid chemistry continue and by the use of modern physical detection techniques, the number of new compounds steadily increases.

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Alkaloids Derived From Ornithine: Pyrrolizidine Alkaloids

13

Helmut Wiedenfeld

Contents

1 Introduction 360
1.1 Chemical Structures of PAs 361
1.2 Biosynthesis 365
2 PA Toxicity in Humans 373
3 Conclusion 374
References 374

Abstract

Pyrrolizidine alkaloids (PAs) occur in about 5% of all flowering plants. They are esters of a basic moiety ("Necine") and esterifying acids ("necic acids"). Up to now, about 500 different PAs are described. PAs can be hazardous to man and domestic animal. They can show cancerogenic, mutagenic, teratogenic, and fetotoxic properties. Many intoxications by PAs are described. The level of the toxicity of each single PA is dependent from its concrete chemical structure. This chapter gives an overview about the chemistry, biosynthesis, and toxicity of PAs.

Keywords

Biosynthesis • Chemical structure • Intoxications • Pyrrolizidine alkaloids • Toxicity and structure

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1 Introduction

The term “pyrrolizidine alkaloids” (PAs) is used for all ester compounds of the hydroxy and/or dihydroxy and/or hydroxymethyl derivatives from pyrrolizidine (hexahydro-1*H*-pyrrolizine) or from 1,2-dehydro-pyrrolizidine (2,3,5,7a-tetrahydro-1*H*-pyrrolizine).

PAs occur in 13 plant families and in about 5% of all flowering plants. Toxic PAs can be found in six plant families: Apocynaceae, Boraginaceae, Asteraceae (Compositae), Leguminosae (Fabaceae), Ranunculaceae, and Scrophulariaceae. These PAs are hepatotoxic, genotoxic, teratogenic, carcinogenic, and pneumotoxic.

Toxic PAs are only those derived from the unsaturated moiety 1,2-dehydropyrrolizidine and are mainly esters of the aminoalcohols (= necines) retronecine, heliotridine, or the untypical aminoalcohol (no bicyclic five-membered system, but an eight-membered monocycle) otonecine. Some few toxic PAs are esters from crotanecine. Of minor toxicity are the esters of the 1-hydroxymethyl-1,2-dehydropyrrolizidine supinidine.

A very large range of pyrrolizidine alkaloids can theoretically be obtained by combining the known necines and the esterifying acids (= necic acids). So far, more than 500 alkaloids have been found and their structures determined. With the exception of the approximately 35 otonecine alkaloids that cannot form N-oxides, if the N-oxides of these alkaloids are taken into consideration, more than 900 structures are known.

The first intoxication was reported by Gilruth who found that a chronic liver disease in livestock was caused by *Senecio jacobaea* [1, 2]. It was further shown that other *Senecio* ssp. as well as *Crotalaria* ssp. led to the same disease [3]. Already in 1920, it was proven that a widespread chronic liver disease in humans was caused by grain contaminated with seeds of *Senecio* ssp. [4, 5]. Several severe intoxications, all caused by the contamination of food (mainly bread), were reported: In the 1950s, severe intoxications in the former USSR were found to be caused by seeds and dust of *Heliotropium lasiocarpum* which contaminated grain [6, 7]; similarly, the same species was the reason for the intoxication of 4,000 people in Tadjikistan [8, 9]. The severest incident was observed in the 1970s in Afghanistan where about 8,000 people were affected from a wheat contamination by seeds of *Heliotropium popovii*, subsp. g*illianum*; more than 3,000 died [10, 11].

It can be assumed that seeds and dust from PA-containing plants are a major source for a human PA exposure [12, 13]. In industrialized countries, grain cleaning methods reduce the PA contamination under a level where acute intoxications can occur, but the dust components are still remaining and there are indications that diseases such as cirrhosis, cancer, and pulmonary arterial hypertension are due to a long-time exposure of low doses of PAs [14].

Besides this, medicinal plants are a further source of a PA intoxication [15–18]. Especially herbal preparations (so-called bush-teas) were found to be the reason for different liver diseases observed in Jamaica and the West Indies as well as in Africa in the 1950s [19–21].

PA intoxications by herbal preparations are mainly observed in developing countries where the use of traditional medicines is common; however, in the last three decades also, in industrial countries like the USA, the UK, Switzerland, Austria, and Germany PA intoxications were reported due to the increased and uncontrolled use of herbal medications. In the 1980s, hand in hand with the so-called green wave, several practitioners claimed that herbal medicine would show only benefits without undesirable side effects which led to several fatal intoxications, mainly in infants who show a higher susceptibility to PA than adults.

Other foods can also be contaminated with PAs and are, therefore, a potential hazard for humans. Milk was shown to be a source for a PA intoxication in a case where the milk-producing animals had access to PA containing feed (hay, silage) [22–30]. Human milk from women exposed to pyrrolizidine alkaloids has caused veno-occlusive disease in neonates and infants [31]. Honey has become of increased importance as it could be shown that, in commercial products, levels of PAs were found which exceeded tolerable values. Here, pollen seems to be the pathway of contamination [31–37].

Recently, in Germany it was found that salads (especially ready-packed rocket salads (*Eruca sativa*), sold in supermarkets) and salad mixtures can be contaminated with PA containing plants, mainly *Senecio vulgaris*, a typical weed of field crops [38].

1,2-dehydropyrrolizidine esters and their N-oxides are carcinogenic, mutagenic, genotoxic, fetototoxic, and teratogenic to varying degrees. The toxicity level of each single PA is dependent on its chemical structure and also physical properties such as lipophilicity, hydrophilicity, and pharmokinetics. PAs prior to metabolic activation show a more or less low acute toxicity, but in vivo they undergo a metabolic toxification process in the liver, which is, as a result, the first target organ for the toxicity. This toxication process is well investigated.

PAs are considered to be toxic contaminants and have no well-recognized uses on their own. In the 1970s, the PA indicine-*N*-oxide was found to show antitumor activities, but on account of severe toxic side effect (especially observed in children) its use was no longer justified [39–41].

1.1 Chemical Structures of PAs

PAs are esters and consist of two parts: the basic aminoalcohols (“necines”) and one or more acids (“necic acids”) that esterify the OH groups of the necines [42–44].

Necines consist of a five-membered bicyclic ring system with a bridgehead nitrogen and – at least – a hydroxymethyl group at position 1 (Fig. 13.1). They can occur as saturated systems or possess a double bond in position 1,2. Most of the PAs show a further OH group at position 7. Further hydroxylation can take place at positions 6 and 2; in the case of saturated PAs a few molecules show hydroxylation at position 1 and in one case the (+) isomer of isoretronecanol (R_3 = ß-H; α-hydroxymethyl at position 1) is reported. Chiral carbons can be at positions 1 and 2 (saturated necines) and 6,7,8 (all necines). Mainly all PAs belong to the 8-α series (only two times the 8-ß position is reported). In nature, the PAs mainly occur in their *N*-oxide form (water-soluble; transport form).

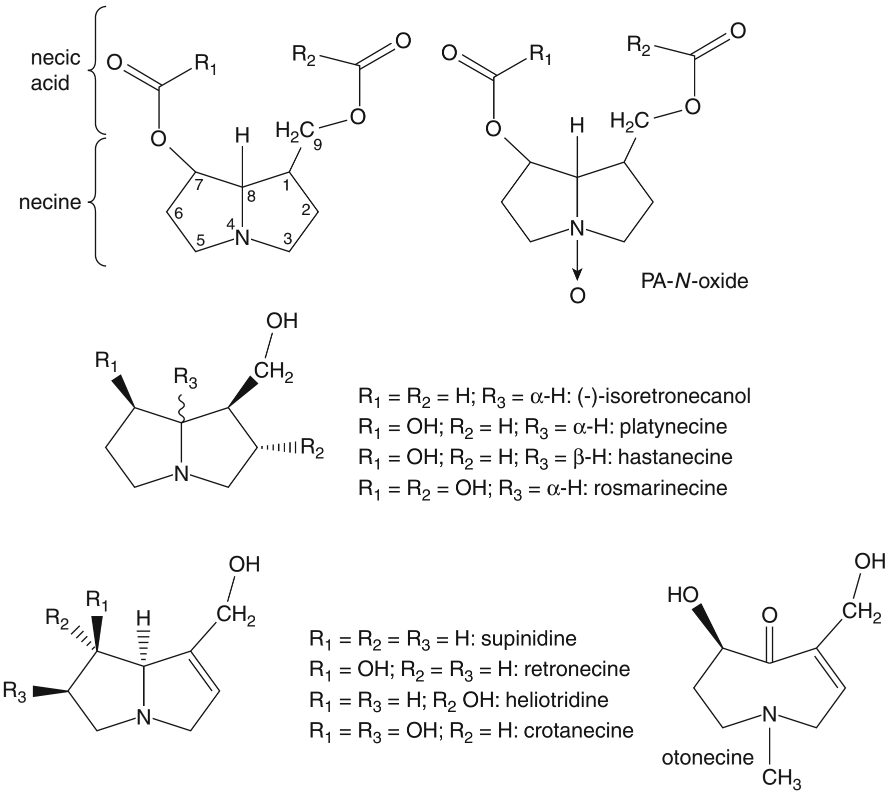

necic acid
necine
R_1
R_2
H_2C
9
H
O
1
2
3
4
5
6
7
8
N
PA-*N*-oxide
OH
CH_2
R_3
$R_1 = R_2 = H; R_3 = \alpha$-H: (-)-isoretronecanol
$R_1 = OH; R_2 = H; R_3 = \alpha$-H: platynecine
$R_1 = OH; R_2 = H; R_3 = \beta$-H: hastanecine
$R_1 = R_2 = OH; R_3 = \alpha$-H: rosmarinecine
$R_1 = R_2 = R_3 = H$: supinidine
$R_1 = OH; R_2 = R_3 = H$: retronecine
$R_1 = R_3 = H; R_2$ OH: heliotridine
$R_1 = R_3 = OH; R_2 = H$: crotanecine
HO
CH_3
otonecine

Fig. 13.1 Structures of necines

An untypical necine can be found in the otonecine PAs: This necine occurs not in form of a bicycle but as a 1-methylazocan-5-one. On account of the results by X-ray analysis, it could be found that there exists a transannular binding between carbonyl and the nitrogen leading to the same behavior as the typical bicycle PAs. All PAs showing a double bond in position 1,2 act as natural toxins.

Necic acids are structurally highly variable. Besides the simple acetic acid they consist mainly of 5–10 carbon atoms. They can form mono-, di-, or macrocyclic diesters and can show more or less branched carbon chains. The carbon atoms can bear different functional groups like hydroxyl, alkoxy, epoxy, and carboxyester groups. Double bonds at different positions are also possible. On account of this situation a theoretically high number of different unique structures are possible, including a large range of stereo isomers [42–44].

Necines with only one OH group can only form PA monoesters like supinine. If there exists a second OH group both can be found: mono as well as diesters as demonstrated for lycopsamine and its acetyl derivative or as in lasiocarpine. These examples demonstrate the variability of the stereochemistry (Fig. 13.2).

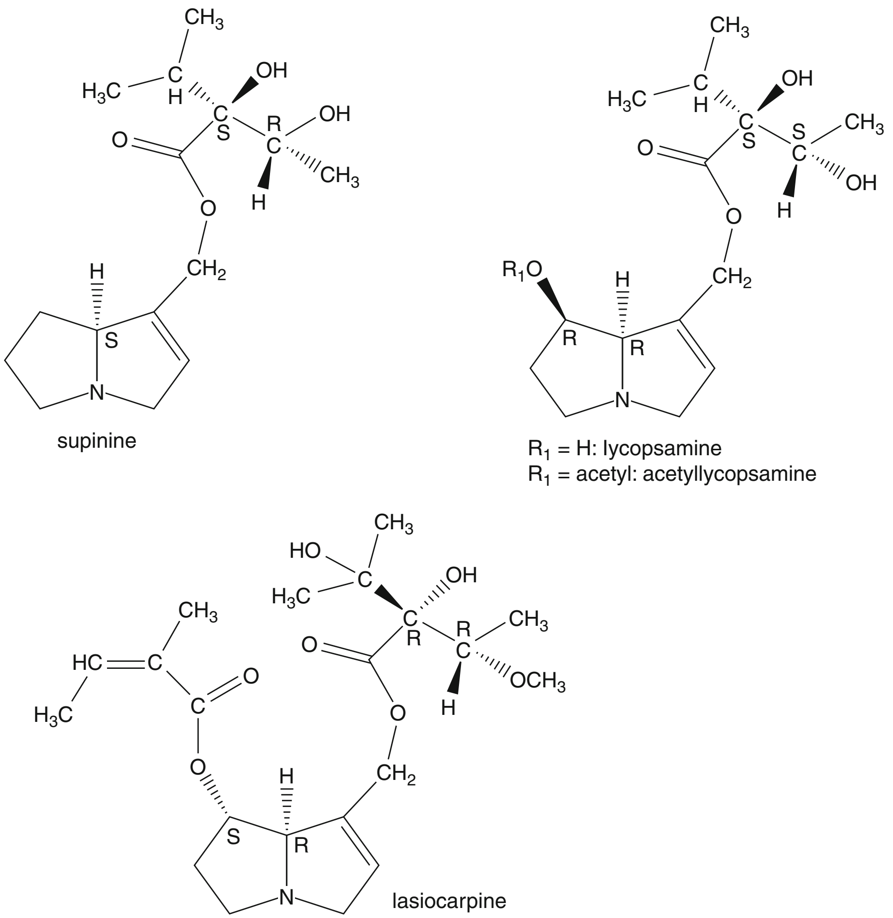


Fig. 13.2 Structures of PA mono- and diesters

With dicarboxyclic acids, the double esterification leads to macrocyclic diesters which can occur as 11–13-membered ring systems. A typical example for an 11-membered PA is monocrotaline, the main PA occurring in *crotalaria* species, or crispatine. Most of the PAs show a 12-membered ring system; this can be found, for example, in senecionine or senkirkine, both contained in many *senecio* species. A typical 13-member ring is demonstrated in the PAs doronenine (also isolated from *senecio*) and in madurensine (from *crotalaria*). Very rare is the 14-member macrocycle: It is found in the PA parsonsine (from *parsonsia*-species); this structure is possible on account of a further ester group within the necic acid chain. It is interesting to mention that until now in all cases of macrocyclic diester PAs only the 7-ß-OH isomer (mainly retronecine) is described and not the α-isomer (heliotridine) (Fig. 13.3).

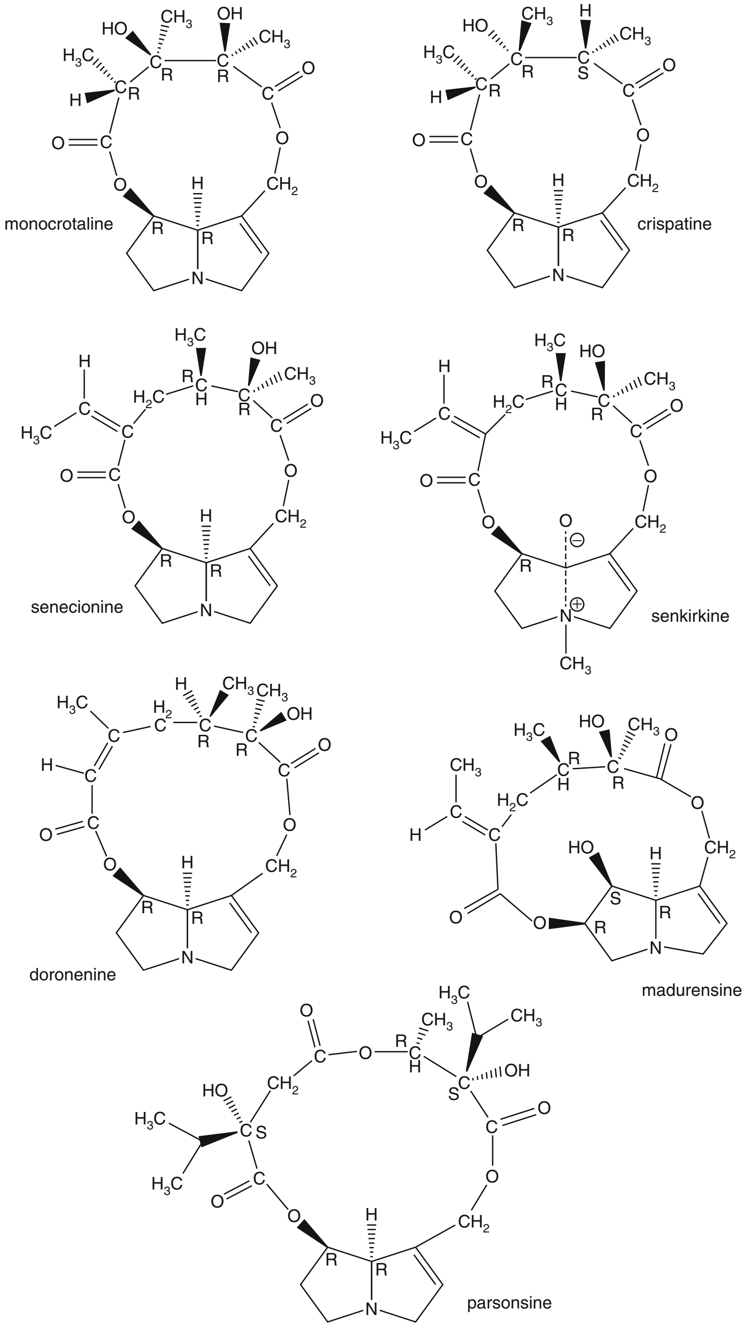

monocrotaline
crispatine
senecionine
senkirkine
doronenine
madurensine
parsonsine

Fig. 13.3 Structures of macrocyclic PA

Theoretically, an innumerable amount of different structures is possible. So far, more than 500 PAs have been isolated and their structures described.

1.2 Biosynthesis

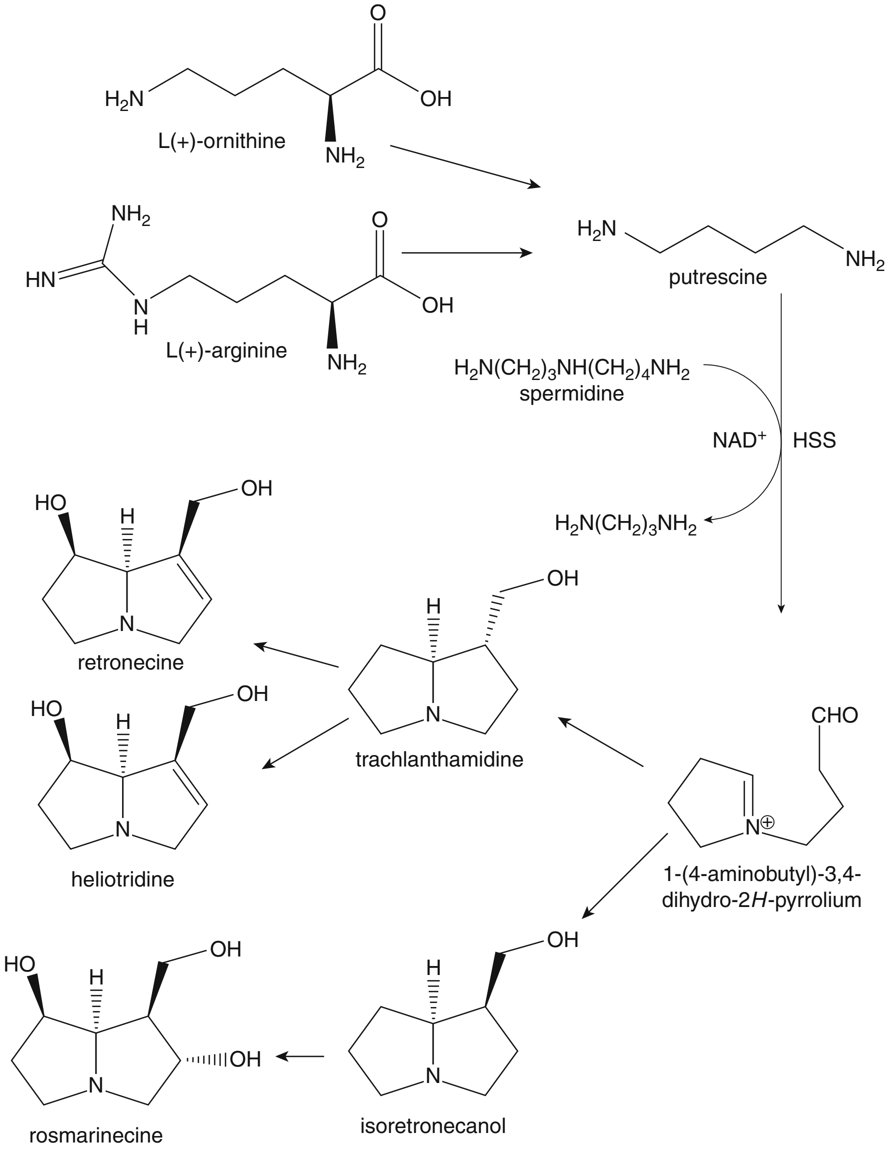
The first experiments concerning the biosynthesis of PAs were done in the 1960s: [2-^{14}C]ornithine, [1-^{14}C]acetate, and [1-^{14}C]propionate were fed to *Crotalaria* species [45]. Similarly, labelled ornithine was incorporated into the alkaloids from *Senecio* species [46, 47]. In all cases, ornithine was incorporated specifically into the basic moiety retronecine, whereas acetate and propionate could be found in the acidic parts of the alkaloids. Bale and Crout used a double isotope technique for a comparison between ornithine and arginine [48] showing that ornithine is the more efficient precursor than arginine. It could be found that ornithine and arginine were incorporated into retronecine via putrescine: Experiments gave evidence for the formation of putrescine from argine in *Heliotropium* species, whereas in *Crotalaria* and *Senecio* species, the main pathway to putrescine is from ornithine [49, 50]. Further experiments came to the conclusion that retronecine is derived from two molecules of putrescine, which can be formed from ornithine or arginine [51, 52]. The use of ^{13}C-^{15}N double-labelled putrescine gave evidence for a further symmetrical intermediate which was then found to be homospermidine. This was proven by feeding experiments with labelled homospermidine which was incorporated into the retronecine moiety of the PAs from *Senecio isatideus* [53–55]. It could be shown that a PA-special enzyme – the homospermidine synthase (HSS) – is essentially involved in the biosynthesis of homospermidine [56–58]. This HSS is responsible for the transfer of the aminobutyl moiety of spermidine into putrescine leading to the symmetric triamine homospermidine; this reaction is NAD$^+$ dependent. Via the 1-(4-aminobutyl)-3,4-dihydro-2*H*-pyrrolium salt the necines trachelanthamidine and further retronecine were generated [59].

Besides, it was shown that trachelanthamidine as well as retronecine were both efficient precursors for the necine otonecine, the base moiety of the PA emiline from *Emilia flammea* [60].

Trachelanthamidine as well as isoretronecanol can be generated from the imminium salt. There exists evidence that the saturated necine rosmarinecine is generated via isoretronecanol and that trachelanthamidine is the precursor for retronecine as well as heliotridine [61]. Isoretronecanol has been shown to be also the precursor of those PAs which have the untypical 8*ß* stereochemistry (instead of 8α) as found in the PAs from *Cynoglossum australe* [62] (Scheme 13.1).

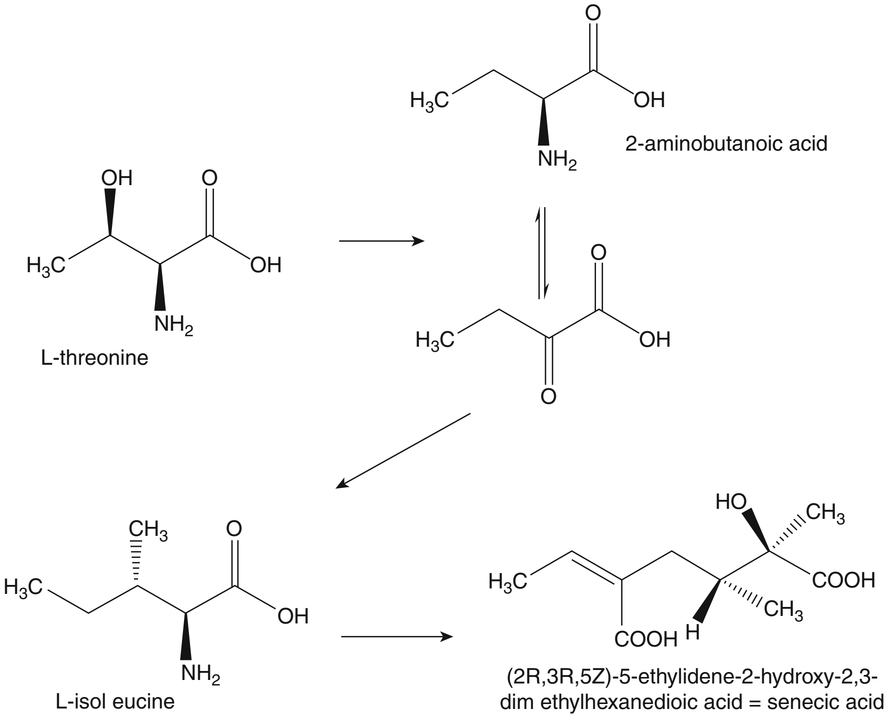
Whereas the biosynthesis of the necines (Scheme 13.1) seems to be clear and intensively investigated, fewer reports can be found for the necic acids. Still some aspects have to be investigated.

In 1966, labelled L-threonine and L-isoleucine were observed to be incorporated into the seneciphyllic acid [63]. Similarly, it was described that labelled DL-valine is a precursor of echimidinic acid [64]. The five-membered angeloylic acid is


L(+)-ornithine
L(+)-arginine
putrescine
$H_2N(CH_2)_3NH(CH_2)_4NH_2$
spermidine
NAD+
HSS
$H_2N(CH_2)_3NH_2$
retronecine
heliotridine
trachlanthamidine
1-(4-aminobutyl)-3,4-dihydro-2H-pyrrolium
rosmarinecine
isoretronecanol

Scheme 13.1 Biosynthetic pathway of necines

found often as a structure part in PAs. L-isoleucine was shown to be a precursor of the angelate component in PAs [65]. Crout et al. reported that in general C_{10} necic acids are derived from isoleucine [66, 67]. This was demonstrated in case of seneciphyllic acid and senecic acid, both forming 12-membered macrocyclic PAs. Monocrotalic acid, the acidic part of monocrotaline – a 11-membered macrocycle – was reported to be built from L-isoleucine and L-threonine [68].


L-threonine
2-aminobutanoic acid
L-isol eucine
(2R,3R,5Z)-5-ethylidene-2-hydroxy-2,3-
dim ethylhexanedioic acid = senecic acid

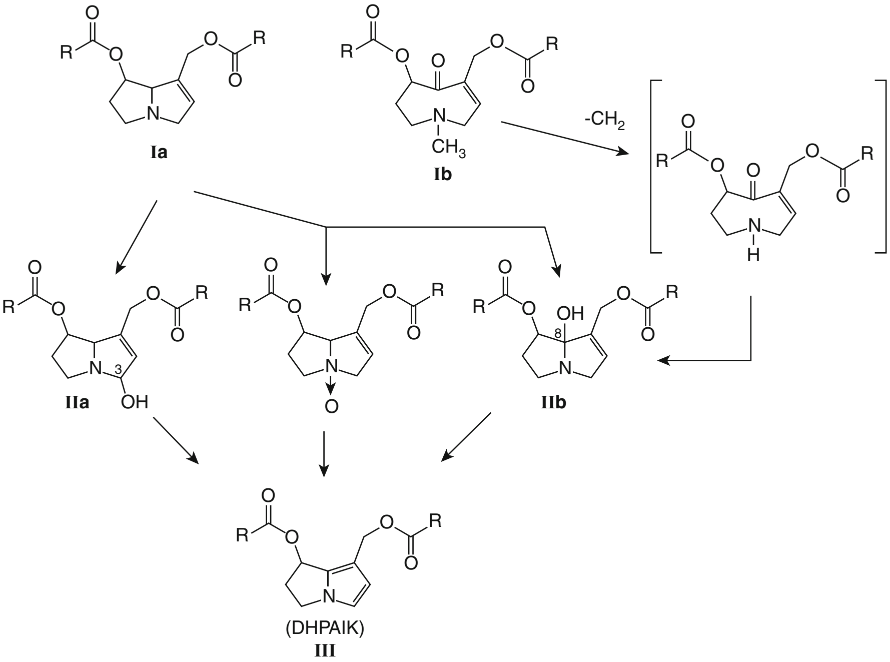
Scheme 13.2 Biosynthetic pathway of senecic acid

Similar findings were reported for the acidic part in the PA strigosine [69] and for angeloylic and tigloylic acid [70]. In the case of the acidic part in the PA monocrotaline, it was shown that not acetate, mevalonate, or glutarate (as reported earlier) are involved but it is only formed via isoleucine [71]. Stereospecific aspects were also studied in the case of senecic and isatinecic acid [72, 73]. The biosynthesis of trichodesmic acid was studied and it was shown that one part of the C_{10} acid was formed by (2*S*)-isoleucine or its biosynthetic precursor (2*S*)-threonine and the other C_5 unit from (2*S*)-leucine or (2*S*)-valine [74]. The complete labeling pattern of senecic acid (the acidic part in the PAs rosmarinine and senecionine) was studied by NMR experiments and it was stated that the biosynthesis of this acid is processed via two molecules of isoleucine (Scheme 13.2) [75].

1.2.1 Metabolic Toxication of PAs

PAs are ester alkaloids derived mainly from the necines retronecine and otonecine. They are carcinogenic, mutagenic, genotoxic, fetotoxic, and teratogenic.

PAs themselves show a more or less low acute toxicity but in vivo they undergo a three-step metabolic toxication process in the liver, which is, as a result, the first target organ for the toxicity.



Scheme 13.3 Metabolism of PAs to Pyrrols

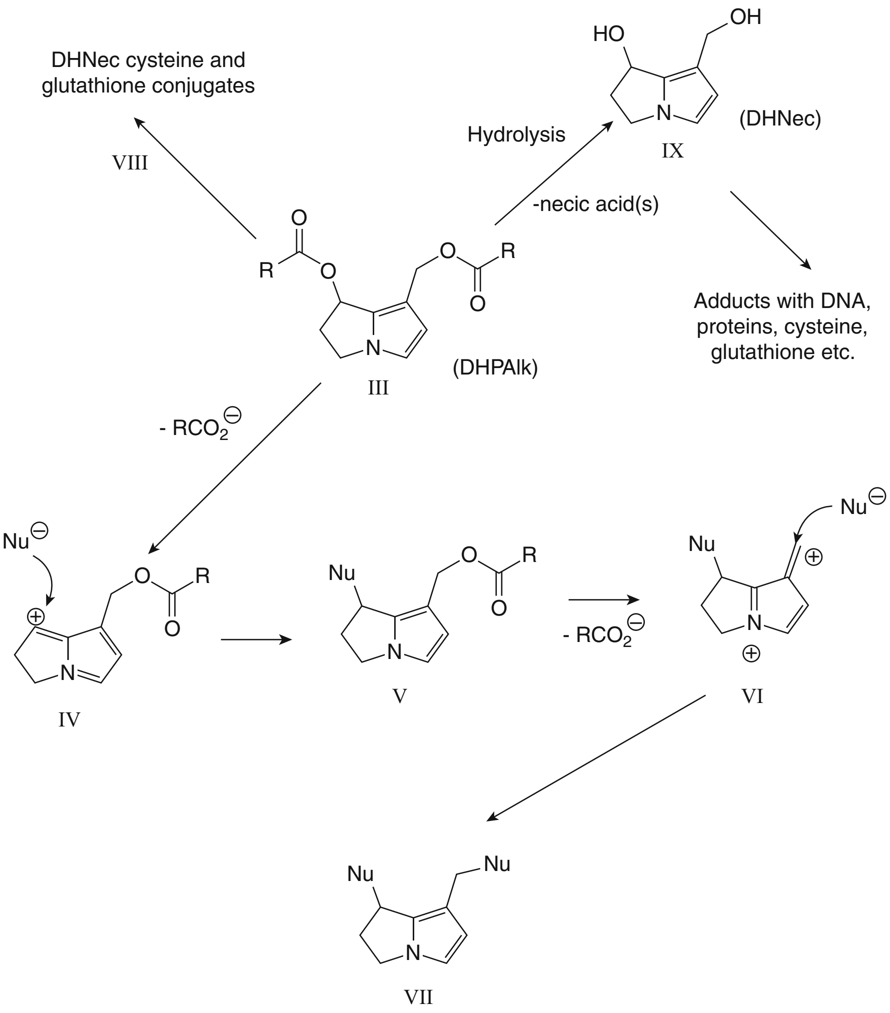
This toxication process is well investigated [12, 75–81].

After oral uptake and absorption of the PAs (Scheme 13.3: Ia and Ib), a hydroxyl-group is introduced adjacent to the nitrogen atom in the necine (position 3 or 8) by the cytochrome P-450 monoxogenase enzyme complex in the liver (Scheme 13.3: IIa and IIb).

These hydroxyl PAs (OHPAs) are unstable and undergo a rapid dehydration to the dehydropyrrolizidine alkaloids (DHPAlk; Scheme 13.3: III). This dehydration results in a second double bond in the necine followed by spontaneous rearrangement to an aromatic pyrrole system III.

PAs occur mainly as their N-oxides in the plants and these cannot be directly converted to the OHPA, but on oral ingestion they are reduced by the gut enzymes or the liver microsomes and NADH or NADPH to the free bases and therefore they show equal toxicity to that of the free bases [82–87].

Otonecine-type PAs (Scheme 13.3: Ib) are metabolized to the OHPAs [80, 88, 89]. These otonecine-PAs possess a methyl function at the nitrogen and a quasi keto function at the bridge carbon 8. After hydroxylation of the N-methyl group it is lost as formaldehyde leaving a NH-function which undergoes condensation with the C8 keto group to produce product IIb (Scheme 13.3) which spontaneously dehydrates to the DHPAlk III.


DHNec cysteine and glutathione conjugates
VIII
Hydrolysis
-necic acid(s)
HO
OH
N
(DHNec)
IX
Adducts with DNA, proteins, cysteine, glutathione etc.
R
O
O
O
R
O
N
(DHPAlk)
III
- RCO2⊖
Nu⊖
⊕
N
O
R
O
IV
Nu
N
O
R
O
V
- RCO2⊖
Nu
Nu⊖
⊕
N
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VI
Nu
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VII

Scheme 13.4 Metabolism of the dehydropyrrolizidine alkaloids

The metabolites III are able to generate stabilized carbonium ions (Scheme 13.4: IV and VI) by loss of hydroxy groups or ester functions as hydroxyl or acid anions. These carbonium ions can react rapidly with nucleophiles (Scheme 13.4: VII).

In the case of necine-diesters (as shown in Ia and Ib, Scheme 13.3), typical of *Senecio* species, the formation of the reactive carbonium ions is facilitated because the necic acid groups provide good leaving groups that facilitate rapid formation of the carbonium ions IV and VI in high yield. Where one of the hydroxy groups at C7 or C1 of the necine is not esterified, formation of the carbonium ions is not so spontaneous. In these cases, the carbonium ions are most readily formed after protonation of the hydroxyls and loss of H_2O [90].

The metabolites IV and VI react rapidly with nucleophilic groups of proteins and the amino groups of the bases in nucleosides like DNA and RNA which leads to abnormal functions showing finally the veno-occlusive disease (VOD) in which the veins are narrowed. Typical macrocyclic diester PAs (like senecionine, seneciphylline, retrorsine, and senkirkine which are PAs commonly found in *Senecio* species) have been shown to produce liver damage due to cross-linking of DNA [84, 91–99]. In case of PA monoesters (e.g., derived from the necine supinidine which lacks a C7 hydroxyl, Fig. 13.2), cross-linking is not possible and they show a lower toxic potential. It has also been shown that the nucleophilic activity at C7 is higher than at C9 resulting in the primary nucleophilic attack at C7 followed by an attack at C9 (Scheme 13.4) [43].

As shown, the DHPAlks can also react with SH groups found in more soluble components like glutathione and cysteine (Scheme 13.4: VIII). High levels of glutathione and cysteine therefore reduce the toxic potential of PAs [88, 100–102].

Furthermore, hydrolysis can take place where the DHPAlks (Scheme 13.4) yield dehydronecine alcohols (DHNecs) (Scheme 13.4: IX) which are more water soluble and less reactive like the DHPAlks but still display a moderate level of alkylating activity [103, 104]. This higher water solubility and lower reactivity can lead to the escape from the liver tissue and subsequent reaction in other organs [12, 105, 106]. DHNecs like dehydroretronecine and dehydroheliotridine have also been shown to produce rhabdomyosarcoma, skin, liver, and lung tumors [24, 107–110].

1.2.2 Detoxication of PA

As well as the metabolic activation, detoxication of PAs also occurs in vivo: Hydrolysis of the ester bonds in PAs from type Ia or Ib by esterases leads to necic acids and to the free necines. Both are nontoxic products and – on account of their higher water solubility – can be renally excreted. The rate of hydrolysis is dependent on the level of steric hindrance of the ester linkages; and it has been shown that the more highly branched necic acids are more resistant to hydrolysis [43, 111]. This means that macrocyclic diesters with more complex acid moieties are more hazardous on account of their lower rate of hydrolytic detoxication.

The N-oxides of PAs (the form occurring most commonly in plant sources) are highly water soluble and can therefore be renally excreted. Besides their natural occurrence, N-oxidation of PAs also takes place in the liver and can be seen as a detoxication process (Scheme 13.3) [76, 78, 82, 112, 113]. However, it has been shown that the N-oxides – besides excretion – can be converted by dehydration or by acetylation followed by elimination of acetic acid to the DHPAlk (Scheme 13.3: III) [43, 90].

In conclusion, it can be stated that the toxicity level of different PAs in non-ruminants is dependent on the following three aspects:

- The efficiency of metabolic activation to form the key intermediate III (Scheme 13.3)
- The efficiency of ester hydrolysis to form nontoxic and water-soluble necines and necic acids
- The efficiency of N-oxidation and excretion via urine

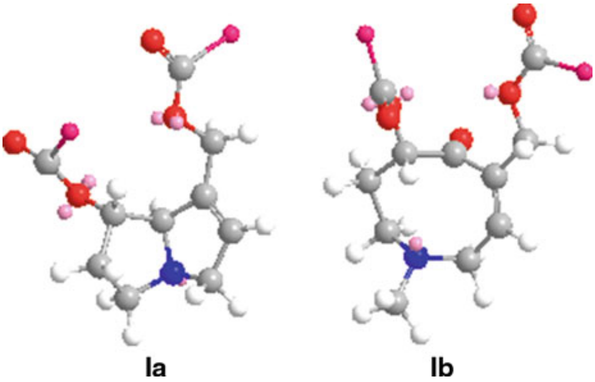


Fig. 13.4 *X-rays of Ia and Ib*

1.2.3 Structure and Toxicity

As mentioned, the key fragment didehydropyrrolizidine (e.g., III) is also generated from pyrrolizidine alkaloids of the otonecine type (e.g., Ib, Scheme 13.3).

These otonecine derivatives do not show a C8-N bond but possess a keto function at C8 and a methyl group at the nitrogen atom. Surprisingly, these seco alkaloids are of identical toxicity as the PA of type Ia (1,2-dehydro-retronecine and heliotridine esters).

On the other hand, for energetic reasons, these seco compounds should occur in a different necine conformation than type Ia PA: The missing C8-N bond should lead to a stable eight-membered macrocycle which generally prevents the metabolization via an intermediate to IIb (Scheme 13.4).

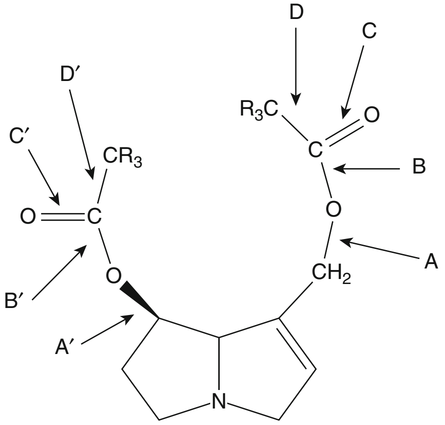
Molecular modeling experiments prove this assumption and show the energy minimized structure as depicted in Fig. 13.3 (energy minimization: Chem 3D ultra; V. 10.0; Cambridge Soft).

Interpretation of the X-ray structure analysis data helps to explain the identical toxicity of PA of type Ia and Ib: In all nine otonecine-type alkaloids measured to date, a necine conformation was found which is identical to those found in those having the C8-N bond (type Ia).

Both the distances between C8 and N are similar and equal values can be found for the plane angles built between plane C1-C3-N-C8 and plane C7-C5-N-C8 (∼125°). This indicates that the seco 1,2-dehydroesters do not exist in the optimal, energy minimized form (Ib, Fig. 13.4) but are of an equal conformation to PA of type Ia, which finally enables the metabolization as shown in Scheme 13.3.

Furthermore, X-ray data show that the dedihydro metabolites (e.g., III, Scheme 13.3) derived from 1,2-dehydrodiester have a high toxic potential compared with the low or missing toxicity of the monoesters of retronecine or heliotridine. As already mentioned, a possible detoxification mechanism is the hydrolysis of the ester bindings by esterases and the subsequent building of dehydronecine (IX, Scheme 13.4) which – due to their higher water solubility – can be easily excreted renally.

Fig. 13.5 Bonds in PAs



On the other hand, the metabolization to C7 as well as to C9 carbonium ions (especially the speed rate of this metabolization) and the subsequent reaction with nucleophiles are seen as a key step concerning the level of toxicity. The concrete binding situation concerning the ester function can be found analyzing the X-ray data. Interpretation of the bond lengths shown in Fig. 13.5 leads to the following results:

In pyrrolizidine alkaloids of type Ia and Ib (1,2-dehydropyrrolizidine diesters) the C-O bonds A and A´ occur in a normal range of 1.45 Å; the following C-O bonds B and B´ are considerably shortened, whereas the keto functions C and C´ show a moderate shortening. The C-C bonds D and D´ show – similar to A and A´ – normal values of about 1.54 Å.

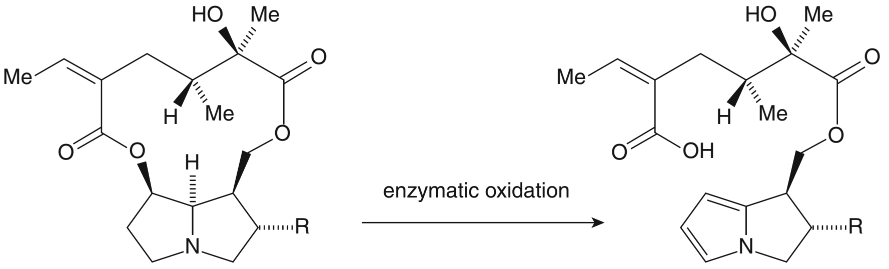
In contrast to these findings, the data for monoesters with retronecine or heliotridine give evidence of a different situation: Here, the A and A´ (C9 as well as C7) bonds are elongated and the corresponding keto bonds C or C´ are of nearly ideal length (1.21 Å).

These results show that in 1,2-dehydro diesters, the ester functions are a conjugated system leading to the conclusion that the bonds C9-O and C7-O (= A and A´) are the target breaking points within the molecule, which makes possible a quick and easy metabolization and further reaction with nucleophiles as described in Scheme 13.4.

Contrary to that, in PA of 1,2-dehydro monoesters, more stable ester bonds are found (no conjugation), which leads to the fact that the building of the didehydrometabolites is more difficult and time consuming. In this case, a hydrolysis by esterases can take place in higher amounts what leads to the detoxification via dehydronecines, which explains the missing or low toxicity of 1,2-dehydro monoester.

1.2.4 Metabolism of 1,2-Saturated Pyrrolizidine Alkaloids

Metabolism of saturated pyrrolizidine alkaloids (necic acid esters of platynecine, hastanecine, rosmarinecine and isoretronecanol (Fig. 13.1)) by mammals has not yet been extensively studied, in part because the saturated ester alkaloids and their necines do not display mammalian toxicity.



R = H : Platyphylline
R = OH : Rosmarinine

Fig. 13.6 Metabolism of saturated PAs

While 1,2-dehydropyrrolizidine alkaloids are metabolized by liver P-450 isozymes into hazardous dihydropyrrolizines (e.g., III) with a pyrrolic A ring, saturated pyrrolizidine alkaloids produce nontoxic, metabolites [79, 111]. The saturated alkaloids platyphylline and rosmarinine (Fig. 13.6), for example, are converted by liver microsomes into pyrrolic metabolites with an aromatic B-ring [79]. These are devoid of biological alkylating properties and are nontoxic.

2 PA Toxicity in Humans

PA poisoning of humans can be described by three dose-related levels: acute, subacute, and chronic. These levels can be progressive resulting in irreversible chronic toxic effects [12, 99, 106, 110, 114–116].

On account of the low toxicity of the PAs themselves, acute poisoning has been reported only in very rare cases; it occurs only in infants and neonates due to their higher susceptibility for a PA poisoning. It is characterized by hemorrhagic necrosis, hepatomegaly, and ascites; death is caused by liver failure [12, 106, 110, 115].

Subacute levels are characterized by hepatomegaly and recurrent ascites; endothelial proliferation and medial hypertrophy leading to an occlusion of hepatic veins, resulting in the so-called veno-occlusive disease (VOD) which can be seen as a characteristic histological sign for PA poisoning [12, 99, 106, 110, 115]. The VOD causes centrilobular congestion, necrosis, fibrosis, and liver cirrhosis, the end-stage of chronic PA intoxication.

As well as the liver VOD, other organs can be affected by PAs. It has been shown that the pyrrolic metabolites (DHPAlks and DHNecs) can escape from the liver into pulmonary arterioles where they can produce damages similar to the VOD changes in the liver [115]. It could be shown that from 62 tested PAs all can produce (dose-dependent) lung lesions [111] and it is speculated that pulmonary damage results from long-term and low-level exposure to PAs [12, 115].

PA intoxication in humans is not only related to the amount and the duration of the exposure but also to age and gender: Males react more sensitive than females, and fetuses and children (especially neonates or infants) show the highest sensitivity to PA poisoning: In 2003, it was shown that the daily uptake of ~ 7 μg PA (from a herbal spice containing comfrey) during pregnancy did not show a toxic effect in the mother's liver but damaged the fetal liver in such a way that the newborn child died after 2 days [117].

It has also been observed that cofactors can exacerbate the PA poisoning: liver damaging agents, bacterial, or viral infections but also medical drugs like barbiturates or metals like copper or mycotoxins like aflatoxins can increase the severity and likelihood of PA liver damage [42, 118–122].

There is a large number of reports in the literature about different liver diseases (mainly VOD) possibly connected with PA poisoning. But in most cases, the connection could not be proven because the outbreak of the liver disease and a possible ingestion of PA-containing material were often separated by a long time period.

3 Conclusion

PAs are widespread in the plant kingdom. They occur in a great variety of different structures. They show no physiological and/or pharmacological properties, but they are of importance on account of possible severe side effects. These side effects are dependent on their chemical structures and range from low or missing toxicity up to strong severe and hazardous problems. PAs themselves are more or less nontoxic but undergo a metabolic toxication process. As a result cancerogenic, mutagenic, teratogenic, and fetotoxic effects can be observed. Many episodes are reported describing intoxications in humans, sometimes even epidemic. These cases were caused mainly by contaminations of food and feed by PA-containing plant material or by the uncontrolled use of herbal medical preparations. Therefore, to avoid those contaminations, special efforts should be made on a global scale to improve food control management.

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14 Alkaloids Derived from Lysine: Quinolizidine (a Focus on Lupin Alkaloids)

Giovanna Boschin and Donatella Resta

Contents

1 Introduction 382
 1.1 Differences Among Quinolizidine Alkaloids 383
 1.2 Alkaloids Fingerprint in Different Lupin Species 386
 1.3 Alkaloid-Poor and Alkaloid-Rich Lupin Species 388
2 Biosynthesis 389
 2.1 Differences in Biosynthesis in Alkaloid-Poor and Alkaloid-Rich Lupin Plants 389
3 Factors Influencing Quinolizidine Alkaloids Synthesis 391
4 Toxicity 393
5 Different Alkaloids in Different Plant Parts 395
6 Biological Activities 397
7 Debittering Processes 398
8 Analytical Methods 399
9 Conclusion 400
References 400

Abstract

Quinolizidine alkaloids (QAs) are usually known as lupin alkaloids because they mainly occur in lupin species and other plants of the Genisteae tribe. They are secondary metabolites synthesized by plants from lysine, for defense against pathogens and other predators. QAs are biosynthesized in green tissues of the plant, transported via phloem and stored in all organs of the plant, including seeds. QAs content depends on genotype, presence of pathogens, and

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pedo-climatic conditions such as environmental effects and soil characteristics. More than 170 QAs have been identified in different *Lupinus* species, being the alkaloid pattern highly variable among species; sparteine and lupanine are the most common ones. QAs show neurotoxicity and for this reason Food Authorities of some countries have fixed a limit of 200 mg kg^{-1} of total QAs in lupin seeds and foods. The level of QAs in lupin seeds can be reduced by debittering processes involving soaking or washing with water; moreover, some lupin varieties producing low levels of QAs have been selected and bred.

Keywords

Lupanine • lupin • *Lupinus* • quinolizidine alkaloids • sparteine

Abbreviations

BW	Body weight
DM	Dry matter
FID	Flame ionization detector
GC-MS	Gas chromatography–mass spectrometry
NACE	Nonaqueous capillary electrophoresis
NOEL	No observed effect level
LD	Lethal dose
MOS	Margin of safety
MRL	Minimal risk level
QA	Quinolizidine alkaloid
SPE	Solid phase extraction
TDI	Tolerable daily intake
TLC	Thin-layer chromatography

1 Introduction

Quinolizidine alkaloids (QAs) are plant secondary metabolites synthesized as part of a defense strategy against predators, such as herbivores and pathogen microorganisms [1–3]. They also have allelopathic functions in relation to a biocenosis, that is, they can be used to influence other plants [2]. Moreover QAs, together with basic amino acids and biogenic polyamines, are nitrogen reserves in plants [3–5].

Their chemical structure is based on quinolizidine ring (Fig. 14.1).

They are prominent among species of the "genistoid alliance," group of legumes, tribes Genisteae, Crotalarieae, Podalyrieae, Liparieae, Thermopsideae, Euchresteae, Brongniarieae, and Sophoreae [6]. QAs are commonly referred to as "lupin alkaloids," since they occur throughout the species of the genus *Lupinus* that belongs to the Fabaceae (or Leguminosae) family, Genisteae tribe. They are the main antinutritional factors that confer unpalatability, in particular bitter taste, and toxicity to lupin seeds.

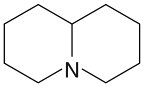


Fig. 14.1 Quinolizidine ring structure

Lupin is a leguminous plant usually used for feed or manure in agriculture practice for its nitrogen fixation capability. Lupin seeds have comparable nutritive characteristics, especially protein content (30–40 %), in respect to soybean. For this reason lupin has been discussed as possible alternative of soybean in human foodstuffs.

From the genus *Lupinus* more than 400 species are known, but only four have been domesticated and are of agronomic and commercial interest: *Lupinus albus* (white lupin), *Lupinus angustifolius* (narrow leaf or blue lupin), *Lupinus luteus* (yellow lupin), and *Lupinus mutabilis* (Andean lupin).

Lupins are mainly distributed in South and North America, whereas only 12 taxa occur in Europe and North Africa. In Europe, especially *L. albus* and *L. luteus* are used as green forage, as manure or are intended for human nutrition. In Australia the main lupin species is *L. angustifolius*.

L. albus plant is annual, more or less pubescent, 30–120 cm high, and has a wide distribution in the Mediterranean regions. It is widely spread as wild plant throughout the southern Balkans, the Italian islands of Sicily and Sardinia, the French island of Corsica, and the Aegean Sea, as well as in Palestine and western Turkey. It occurs in meadows, pastures, and grassy slopes, predominantly on sandy and acid soils. It is cultivated over all the Mediterranean regions and also in Egypt, Sudan, Ethiopia, Syria, Central and Western Europe, the USA and South America, Tropical and Southern Africa.

L. angustifolius is native to Europa, Asia, and Northern Africa, but it is cultivated also in North America and Australia, that is nowadays the major producing country.

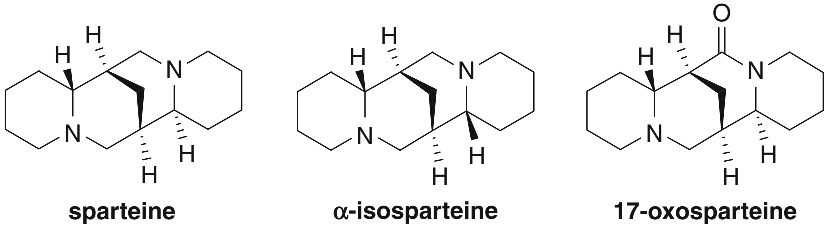
L. luteus is known as European yellow lupine. It is native to the Mediterranean regions of Southern Europe; it grows in the Mediterranean countries, including Morocco, Portugal, Spain, Italy, Greece, and Egypt.

L. mutabilis is cultivated in tropical and subtropical zones and it is still cultivated using traditional methods by the native peoples of Ecuador, Peru, and Bolivia.

Each of the about 450 lupin species which have been identified differs in both content and composition of QAs. The problem of great difference of QAs content is further complicated as it can fluctuate among the different genetic varieties of the same species. QAs content depends also from environmental conditions, soil characteristics, drought, etc. Moreover, substantial variations are found in the alkaloid patterns of different organs: seeds, leaves, pods, roots, flowers.

1.1 Differences Among Quinolizidine Alkaloids

More than 170 alkaloids of the quinolizidine group have been identified in different *Lupinus* species [7]. They can be divided into different classes in according to their chemical structure: sparteine and its derivatives (Fig. 14.2), lupanine and its derivatives

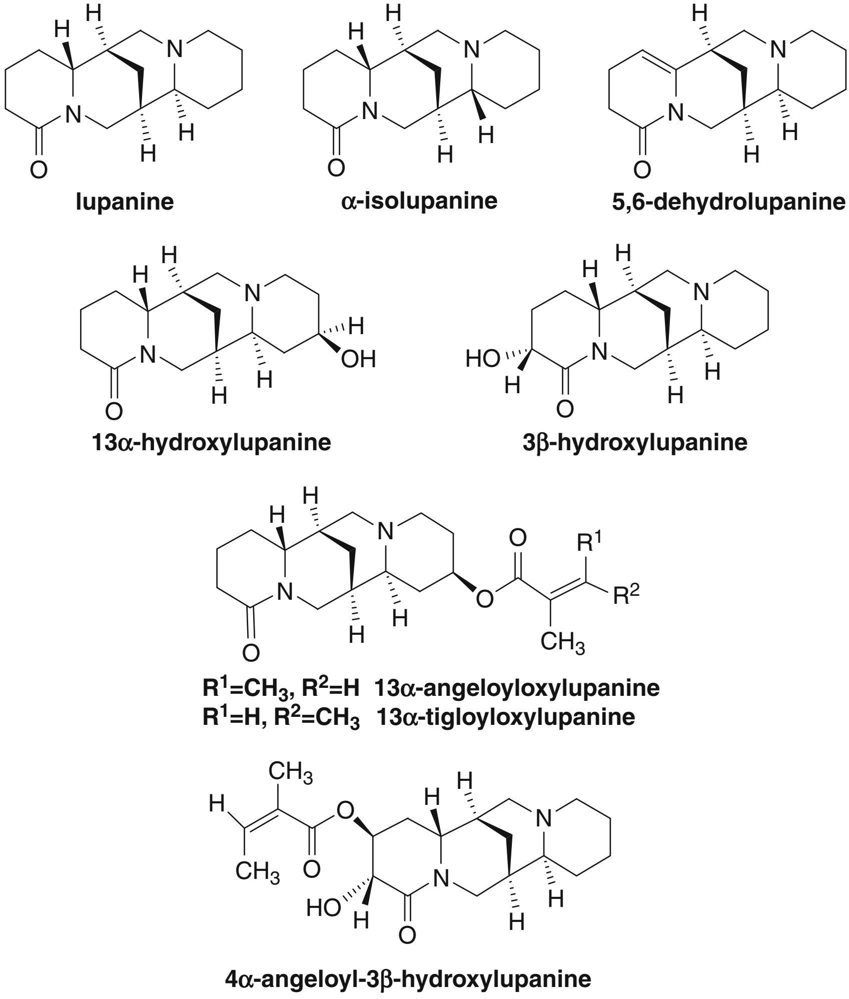


sparteine

α-isosparteine

17-oxosparteine

Fig. 14.2 Chemical structure of sparteine and its derivatives



lupanine

α-isolupanine

5,6-dehydrolupanine

13α-hydroxylupanine

3β-hydroxylupanine

$R^1=CH_3$, $R^2=H$ 13α-angeloyloxylupanine

$R^1=H$, $R^2=CH_3$ 13α-tigloyloxylupanine

4α-angeloyl-3β-hydroxylupanine

Fig. 14.3 Chemical structure of lupanine and its main derivatives

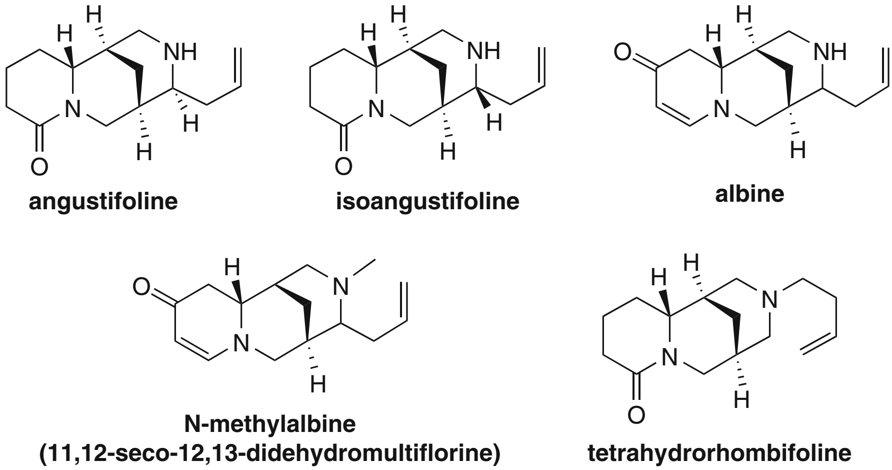

angustifoline
isoangustifoline
albine
N-methylalbine
(11,12-seco-12,13-didehydromultiflorine)
tetrahydrorhombifoline

Fig. 14.4 Chemical structure of angustifoline and its derivatives

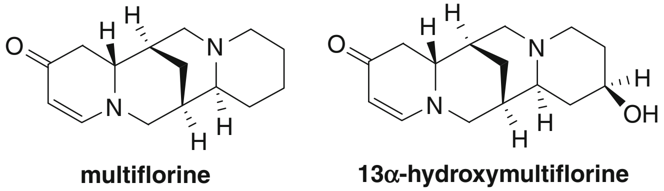

multiflorine
13α-hydroxymultiflorine

Fig. 14.5 Chemical structure of multiflorine and its derivatives

(Fig. 14.3), angustifoline and its derivatives (Fig. 14.4), multiflorine and its derivatives (Fig. 14.5), aphylline and its derivatives (Fig. 14.6), lupinine (Fig. 14.7).

Sparteine is a tetracyclic QA formed by two quinolizidine rings; lupanine is similar to sparteine but it has a carbonyl group on the C1 of the first ring. Its main derivative is hydroxylupanine, in particular 13-hydroxylupanine (3-hydroxylupanine is less abundant) that is the precursor of a large number of esters. Angustifoline, tetrahydrorhombifoline, and albine (Fig. 14.4) are tricyclic molecules because the fourth ring does not exist. Multiflorine (Fig. 14.5) is characterized by a conjugated carbonyl group on the third carbon atom of the first ring. Aphylline and its derivatives are characterized by a carbonyl group on the second ring of the molecule (Fig. 14.6).

Tetracyclic QAs such as sparteine (Fig. 14.2), lupanine, and 13α-hydroxylupanine (Fig. 14.3) are the major alkaloids present in almost all *Lupinus* species [8, 9].

Angustifoline (Fig. 14.4) occurs as a major alkaloid only in some lupin species such as *L. angustifolius*. Quite often angustifoline is seen as a minor component in Old World species (*L. albus*, *L. micranthus*), in South American species (*L. mutabilis*, *L. gibertianus*), and in North American species (*L. elegans*, *L. leucophyllus*, *L. perennis*).

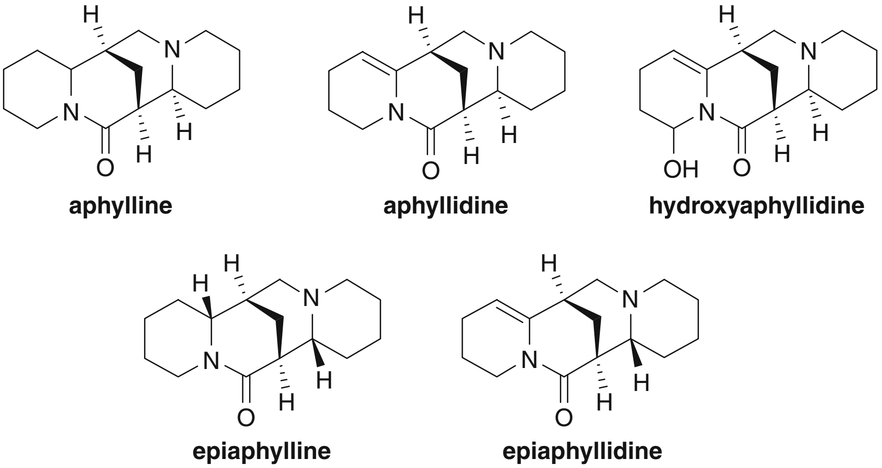


Fig. 14.6 Chemical structure of aphylline and its derivatives

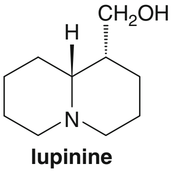


Fig. 14.7 Chemical structure of lupinine

Multiflorine (Fig. 14.5) and its derivatives have a restricted distribution and were found in Old World lupins such as *L. albus L. atlanticus*, *L. cosentinii*, *L. varius*, and also in some American taxa such as *L. albescens*; North American lupins do not accumulate these alkaloids in substantial amounts.

Aphylline (Fig. 14.6) and its derivatives occur as major alkaloids in a limited number of North American lupins such as *L. campestris*, *L. argenteus*, and *L. leucophyllus*.

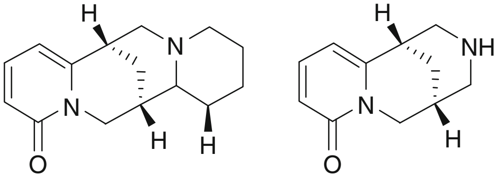
Lupinine (Fig. 14.7), a byciclic QA, is characteristic of *L. luteus* and it is abundant in Old World lupins such as *L. luteus*, *L. hispanicus*, *L. cosentinii*, *L. pilosus*, and *L. varius*.

QAs of the α-pyridone skeleton such as anagyrine and cytisine (Fig. 14.8) are typical for many genera of Papilionoideae, but usually absent in lupins, even if some few taxa can accumulate them.

Some lupin taxa produce also piperidine alkaloids such as ammodendrine (Fig. 14.9) that is abundant in *L. elegans*, *L. sulphureus*, and *L. bakeri*.

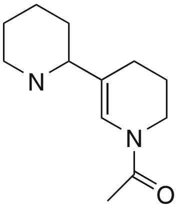
1.2 Alkaloids Fingerprint in Different Lupin Species

The alkaloid profiles are typical and characteristic for a given lupin species producing the so-called alkaloid fingerprint.



anagyrine **cytisine**

Fig. 14.8 Chemical structure of anagyrine and cytisine



ammodendrine

Fig. 14.9 Chemical structure of ammodendrine

Generally, Old World and South American lupins have profiles without much intraspecific variation, whereas a lot of lupins from North America present a high degree of intraspecific variation [10].

Great differences can also occur among different organs of the plant such as seeds and leaves (see the Sect. 5) [8].

A work by Wink and coworkers (1995) describes the complete alkaloid pattern of the main lupin species [8]. The QAs composition of the four main lupin species is reported above.

The main QAs of *L. albus* seeds are albine, lupanine, multiflorine, 13α-hydroxylupanine; minor quantities of sparteine, tetrahydrocytisine, isoangustifoline, tetrahydrorhombifoline, angustifoline, α-isolupanine, 5,6-dehydrolupanine, 11,12-seco-12,13-didehydromultiflorine (formerly *N*-methylalbine), 17-oxolupanine, 13α-hydroxymultiflorine, and different esters of 13-hydroxylupanine such as 13-propyloxylupanine, 13-isobutyryloxylupanine, 13-butyryloxylupanine, 13-isovaleroyloxylupanine, 13-angeloyloxylupanine, 13-tigloyloxylupanine, and of 13-hydroxymultiflorine such as 13-angeloyloxymultiflorine, 13-tigloyloxymultiflorine are detected [8].

In *L. angustifolius* seeds the main QAs are angustifoline, lupanine, 13α-hydroxylupanine; minor quantities of sparteine, isoangustifoline, tetrahydrorhombifoline, α-isolupanine, 17-oxolupanine are usually detected [8].

The main QAs in *L. luteus* are lupinine and its esters (E/Z isomers of (4′-hydroxy-3′-methoxycinnamoyl) lupinine) and ((4′-hydroxycinnamoyl)lupinine) and sparteine; minor amounts of β-isosparteine, tetrahydrorhombifoline, 17-oxosparteine, lupanine, feruloyllupinine are present [8, 11].

In *L. mutabilis* seeds there are sparteine, 11,12-dehydrosparteine, ammodendrine, tetrahydrorhombifoline, angustifoline, lupanine, 3β-hydroxylupanine (formerly nuttaline or 4-hydroxylupanine), 13α-hydroxylupanine, 13α-angeloyloxylupanine, 13α-tigloyloxylupanine as main alkaloids. Minor amounts of 17-oxosparteine, α-isolupanine, multiflorine, 3-propionyloxylupanine, 13-benzoyloxylupanine, 3,13-dihydroxylupanine, *cis* 13α-cinnamoyloxylupanine, *trans* 13α-cinnamoyloxylupanine are detected [8, 12].

1.3 Alkaloid-Poor and Alkaloid-Rich Lupin Species

Some cultivars belonging to *L. albus*, *L. angustifolius*, *L. luteus*, and *L. mutabilis* have been selected and bred to produce varieties with low concentration of QAs and, consequently, produce seeds that are less toxic and more palatable. These varieties are also known in literature as low-alkaloid or feed or alkaloid-free or sweet lupin, definition that was originally based on their taste. These varieties contain low levels of QAs in vegetative parts as well as seed and, not surprisingly, have considerably lower resistance to disease and predation compared with "bitter," wild germplasm [3].

The bitter and sweet cultivars are similar in the case of traits such as flowering time and pod shattering, but differ for the alkaloids content. The bitter and the sweet cultivars have a similar background, but the sweet cultivars are homozygous for the mutant iuc allele, which results in low-alkaloid phenotypes [13].

In agriculture practice, the QAs content of 0.05 % DM (Dry Matter) of seed is taken as the division between alkaloid-rich "bitter" and alkaloid-poor "sweet" white lupins [14].

In the case of white lupin, von Baer and Perez [14] proposed a classification according to which the alkaloid content of the sweet lupin is less than 0.05 % DM, the semisweet is in the range 0.05–0.10 % DM, the semibitter in the range 0.10–0.20 % DM, and the bitter lupin over 0.2 % DM. This definition of "sweet," however, appears to be rather misleading from a toxicological viewpoint [15].

Considering all the physiological experience up to now, it was proposed that for human consumption lupin seed should not exceed an alkaloid content of 0.02 % [16].

The Food Authorities of some countries, such as Australia and New Zealand [17], France [18], and Great Britain [19] have decided to fix at 0.2 mg/g the minimal risk level (MRL) of alkaloids in lupin flour and food products (see Sect. 4).

The total QAs content is only a crude indicator and it is not enough to induce whether a particular lupin is fit for direct use in human nutrition. In fact it is important to know the content of particular alkaloids such as anagyrine (Fig. 14.8), cytisine (Fig. 14.8), and ammodendrine (Fig. 14.9). Anagyrine and cytisine are α-pyridone alkaloids, whereas ammodendrine is a piperidine alkaloid. They are usually extracted and analyzed with quinolizidine ones. All of them have shown teratogenic activity (see Sect. 4) [8].

Considering the great variability of the concentration of the alkaloids in commercial cultivars, the increasing use of lupin-based ingredients in food manufacturing encourages to establish a homogeneous regulation at least in all countries were lupin is produced or utilized.

2 Biosynthesis

In general, alkaloids derive from the metabolism of amino acids such as phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), ornitine (Orn), or lysine (Lys). Quinolizidine alkaloids derived from L-lysine. Its decarboxylation by means of the enzyme lysine decarboxylase gives cadaverine (Cad), the first detectable intermediate of this biosynthetic pathway (Scheme 14.1).

Subsequently, the quinolizidine byciclic system is formed from the cyclization of two molecules of cadaverine; from this intermediate all the bicyclic alkaloids (e.g., lupinine) are formed.

The cyclization of three or more molecules of cadaverine yields the formation of tetracyclic alkaloids (e.g., lupanine and sparteine). These cyclic alkaloids are then transformed by cellular enzymes through dehydrogenation, oxygenation, hydroxylation, glycosydation, or esterification to yield a variety of alkaloids [20–23].

QAs are biosynthesized in the green tissues of the plant, in the mesophyll chloroplasts, then transported via the phloem and stored in all organs of the plant, including seeds in which they cause the bitter taste.

Recently, the biochemical localization of two acyltransferases that catalyze the last two acylations of (+)-p-coumaroylepilupinine and (−)-13α-tigloyloxymultiflorine showed that one enzyme occurs in the cytoplasm, whereas the other resides in the mitochondria of *L. albus* [24].

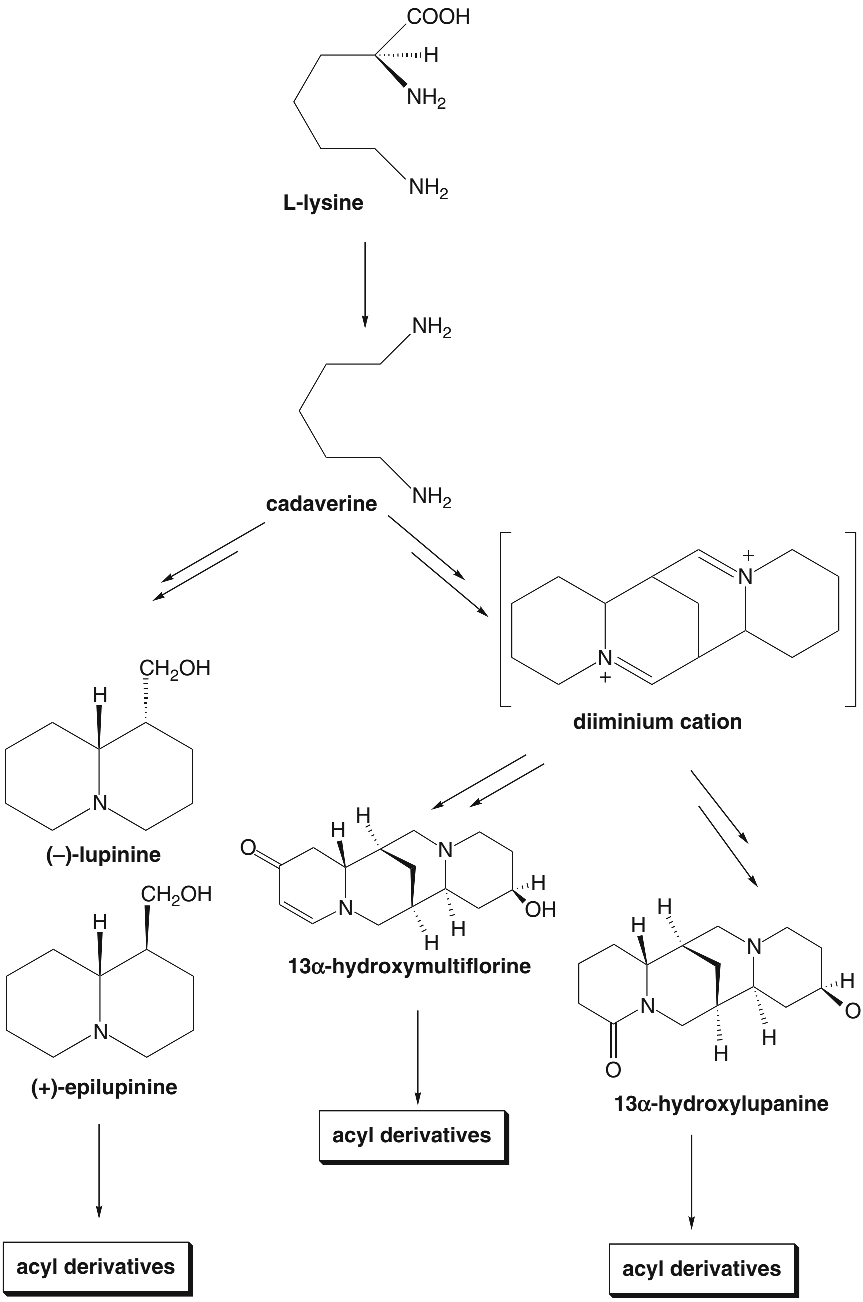
Although the quinolizidine nucleus appears to be synthesized in the stroma of leaf chloroplasts, subsequent modifications can occur only after alkaloid intermediates are transported to the cytosol and mitochondria. QAs are thought to accumulate in vacuoles of lupin epidermal cells, where their defensive properties are most effective [24].

Like many secondary metabolites, QAs are not the end product of the metabolism, but show a high degree of turnover. As alkaloid synthesis takes place in the chloroplasts, it follows a diurnal light-regulated cycle, with a stimulated period during the day and low values during the night [25].

Then they are transported via the phloem and stored in vacuoles in all the organs of the plant [26], preferentially in epidermal and subepidermal tissues of stems and leaves; the seeds are especially rich in alkaloids. Alkaloids tend to disappear during maturation from all parts except the seeds; the concentration dropped 90 % during senescence.

2.1 Differences in Biosynthesis in Alkaloid-Poor and Alkaloid-Rich Lupin Plants

The total content of QAs in alkaloid-poor plants is uniformly lower than that of the bitter ones, suggesting that the differences of alkaloid pattern between bitter and sweet plants are quantitative, but not qualitative.


COOH
H
NH2
NH2
L-lysine
NH2
NH2
cadaverine
diiminium cation
CH2OH
H
N
(–)-lupinine
CH2OH
H
N
(+)-epilupinine
13α-hydroxymultiflorine
13α-hydroxylupanine
acyl derivatives
acyl derivatives
acyl derivatives

Scheme 14.1 Biosynthetic pathway of quinolizidine alkaloids

The limiting step of the biosynthetic pathway in sweet plants, causing a sweet phenotype, is the reaction from cadaverine to the first cyclic alkaloid in both *L. luteus* and *L. albus* [20].

It is not surprising that the concentration of lysine does not change in bitter and sweet plants because protein-amino acid biosynthesis is strictly regulated in the plant cells.

Contents of cadaverine are much lower than those of lysine and are similar in bitter and sweet plants. This suggests that lysine decarboxylase responsible for the formation of cadaverine is controlled in the same manner and does not substantially change its activity in bitter and sweet plants.

The enzyme or the enzymes involved in the formation of initial cyclic alkaloids (lupinine and lupanine type) are suppressed in the sweet plants to cause low alkaloid accumulation. The same amounts of acyltransferase activities of post-ring closure steps are detected in sweet plants as well as in bitter ones, while the concentrations of lupinine and 13α-hydroxylupanine decreased in sweet plants [27].

These results suggest that the suppressed step for formation of alkaloids in sweet plants is the ring closure reaction from cadaverine to initial bicyclic and tetracyclic alkaloids, but neither the steps prior to the formation of cadaverine nor the specific reaction of cyclic alkaloids.

Moreover enzymatic activities of a post-ring cyclization step are still intact in sweet plants as well in the bitter ones [20, 27].

3 Factors Influencing Quinolizidine Alkaloids Synthesis

QAs are constitutive metabolites and their presence and abundance can vary during growth stage and depend on plant organ [1, 28, 29].

Seed alkaloids concentration may fluctuate depending both on genotype and environment and important variations in alkaloid content between different lots of lupin can be unpredictable [15, 30]. This variability is a potential hindrance to the wider use of lupin for human consumption and a toxicological topic for consumers safety.

During the vegetation period, alkaloid content undergoes changes, the peak coinciding with the flowering. At the end of vegetation, alkaloids accumulate mainly in seeds.

Some factors can affect alkaloid production: environmental effects such as drought, state of sunlight, temperature, season, site elevation, soil characteristics (moisture and levels of fertilizing), or presence of pathogens, such as insects or herbivores.

Significant environmental effects on QAs content are reported both on *L. angustifolius* [31] and *L. albus* cultivars [15]. In the first paper, large environmental effects were reported, substantially unrelated to rainfall amount. In particular, early drought stress tends to increase the alkaloid content, whereas terminal stress has the opposite effect; these effects are mainly observed when stress was present during the vegetative growth stage [32]. The results gained in Boschin et al. [15] on 48 ecotypes from 11 regions, show that the selective pressure that leads to greater or lower alkaloid content, varied markedly within each region.

Regarding sweet cultivars, a major environmental effect was consistent between cultivars grown in the subcontinental climate site relative to the Mediterranean location. The main difference between these cropping environments concerned the extent of low temperatures during winter [15].

One of the factors that may affect seed alkaloids content is soil nutrient deficiency. For example, K deficiency may induce high alkaloid contents in seeds both of sweet and bitter varieties, while it seems not to affect alkaloid production in the vegetative portions of the plant [33].

High alkaloid levels of seeds of both sweet and bitter lupins grown in glass house are observed, compared with seeds produced in field experiments. This may be due to the synergistic effect of other environmental stresses, such as light energy and air temperatures in the glasshouse, together with the limited rooting space available in the pots [33].

Also the differences in nitrogen forms used as fertilizer can influence alkaloid production [34]. In *L. albus*, differences are observed between sweet and bitter cultivars in relation to the different forms of nitrogen used for soil fertilization. For a sweet variety, the highest accumulation is observed when nitrogen is uptaken in NO_3^-, NH_4^+, or N_2 form; whereas, for other N forms, the alkaloid production is lower. On the contrary for a bitter cultivar, only uptaken in NO_3^- or N form cause less alkaloid production than the control [34].

Soil P deficiency reduced seed alkaloid concentration in sweet, but not in bitter varieties [35]. Furthermore, sweet varieties change their alkaloid pattern mimicking the bitter ones. That is 13α-hydroxylupanine content is larger than lupanine one. With adequate or abundant P, lupanine is the predominant alkaloid in sweet varieties.

Germination involves a great number of physiological changes, including synthesis, degradation, and transformation of different compounds. This process can reduce the presence of antinutrients such as α-galactosides or phytic acid in legume seeds, but also alkaloid content [29].

During germination, esterification reactions take place. In *L. albus*, for example, levels of albine and 13α-hydroxylupanine decreased, whereas lupanine and its ester 13α-tigloyloxylupanine increased [21]; the latter one also increased during germination of *L. angustifolius*.

Lupanine is transformed into 13α-hydroxylupanine both in *L. angustifolius* and in *L. albus*, but the reaction is more active in the first one. In *L. albus*, the synthesis of lupanine is more intense than its transformation, since its level increase during germination. The decrease of 13α-hydroxylupanine in *L. albus* and *L. angustifolius* indicates a subsequent progressive degradation of this compound during the germination process.

In *L. angustifolius*, lupanine is highly transformed into angustifoline, isoangustifoline and its direct derivatives, 13α-hydroxylupanine, and 5,6-dehydrolupanine.

On the contrary, in *L. albus*, lupanine content is very high with less transformation into its derivatives [29].

In *L. campestris*, hydroxyaphylline and hydroxyaphyllidine increased at the expense of epihydroaphylline and dehydroepihydroxyaphylline. The optimal germination period seems to be three days in order to minimize the presence of antinutritive factors and avoid formation of quinolizidine esters [36].

Defensive chemicals, mainly secondary metabolites, can be constitutively present, but can be de novo synthesized or their concentration can increase, triggered by external stimuli, that is, chemical induction. Traditional bitter varieties have inductive increases in their alkaloid concentration after damage. In particular, lupanine is the most abundant alkaloid also after the induction, whereas minor alkaloids changed scarcely after damage.

QAs production can be stimulated by applying abiotic, for example, $CuCl_2$, or biotic elicitors, so-called phytoalexins, prepared, for example, from cells of fungal mycelium [37].

Changes in alkaloid profile have been detected for *L. albus* and *L. angustifolius* cultivars as a response to mechanical damage, such as biomass removal mimicking large herbivore action on aerial parts of the plants [38]. Both sweet and bitter varieties showed an increase in total alkaloids content, but sweet varieties increase is greater than bitter varieties one: from 58.8 % to 67.9 % versus from 22 % to 32.8 %, respectively.

Seed multiplication is exposed to risk of genetic shift toward higher alkaloid content due to pollen flow from bitter material, which increases over generations of multiplication as a consequence of higher advantage of greater bitterness under natural selection. In this context, the need to carefully monitor the alkaloid content of seeds is of outmost importance. This control is necessary not only in production lots, but also across the various stages of seed multiplication of the varieties; it is also important to consider the different environmental conditions of their cultivation.

4 Toxicity

The majority of the acute studies has been performed on the common lupin alkaloids sparteine and lupanine [39–41]. They both display moderate acute toxicity, the former being the more toxic one. The observed symptoms suggest that alkaloids cause neurological effects leading to loss of motor coordination and muscular control. The effects are generally reversible.

Lupanine and other lupin alkaloids show moderate toxicity in vertebrates. In mammals, acute intoxications with sparteine, lupanine, or angustifoline cause convulsions, shaking and trembling, and death from respiratory and cardiac arrest [15].

QAs exert a blocking effect on the nicotinic cholinergic receptor and they are weak antagonists at the muscarinic cholinergic receptor [42], displaying similar agonistic activities as the alkaloid nicotine. Neurological (weakness, dizziness, mydriasis, anxiety, confusion, malaise, loss of coordination, visual disturbances, and dry mouth), cardiovascular (dysrhythmias) and gastrointestinal (nausea, vomiting) symptoms are due to these anticholinergic effects.

Some QAs can inhibit Na^+ and K^+ channels, inducing gastrointestinal, nervous, and respiratory symptoms in humans and animals.

Sparteine and lupanine, when administered by intravenous rout, inhibit ganglionic transmission of the sympathetic nervous system, assessing their neurotoxicity [17].

L. angustifolius alkaloids have been found to be neither carcinogenic nor teratogenic and their LD_{50} levels are above the levels that would be consumed in a normal diet.

In a 90-day feeding study, rats were fed with diets supplemented with 20 % lupin protein from sweet varieties of *L. albus* and *L. luteus*, containing 0.05 % (0.5 g kg^{-1}) and 0.09 % (0.9 g kg^{-1}) of lupin alkaloids, respectively. The actual levels of alkaloids present in the diets were not reported, but taking into account the protein levels in sweet lupins of 35 % and 39 % and assuming full incorporation of the alkaloids into the diet, the lupin flour dietary levels would amount to about 290 and 460 mg alkaloids kg^{-1} diet, respectively, resulting in dietary intakes of about 14.5 and 23 mg lupin alkaloids kg^{-1} bw day^{-1}. No adverse effects were observed in terms of food intake, organ weight, and microscopic examinations [43].

The acute oral LD_{50} (lethal dose for 50 % of the population) values in rats for lupanine is 1,664 mg kg^{-1} bw [44].

At high doses, lupanine showed a moderate acute oral toxicity due to neurological effects leading to loss of motor coordination and muscular control; in mammals, the intoxication is characterized by trembling, shaking, excitation, and convulsion [45].

Effects on human health reflecting neurological, cardiovascular, or gastrointestinal symptoms can be observed either if lupin seeds are eaten unripe or not debittered. Symptoms of intoxication after ingestion of lupin seeds with high alkaloids content appear 1–14 h after consumption and include dry mouth, muscular weakness, disturbed balance, sweating, palpitation, blurred vision, mydriasis (i.e., dilated pupils), urine retention, gastric and intestinal troubles, and abundant ventricular extrasystoles [45].

A few poisoning cases have been reported in literature, all related to the consumption of raw lupin seeds [46, 47]. There are some cases of acute toxicity in humans who ate lupin beans, which had not been previously debittered. These subjects suffered from blurry vision, dry mouth, facial flushing, and confusion [46, 47].

Marquez et al. [48] reported a single case of a young man who drank 0.5 L of water that had been used for lupin seeds debittering process. He suffered from sudden weakness, palpitations, extrasystoles, and different anticholinergic symptoms.

Very little research has been done on chronic QAs toxicity. It is generally assumed that, being them water soluble, an organism can easily eliminate them and therefore prevent any cumulative toxic effect. Little data is available on the metabolism and excretion of alkaloids in animals and humans. In one human study, orally administered lupanine was excreted unchanged in the urine with a half-life of 6–7 h [17].

A study on rats fed with *L. albus* flour (with 0.025 % lupanine) for two generations showed no harmful effects. In addition, rats which survive a lethal dose, recover completely, manifest no signs of clinical abnormality and subsequently attain normal weight and physiological maturity, comparable to those of untreated rats [49].

Tolerable daily intake (TDI) values for QAs have not been established, although taking the NOAEL (no observed adverse effect level) of 12.5 mg kg^{-1} bw day^{-1} and a MOS (margin of safety) of 100, it may conclude that a daily intake of 0.125 mg lupin alkaloids kg^{-1} bw day^{-1} would not be of safety concern.

This value is in the same range as the provisional tolerable daily intake of 0.035 mg lupin alkaloids kg^{-1} bw day^{-1} derived by ANZFA (Australia and New Zealand Food Authority) based on traditional use of lupin seeds. ANZFA concluded that traditional use of lupin seeds in Europe suggests that a daily dose of 0.35 mg lupin alkaloids kg^{-1} bw (20 mg lupin alkaloids day^{-1}) can be tolerated in human adults without adverse effects. If an uncertainty factor of 10 is applied to account for the uncertainties in the data and particularly to take into account likely human variation, the provisional tolerable daily intake for humans is 0.035 mg lupin alkaloids kg^{-1} bw day^{-1} [17].

This would imply that for a 60 kg person, a daily intake of 2.1–7.5 mg alkaloids can be tolerated.

Given that the seeds of modern cultivars contain 200 mg alkaloids kg^{-1}, this would imply a daily consumption of at most 10.7–37.2 g of sweet lupin seeds. This estimate suggests that good quality control of lupin-containing foods is necessary.

Regulations in France [18], Great Britain [19], and Australia and New Zealand [17] demand to comply with a maximum contamination of 200 μg g^{-1} (i.e., mg kg^{-1}) alkaloids in lupin-based foods.

A different matter is the toxicity of other alkaloids occurring in some lupin species like α-pyridone alkaloids, such as cytisine and anagyrine, or piperidine alkaloids, such as ammodendrine. All of them have a high toxicity, mainly teratogenicity [50]. In particular, anagyrine and ammodendrine are responsible of the so-called crooked calf disease.

Historically, acute intoxication from lupin was a major cause of sheep death [51]. Ingestion of lupin by cattle was first reported to cause congenital birth defects in calf, called crooked calf disease, in the late 1950s. This disease was described as a condition in which calves were born with some deformities such as scoliosis, arthrogryposis, torticollis, and cleft palate.

Anagyrine and some piperidine alkaloids such as ammodendrine were shown to reduce fetal movement during a period of gestation causing the spine and limbs to develop in contracted or misaligned positions. *L. leucophyllus*, *L. sulphureus*, and *L. sericeus* can contain anagyrine, but it does not occur in the domesticated lupin varieties [51].

5 Different Alkaloids in Different Plant Parts

Alkaloid profiles generally show variations among plant parts. As already reported, QAs synthesis occurred in leaves and, via phloem and xylem, they are translocated in the other parts of the plant [27]. The highest concentration of alkaloids was revealed in stems, leaves, flowers, and pods, lowest concentration in roots [27].

A higher diversity and concentration of QAs is revealed in seeds in comparison with leaves; in fact, seeds are the reproduction organs of plants, so they need higher protection.

Usually leaves have different alkaloid profiles than seeds. Generally the hydroxylated alkaloids predominate in seeds, whereas esters of 13-hydroxylupanine, 3-hydroxylupanine, and 13-hydroxymultiflorine are abundant in leaves. In particular, these esters are generated by specific acyltransferases during germination.

In *L. albus* leaves the main QAs are albine, angustifoline, and lupanine; other alkaloids usually present are sparteine, 11,12-dehydrosparteine, isoangustifoline, tetrahydrorhombifoline, 17-oxosparteine, α-isolupanine, 5,6-dehydrolupanine, 13-angeloyloxymultiflorine, 13-tigloyloxymultiflorine. 13-Hydroxylupanine that is usually very abundant in seeds, in leaves is almost totally esterified [8].

For *L. angustifolius*, the main QAs of leaves are angustifoline, lupanine, 13-hydroxylupanine, 13-*cis*-cinnamoyloxylupanine; other QAs are isoangustifoline, tetrahydrorhombifoline, α-isolupanine, 17-oxolupanine, 13-benzoyloxylupanine, dihydroxyaphyllidine, dihydroxylupanine, dehydrolupanine [8].

In *L. luteus* leaves the main QAs are lupinine, sparteine, feruloyllupinine, p-coumaryllupinine; other QAs are gramine, α-isosparteine, 11,12-dehydrosparteine, tetrahydrorhombifoline, 17-oxosparteine, lupanine [8].

L. mutabilis leaves mainly contain sparteine, tetrahydrorhombifoline, angustifoline, lupanine, 3β-hydroxylupanine (formerly nuttaline or 4-hydroxylupanine), 13-hydroxylupanine, 13-isovaleroyloxylupanine, 13-tigloyloxylupanine. Other QAs are 11,12-dehydrosparteine, 17-oxosparteine, α-isolupanine, 13-valeroyloxylupanine, 13-benzoyloxylupanine, 13-*cis*-cinnamoyloxylupanine, 13-*trans*-cinnamoyloxylupanine, dihydroxylupanine, 3,13-dihydroxylupanine [8].

Lupinus aschenbornii different organs were recently analyzed obtaining different results for seeds, stems, flowers, and leaves [52]. The authors do not detect QAs in roots, whereas pods show sparteine as the main alkaloid. Total QA contents of different organs are very different: 3.3 mg DM^{-1} in seeds, 2.8 mg in flowers, 1.9 mg in leaves, 1.5 mg in stems, and 1.4 mg in pods. These results confirm that QAs accumulate in the reproductive organs, playing an important role in its defense, and also contribute to N metabolism [5].

Also the alkaloid profile is very different: seeds of *L. aschenbornii* contain mainly *N*-formylangustifoline, whereas stems, leaves and flowers contain mainly sparteine. The explanations proposed by the authors for this diversity are: (1) “one predator, one organ,” that is a variability of QAs can confer to lupin plants higher possibility to survive and reproduce in an adverse environment and (2) the transport of QAs through the plant is uneven [52].

Few data are available for plant roots. In roots of *L. albus* mainly lupanine and its ester 13-tigloyloxylupanine were detected followed by minor amounts of angustifoline, 11,12-seco-12,13-didehydromultiflorine, and 17-oxolupanine [37].

6 Biological Activities

Over 12,000 alkaloids are known, most of all displaying potent pharmacological activities, and several widely used as pharmaceuticals. There are a large number of reports on the biological activities of lupin alkaloids having antipyretic, hypoglycemic, cardiotonic, and antiulcerogenic activity, inhibitors of edema, inhibitors of natural killer cell growth and of acetylcholinesterase [53–55].

Antimicrobial activity is well known. For example, sparteine is reported to possess antimicrobial activity against bacteria and phytopathogenic fungi. In particular, antibacterial and antifungal activity of lupin leaves extracts were shown on *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *C. albicans* [55].

L. albus seeds are used as diuretic, anthelmintic, and tonic in folk medicine; they are also used in the treatment of liver disorders, diabetes, hemorrhoids, and eczema [55].

Oral administration of *L. termis* and *L. angustifolius* reduces high blood pressure and hyperglycemia in rabbits, rats, and mice [54].

The addition of lupin seeds to the food of diabetic-hypercholesterolemic rabbits decreases cholesterol levels and postprandial hyperglycemia. Sparteine sulfate administered by intravenous infusion to normal men increases either basal or glucose-induced secretion; sparteine administration to patients with type 2 diabetes causes a fall in plasma glucose levels [56].

QAs show a wide range of biological activities: they can inhibit the multiplication of viruses, the proliferation of bacteria, and the growth of some fungi.

Some allelopathic (phytotoxic) effects of QAs have been described, including the inhibition of the growth of competing plants. They can also deter number of herbivores (nematodes, caterpillars, beetles, aphids, locusts, snails, rabbits, and cows), but also pollinators such as bees.

Natural compounds such as alkaloids derived from wild plants may represent interesting alternative bio-rational pesticides against phytophagous insects. QAs can be used as insecticides either alone or in combination with other classes of pesticides such as pyrethroids. They can be rapidly degraded by UV and hence posses low persistence in the environment. Moreover, some of them are very specific as insecticides and/or deterrents [1].

Sparteine, lupanine, and 13-tigloyloxylupanine were investigated in detail and sparteine was found to have toxic as well feeding deterrent properties for some Lepidoptera.

Extracts from leaves of *L. montanus*, *L. aschenbornii*, *L. stipulatus* grown in Mexico showed insecticidal activity toward larvae of the Fall Armyworm (*Spodoptera frugiperda*) [57]. The extracts of *L. montanus* and *L. aschenbornii* contain sparteine as the major constituent, while the major alkaloids in *L. stipulatus* extract are aphylline and an epiaphylline-like alkaloid. This last one was found to be the most effective against larvae of *S. frugiperda*. Extracts of *L. montanus* and *L. aschenbornii* were found to have toxicity similar to sparteine [57].

The differences in alkaloid composition between species indicate a broad range of defense strategies against predation. Moreover, the fact that some intraspecific variations in alkaloid composition occur with the season and the altitude at which the plants grow, may explain why the proportions and sometimes even the composition of alkaloid, vary among published studies.

7 Debittering Processes

As previously described, by mean of hybridization it is possible to reduce the alkaloid level and to develop alkaloid-poor varieties also known in literature as sweet varieties or alkaloid-free lupins.

Unfortunately, sweetness is a genetically recessive characteristic, hence in the regions where lupin is spontaneous, a progressive cross-pollination induce rebittering, and residual levels of alkaloids in the new varieties can tend to increase [58].

Already in ancient times it was known that when lupins are soaked in water, the water-soluble alkaloids can be leached out. This old finding was already practice in ancient Greece and by Indians in the Andes.

As an alternative, treatments such as cooking, soaking, germination, and fermentation are known to eliminate QAs. To remove significant amounts of alkaloids, it is necessary to cook and wash the seeds for several days; this preparation eliminates the toxicity and makes the seed more palatable for human consumption. Usually, the seeds are soaked in running water for a few days and afterward they are cooked.

In particular, aqueous, alkaline, and acid thermal treatments are tested for debittering processes [49]. In the case of aqueous thermal treatment, a 56 % decrease is obtained after 3 h, whereas in the case of alkaline treatment a greater decrease (76.5 %) is observed.

In alkaline environment, the seed hydration was greater than in aqueous one (78 % and 62 %, respectively). After 5 h of alkaline treatment, the total alkaloid concentration is 0.03 %, which means that about 99 % of alkaloids has been eliminated. For aqueous treatment, this level was obtained only after 6 h. The final concentration of QAs in debittered seeds with alkaline thermal treatment is 0.002 %, which represents 99.9 % of QAs elimination. After technological treatment, QAs content decreased by 89 % in the aqueous thermal treatment, 88 % in the acidic treatment, and 95 % in the alkaline treatment [59]. During both the steps of soaking and cooking, the total content of QAs in seeds diminished by 55 % and 35 % for *L. campestris* and *L. mutabilis*, respectively. Seeds that are soaked, cooked, and fermented for 24 h showed a decrease of 70 % and 81 %, of total QAs, respectively. The greater total QAs losses occur during soaking and cooking of the seed (>50 %).

Fermentation causes a general improvement in the nutritional value of legume seeds and may result in the breakdown of some of the antinutritional endogenous compounds. For example, *Rhizopus oligosporus*, used for fermentation step during the production of tempeh, decreases QAs content from seeds of *L. campestris* and *L. mutabilis* at levels of 95 % and 91 %, respectively [60]. In conclusion, the fermentation method could be a good alternative for debittering of lupines seed since it is faster, less expensive, and without environmental contamination.

Other scientists proposed the removal of QAs from *L. albus* flours using bacteria able to catabolize lupanine and to degrade other lupin alkaloids in culture media [58]. The obtained results suggest that enzymatically active bacterial lysates or purified enzyme complex may possess a high potential for the detoxification of QAs, being an alternative debittering process.

8 Analytical Methods

The extraction of alkaloids from vegetative samples (seeds, leaves, pods, etc.) or food ingredients is usually performed in acidic aqueous medium; then the solution is basified, and the QAs are extracted with unpolar solvents, usually dichloromethane.

In alternative to solvent extraction, a SPE (Solid Phase Extraction) on Extrelut© columns is performed in order to avoid the formation of resistant emulsions and, at the end, to maximize and standardize QAs extraction [15].

The chromatography of QAs on thin-layer plates (Thin Layer Chromatography, TLC) and their following detection by Dragendorff reagent has been used in many studies, especially in older ones [7]. This technique has some limitation in the analyses of mixture of alkaloids in which there are both major and minor QAs.

A good alternative can be the gas chromatography equipped with FID, that is, flame ionization detector [61] or with mass spectrometer, used in the majority of papers. Wink and coworkers [8], have studied the QAs profile of 56 species of lupin by GC-MS reporting Kovat’s index and fragmentation ions of more than 100 quinolizidine alkaloids.

Usually nonpolar or low-polarity columns were used, as a consequence of the low polarity of QAs. No derivatization is required, even if some papers used it when the analysis was hampered by matrix peaks or lack of analytes detectability without derivatization [61].

Very few papers use HPLC for alkaloids quantitation [9, 62]. An alternative to these methods could be the nonaqueous capillary electrophoresis (NACE) coupled with UV or MS detection; this technique shows high separation efficiency, economic performance, and versatility [63].

The quantification of QAs is complicated by the fact that commercial standard of QAs are not easily available; for this reason the isolation of alkaloids from extracts is necessary to obtain single alkaloids.

Usually, single alkaloid contents were expressed in percentage respect to total alkaloid content, or in absolute values using calibration curve of lupanine or sparteine depending on the chemical structure and adjusting for molecular weight of single alkaloids [30, 37, 58, 64].

The identification of a single structure after isolation is mainly performed with spectral analysis: mass spectrum, IR and Raman spectra, NMR spectra both of ^{1}H and ^{13}C, both mono- and bi-dimensional ones [36].

9 Conclusion

QAs are a large family of molecules, generally known as lupin alkaloids as they occur in lupin and other plants of the Leguminosae family. They act as a defense against biotic and abiotic stresses, such as predators, pathogens, or adverse climatic events. They are biosynthesized in the vegetative parts of the plants, but they accumulate in seeds that are the raw material for the production of lupin ingredients to be included in human foodstuffs. As these ingredients are gaining outmost importance as source of proteins, a maximum limit of total QAs content has been fixed in some countries, in order to assure the safety of lupin foods.

A lot of chemical and analytical work has been performed on QAs in order to determine chemical structures of single compounds and of biochemical work to determine pharmacological or biological activities of both single compounds and complex extracts. Moreover, this issue has yet great potentiality both for analytical and synthetic chemists, biologists, and phytopharmacological scientists.

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Alkaloids Derived from Tyrosine: Modified Benzyltetrahydroisoquinoline Alkaloids 15

Feroz Khan, Tabish Qidwai, Rakesh K. Shukla, and Vikrant Gupta

Contents

1 Introduction 407
1.1 Natural Product 408
2 Secondary Metabolites 408
2.1 Classification of Secondary Metabolites 409
3 Alkaloids 410
4 Classification of Alkaloids 411
5 Biosynthesis of Alkaloids 418
5.1 Pyrrolizidine Alkaloids 419
5.2 Isoquinoline Alkaloids 420
5.3 Isoquinoline Alkaloid Biosynthesis 420
6 Intermediates of Morphine Biosynthetic Pathway 428
6.1 The Opium Alkaloids 433
6.2 Papaverine 440
7 Other Compounds 441
7.1 Meconic Acid 441
7.2 Morphine-6-Glucuronide 441
7.3 Narcotoline 442
7.4 Pseudomorphine 442
7.5 Laudanosine 443
7.6 Berberine 444
7.7 Sanguinarine 445
7.8 Coptisine 445
7.9 Cycloartenol 445
7.10 Glaucine 446
7.11 Oripavine 446

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7.12 Reticuline 447
7.13 Salutaridine 448
7.14 Sanguinarine 448
7.15 Scoulerine 449
7.16 Stepholidine 449
7.17 Structure and Properties of Alkaloids 449
8 Use of Alkaloids in Medicine 451
8.1 Antimalarial Properties of Alkaloids 455
8.2 Anticancer Properties of Alkaloids 457
9 Conclusion 458
References 458

Abstract

Secondary metabolites are produced by plants in response to biotic or abiotic interactions with their environment and confer protection through a variety of antimicrobial, pesticidal, and pharmacological properties. Alkaloids are a class of plant secondary metabolites that traditionally have been classified as basic compounds derived from amino acids that contain one or more heterocyclic nitrogen atom. About 20 % of plant species accumulate alkaloids, which are mostly derived from amino acids, e.g., phenylalanine, tyrosine, tryptophan, and lysine. The alkaloids are popular for their medicinal importance. The pharmaceutically important representatives of secondary metabolites are mostly alkaloids derived from tyrosine. In this chapter, we summarized the prior information, basic knowledge about the alkaloids, origin, physicochemical properties, uses, classification, biosynthetic reactions, and distribution of tyrosine-derived alkaloids especially opium alkaloids and their biosynthetic pathways in plants. We have also reviewed different web resources related to alkaloids and secondary metabolic pathway databases such as KEGG.

Keywords

Anticancer • antimalarial • classification of alkaloids • isoquinoline alkaloids • KEGG • tyrosine alkaloid biosynthesis

Abbreviations

BIA	Benzylisoquinoline alkaloids
CNMT	(S)-coclaurine-*N*-methyltransferase
Cor	Codeinone reductase
CYP	Cytochrome P
DOPA	Dihydroxy phenylalanine
KEGG	Kyoto Encyclopedia of Genes and Genomes
M6G	Morphine-6-glucuronide
NCS	(S)-norcoclaurine synthase
OMT	(R,S)-reticuline 7-*O*-methyltransferase
SalR	Salutaridine reductase

SDR	Short-chain dehydrogenase/reductase
tyrDC	Tyrosine decarboxylase
UGT2B7	UDP-Glucuronosyltransferase-2B7

1 Introduction

Plants produce a vast array of secondary metabolites in response to biotic or abiotic interactions with their environment, which impart flavor, color, and fragrance and confer protection through a variety of antimicrobial, pesticidal, and pharmacological properties. About 20 % of plant species accumulate alkaloids, which are mostly derived from amino acids, e.g., Phe, Tyr, Trp, and Lys. In addition, the monoterpenoid indole alkaloids, which form a large class of complex compounds, are derived from tryptophan and terpenoid precursors [1]. They are assumed to play indispensable roles for the survival of plants producing these metabolites as defense compounds against pathogenic organisms or predators and allelopathic metabolites for competing with other plants. These are natural products of non-peptidic origin containing nitrogen that have had the major impact throughout history on the economic, medical, political, and social affairs of humans. In fact, they are the skeleton framework of about 60 % of the modern drugs, such as atropine for tropicamide, quinine for chloroquine, and cocaine for procaine and tetracaine, that are available today. Thus, there has been a resurgence in the screening of plant extracts for pharmacological activities as part of industrial drug development programs in recent years.

Their basic character is reflected in the name derived from alkaline which means basic. The ease with which they can be extracted from plants, fungi, or insects where they occur naturally coupled with their sometimes profound biological effects on human have made them a very early focus of systematics chemical research. Given the commercial value of the alkaloids, the processes and genes involved in their biosynthesis and secretion are attractive targets for genetic engineering. In the course of studying the biosynthesis of these metabolites at biochemical, genomic, and systematic level, life scientists recently listed the following questions: (a) to what extent is the genomic pathways conserved among different species? (b) is there a minimal set of pathways that are required by all organisms? (c) how are organisms related in terms of the distance between pathways rather than at the level of DNA sequence similarity? At the core of such questions lies the identification of pathways in different organisms. So in a way pathway knowledge in public databases enables us to examine how individual metabolites are connected via chemical reactions and what genes are implicated in those processes. Given the commercial value of the alkaloids and the processes and genes involved in their biosynthesis and secretion which are attractive targets for genetic engineering, very little is known about how plants synthesize these substances and so far little is known about how the synthesis is regulated at the genetic level. Moreover, owing to the cost of sequencing and their relatively large size, the full genome sequences of only a few plants are currently available. Recent progress in the many plant sequencing projects; there is an imperative need to integrate functional genomics data to obtain a more comprehensive system-biology view of the results. Besides, biochemical pathway maps composed of genes, proteins, and metabolites are powerful tools around which one can compile the biological context of functional genomics datasets. Several metabolic pathway databases are available to facilitate our understanding of transcriptome and metabolome data such as KEGG (http://www.genome.ad.jp/kegg), MetaCyc (http://metacyc.org), AraCyc (http://www.arabidopsis.org/tools/aracyc), BioCyc (http://biocyc.org), and Reactome (http://reactome.org), but the pathways and reactions involved in alkaloid biosynthesis are not well represented and the information contained in these databases does not meet the unique data requirements for plant researchers, especially those involved in plant alkaloid metabolism studies. Alkaloids have an enormous structural and biosynthetic diversity, and these are identified and quantified using a wide range of technology platforms. In this chapter, alkaloid structure, classification, biosynthesis, and function and their role in disease therapy like malaria and cancer have been covered.

1.1 Natural Product

A natural product is a chemical compound or substance produced by a living organism found in nature that usually has a pharmacological or biological activity for use in pharmaceutical drug discovery and drug design. Not all natural products can be fully synthesized, and many natural products have very complex structures that are too difficult and expensive on an industrial scale such as penicillin, morphine, paclitaxel, and vincristine. To avoid side effects of many nonnatural and synthetic drugs, active molecules derived from secondary metabolites are harvested through medicinal plants. Figure 15.1 represents the synthesis of natural products derived from plants. Prior reports describe the biosynthesis of different natural medicinal products [2].

2 Secondary Metabolites

Because of their sessile way of life, plants have developed a rich arsenal of chemicals, encompassing some 200,000 known compounds [2]. These metabolites participate in all kinds of biotic and abiotic interactions with the environment, predominantly with regard to defense against herbivores and pathogens, and are considered “secondary metabolites” because they are not involved in the primary processes of growth and development [3, 4]. They have traditionally been of interest only due to their pronounced and various physiological activities in animals and humans [5]. They are low molecular weight organic compounds that possess

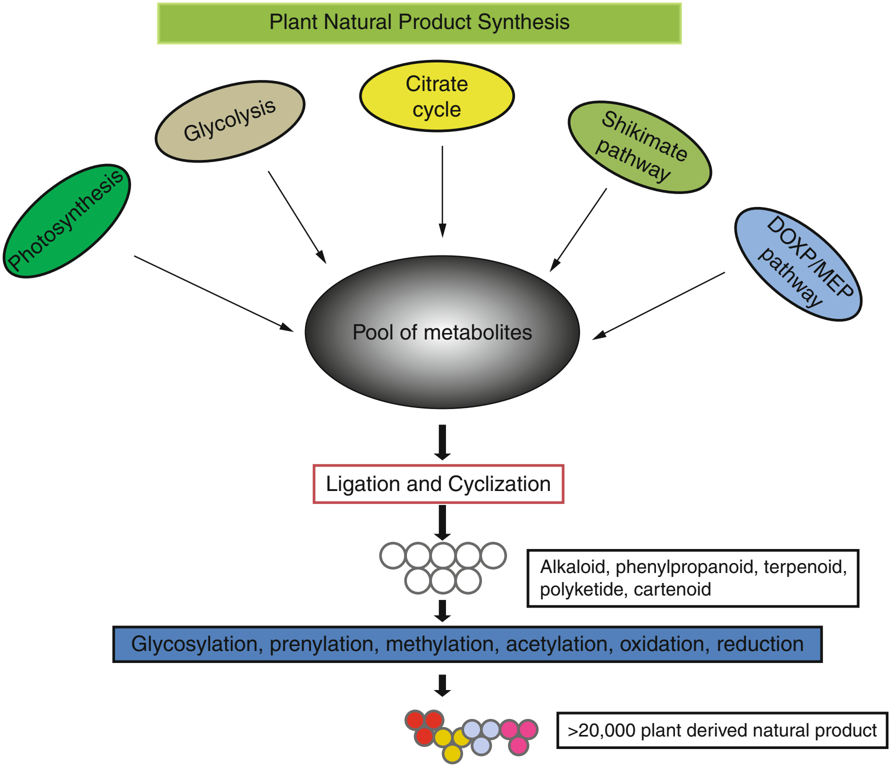

Plant Natural Product Synthesis
Glycolysis
Citrate cycle
Shikimate pathway
Photosynthesis
DOXP/MEP pathway
Pool of metabolites
Ligation and Cyclization
Alkaloid, phenylpropanoid, terpenoid, polyketide, cartenoid
Glycosylation, prenylation, methylation, acetylation, oxidation, reduction
>20,000 plant derived natural product

Fig. 15.1 Synthesis of natural products derived from plants

interesting biological activities and fine applications, such as pharmaceuticals, insecticides, dyes, flavors, and fragrances. Higher plants produce a wide variety of secondary metabolites, including more than 25,000 terpenoids, about 8,000 phenolic compounds, and about 12,000 alkaloids [6]. One of the most conspicuous features of secondary metabolites is that they are often restricted to individual species or related groups of species, rather than being broadly distributed in the plant kingdom. Figure 15.2 represents the biosynthetic relationships of major groups of secondary compounds.

2.1 Classification of Secondary Metabolites

Most of the secondary metabolites of interest belong to the following categories. These categories are broad categories which classify secondary metabolites based on their biosynthetic origin.

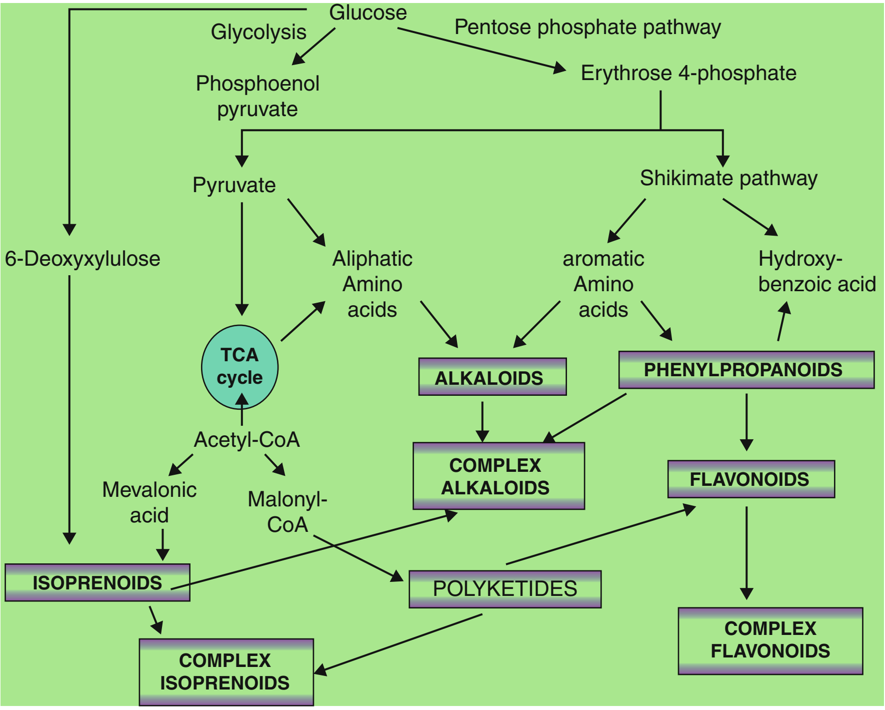

Glucose
Glycolysis
Pentose phosphate pathway
Phosphoenol pyruvate
Erythrose 4-phosphate
Pyruvate
Shikimate pathway
6-Deoxyxylulose
Aliphatic Amino acids
aromatic Amino acids
Hydroxy-benzoic acid
TCA cycle
ALKALOIDS
PHENYLPROPANOIDS
Acetyl-CoA
COMPLEX ALKALOIDS
FLAVONOIDS
Mevalonic acid
Malonyl-CoA
ISOPRENOIDS
POLYKETIDES
COMPLEX ISOPRENOIDS
COMPLEX FLAVONOIDS

Fig. 15.2 Biosynthetic relationships of major groups of secondary compounds

Alkaloids (derived from amino acids):

- Hyoscyamine, present in *Datura stramonium*
- Atropine, present in *Atropa belladonna*
- Cocaine, present in *Erythroxylon coca*
- Codeine and morphine, present in *Papaver somniferum*

Terpenoids (come from semiterpene oligomerization):

- Azadirachtin, present in seeds of *Azadirachta indica* (neem)
- Artemisinin, present in *Artemisia annua*
- Tetrahydrocannabinol, present in *Cannabis sativa*

Steroids (terpenes with a particular ring structure)
Glycosides (heavily modified sugar molecules)
Phenols
Phenazines

3 Alkaloids

Alkaloids are a class of secondary plant metabolites that traditionally have been classified as basic compounds derived from amino acids that contain one or more heterocyclic nitrogen atom. Although this definition holds for most known alkaloids recently, any *N*-containing secondary compound is considered an alkaloid if it cannot readily be classified otherwise – i.e., not an amine, cyanogenic glycoside, glucosinolate, etc. The original definition for alkaloids is pharmacologically active, *N*-containing basic compounds of plant origin. Ergot alkaloids are toxins and important pharmaceuticals and have been identified in two orders of fungi and three families of higher plants [7]. The most important producers are fungi of the genera Claviceps, Penicillium, and Aspergillus (all belonging to the Ascomycota). Chemically, ergot alkaloids are characterized by the presence of a tetracyclic ergoline ring and can be divided into three classes according to their structural features, i.e., amide or peptide-like amide derivatives of D-lysergic acid and the clavine alkaloids. Significant progress has been achieved on the molecular biological and biochemical investigations of ergot alkaloid biosynthesis in the last decade. By gene cloning and genome mining, gene clusters for ergot alkaloid biosynthesis have been identified in at least 8 different ascomycete species. The functions of most structure genes have been assigned to reaction steps in the biosynthesis of ergot alkaloids by gene inactivation experiments or biochemical characterization of the overproduced proteins. An overview of the studies related to the biosynthesis of alkaloids has been published [8]. Several biologically active alkaloids (1–4, 6), including a new quinazoline-6-carboxylic acid (1), were isolated from the medicinal plant Zanthoxylum rhetsa, an evergreen tree, native to subtropical areas [9]. Whereas the pharmacological properties of the plant extract and single constituents have been widely tested, we now show that all of the metabolites have anti-algal activities, all but 6 are antibacterial, and 6 and the reduction product 5 (derived from 4) are also antifungal. A detailed account on the biological activities of structurally diverse secondary metabolites from marine sponges having 2-aminoimidazole, glycociamidine, and/or 2-thiohydantoin ring functions has been analyzed by Kumar et al. [10].

4 Classification of Alkaloids

The alkaloids, as an important and enormously large conglomerate of naturally occurring nitrogen-containing plant substances having very specific as well as most diversified pharmacological properties, may be classified in a number of modes and means. Hegnauer [11] conveniently classified alkaloids into six important groups, corresponding to the six amino acids legitimately considered as the starting points for their biosynthesis, such as anthranilic acid, histidine, lysine, ornithine phenylalanine, and tryptophan. Price [12] further took a leading clue from the earlier observation and considered in details the alkaloids present in one of the families (Rutaceae) and logically placed them in the following *nine* chemical-structural categories, namely, acridines, amides, amines, benzylisoquinolines, canthinones, imidazoles, indolquinazolines, furoquinolines, and quinazolines. Another school of thought classifies alkaloids in the following *four* heads, namely:

(a) *Biosynthetic Classification* – In this particular instance, the significance solely lies to the precursor from which the alkaloids in question are produced in the plant biosynthetically. Therefore, it is quite convenient and also logical to group together all alkaloids having been derived from the same precursor but possessing different taxonomic distribution and pharmacological activities.

Examples

1. Indole alkaloids derived from tryptophan
2. Piperidine alkaloids derived from lysine
3. Pyrrolidine alkaloids derived from ornithine
4. Phenylethylamine alkaloids derived from tyrosine
5. Imidazole alkaloids derived from histidine

(b) *Chemical Classification* – It is probably the most widely accepted and common mode of classification of **alkaloids** for which the main criterion is the presence of the basic heterocyclic nucleus (i.e., the **chemical entity**).

Examples

1. Pyrrolidine alkaloids (e.g., hygrine)
2. Piperidine alkaloids (e.g., lobeline)
3. Pyrrolizidine alkaloids (e.g., senecionine)
4. Tropane alkaloids (e.g., atropine)
5. Quinoline alkaloids (e.g., quinine)
6. Isoquinoline alkaloids (e.g., morphine)
7. Aporphine alkaloids (e.g., boldine)
8. Indole alkaloids (e.g., ergometrine)
9. Imidazole alkaloids (e.g., pilocarpine)
10. Diazocin alkaloids (e.g., lupanine)
11. Purine alkaloids (e.g., caffeine)
12. Steroidal alkaloids (e.g., solanidine)
13. Amino alkaloids (e.g., ephedrine)
14. Diterpene alkaloids (e.g., aconitine)

(c) *Pharmacological Classification* – Interestingly, the **alkaloids** exhibit a broad range of pharmacological characteristics. This method is also used as a strong basis for the general classification of the wide spectrum of alkaloids derived from the plant kingdom, such as analgesics, cardiovascular drugs, CNS stimulants and depressants, dilation of pupil of eye, mydriatics, anticholinergics, sympathomimetics, antimalarials, and purgatives. However, such a classification is not quite common and broadly known.

Examples

1. Morphine as narcotic analgesic
2. Quinine as antimalarial
3. Strychnine as reflex excitability
4. Lobeline as respiratory stimulant
5. Boldine as choleretics and laxatives
6. Aconitine as neuralgia as an external agent in neuralgia and rheumatism
7. Pilocarpine as antiglaucoma agent and miotic
8. Ergonovine as oxytocic
9. Ephedrine as bronchodilator
10. Narceine as analgesic (narcotic) and antitussive

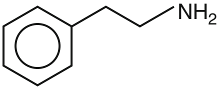
(d) *Taxonomic Classification* – This particular classification essentially deals with the "Taxon," i.e., the taxonomic category. The most common *taxa* are the genus, subgenus, species, subspecies, and variety. Therefore, the taxonomic classification encompasses the plethora of alkaloids exclusively based on their respective distribution in a variety of plant families, sometimes also referred to as the "natural order." A few typical examples of plant families and the various species associated with them are stated below, namely:

1. *Cannabinaceous Alkaloids:* e.g., *Cannabis sativa* Linn. (Hemp, Marijuana)
2. *Rubiaceous Alkaloids:* e.g., *Cinchona Sp*. (Quinine), *Mitragyna speciosa* Korth (Katum, Kratum, Kutum), *Pausinystalia johimbe* (K. Schum) (Yohimbe)
3. *Solanaceous Alkaloids:* e.g., *Atropa belladona* L. (Deadly Nightshade, Belladona), *Brunfelsia uniflorus* (Pohl) D. Don (Manaca, Manacan), *Capsicum annuum* L. (Sweet Peppers, Paprika), *Datura candida* (Pers.) Saff. (Borrachero, Floripondio), *Duboisia myoporoides* R. Br. (Corkwood Tree, Pituri), *Hyoscyamus niger* L. (Henbane, Henblain, Jusquaime), *Mandragora officinarum* L. (Mandrake, Loveapple), *Nicotiana glauca* R. Grah. (Tree Tobacco), *Seopolia carniolica* Jacq. (Scopolia), *Solanum dulcamara* L. (Bittersweet, Bitter Nightshade, Felonwood), *Withania somniferum* (L.) Dunal (Ashwagandha), etc.

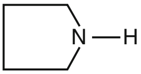
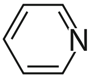
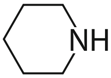
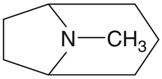
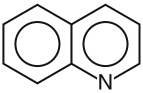
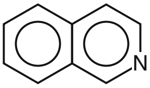
Invariably, they are grouped together according to the name of the *genus* wherein they belong to, such as coca, cinchona, and ephedra. Some "phytochemists" have even gone a step further and classified the alkaloids based on their chemotaxonomic classification.

In the recent past, the alkaloids have been divided into *two* major categories based on the analogy that one contains a *non-heterocyclic nucleus* while the other has the *heterocyclic nucleus*. These two classes of alkaloids shall be discussed briefly as under:

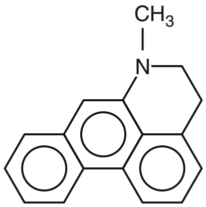
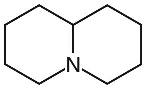
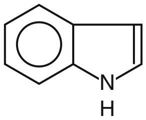
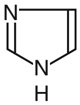
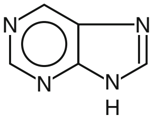
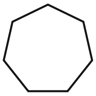
(a) *Non-heterocyclic Alkaloids* – A few typical alkaloids having a non-heterocyclic nucleus are enumerated below with other related information:

S.No.	Basic ring structure	Alkaloid	Botanical origin	Family
1	Phenylethylamine 	Ephedrine	*Ephedra vulgaris*	Gnetaceae
		Hordenine	*Hordeum vulgare*	Graminae
		Capsaicin	*Capsicum annuum*	Solanaceae
		Mescaline	*Laphophora williamsii*	Cactaceae
		Narceine	*Papaver somniferum*	Papaveraceae

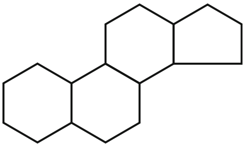
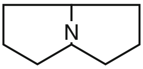
(b) *Heterocyclic Alkaloids* – A large number of specific alkaloids possessing heterocyclic nucleus are stated below with other related information

S.No.	Basic ring structure	Alkaloid	Botanical origin	Family
1	Pyrrolidine 	Hygrine	*Erythroxylon coca*	Erythroxylaceae
		Stachydrine	*Stachys tuberifera*	Labiatae
2	Pyridine 	Arecoline	*Areca catchu*	Palmaceae
		Ricinine	*Ricinus communis*	Euphorbiaceae
		Trigenelline	*Trigonella foenumgraecum*	Leguminosae
3	Piperidine 	Connine	*Conium maculatum*	Umbelliferae
		Lobeline	*Lobelia inflata*	Lobeliaceae
		Pelletierine	*Punica granatum*	Punicaceae
4	Tropane [piperidine-pyrrolidine(*N*-methyl)] 	Atropine	*Atropa belladona*	Solanaceae
		Cocaine	*Datura stramonium*	Solanaceae
		Hyoscyamine	*Erythroxylon coca*	Erythroxylaceae
			Atropa belladona	Solanaceae
5	Quinoline 	Quinine, Quinidine	*Cinchona officinalis*	Rubiaceae
		Cuspareine	*Cusparia trifoliata*	Rutaceae
6	Isoquinoline 	Papaverine	*Papaver somniferum*	Papaveraceae
		Berberine	*Hydrastis canadensis*	Berberidaceae
		Emetine	*Uragoga ipecacuanha*	Rubiaceae
		Corydaline	*Corydalis aurea*	Fumariaceae
			Corydalis solida	Fumariaceae
		Tubocurarine	*Chondodendron tomentosum*	Menispermaceae

(*continued*)

S.No.	Basic ring structure	Alkaloid	Botanical origin	Family
7	Aporphine Isoquinoline Phenanthrene  	Boldine	*Peumus boldus*	Monimiaceae
8	Norlupinane  	Sparteine	*Lupinus luteus*, *Lupinus niger*, *Cytisus scoparius*, *Anagyris foetida*	Leguminosae
		Lupinine	*Lupinus luteus*	Leguminosae
			Anabasis aphylla	Chenopodiaceae
9	Indole (benzopyrrole)  	Ergotamine, Ergometrine	*Claviceps purpurea*	Hypocreales
		Physostigmine	*Physostigma venenosum*	Leguminosae
		Reserpine	*Rauwolfia serpentina*	Apocynaceae
		Yohimbine	*Coryanthe johimbe*	Rubiaceae
			Rauwolfia serpentina	Apocynaceae
		Vinblastine	*Vinca rosea*	Apocynaceae
		Strychnine	*Strychnos nux-vomica*	Loganiaceae
10	Imidazole (glyoxaline)  	Pilocarpine	*Pilocarpus jaborandi*	Rutaceae
11	Purine (pyrimidine-imidazole)  	Caffeine	*Thea sinensis*	Ternstroemiaceae
			Camellia sinensis	
			Coffea arabica	Rubiaceae
			Theobroma cacao	Sterculiaceae
12	Tropolone 	Colchicine	*Colchicum autumnale*	Liliaceae

(*continued*)

S.No.	Basic ring structure	Alkaloid	Botanical origin	Family
13	Steroid 	Conessine	*Holarrhena antidysenterica*	Apocynaceae
		Funtumine	*Funtumia latifolia*	Apocynaceae
		Solanidine	*Solanum spp.*	Solanaceae
		Veratramine	*Veratrum grandiflorum, Veratrum viride*	Liliaceae
14	Terpenoid (diterpene)	Aconine	*Aconitum napellus*	Ranunculaceae
		Aconitine (Glycoside)	*Aconitum napellus*	Ranunculaceae
		Atisine	*Aconitum heterophyllum, Aconitum anthora*	Ranunculaceae
		Lycoetonine	*Aconitum lycoctonum*	Ranunculaceae
15	Pyrrolizidine 	Senecionine	*Senecio vulgaris*	Compositae
		Seneciphylline	*Senecio platyphyllus*	Compositae

It is pertinent to mention that the enormous volume of authentic information accumulated so far with regard to the isolation of alkaloids from a variety of plant species and their subsequent characterization by the help of latest analytical techniques they may be classified as follows:

(a) Alkaloids derived from amination reactions

1. Acetate-derived alkaloids
2. Phenylalanine-derived alkaloids
3. Terpenoid alkaloids
4. Steroidal alkaloids

(b) Alkaloids derived from anthranilic acid

1. Quinazoline alkaloids
2. Quinoline alkaloids
3. Acridine alkaloids

(c) Alkaloids derived from histidine

Imidazole alkaloids

(d) Alkaloids derived from lysine

1. Piperidine alkaloids
2. Quinolizidine alkaloids
3. Indolizidine alkaloids

(e) Alkaloids derived from nicotinic acid

Pyridine alkaloids

(f) Alkaloids derived from ornithine

1. Pyrrolidine alkaloids
2. Tropane alkaloids
3. Pyrrolizidine alkaloids

(g) Alkaloids derived from tyrosine

1. Phenylethylamine alkaloids
2. Simple tetrahydroisoquinoline alkaloids
3. Modified benzyl tetrahydroisoquinoline alkaloids

(h) Alkaloids derived from tryptophan

1. Simple indole alkaloids
2. Simple b-carboline alkaloids
3. Terpenoid indole alkaloids
4. Quinoline alkaloids
5. Pyrroloindole alkaloids
6. Ergot alkaloids (Table 15.1)

Table 15.1 Distribution of pathways according to alkaloids

S.No.	Pathway name	Alkaloids
1	Alkaloidal amines biosynthesis	Dopamine, colchicine, ephedrine, methaphetamine
2	Tropane alkaloid Biosynthesis	Scopolamine, nicotine, hygrine, calystegines B1, calystegines B2, spermidine, spermine, cocaine, tropinone, littorine, tropine
3	Quinoline alkaloid biosynthesis	Quinine, quinidine
4	Indole alkalod biosynthesis	Tryptamine, sarpagine, ajmaline, vinblastine, vindoline, catharanthine, serpentine, tetrahydro alstonine, ajmalicine
5	Quinolizidine alkaloid biosynthesis	Cadaverine, (+)-P- coumaroylepilupinine/lupinine, (−)-13a-tigloyloxymutiflorine/lupanine
6	Purine alkaloid biosynthesis	Xanthine, 1,3,7-trimethylxanthine(caffeine), 3,7-dimethylxanthine(theobromine), 1,3-dimethylxanthine (theophylline)
7	Steroidal alkaoid biosynthesis	Solanine, chaconine, solasodine
8	Isoquinoline alkaloid biosynthesis	Dopamine, sanguinarine, morphine, laudanine, berbamunine, guatteguaumerine, 2′-Norberbamunine, 10-hydroxychelerythrine, macarpine, (S)canadine, berberine, 7,8-dihydroberberine, thebaine, methopapaverberbine, macrantaline, narcotolinediol, narcotinediol, narcotoline, noscapine
9	Pyrrolizidine alkaloid biosynthesis	Senecionine *N*-oxide
10	Pyridine-piperidine alkaloid biosynthesis	Cadaverine, lupinate, L-pipecolate, *N*-methyl pelletierine, anapheline, anatabine, nicotine, nornicotine, *N*-formylnornicotine, nicotyrine, myosrnine, cotinine, tropinone, *N*-methyl pelletierine, anapheline, anatalline, anabasine

5 Biosynthesis of Alkaloids

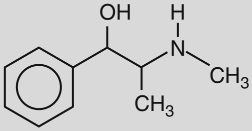
Alkaloids derived from tyrosine include phenylethylamine alkaloids, simple tetrahydroisoquinoline alkaloids, and modified benzyl tetrahydroisoquinoline alkaloids. The chemistry of Erythrina and related alkaloids from 1996 to mid-2009 has been reviewed, with a particular focus on the preparation of Erythrina alkaloids possessing an aromatic "D" ring [13].

Phenylethylamine Alkaloids

The important alkaloids belonging to this category are, namely, **ephedrine**, **hordenine**, **mescaline**, and **narceine**.

Ephedrine

It occurs in *Ephedra vulgaris* Hook. F. (*E. gerardiana* Wall), *Ephedra sinica* Stapf. (1–3 %), and *Ephedra equisetina* Bunge (2 %) belonging to the natural order *Gentaceae* and several other Ephedra species. Besides, it is also found in the roots of *Aconitum napellus* L. (*Ranunculaceae*) (Aconite, Monkshood, Blue Rocket); and *Ephedra nevadensis* S. Wats. (*Ephedraceae*) (Mormon Tea, Nevada Jointfir).

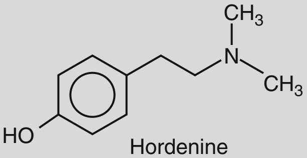


Ephedrine

α-[1-(Methylamino)-ethyl] benzene-methanol; ($C_{10}H_{15}NO$)

Hordenine

It is obtained from the plant of *Lophophora williamsii* (Lamaire) Coult. (*Catctaceae*) (Peyote) and *Selenicereus grandiflorus* Britt and Rose (*Coctaceae*) (Night Blooming Cereus).



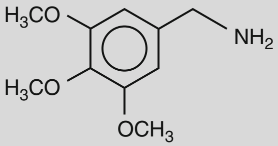
Hordenine

4-[2-Dimethylamino) ethyl] phenol; ($C_{10}H_{15}NO$)

It is very soluble in chloroform, ethanol, and ether; 7 g dissolves in 1 L of water; practically insoluble in petroleum ether; and sparingly soluble in benzene, xylene and toluene.

Mescaline

It is obtained from *Peyote* (Mescal Buttons) the flowering heads of *Lophophora williamsii* (Lemaire) Coult. (*Coctaceae*) and the cactus *Trichocereus pachanoi* Britton and Rose (*Cactaceae*) (Achuma, San Pedro Aguacolli).



Mescaline

3, 4, 5-Trimethoxybenzeneethanamine; ($C_{11}H_{17}NO_3$)

5.1 Pyrrolizidine Alkaloids

The system of pyrrolizidine alkaloids has proven to be a powerful system for studying the evolution of a biosynthetic pathway in plant secondary metabolism. Pyrrolizidine alkaloids are typical plant secondary products produced by the plant as a defense against herbivores. The first specific enzyme, homospermidine synthase, has been shown to have evolved by duplication of the gene encoding deoxyhypusine synthase, which is involved in primary metabolism. Despite the identical function of the homospermidine synthase for pyrrolizidine alkaloid biosynthesis in the various plant lineages, this gene duplication has occurred several times independently during angiosperm evolution. After duplication, these gene copies diverged with respect to gene function and regulation. In the diverse plant lineages producing pyrrolizidine alkaloids, homospermidine synthase has been shown to be expressed in a variety of tissues, suggesting that the regulatory elements were recruited individually after the duplication of the structural gene [14].

Pyrrolizidine alkaloids are one of the best-studied examples of a plant's defense system that has been recruited by several insect lineages for their own chemical defense. In each case, this recruitment requires sophisticated mechanisms of adaptations, e.g., efficient excretion, transport, suppression of toxification, or detoxification. Here, we briefly summarize the detoxification mechanism known for pyrrolizidine alkaloids and focus on pyrrolizidine alkaloid *N*-oxidation as one of the mechanisms allowing insects to accumulate the sequestered toxins in an inactivated protoxic form. Recent research into the evolution of pyrrolizidine alkaloid *N*-oxygenases of adapted arctiid moths (Lepidoptera) has shown that this enzyme originated by the duplication of a gene encoding a flavin-dependent monooxygenase of unknown function early in the arctiid lineage. The available data suggest several similarities in the molecular evolution of this adaptation strategy of insects to the mechanisms described previously for the evolution of the respective pathway in plants [15].

5.2 Isoquinoline Alkaloids

Isoquinoline alkaloids are tyrosine-derived plant alkaloids with an isoquinoline skeleton. Among them, benzylisoquinoline alkaloids form an important group with potent pharmacological activity, including analgesic compounds of morphine and codeine and antiinfective agents of berberine, palmatine, and magnoflorine. Biosynthesis of isoquinoline alkaloids proceeds via decarboxylation of tyrosine or DOPA to yield dopamine, which together with 4-hydroxyphenylacetaldehyde, an aldehyde derived from tyrosine, is converted to reticuline, an important precursor of various benzylisoquinoline alkaloids.

5.3 Isoquinoline Alkaloid Biosynthesis

Isoquinoline alkaloid biosynthesis is one of the well-characterized pathways in the secondary metabolism of plant cells which comprises some of the most important drugs for therapy and euphoria (e.g., morphine and its chemical derivatives, papaverine, berberine, dimeric bisbenzylisoquinolines).

The benzylisoquinoline alkaloid class includes several important medicinal compounds such as analgesic morphine, codeine, papaverine, berberine, tubocurarine, and antimicrobial sanguinarine. Enzymes involved in the biosynthesis of at least two tetrahydrobenzylisoquinoline alkaloids, the benzophenanthridine alkaloid sanguinarine and the bisbenzylisoquinoline alkaloid berbamunine, have been reported. The branch point that differentiates the biosynthetic pathway from that which leads to (S)-reticuline-derived benzylisoquinoline alkaloids is shown to be (S)-*N*-methylcoclaurine. All benzylisoquinoline alkaloids share a common biosynthetic origin beginning with a lattice of decarboxylations, ortho-hydroxylations, and deaminations that convert L-tyrosine into both dopamine and 4-hydroxyphenylacetaldehyde. Dopamine and 4-hydroxyphenylacetaldehyde condense to form the trihydroxylated (S)-reticuline which is a key branch-point intermediate in the biosynthesis of most benzylisoquinoline alkaloids, including those with a morphinan (e.g., morphine), benzophenanthridine (e.g., sanguinarine), or protoberberine (e.g., berberine) nucleus and are found mainly in species of the Papaveraceae, Monimiaceae, Ranunculaceae, Berberidaceae, and Menispermaceae. (S)-Reticuline is clearly one of the most versatile molecules in plant secondary metabolism. Five enzymes of alkaloid formation are reported: (R,S)-3-hydroxy-*N*-methylcoclaurine 4-*O*-methyltransferase central to the biosynthesis of tetrahydroisoquinoline-derived alkaloids, the berberine bridge enzyme of the sanguinarine pathway, (R,S)-reticuline 7-*O*-methyltransferase specific to laudanosine formation, and salutaridinol 7-*O*-acetyltransferase and codeinone reductase, which lead to morphine. The conversion of (S)-reticuline to (S)-scoulerine via the berberine bridge enzyme (BBE) represents the first committed step in benzophenanthridine and protoberberine alkaloid biosynthesis. Plant *O*-methyltransferases: molecular analysis, common signature and classification have been studied by Ibrahim et al. [16].

Morphine and codeine are members of the large and diverse group of benzylisoquinoline alkaloids, of which morphine and sanguinarine share a common biosynthetic pathway, beginning with the condensation of two L-Tyr derivatives to produce the central precursor (S)-norcoclaurine yields (S)-reticuline, the last common intermediate in the biosynthesis of both sanguinarine and morphine. Berberine bridge enzyme (BBE) catalyzes the conversion of (S)-reticuline to (S)-scoulerine, the first committed step in the sanguinarine pathway. Alternatively, (S)-reticuline can be isomerized to its (R)-epimer as the first step in the formation of morphine. Since the pathway from tyrosine to (S)-reticuline is also known at the enzyme level, the conversion of L-tyrosine to macarpine involves a total of 19 enzymes which are now at least partially characterized.

The isoquinoline pathway's enzyme orthologs in other plants showed that genes of methyltransferase family such as (R,S)-reticuline 7-*O*-methyltransferase, coclaurine 4′-*O*-methyltransferase, (S)-norcoclaurine 6-*O*-methyltrasferase, columbamine *O*-methyltransferase, coclaurine *N*-methyltransferase, putrescine *N*-methyltransferase responsible for the production of reticuline, coclaurine, norcoclaurine, columbaine have been reported from *Papaver somniferum, Coptis japonica, Thalictrum flavum, Thalictrum tuberosum, Coffea liberica, Coffea arabica, Coffea canephora, Nicotiana tabacum, Solanum tuberosum, Datura stramonium, Hyoscyamus niger*, and *Atropa belladonna*. Pathway contains information of about four alkaloids such as dopamine, colchicine, ephedrine, and methamphetamine and identified two missing links.

Eschscholzia californica produces various types of isoquinoline alkaloids. The structural diversity of these chemicals is often due to cytochrome P450 (P450) activities. Members of the CYP719A subfamily, which are found only in isoquinoline alkaloid-producing plant species, catalyze methylenedioxy bridge-forming reactions. In this study, four kinds of CYP719A genes from *E. californica* have been characterized. These four cDNAs encoded amino acid sequences that were highly homologous to *Coptis japonica* CYP719A1 and *E. californica* CYP719A2 and CYP719A3, which suggested that these gene products may be involved in isoquinoline alkaloid biosynthesis in *E. californica*, especially in methylenedioxy bridge-forming reactions. Expression analysis of these genes showed that two genes (CYP719A9 and CYP719A11) were preferentially expressed in plant leaf, where pavine-type alkaloids accumulate, whereas the other two showed higher expression in the root than in other tissues [17] (Fig. 15.3, Tables 15.2–15.5).

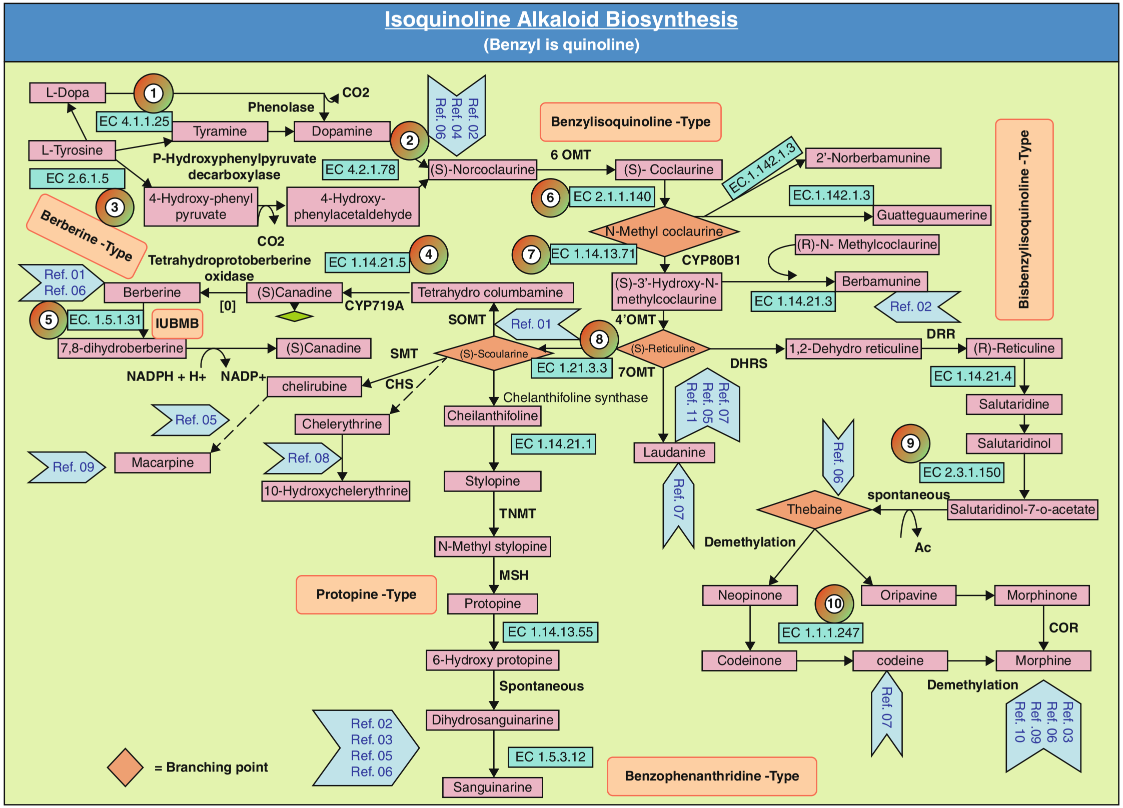
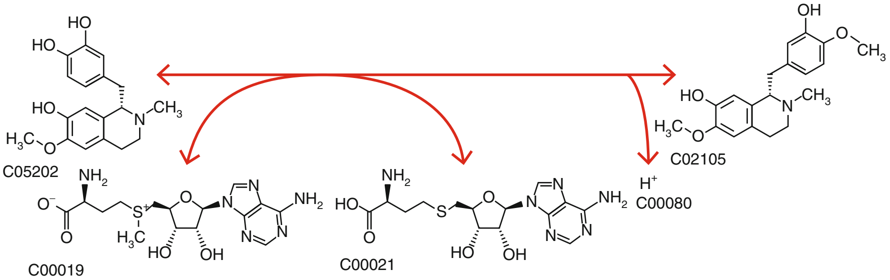

Isoquinoline Alkaloid Biosynthesis
(Benzyl is quinoline)
L-Dopa
1
CO2
Phenolase
EC 4.1.1.25
Tyramine
Dopamine
2
Ref. 02
Ref. 04
Ref. 06
L-Tyrosine
P-Hydroxyphenylpyruvate
decarboxylase
EC 4.2.1.78
(S)-Norcoclaurine
6 OMT
(S)- Coclaurine
EC 2.6.1.5
3
4-Hydroxy-phenyl pyruvate
4-Hydroxy-phenylacetaldehyde
6
EC 2.1.1.140
EC.1.142.1.3
2'-Norberbamunine
EC.1.142.1.3
Guatteguaumerine
Benzylisoquinoline -Type
Bisbenzylisoquinoline -Type
Berberine -Type
CO2
Tetrahydroprotoberberine
oxidase
EC 1.14.21.5
4
7
EC 1.14.13.71
N-Methyl coclaurine
CYP80B1
(R)-N- Methylcoclaurine
Berbamunine
Ref. 01
Ref. 06
Berberine
(S)Canadine
[0]
CYP719A
Tetrahydro columbamine
(S)-3'-Hydroxy-N-methylcoclaurine
EC 1.14.21.3
Ref. 02
5
EC. 1.5.1.31
IUBMB
SOMT
Ref. 01
8
4'OMT
DRR
7,8-dihydroberberine
(S)Canadine
SMT
(S)-Scoularine
(S)-Reticuline
1,2-Dehydro reticuline
(R)-Reticuline
NADPH + H+
NADP+
chelirubine
CHS
EC 1.21.3.3
7OMT
DHRS
EC 1.14.21.4
Chelanthifoline synthase
Ref. 07
Ref. 05
Ref. 11
Salutaridine
Ref. 05
Chelerythrine
Cheilanthifoline
EC 1.14.21.1
Laudanine
9
Salutaridinol
Ref. 09
Macarpine
Ref. 08
Stylopine
Ref. 06
EC 2.3.1.150
10-Hydroxychelerythrine
TNMT
Ref. 07
spontaneous
Thebaine
Salutaridinol-7-o-acetate
Demethylation
Ac
N-Methyl stylopine
MSH
Protopine -Type
Protopine
Neopinone
10
Oripavine
Morphinone
EC 1.14.13.55
EC 1.1.1.247
COR
6-Hydroxy protopine
Codeinone
codeine
Morphine
Spontaneous
Ref. 07
Demethylation
Ref. 03
Ref. 06
Ref .09
Ref. 10
Dihydrosanguinarine
Ref. 02
Ref. 03
Ref. 05
Ref. 06
EC 1.5.3.12
Benzophenanthridine -Type
= Branching point
Sanguinarine

Fig. 15.3 Isoquinoline alkaloid biosynthesis pathway represented in DbAlBioPath database (Available at intranet, CSIR-CIMAP, Lucknow, India)

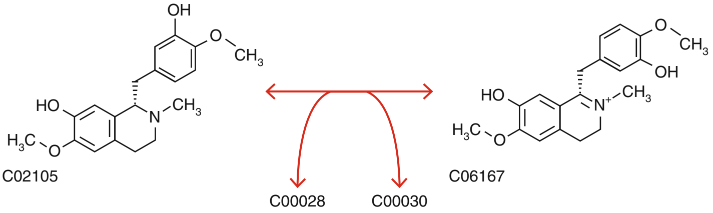
Table 15.2 Details of KEGG pathway and enzyme name and equation catalyzed by the enzyme during the biosynthesis of isoquinoline alkaloids

KEGG reaction entry	R03832
Name	S-adenosyl-L-methionine:3′-hydroxy-*N*-methyl-(S)-coclaurine 4′-*O*-methyltransferase
Definition	S-adenosyl-L-methionine + 3′-hydroxy-*N*-methyl-(S)-coclaurine < = > S-adenosyl-L-homocysteine + (S)-reticuline + H+
Equation	C00019 + C05202 < = > C00021 + C02105 + C00080



Enzyme	2.1.1.116; 3′-hydroxy-*N*-methyl-(S)-coclaurine 4′-O-methyltransferase; transferases
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)
Orthology	K13386; 3′-hydroxy-*N*-methyl-(S)-coclaurine 4′-O-methyltransferase [EC:2.1.1.116]

KEGG reaction entry	R03833
Definition	(S)-reticuline + acceptor < = > 1,2-dehydroreticuline + reduced acceptor
Equation	C02105 + C00028 < = > C06167 + C00030

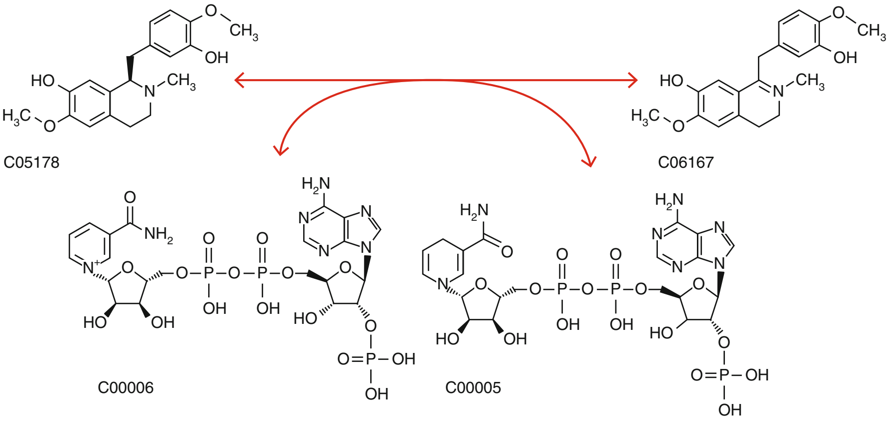


Enzyme	1.5.-.-; Oxidoreductases; acting on the CH-NH group of donors
Pathway	Isoquinoline alkaloid biosynthesis
	Metabolic pathways
	Biosynthesis of secondary metabolites

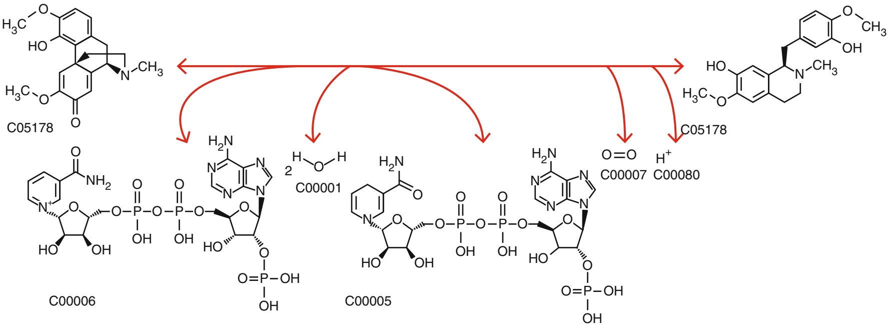
KEGG reaction entry	R04695
Name	(R)-reticuline:NADP + oxidoreductase
Definition	(R)-reticuline + NADP + <= > 1,2-dehydroreticuline + NADPH
Equation	C05178 + C00006 < = > C06167 + C00005

(*continued*)

Table 15.2 (continued)



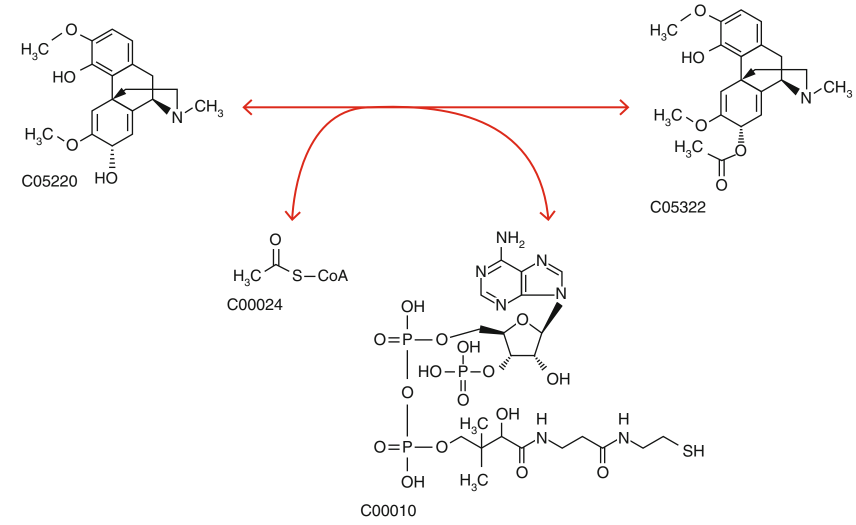
Enzyme	1.5.1.27; 1,2-dehydroreticulinium reductase (NADPH); 1,2-dehydroreticulinium ion reductase
Pathway	Isoquinoline alkaloid biosynthesis
KEGG reaction entry	R04696
Name	(R)-reticuline, NADPH:oxygen oxidoreductase (C-C phenol-coupling)
Definition	Salutaridine + NADP+ + 2 H2O < = > (R)-reticuline + NADPH + oxygen + H+
Equation	C05179 + C00006 + 2 C00001 < = > C05178 + C00005 + C00007 + C00080



Enzyme	1.14.21.4; salutaridine synthase; (R)-reticuline oxidase (C-C phenol-coupling) (oxidoreductases)
Pathway	Isoquinoline alkaloid biosynthesis
Orthology	Cytochrome P450, family 719, subfamily B, polypeptide 1 (salutaridine synthase) [EC:1.14.21.4] (K13391)
KEGG reaction entry	R04723
Name	Acetyl-CoA:salutaridinol 7-*O*-acetyltransferase
Definition	Acetyl-CoA + 5,6,8,14-Tetradehydro-3,6-dimethoxy-17-methyl-morphinan-4,7-diol < = > CoA + 7-*O*-acetylsalutaridinol
Equation	C00024 + C05220 < = > C00010 + C05322

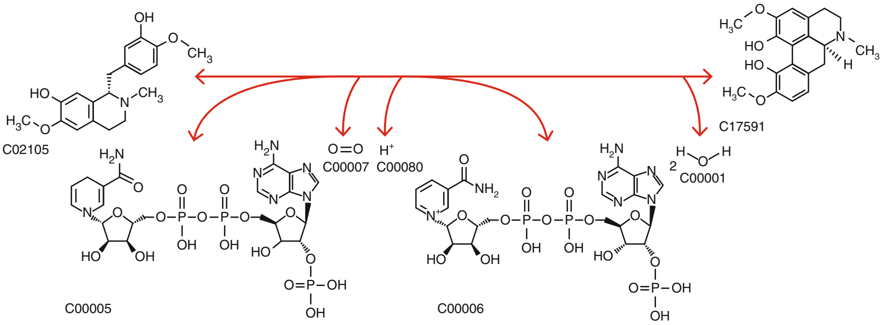
(*continued*)

Table 15.2 (continued)


C05220
C05322
C00024
C00010
S–CoA

Enzyme	2.3.1.150; Salutaridinol 7-*O*-acetyltransferase; class: transferases; acyltransferases
Pathway	Isoquinoline alkaloid biosynthesis
Orthology	Salutaridinol 7-*O*-acetyltransferase [EC:2.3.1.150] (K13393)
Enzyme	Spontaneous (following salutaridinol 7-*O*-acetyltransferase [2.3.1.150] reaction)
Pathway	Isoquinoline alkaloid biosynthesis

KEGG reaction entry	R08790
Name	(S)-reticuline,NADPH:oxygen oxidoreductase (C-C phenol-coupling)
Definition	(S)-Reticuline + NADPH + Oxygen + H + <= > (S)-Corytuberine + NADP+ + 2 H2O
Equation	C02105 + C00005 + C00007 + C00080 < = > C17591 + C00006 + 2 C00001


C02105
C17591
O=O
C00007
H+
C00080
2
C00001
C00005
C00006

Comment	CYP80G2
Enzyme	1.14.21.-; Oxidoreductases

(*continued*)

Table 15.2 (continued)

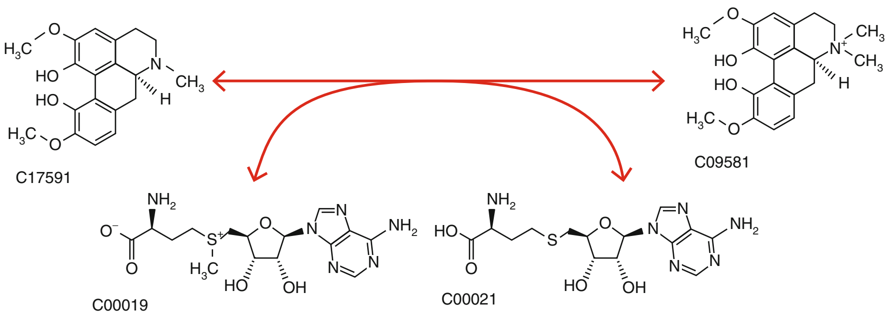
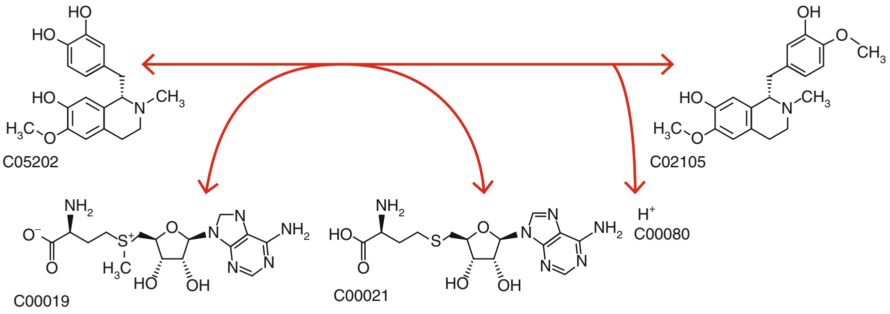
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)
Orthology	K13389; cytochrome P450, family 80, subfamily G, polypeptide 2 (corytuberine synthase) [EC:1.14.21.-]
KEGG reaction entry	R08791
Name	S-adenosyl-L-methionine:(S)-corytuberine N-methyltransferase
Definition	(S)-corytuberine + S-adenosyl-L-methionine < = > magnoflorine + S-adenosyl-L-homocysteine
Equation	C17591 + C00019 < = > C09581 + C00021
	
Comment	coclaurine *N*-methyltransferase
Enzyme	2.1.1.-
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)
KEGG reaction entry	R03832
Name	S-adenosyl-L-methionine:3′-hydroxy-*N*-methyl-(S)-coclaurine 4′-O-methyltransferase
Definition	S-adenosyl-L-methionine + 3′-Hydroxy-*N*-methyl-(S)-coclaurine < = > S-adenosyl-L-homocysteine + (S)-reticuline + H+
Equation	C00019 + C05202 < = > C00021 + C02105 + C00080
	
Enzyme	2.1.1.116; 3′-hydroxy-*N*-methyl-(S)-coclaurine 4′-O-methyltransferase; transferases
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)
Orthology	K13386; 3′-hydroxy-*N*-methyl-(S)-coclaurine 4′-O-methyltransferase [EC:2.1.1.116]

Table 15.3 The distribution of pathways in DbAlBioPath based on the number of alkaloids, enzymes, reactions, reactions with missing annotation and branching points

Pathways	Alkaloids	Total number of alkaloids	Total number of enzymes	Number of reactions	Reaction with missing annotations
Isoquinoline alkaloid biosynthesis	Dopamine, sanguinarine, morphine, laudanine, berbamunine, guatteguaumerine, 2′-norberbamunine, 10-hydroxychelerythrin, macarpine, (S)canadine, berberine, 7,8-dihydroberberine, thebaine, methopapaverberbine, macrantaline, narcotolinediol, narcotinediol, narcotoline, noscapine	19	32	>52	2

Table 15.4 Predicted orthologs of isoquinoline alkaloid biosynthesis pathway enzymes (benzylisoquinoline) in other plants

S.No.	*Plants*	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15
1	*Berberis stolonifera*	+		+											+	+
2	*Coptis japonica var. dissecta*	+						+	+		+					+
3	*Papaver somniferum*	+	+	+	+		+	+	+		+		+	+		+
4	*Eschscholzia californica*	+		+						+	+			+	+	+
5	*Coptis chinensis*	+														+
6	*Vitis vinifera*	+	+	+		+	+		+		+		+			+
7	*Thalictrum flavum subsp. glaucum*	+		+			+		+		+					+
8	*Catharanthus roseus*	+	+				+								+	
9	*Arabidopsis thaliana*	+	+	+		+	+		+		+		+			+
10	*Pastinaca sativa*	+														+
11	*Oryza sativa Japonica Group*	+	+	+		+	+	+	+		+		+			+
12	*Oryza sativa Indica group*	+		+		+	+	+	+		+		+			+

Note: "+" refers presence of homolog

Table 15.5 Details of enzymes detected for orthologs in other plants related to isoquinoline alkaloid biosynthesis pathway

S.No.	Enzyme code	Enzymes	Genes	Organism
1	E1	Berbamunine synthase	CYP, gfh, TT7, IMT, tht	*Berberis stolonifera*
2	E2	Salutaridinol 7-*O*-acetyltransferase	MAT, SALAT, ACT, AAT, Pun, BEAT, DAT, catf, hct, HQT, AsHHT	*Vitis vinifera*
3	E3	Reticuline oxidase	BBE, CBDAS	*Berberis stolonifera*
4	E4	Salutaridine synthase	Not reported	*Papaver somniferum*
5	E5	Tyrosine transaminase	SUR, TAT, naat, CORi	*Arabidopsis thaliana*
6	E6	Tyrosine decarboxylase	eli5, TYDC, AADC, PAAS, tyrDC	*Arabidopsis thaliana*
7	E7	(S)-norcoclaurine synthase	NCS, betv, PR10, picg	*Papaver somniferum*
8	E8	(S)-coclaurine-*N*-methyltransferase	CNMT	*Thalictrum flavum subsp. glaucum*
9	E9	(S)-stylopine synthase	Not reported	*Eschscholzia californica*
10	E10	(S)-canadine synthase	CYP, EcCYP	*Thalictrum flavum subsp. glaucum*
11	E11	Berberine reductase	Not reported	*Corydalis cava*
12	E12	Codeinone reductase	Cor, PKR, AKR	*Papaver somniferum*
13	E13	Dihydrobenzophenanthridine oxidase	Not reported	*Eschschoizia californica*
14	E14	Protopine 6-monooxygenase	Not reported	*Chelidonium majus*
15	E15	*N*-methylcoclaurine 3′-monooxygenase	CYP, IMT, Hf1	*Eschscholzia californica*

6 Intermediates of Morphine Biosynthetic Pathway

Poppies (Papaver somniferum, Papaveraceae) have long been used as medicinal plants, food plants, and drugs of abuse. Morphine alkaloids are found in many members of the genus Papaver, but codeine and morphine are only found in *P. somniferum*. Another species, Papaver bracteatum contains thebaine. Morphine alkaloids are derived from (−)- or (R)-reticuline by series of reactions involving an oxidative coupling reaction. Radioactive labelling experiments have established the series of reactions from thebaine to codeine to morphine. The most important compound from a biosynthetic point of view is (+)-reticuline. This alkaloid is a precursor of several other groups of alkaloids. (+)- or (S)-reticuline is converted to (−)- or (R)-reticuline, which is, in turn, a major precursor of another alkaloid group. For morphinan alkaloid biosynthesis, (S)-reticuline undergoes an inversion of stereochemistry to (R)-reticuline (an isoquinoline alkaloid) catalyzed by (R,S)-reticuline 7-*O*-methyltransferase (OMT), a member of the short-chain dehydrogenase/reductase (SDR) protein family. (S)-reticuline formed in the poppy plant is converted by means of 1,2-dehydroreticuline to (R)-reticuline, which in turn is then transformed into morphine. cDNAs have been isolated for all of the enzymes leading to (S)-reticuline, as well as those involved in the conversion of (R)-reticuline to salutaridine-7-*O*-acetate. Recently, the short-chain dehydrogenase/reductase (SDR) implicated in morphine biosynthesis was cloned from Papaver somniferum. The biosynthetic pathways and the participating enzymes or cDNAs are characterized only for a few selected members, whereas the biosynthesis of the majority of the compounds is still largely unknown [18].

The benzylisoquinoline alkaloids (BIA) comprise a large and diverse group of nitrogen-containing secondary metabolites with about 2,500 compounds identified in plants. BIA biosynthesis begins with the condensation of the tyrosine-derived precursors dopamine and *p*-hydroxyphenylacetaldehyde to (S)-norcoclaurine. Subsequent regiospecific *O*- and *N*-methylations and aromatic ring hydroxylation lead to (S)-reticuline, which is the central intermediate for almost all BIAs. For morphinan alkaloid biosynthesis, (S)-reticuline undergoes an inversion of stereochemistry to (R)-reticuline, followed by C-C phenol coupling catalyzed by a unique cytochrome P450-dependent monooxygenase to yield salutaridine. The cDNA sequence of enzymes leading to (S)-reticuline, as well as those involved in the conversion of (R)-reticuline to salutaridine-7-*O*-acetate is already characterized. The inversion of (S)-reticuline to (R)-reticuline represent the important steps in morphine biosynthesis. Wound induced transcript accumulation in Papaver reveals a novel wound inducible EST (NCBI DbEST: GO238757) showing homology with (R,S)-reticuline 7-*O*-methyltransferase (ID:Q6WUC2) isolated from Papaver somniferum. Researchers compared the substrate binding homology of this novel wound inducible (R,S)-reticuline 7-*O*-methyltransferase (7-OMT) using template of P. somniferum (Q6WUC2; gb|AAQ01668) as experimental control. Homology modeling with 70 % identity and 85 % similarity with the catalytic site of template protein, i.e., (Q6WUC2) short-chain dehydrogenase/reductase (SDR), showed docking energy −69.9 and −75.8 kcal/mol with (S)-reticuline (CID:439653) and (R)-reticuline (CID:440586), respectively, which are comparable with experimental control binding site interaction energies. Docking of S- and R-reticuline into the active site revealed eight amino acids, namely, (F(5), E(18), W(24), C(47), F(44), P(45), C(46), and I(47), presumably responsible for the high substrate specificity of (R,S)-reticuline 7-*O*-methyltransferase.

The concept of phenolic oxidative coupling is a crucial theme in modifying the basic benzyltetrahydroisoquinoline skeleton to many other types of alkaloid. Tetrandrine and tubocurarine represent the coupling of two benzyltetrahydroisoquinoline molecules by ether bridges, but this form of coupling is perhaps less frequent than that involving carbon–carbon bonding between aromatic rings. The principal opium alkaloids **morphine**, **codeine**, and **thebaine** are derived from this type of coupling, though the subsequent reduction of one aromatic ring to some extent disguises their benzyltetrahydroisoquinoline origins. (*R*)-**Reticuline** is firmly established as the precursor of these morphinan alkaloids. (*R*)-Reticuline is the substrate for one-electron oxidations via the phenol group in each ring, giving the diradical. Coupling *ortho* to the phenol group in the tetrahydroisoquinoline, and *para* to the phenol in the benzyl substituent, then yields the dienone **salutaridine**, found as a minor alkaloid constituent in the opium poppy *Papaver somniferum* (Papaveraceae). The alkaloid **thebaine** is obtained by way of **salutaridinol**, formed from salutaridine by stereospecific reduction of the carbonyl group. Ring closure to form the ether linkage in thebaine would be the result of nucleophilic attack of the phenol group on to the dienol system and subsequent displacement of the hydroxyl. This cyclization step can be demonstrated chemically by treatment of salutaridinol with acid. Subsequent reactions involve conversion of thebaine into **morphine** by way of **codeine**, a process which modifies the oxidation state of the diene ring, but most significantly removes two *O*-methyl groups. One is present as an enol ether, removal generating **neopinone**, which gives **codeinone** and then codeine by allylic isomerization and reduction, respectively. The last step, demethylation of the phenol ether codeine to the phenol morphine, is the type of reaction only achievable in the laboratory by the use of powerful and reactive demethylating agents, e.g., HBr or BBr3. Because of the other functional groups present, chemical conversion of codeine into morphine is not usually a satisfactory process. However, the enzyme-mediated conversion in *P. somniferum* proceeds smoothly and efficiently. The enzymic demethylations of both the enol ether and the phenol ether probably involve initial hydroxylation followed by the loss of the methyl groups as formaldehyde.

The involvement of these *O*-demethylation reactions is rather unusual; secondary metabolic pathways tend to increase the complexity of the product by adding methyls rather than removing them. In this pathway, it is convenient to view the methyl groups in reticuline as protecting groups, which reduce the possible coupling modes available during the oxidative coupling process, and these groups are then removed towards the end of the synthetic sequence. There is also some evidence that the later stages of the pathway are modified in some strains of opium poppy. In such strains, thebaine is converted by way of **oripavine** and **morphinone**, this pathway removing the phenolic *O*-methyl before that of the enol ether, i.e., carrying out the same steps but in a different order. The enzymic transformation of thebaine into morphine and the conversion of (*R*)-reticuline into salutaridinol have also been observed in mammalian tissues, giving strong evidence that the trace amounts of morphine and related alkaloids which can sometimes be found in mammals are actually of endogenous origin rather than dietary. Detection of substrate binding motifs for morphine biosynthetic pathway intermediates has been done by Sonal Mishra et al. [19].

A minor constituent of *P. somniferum* is the aporphine alkaloid **isoboldine**. Other species of poppy, e.g., *Papaver orientale* and *P. pseudoorientale*, are known to synthesize aporphine alkaloids as principal constituents rather than morphinan structures. (*S*)-Isoboldine is readily appreciated to be the product of oxidative coupling of (*S*)-**reticuline**, coupling *ortho* to the phenol group in the tetrahydroisoquinoline and *para* to the phenol of the benzyl substituent. Some

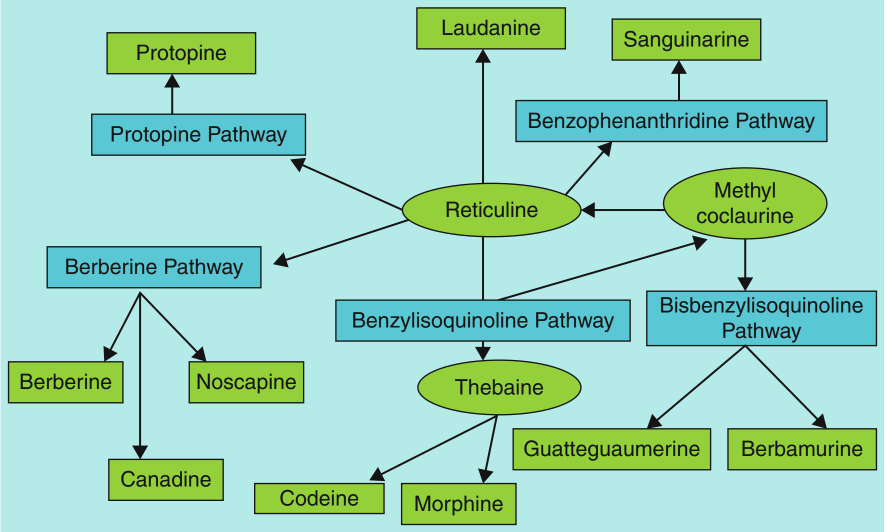

Laudanine
Protopine
Sanguinarine
Protopine Pathway
Benzophenanthridine Pathway
Reticuline
Methyl coclaurine
Berberine Pathway
Benzylisoquinoline Pathway
Bisbenzylisoquinoline Pathway
Berberine
Noscapine
Thebaine
Guatteguaumerine
Berbamurine
Canadine
Codeine
Morphine

Fig. 15.4 Flowchart representing the branching points and links to different pathways in isoquinoline biosynthesis pathways

structures, e.g., **isothebaine** from *P. orientale*, are not as easily rationalized. (*S*)-**Orientaline** is a precursor of isothebaine (Fig. 15.4). This benzyltetrahydroisoquinoline, with a different methylation pattern to reticuline, is able to participate in oxidative coupling, but inspection of the structures indicates a phenol group is lost in the transformation. The pathway involves an unexpected rearrangement process, however. Thus, oxidative coupling *ortho–para* to the phenol groups gives a dienone **orientalinone** (compare the structure of salutaridine). After reduction of the carbonyl group, a rearrangement occurs, restoring aromaticity and expelling the hydroxyl (originally a phenol group) to produce **isothebaine**. This type of rearrangement, for which good chemical analogies are available, is a feature of many other alkaloid biosynthetic pathways and occurs because normal keto–enol tautomerism is not possible for rearomatization when coupling involves positions already substituted. The process is fully borne out by experimental evidence, including the subsequent isolation of orientalinone and orientalinol from *P. orientale*.

Stephanine from *Stephania* species (Menispermaceae) is analogous to isothebaine and shares a similar pathway, though from (*R*)-**orientaline**. The different substitution pattern in stephanine compared to isothebaine is a consequence of the intermediate dienol suffering migration of the alkyl rather than aryl group. **Aristolochic acid** is a novel modified aporphine containing a nitro group and is produced from stephanine by oxidative reactions leading to ring cleavage. Aristolochic acid is present in many species of *Aristolochia* (Aristolochiaceae) used in traditional medicine, e.g., snake root *A. serpentina*. However, because aristolochic acid is now known to be nephrotoxic and to cause acute kidney failure, the use of *Aristolochia* species in herbal medicines, especially Chinese remedies, has been banned in several countries.

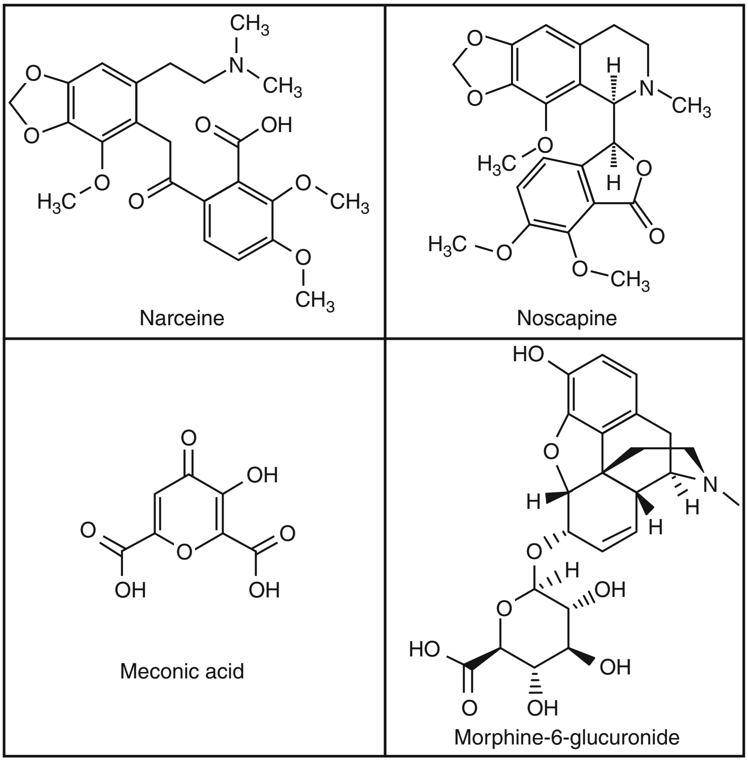


Fig. 15.5 Chemical structure of opium alkaloids

The alkaloid **berberine** is found in many members of the Berberidaceae (e.g., *Berberis*, *Mahonia*), the Ranunculaceae (e.g., *Hydrastis*), and other families. Berberine has antiamoebic, antibacterial, and antiinflammatory properties, and plants containing berberine have long been used in traditional medicine. Its tetracyclic skeleton is derived from a benzyltetrahydroisoquinoline system with the incorporation of an extra carbon atom, supplied from *S*-adenosylmethionine via an *N*-methyl group. This extraskeletal carbon is known as a "berberine bridge." Formation of the berberine bridge is readily rationalized as an oxidative process in which the *N*-methyl group is oxidized to an iminium ion, and a cyclization to the aromatic ring occurs by virtue of the phenolic group.

The oxidative cyclization process is analogous to the formation of a methylenedioxy group, while the mechanism of cyclization is exactly the same as that invoked in the formation of a tetrahydroisoquinoline ring, i.e., a Mannich-like reaction. The product from the enzymic transformation of (*S*)-**reticuline** is the protoberberine alkaloid (*S*)-**scoulerine**, the berberine bridge enzyme requiring molecular oxygen as oxidant and releasing H_2O_2 as by-product. Its role in the cyclization reaction completed, the phenol group in scoulerine is then methylated, and **tetrahydrocolumbamine** is oxidized further to give the quaternary isoquinoline system in **columbamine**. This appears to involve two separate oxidation steps, both requiring molecular oxygen, though H_2O_2 and H_2O are produced in the successive processes. The mechanistic sequence through an iminium ion has been suggested to account for these observations. Finally, **berberine** is produced by transformation of the *ortho*-methoxyphenol to a methylenedioxy group, via the O^2-, NADPH-, and cytochrome P450-dependent enzyme. The protoberberine skeleton of scoulerine may be subjected to further modifications. Cleavage of the heterocyclic ring systems adjacent to the nitrogen atom as shown give rise to new skeletal types: protopine, e.g., **protopine** from *Chelidonium majus* (Papaveraceae); phthalideisoquinoline, e.g., **hydrastine** from *Hydrastis canadensis* (Ranunculaceae); and benzophenanthridine, e.g., **chelidonine**, also from *Chelidonium majus*. The non-heterocyclic system seen in the opium alkaloid **narceine** from *Papaver somniferum* can be visualized as the result of cleavage of two of these bonds (Fig. 15.5). Some alkaloids of the phthalide type are medicinally important. **Noscapine** is one of the opium alkaloids, and although it lacks any analgesic activity, it is an effective cough suppressant (Fig. 15.5). **Hydrastine** is beneficial as a traditional remedy in the control of uterine bleeding. *Hydrastis* also contains berberine, indicating the close biosynthetic relationship of the two types of alkaloid. **Bicuculline** from species of *Corydalis* and *Dicentra* (Fumariaceae) and its quaternary methiodide have been identified as potent GABA (γ-aminobutyric acid) antagonists and have found widespread application as pharmacological probes for convulsants acting at GABA neuroreceptors. Figure 15.4 represents the branching points and links to different pathways in isoquinoline biosynthesis pathways.

6.1 The Opium Alkaloids

More than 12,000 alkaloids are known in plants, mostly used as medicine with a world market value of about 4 billion US$. Opium poppy, *Papaver somniferum*, is the most important economic source of morphinane alkaloids such as morphine, codeine, thebaine, narcotine, and papaverine that are exploited by the pharmaceutical industry as analgesics, antitussives, and antispasmodics. Opium is the air-dried milky exudates, or latex, obtained by incising the unripe capsules of the opium poppy *Papaver somniferum* (Papaveraceae). The plant is an annual herb with large solitary flowers, of white, pink, or dull red-purple color. For opium production, the ripening capsules, which are just changing color from blue-green to yellow, are carefully incised with a knife to open the latex tubes, but not to cut through to the interior of the capsule. These latex tubes open into one another, so it is not necessary to incise them all. Cuts are made transversely or longitudinally according to custom. The initially white milky latex quickly oozes out but rapidly turns brown and coagulates. This material, the raw opium, is then removed early the following morning, being scraped off and moulded into balls or blocks. Typically, these are wrapped in poppy leaves and shade-dried. The blocks may be dusted with various plant materials to prevent cohering. Fresh opium is pale to dark brown and plastic, but it becomes hard and brittle when stored. Crude opium has been used as an analgesic and sleep inducer (narcotic) and for the treatment of coughs. Opium has traditionally been smoked for pleasure, but habitual use develops a craving for the drug followed by addiction. An unpleasant abstinence syndrome is experienced if the drug is withdrawn [20].

The six opium alkaloids which occur naturally in the largest amounts are morphine, narcotine, codeine, thebaine, papaverine, and narceine. Of these, three are phenanthrene alkaloids and are under international control; these are morphine, codeine, and thebaine. They are all three used in the drug industry, thebaine usually for conversion into some derivative which is more useful medically. Of the other three, not under international control, narcotine and narceine have scarcely any medical or other uses. Consequently, the four economically significant alkaloids of opium are morphine, codeine, thebaine, and papaverine. About twenty other alkaloids exist in opium, but they have little or no significance medically or economically up to the present time. The relative proportions of the different alkaloids vary greatly, however, in different kinds of opium and certainly also in different varieties of the poppy. All varieties, however, belong to one species of poppy, *P. somniferum*. Figure 15.6 represents the biosynthesis of opium alkaloids.

In modern medicine, only the purified opium alkaloids and their derivatives are commonly employed. Although the ripe poppy capsule can contain up to 0.5 % total alkaloids, opium represents a much concentrated form and up to 25 % of its mass is composed of alkaloids. Of the many (>40) alkaloids identified, some six represent almost all of the total alkaloid content. Actual amounts vary widely, e.g., morphine (4–21 %), codeine (0.8–2.5 %), thebaine (0.5–2.0 %), papaverine (0.5–2.5 %), noscapine (narcotine) (4–8 %), and narceine (0.1–2 %). A typical commercial sample of opium would probably have a morphine content of about 12 %. Powdered opium is standardized to contain 10 % of anhydrous morphine, usually by dilution with an approved diluent, e.g., lactose or cocoa husk powder. The alkaloids are largely combined in salt form with meconic acid, opium containing some 3–5 % of this material. Meconic acid is invariably found in opium but, apart from its presence in other Papaver species, has not been detected elsewhere. It gives a deep red-colored complex with ferric chloride, and this has thus been used as a rapid and reasonably specific test for opium. Of the main opium alkaloids, only morphine and narceine display acidic properties, as well as the basic properties due to the tertiary amine. Narceine has a carboxylic acid function, while morphine is acidic due to its phenolic hydroxyl. This acidity can be exploited for the preferential extraction of these alkaloids (principally morphine) from an organic solvent by partitioning with aqueous base (Table 15.6).

6.1.1 Morphine

Morphine is the main alkaloid of opium both in amount and in medical importance (UNODC-Bulletin on Narcotics). The expression of the morphine content of opium as a percentage depends in part on the moisture content. When the government

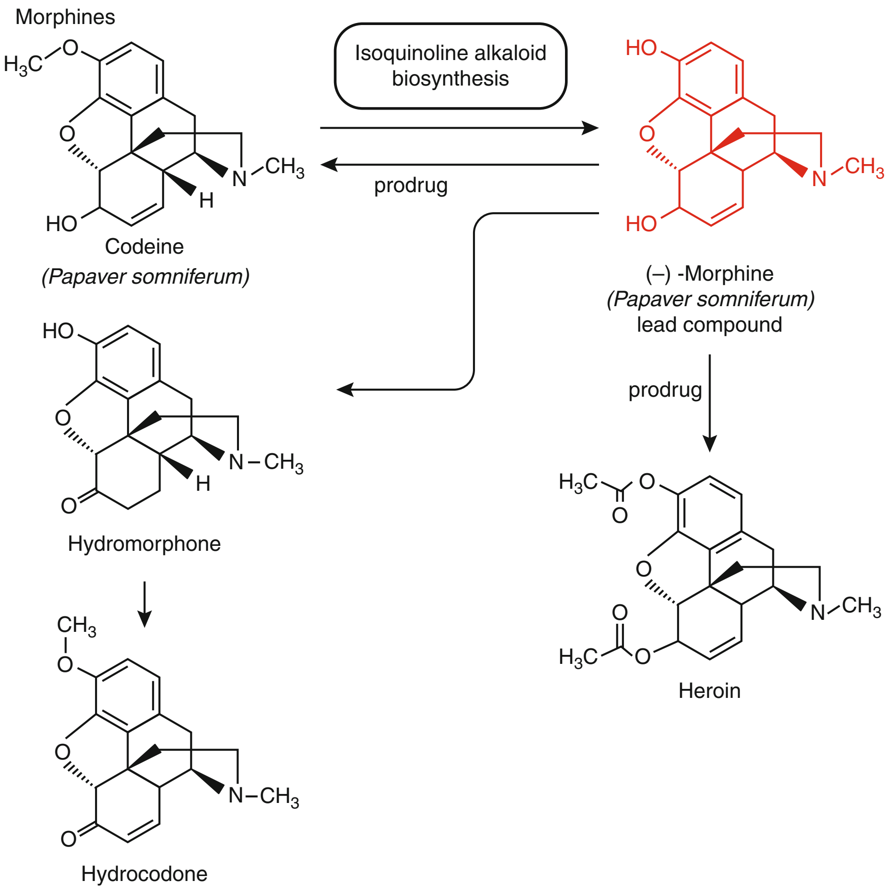

Morphines
Isoquinoline alkaloid biosynthesis
prodrug
Codeine
(Papaver somniferum)
(–) -Morphine
(Papaver somniferum)
lead compound
prodrug
Hydromorphone
Heroin
Hydrocodone
H3C
O
HO
N
CH3
H

Fig. 15.6 Biosynthesis of opium alkaloids

purchases the opium as soon as practicable after it is collected, the moisture content is then usually about 30 percent (%). Commercial opium usually has around 15–10 % moisture. Opium apparently dry at ordinary temperatures still retains considerable moisture, usually about 6 % which can be driven off at about 103° centigrade. Normal, unadulterated opium of any type, in the air-dry condition, usually has between 8 and 19 % of morphine. The principal commercial opiums generally have approximately the following morphine contents: Yugoslavia 15 %, Turkey 13 %, Iran 11 %, and India 11 %. The quantity of morphine produced by poppy plants in the form of opium depends on two factors: the percentage of morphine in the opium and the quantity of opium produced. The latter factor in turn depends in part on whether each capsule is bled several times, or once only. In Turkey, Yugoslavia, Greece, and Bulgaria, it is customary to bleed each capsule only once, but in most other opium-producing

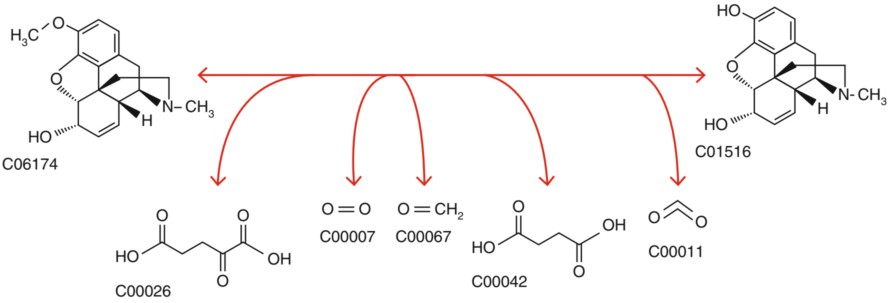
Table 15.6 Representation of chemical category and component of opium alkaloids

Chemical category	Opium components
Alkaloids	16-Hydroxythebaine, berberine, canadine, codamine, coptisine, coreximine, cycloartenol, cycloartenone, cyclolaudenol, dehydroreticuline, dihydrosanguinarine, glaucine, isoboldine, isocorypalmine, laudanidine, magnoflorine, narceine, narceinone, norlaudanosoline, norsanguinarine, oripavine, oxysanguinarine, palaudine, papaverrubine B (*O*-methyl-porphyroxine), papaverrubine C (epiporphyroxine), reticuline, salutaridine (sinoacutine), sanguinarine, scoulerine, somniferine, stepholidine
Morphine group (phenanthrenes, opioids)	Codeine, morphine, narcotoline, neopine, perparin, papaverrubine D (porphyroxine), pseudocodeine, pseudomorphine, thebaine
Isoquinolines	Cotarnine, eupaverine, hydrocotarnine, laudanosine, laudanine, noscapine (narcotine), papaverine, papaveraldine, xanthaline
Protopine group	α-Allocryptopine, α-fagarine, corycavamine, corycavine, cryptopine, protopine
Tetrahydroprotoberberine group	Corydaline, corybulbine, isocorybulbine, capaurine
Aporphine group	Dicentrine, glaucine, corytuberine, cularine, corydine, isocorydine, bulbocapnine
Phtalide-isoquinolines	Adulmine, bicuculline, bicucine, corlumine
α-Naphthaphenanthridines	Chelidonine, β-homochelidonoine, chelerythrine, sanguinarine
Other components	Meconic acid

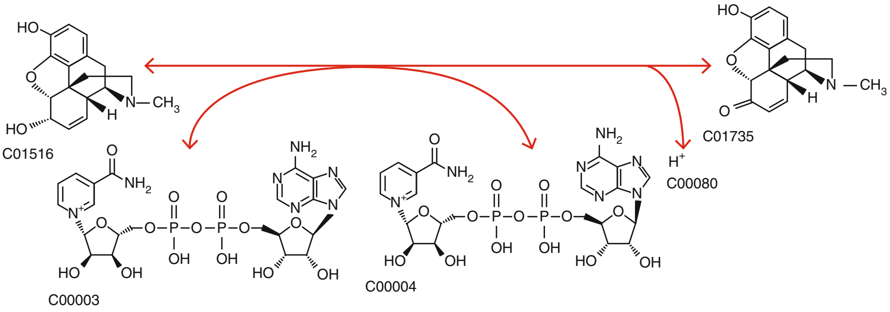
countries, the capsules are incised repeatedly, often four or five times on different days, until they will yield no more latex.

The quantity of latex falls off rapidly with later incisions, and so does the morphine content, as was shown by Annett in India (1). Usually, all the opium so obtained is mixed together. This is probably the chief reason for the lower morphine content of Iranian and Indian opiums as compared with Turkish and Yugoslav opiums, although it must also be recognized that there are low-yielding and high-yielding strains of the poppy, one or the other of which may predominate in a given district. Samples of opium assaying some 15 % morphine from Japan, Indochina, and Afghanistan, as well as from Turkey, Greece, and Yugoslavia, have been examined by the Secretariat. Afghanistan at one time exported two grades of opium, one of about 15 % morphine and the other about 10 %. The morphine content of dry capsule-chaff is about 0.25–0.5 % when not washed out by rain. Here again there are low-yielding and high-yielding varieties, but proper agricultural selection of poppies for morphine production means taking into account not only the percentage yield of morphine but also the total weight of the capsule-chaff produced per hectare, the poppy seed production per hectare, and other factors. Morphine is used to manufacture apomorphine (not subject to the conventions), codeine, ethylmorphine, beta-4-morpholinylethylmorphine, benzylmorphine, diacetylmorphine, dihydromorphine, hydromorphone (dihydromorphinone), metopon, morphine-*N*-oxide, desomorphine (dihydrodesoxymorphine), and *N*-allyl-normorphine.

KEGG reaction entry	R09402
Name	Codeine, 2-oxoglutarate:oxygen oxidoreductase (3-*O*-demethylating)
Definition	Codeine + 2-oxoglutarate + oxygen < = > morphine + formaldehyde + succinate + CO2
Equation	C06174 + C00026 + C00007 < = > C01516 + C00067 + C00042 + C00011



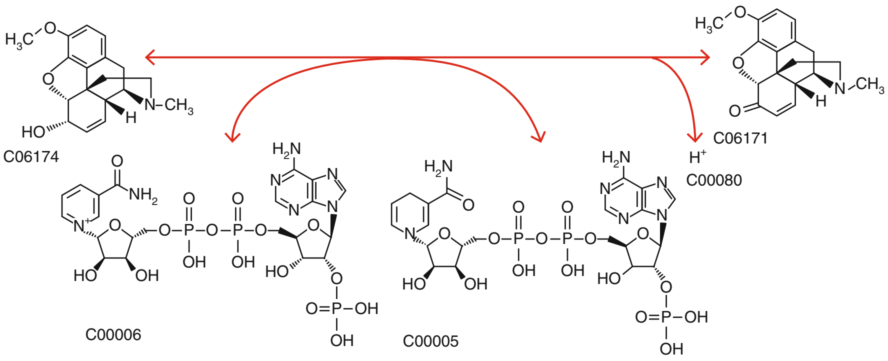
Enzyme	1.14.11.32; codeine 3-*O*-demethylase; codeine *O*-demethylase; CODM; oxidoreductases; codeine,2-oxoglutarate:oxygen oxidoreductase (3-*O*-demethylating)
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)
KEGG reaction Entry	R03591
Name	Morphine: NAD + 6-oxidoreductase
Definition	Morphine + NAD + < = > morphinone + NADH + H+
Equation	C01516 + C00003 < = > C01735 + C00004 + C00080



Enzyme	1.1.1.218; Morphine 6-dehydrogenase; naloxone reductase; reductase, naloxone; oxidoreductases
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)

6.1.2 Codeine

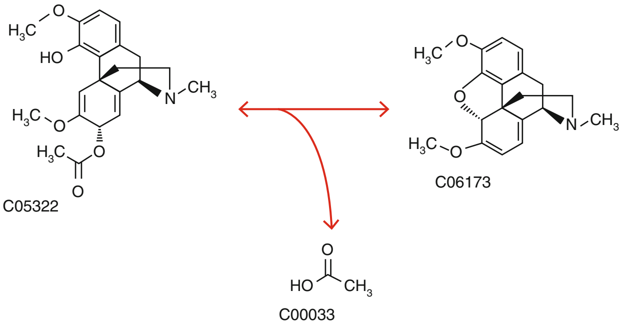
The codeine content of opium is related inversely to the morphine content but only in a general way. The codeine content is closely related to the type of opium produced in a given district or even in some cases in an entire country. The opiums of the principal exporting countries have approximately the following percentages of codeine: Yugoslavia 1.25 %, Turkey 1.25 %, Iran 3.4 %, and India 3.0 %. The manufacturer's statistics do not ordinarily show all the codeine obtained from opium. Some of its coprecipitates with the morphine, and there is no necessity of purifying the morphine completely on its codeine content, especially if it is to be used to manufacture more codeine. Codeine is used to manufacture dihydrocodeine and acetyldihydrocodeine and may also be used to manufacture the drugs ordinarily made by conversion of thebaine.

KEGG reaction entry	R05124
Name	Codeine: NADP + oxidoreductase
Definition	Codeine + NADP + < = > codeinone + NADPH + H+
Equation	C06174 + C00006 < = > C06171 + C00005 + C00080
	
Enzyme	1.1.1.247; Codeinone reductase (NADPH); oxidoreductases; codeine-NADP + oxidoreductase
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)

6.1.3 Thebaine

The Secretariat is currently engaged in a survey, the most extensive ever attempted in this field, of opium samples from different regions for their thebaine and papaverine percentages. As yet, it is premature for general conclusions. However, the highest thebaine percentages found (nearly 5 %) were in some samples from Indochina, which at the same time had virtually no papaverine. Both thebaine and papaverine have been higher in the most Iranian samples run. Papaverine is low in some Afghan and Indian opiums. Thebaine is the most poisonous opium alkaloid and is scarcely used medically. It is even omitted from some of the preparations of mixed opium alkaloids which are used as soluble substitutes for opium. However, it is converted into several other narcotics which have medical use: hydrocodone (dihydrocodeinone), acetyldihydrocodeinone, and oxycodone (dihydrohydroxy-codeinone). Papaverine has a considerable medical use, so much so that supplies available from opium have sometimes run short. It is then manufactured synthetically.

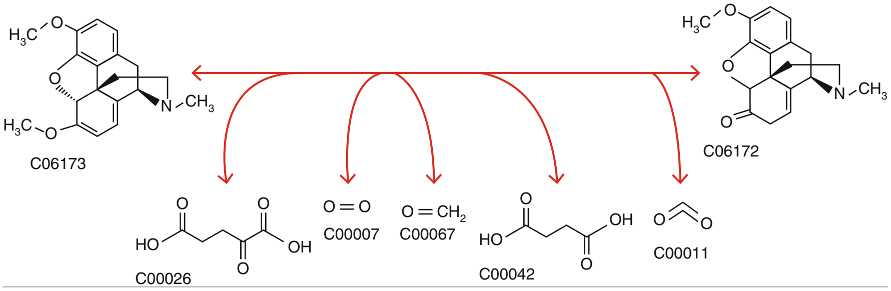
KEGG reaction entry	R04769
Definition	7-*O*-Acetylsalutaridinol < = > thebaine + acetate
Equation	C05322 < = > C06173 + C00033



Enzyme	Spontaneous (following salutaridinol 7-*O*-acetyltransferase [2.3.1.150] reaction)
Pathway	Isoquinoline alkaloid biosynthesis

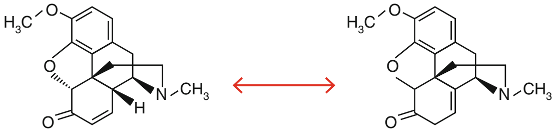
Thebaine converted into **morphine** by way of **codeine**, a process which modifies the oxidation state of the diene ring, but most significantly removes two *O*-methyl groups. One is present as an enol ether, removal generating **neopinone**, which gives **codeinone**.

KEGG reaction entry	R05126
Name	Thebaine,2-oxoglutarate:oxygen oxidoreductase (6-*O*-demethylating)
Definition	Thebaine + 2-oxoglutarate + oxygen < = > neopinone + formaldehyde + succinate + CO_2
Equation	C06173 + C00026 + C00007 < = > C06172 + C00067 + C00042 + C00011



Enzyme	1.14.11.31; Thebaine 6-*O*-demethylase; T6ODM; oxidoreductases; thebaine,2-oxoglutarate:oxygen oxidoreductase (6-*O*-demethylating)
Pathway	Isoquinoline alkaloid biosynthesis (rn00950), metabolic pathways (rn01100), biosynthesis of secondary metabolites (rn01110)
KEGG reaction entry	R05125

(*continued*)

Definition	Codeinone < = > neopinone
Equation	C06171 < = > C06172
	 C06171 C06172
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)

6.2 Papaverine

Papaverine is a benzylisoquinoline alkaloid and is structurally very different from the morphine, codeine, and thebaine group of alkaloids (morphinans). It has little or no analgesic or hypnotic properties put possesses spasmolytic and vasodilator activity. It has been used in some expectorant preparations and in the treatment of gastrointestinal spasms, but its efficacy was not substantiated. It is sometimes used as an effective treatment for male impotence, being administered by direct injection to achieve an erection of the penis.

Papaverine is an opium alkaloid antispasmodic drug, used primarily in the treatment of visceral spasm, vasospasm (especially those involving the heart and the brain), and occasionally in the treatment of erectile dysfunction. While it is found in the opium poppy, papaverine differs in both structure and pharmacological action from the analgesic (morphine-related) opium alkaloids (opioids). Papaverine is approved to treat spasms of the gastrointestinal tract, bile ducts, and ureter and for use as a cerebral and coronary vasodilator in subarachnoid hemorrhage (combined with balloon angioplasty) and coronary artery bypass surgery. Papaverine may also be used as a smooth muscle relaxant in microsurgery where it is applied directly to blood vessels.

Papaverine is used as an erectile dysfunction drug. Papaverine, when injected in penile tissue, causes direct smooth muscle relaxation and consequent filling of the corpus cavernosum with blood, resulting in erection. It is also commonly used in cryopreservation of blood vessels along with the other glycosaminoglycans and protein suspensions. It functions as a vasodilator during cryopreservation when used in conjunction with verapamil, phentolamine, nifedipine, tolazoline, or nitroprusside. Papaverine is also being investigated as a topical growth factor in tissue expansion with some success.

6.2.1 Narceine

Narceine is an opium alkaloid produced by the *Papaver somniferum* (opium poppy) plant. It is a bitter, crystalline compound with narcotic effects and was formerly used as a substitute for morphine (Fig. 15.5).

6.2.2 Noscapine

Noscapine was first isolated and characterized in the chemical breakdown and properties in 1817 under the denomination of “Narcotine” by Pierre Robiquet, a French chemist in Paris. Noscapine’s antitussive effects appear to be primarily mediated by its sigma receptor agonist activity. Noscapine is currently under investigation for use in the treatment of several cancers and hypoxic ischemia in stroke patients. Noscapine can survive the manufacturing processes of heroin and can be found in street heroin.

Noscapine is a member of the phthalideisoquinoline alkaloids and provides a further structural variant in the opium alkaloids. Noscapine has good antitussive and cough suppressant activity comparable to that of codeine, but no analgesic or narcotic action. Its original name “narcotine” was changed to reflect this lack of narcotic action. Despite many years of use as a cough suppressant, the finding that noscapine may have teratogenic properties (i.e., may deform a fetus) has resulted in noscapine preparations being deleted. In recent studies, antitumour activity has been noted from noscapine, which binds to tubulin as do podophyllotoxin and colchicine, thus arresting cells in mitosis. The chemotherapeutic potential of this orally effective agent merits further evaluation. Noscapine (also known as narcotine, nectodon, nospen, anarcotine, and (archaic) opiane) is a benzylisoquinoline alkaloid from plants of the Papaveraceae family, without significant painkilling properties. This agent is primarily used for its antitussive (cough-suppressing) effects. It has also been shown to have anticancer activity (Fig. 15.5).

7 Other Compounds

7.1 Meconic Acid

Meconic acid, also known as acidum meconicum and poppy acid, is a chemical substance found in certain plants of the Papaveraceae family (poppy) such as Papaver somniferum (opium poppy) and Papaver bracteatum. Meconic acid constitutes about 5 % of opium and can be used as an analytical marker for the presence of opium. Meconic acid has erroneously been described as a mild narcotic, but it has little or no physiological activity, and is not used medicinally. Meconic acid forms salts with alkaloids and metals. These salts as well as meconic acid esters are called meconates. Meconic acid was first isolated by Friedrich Sertürner in 1805 (Fig. 15.5).

7.2 Morphine-6-Glucuronide

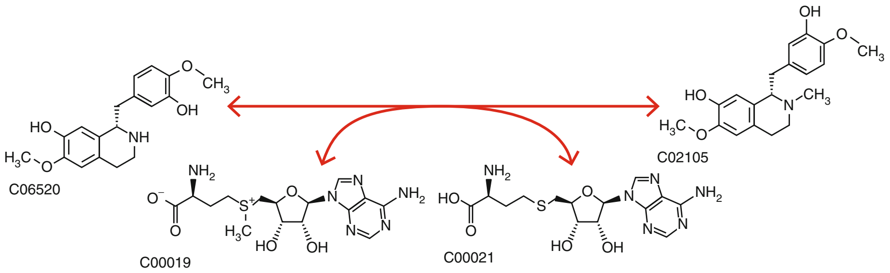
Morphine-6-glucuronide (M6G) is a major active metabolite of morphine and as such is the molecule responsible for much of the pain-relieving effects of morphine (and thus heroin). M6G is formed from morphine by the enzyme

UDP-glucuronosyltransferase-2B7 (UGT2B7). M6G can accumulate to toxic levels in kidney failure. The opioid receptor subtype 3 appears to be activated (agonized) by morphine-6β-glucuronide but not morphine itself. This finding is also true of certain heroin metabolites (6-MAM) but not morphine proper. This analgesic activity of M6G (in animals) was first noted by Yoshimura (Fig. 15.5).

7.3 Narcotoline

Narcotoline is an opiate alkaloid chemically related to noscapine. It binds to the same receptors in the brain as noscapine to act as an antitussive and has also been used in tissue culture media. It can be obtained from the opium poppy, Papaver somniferum. It is present at much higher levels in culinary strains (cultivars) of *P. somniferum* used for poppy seed production than in high-morphine pharmaceutical strains used for opium production.

KEGG reaction entry	R05211
Name	S-adenosyl-L-methionine:(RS)-1-benzyl-1,2,3,4-tetrahydroisoquinolie *N*-methyltransferase
Definition	S-adenosyl-L-methionine + (S)-norreticuline < = > S-adenosyl-L-homocysteine + (S)-reticuline
Equation	C00019 + C06520 < = > C00021 + C02105



Enzyme	2.1.1.115; (RS)-1-benzyl-1,2,3,4-tetrahydroisoquinoline *N*-methyltransferase; norreticuline *N*-methyltransferase; transferases
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)

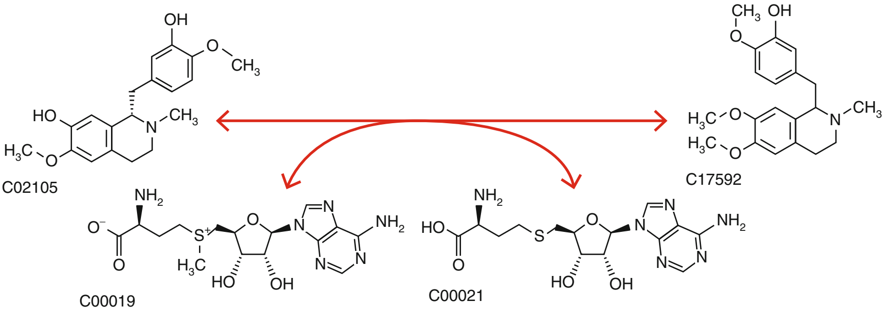
7.4 Pseudomorphine

Pseudomorphine (also known as oxydimorphine or dehydromorphine) is a natural dimerization product of the morphine molecule in tandem and thus a common impurity in morphine concentrations. It was first described by Pelletier in 1835. This compound may be synthesized by the oxidative coupling of morphine by potassium ferricyanide.

7.5 Laudanosine

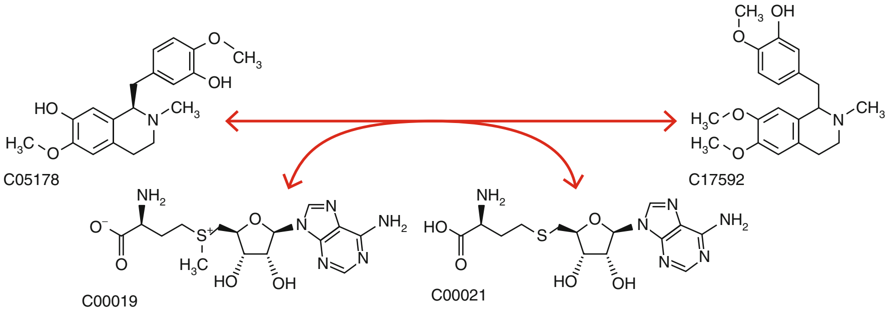
Laudanosine or *N*-methyltetrahydropapaverine is a recognized metabolite of atracurium and cisatracurium. Laudanosine decreases the seizure threshold, and thus, it can induce seizures if present at sufficient threshold concentrations; however, such concentrations are unlikely to be produced consequent to chemodegradable metabolism of clinically administered doses of cisatracurium or atracurium. Laudanosine also occurs naturally in minute amounts (0.1 %) in opium, from which it was first isolated in 1871. Partial dehydrogenation of laudanosine will lead to papaverine, the alkaloid found in the opium poppy plant (Papaver somniferum). Laudanosine is a benzyltetrahydroisoquinoline alkaloid. It has been shown to interact with GABA receptors, opioid receptors, and nicotinic acetylcholine receptors, but not benzodiazepinergic or muscarinic receptors which are also involved in epilepsy and other types of seizures.

KEGG reaction entry	R08787
Name	S-adenosyl-L-methionine:(S)-reticuline methyltransferase 7-O-methyltransferase
Definition	(S)-reticuline + S-Adenosyl-L-methionine < = > laudanine + S-adenosyl-L-homocysteine
Equation	C02105 + C00019 < = > C17592 + C00021



Comment	(R,S)-reticuline 7-*O*-methyltransferase
Enzyme	2.1.1.-
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)
Orthology	(R,S)-reticuline 7-*O*-methyltransferase [EC:2.1.1.-]
KEGG reaction entry	R08788
Name	S-adenosyl-L-methionine:(R)-reticuline methyltransferase 7-*O*-methyltransferase
Definition	(R)-reticuline + S-adenosyl-L-methionine < = > Laudanine + S-adenosyl-L-homocysteine
Equation	C05178 + C00019 < = > C17592 + C00021

(*continued*)



Comment	(R,S)-reticuline 7-*O*-methyltransferase
Enzyme	2.1.1.-
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)
Orthology	(R,S)-reticuline 7-*O*-methyltransferase [EC:2.1.1.-]

7.6 Berberine

Berberine is a quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids. It is found in such plants as Berberis (e.g., Berberis aquifolium (Oregon grape), Berberis vulgaris(Barberry), and Berberis aristata (Tree Turmeric)),Hydrastis canadensis (Goldenseal), Phellodendron amurense (Amur Cork Tree, Huang Bai, Huang Po, Po Mu) and Coptis chinensis (Chinese Goldthread, Huang-Lian, Huang-Lien), and Tinospora cordifolia, and to a smaller extent in Argemone mexicana (Prickly Poppy) and Eschscholzia californica (Californian Poppy). Berberine is usually found in the roots, rhizomes, stems, and bark. Berberine is strongly yellow colored, which is why in earlier times Berberis species were used to dye wool, leather, and wood. Wool is still today dyed with berberine in northern India. Under ultraviolet light, berberine shows a strong yellow fluorescence. Because of this, it is used in histology for staining heparin in mast cells. As a natural dye, berberine has a Colour Index (CI) of 75160.

As a traditional medicine or dietary supplement, berberine has shown some activity against fungal infections, Candida albicans, yeast, parasites, and bacterial/viral infections. Berberine seems to exert synergistic effects with fluconazole even in drug-resistant Candida albicans infections. Some research has been undertaken into possible use against MRSA infection. Berberine is considered antibiotic. When applied in vitro and in combination with methoxyhydnocarpin, an inhibitor of multidrug resistance pumps, berberine inhibits growth of Staphylococcus aureus and Microcystis aeruginosa, a toxic cyanobacterium. Berberine is a component of some eye drop formulations. There is some evidence it is useful in the treatment of trachoma, and it has been a standard treatment for leishmaniasis. Berberine prevents and suppresses proinflammatory cytokines, E-selectin, and genes, and increases adiponectin expression which partly explains its versatile health effects. Berberine is a nucleic acid-binding isoquinolone alkaloid with wide potential therapeutic properties. Berberine is produced by transformation of the *ortho*-methoxyphenol to a methylenedioxy group, via the O_2^-, NADPH$^-$, and cytochrome P450-dependent enzyme.

7.7 Sanguinarine

Sanguinarine is a quaternary ammonium salt from the group of benzylisoquinoline alkaloids. It is extracted from some plants, including bloodroot (Sanguinaria canadensis), Mexican prickly poppy Argemone mexicana, Chelidonium majus and Macleaya cordata. It is also found in the root, stem and leaves of the opium poppy but not in the capsule. Sanguinarine is a toxin that kills animal cells through its action on the Na^+-K^+ -ATPase transmembrane protein. Epidemic dropsy is a disease that results from ingesting sanguinarine. If applied to the skin, sanguinarine kills cells and may destroy tissue. In turn, the bleeding wound may produce a massive scab, called an eschar. For this reason, sanguinarine is termed an escharotic. In plants, sanguinarine is synthesized from dihydrosanguinarine through the action of dihydrobenzophenanthridine oxidase (EC 1.5.3.12).

7.8 Coptisine

Coptisine is an alkaloid found in Chinese goldthread (Coptis chinensis). Famous for the bitter taste that it produces, it is used in Chinese herbal medicine along with the related compound berberine for treating digestive disorders caused by bacterial infections. It is also found in Greater Celandine and has also been detected in opium. Coptisine has been found to reversibly inhibit Monoamine oxidase A in mice, pointing to a potential role as a natural antidepressant. However, this may also imply a hazard for those taking other medications or with a natural functional disorder in Monoamine oxidase A. Coptisine was found to be toxic to larval brine shrimp and a variety of human cell lines, potentially implying a therapeutic effect on cancer or alternatively a generally toxic character. The same authors illustrate a four-step process to produce Coptisine from Berberine.

7.9 Cycloartenol

Cycloartenol is an important type of stanol found in plants. The biosynthesis of cycloartenol starts from the triterpenoid squalene. It is the first precursor in the biosynthesis of other stanols and sterols, referred to as phytostanols and phytosterols in photosynthetic organisms and plants. The identities and distribution of phytostanols and phytosterols is characteristic of a plant species. One notable product of cycloartenol biosynthesis is the triterpenoid lanosterol.

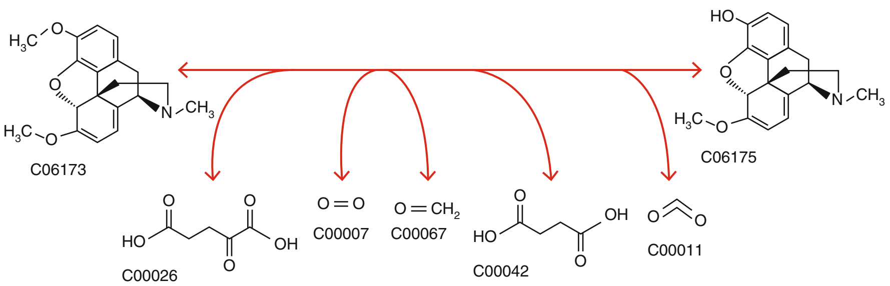
7.10 Glaucine

Glaucine is an alkaloid found in several different plant species such as Glaucium flavum, Glaucium oxylobum, Croton lechleri and Corydalis yanhusuo. It has bronchodilator and antiinflammatory effects, acting as a PDE4 inhibitor and calcium channel blocker, and is used medically as an antitussive in some countries. Glaucine may produce side effects such as sedation, fatigue, and a hallucinogenic effect characterized by colorful visual images, and has recently been detected as a novel recreational drug.

7.11 Oripavine

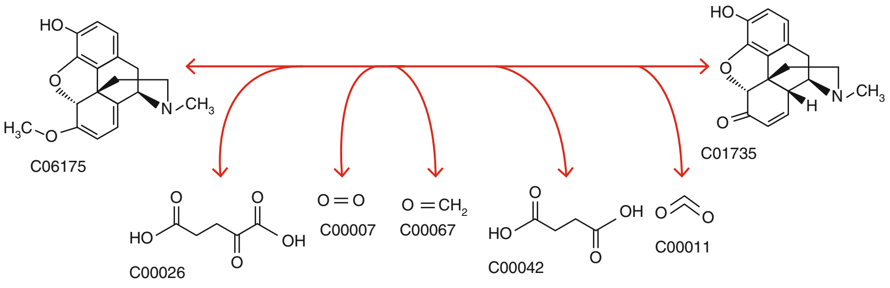
Oripavine is an opiate and the major metabolite of thebaine. It is the parent compound from which a series of semisynthetic opioids are derived, which includes the compounds etorphine and buprenorphine. Although its analgesic potency is comparable to morphine, it is not used clinically due to its severe toxicity and low therapeutic index. Oripavine possesses an analgesic potency comparable to morphine; however, it is not clinically useful due to severe toxicity and low therapeutic index. In both mice and rats, toxic doses caused tonic-clonic seizures followed by death, similar to thebaine. Oripavine has a potential for dependence which is significantly greater than that of thebaine but slightly less than that of morphine.

KEGG reaction entry	R05127
Name	thebaine,2-oxoglutarate:oxygen oxidoreductase (3-*O*-demethylating)
Definition	Thebaine + 2-oxoglutarate + oxygen < = > oripavine + formaldehyde + succinate + CO2
Equation	C06173 + C00026 + C00007 < = > C06175 + C00067 + C00042 + C00011



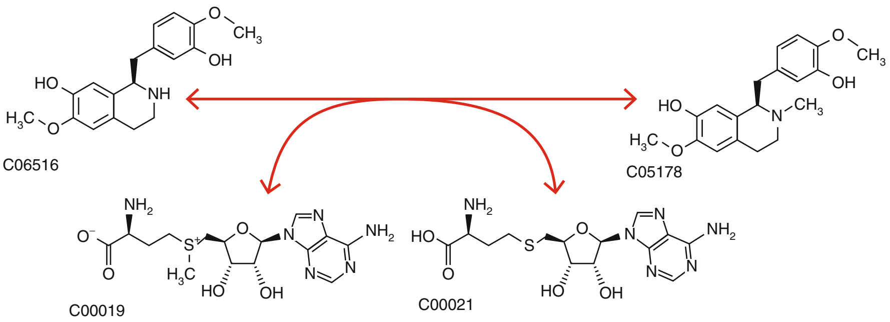
Enzyme	1.14.11.32; codeine 3-*O*-demethylase; codeine *O*-demethylase; CODM; oxidoreductases; codeine,2-oxoglutarate:oxygen oxidoreductase (3-*O*-demethylating)
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)
KEGG Reaction Entry	R03698
Name	Oripavine,2-oxoglutarate:oxygen oxidoreductase (6-*O*-demethylating)
Definition	Oripavine + 2-oxoglutarate + oxygen < = > morphinone + formaldehyde + succinate + CO2

(*continued*)

Equation	C06175 + C00026 + C00007 < = > C01735 + C00067 + C00042 + C00011
	 C06175 C01735 C00026 C00007 C00067 C00042 C00011
Enzyme	1.14.11.31; thebaine 6-*O*-demethylase; T6ODM; oxidoreductases
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)

7.12 Reticuline

Reticuline is a chemical compound that can be found in Lindera aggregata. It is also one of the alkaloids found in opium. Metabolism showed that 3′-hydroxy-*N*-methyl-(S)-coclaurine 4′-*O*-methyltransferase uses *S*-adenosyl methionine and 3′-hydroxy-*N*-methyl-(S)-coclaurine to produce S-adenosylhomocysteine and (S)-reticuline. Reticuline oxidase uses (S)-reticuline and O_2 to produce (S)-scoulerine and H_2O_2. Salutaridine synthase uses (R)-reticuline, NADPH, H^+, and O_2 to produce salutaridine, NADP$^+$, and H_2O. 1,2-dehydroreticulinium reductase (NADPH) uses (R)-reticuline and NADP + to produce 1,2-dehydroreticulinium, NADPH, and H^+.

KEGG Reaction Entry	R05216
Name	S-adenosyl-L-methionine:(RS)-1-benzyl-1,2,3,4-tetrahydroisoquinolie *N*-methyltransferase
Definition	S-adenosyl-L-methionine + (R)-Norreticuline < = > S-adenosyl-L-homocysteine + (R)-reticuline
Equation	C00019 + C06516 < = > C00021 + C05178
	

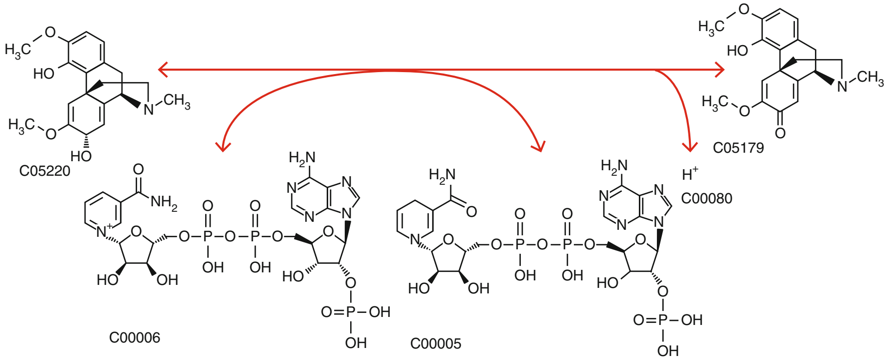
(*continued*)

Enzyme	2.1.1.115; (RS)-1-benzyl-1,2,3,4-tetrahydroisoquinoline N-methyltransferase; norreticuline N-methyltransferase; transferases
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)

7.13 Salutaridine

Salutaridine is an alkaloid that is present in the morphinan alkaloid pathway of opium poppy. Its precursor is the alkaloid (R)-reticuline. (R)-reticuline is converted to salutaridine by the enzyme salutaridine synthase. Salutaridine is converted to salutaridinol by the enzyme salutaridine reductase (SalR), with the reduction of NADPH to NADP$^+$.

KEGG Reaction Entry	R04697
Name	Salutaridinol: NADP + 7-oxidoreductase
Definition	5,6,8,14-Tetradehydro-3,6-dimethoxy-17-methyl-morphinan-4,7-diol + NADP + < = > salutaridine + NADPH + H+
Equation	C05220 + C00006 < = > C05179 + C00005 + C00080



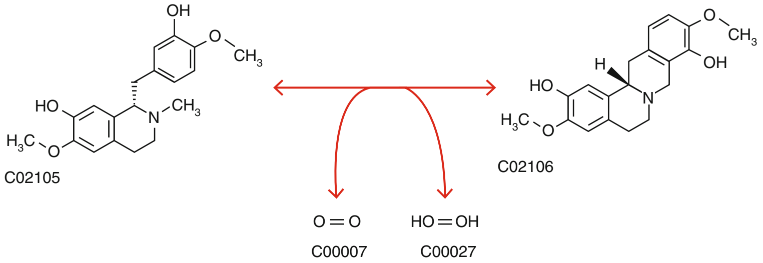
1.1.1.248; Salutaridine reductase (NADPH); oxidoreductases
Isoquinoline alkaloid biosynthesis (rn00950)
Salutaridine reductase (NADPH) [EC:1.1.1.248] (K13392)

7.14 Sanguinarine

Sanguinarine is a quaternary ammonium salt from the group of benzylisoquinoline alkaloids. It is extracted from some plants, including bloodroot (Sanguinaria canadensis), Mexican prickly poppy Argemone mexicana, Chelidonium majus, and Macleaya cordata. It is also found in the root, stem, and leaves of the opium poppy but not in the capsule. Sanguinarine is a toxin that kills animal cells through its action on the Na$^+$-K$^+$-ATPase transmembrane protein. Epidemic dropsy is a disease that results from ingesting sanguinarine. If applied to the skin, sanguinarine kills cells and may destroy tissue. In turn, the bleeding wound may produce a massive scab, called an eschar. For this reason, sanguinarine is termed an escharotic. In plants, sanguinarine is synthesized from dihydrosanguinarine through the action of Dihydrobenzophenanthridine oxidase (EC 1.5.3.12).

7.15 Scoulerine

Scoulerine, also known as discretamine and aequaline, is an alkaloid found in the opium poppy, Croton flavens, and certain plants in the Erythrina genus. Studies show that scoulerine is an antagonist at the α2-adrenoceptor, α1D-adrenoceptor, and 5-HT receptor. It has also been found to be a GABAA receptor agonist.

KEGG reaction entry	R03831
Name	(S)-reticuline:oxygen oxidoreductase (methylene-bridge-forming)
Definition	(S)-reticuline + oxygen < = > (S)-scoulerine + hydrogen peroxide
Equation	C02105 + C00007 < = > C02106 + C00027
	 OH O CH3 HO N CH3 H3C O C02105 O=O C00007 HO—OH C00027 O CH3 H OH HO N H3C O C02106
Enzyme	1.21.3.3; Reticuline oxidase; BBE; berberine bridge enzyme; berberine-bridge-forming enzyme; tetrahydroprotoberberine synthase; oxidoreductases
Pathway	Isoquinoline alkaloid biosynthesis (rn00950)
Orthology	Reticuline oxidase [EC:1.21.3.3]

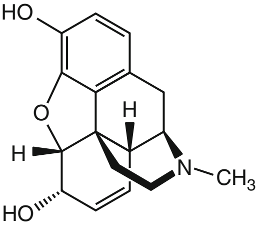
7.16 Stepholidine

(−)-Stepholidine is a naturally occurring chemical compound found in the herb Stephania intermedia. Stepholidine is a dual D2 receptor antagonist and D1 receptor agonist, and has shown antipsychotic activity in animal studies.

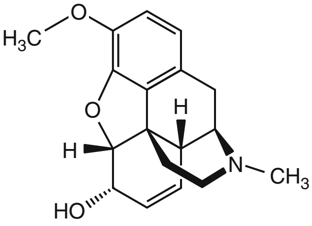
7.17 Structure and Properties of Alkaloids

The different types of alkaloids have different structures and thus have different properties. The structures and properties of alkaloids have been represented in Table 15.7 and 15.8

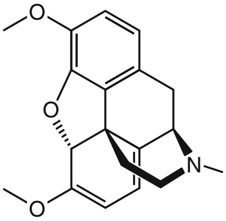
Table 15.7 Comparison of the properties of different opium alkaloids



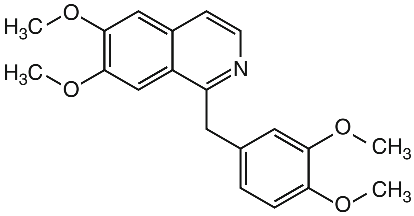
Morphine	
Bioavailability	~25 % (Oral); 100 % (IV)
Protein binding	30–40 %
Metabolism	Hepatic, 90 %
Half-life	2–3 h
Excretion	Renal 90 %, biliary 10 %
CAS number	57-27-2, 64-31-3, 52-26-6
PubChem	CID 5288826
DrugBank	DB00295
ChemSpider	4450907
KEGG	D08233
ChEBI	CHEBI:17303
ChEMBL	CHEMBL70
Formula	$C_{17}H_{19}NO_3$
Mol. mass	285.34



Codeine	
Bioavailability	~90 % Oral
Metabolism	Hepatic, via CYP2D6 (cytochrome P450 2D6)
Half-life	2.5–3 h
CAS number	76-57-3
PubChem	CID 5284371
DrugBank	APRD00120
ChemSpider	4447447
KEGG	C06174
ChEBI	CHEBI:16714
ChEMBL	CHEMBL485
Formula	$C_{18}H_{21}NO_3$
Mol. mass	299.364 g/mol



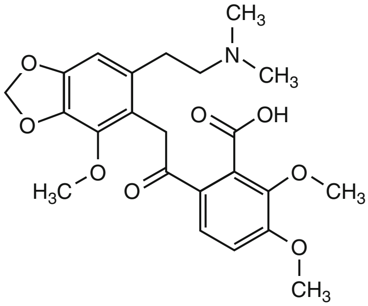
Thebaine	
CAS number	115-37-7
PubChem	5324289
ChemSpider	4481822, 4479543
KEGG	C06173
ChEBI	CHEBI:9519
ChEMBL	CHEMBL403893
Molecular formula	$C_{19}H_{21}NO_3$
Molar mass	311.37 g mol^{-1}
Metabolism	*O*-demethylation

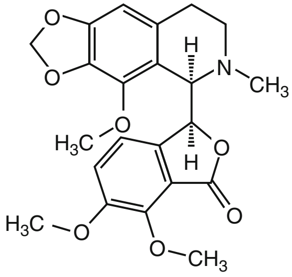


Papaverine	
Bioavailability	80 %
Protein binding	~90 %
Metabolism	Hepatic
Half-life	1.5–2 h
Excretion	Renal
CAS number	58-74-2, 61-25-6
PubChem	CID 4680
DrugBank	APRD00628
ChemSpider	4518
KEGG	D07425
ChEMBL	CHEMBL19224
Formula	$C_{20}H_{21}NO_4$
Mol. mass	339.385 g/mol

(*continued*)

Table 15.7 (continued)





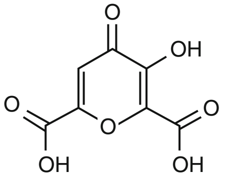
Narceine	
CAS number	131-28-2
PubChem	8564
Molecular formula	$C_{23}H_{27}NO_8$
Molar mass	445.46 g mol^{-1}

Noscapine	
Bioavailability	∼30 %
Half-life	1.5 to 4 h (mean 2.5)
CAS number	128-62-1
PubChem	CID 275196
KEGG	D01036^{Y}
ChEMBL	CHEMBL364713^{Y}
Synonyms	Narcotine
Formula	$C_{22}H_{23}NO_7$
Mol. mass	413.421

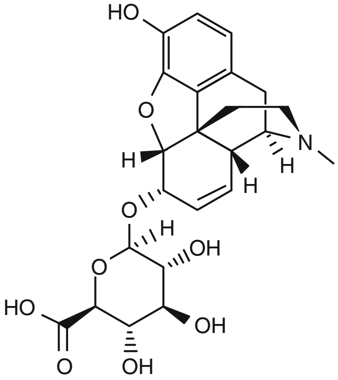
8 Use of Alkaloids in Medicine

Natural compounds, mostly from plants, have been the mainstay of traditional medicine for thousands of years [21]. They have also been the source of lead compounds for modern medicine, but the extent of mining of natural compounds for such leads decreased during the second half of the twentieth century. The advantage of natural compounds for the development of drugs derives from their innate affinity for biological receptors. Natural compounds have provided the best antimalarials known to date. Recent surveys have identified many extracts of various organisms (mostly plants) as having anti-plasmodial activity. Huge libraries of fractionated natural compounds have been screened with impressive hit rates. Importantly, many cases are known where the crude biological extract is more efficient pharmacologically than the most active purified compound from this extract. This could be due to synergism with other compounds present in the extract, which as such have no pharmacological activity. Indeed, such compounds are best screened by cell-based assay where all potential targets in the cell are probed and possible synergies identified. Traditional medicine uses crude extracts. These have often been shown to provide many concoctions that deal better with the overall disease condition than with the causative agent itself. Traditional medicines are used by ∼80 % of Africans as a first response to ailment. Many of the traditional medicines have demonstrable anti-plasmodial activities. It is suggested that rigorous evaluation of traditional medicines involving controlled clinical trials in

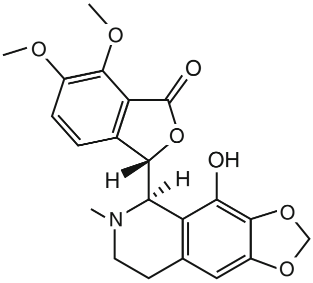
Table 15.8 Details of compounds similar to opium alkaloids



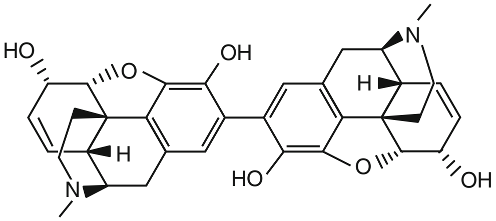
Meconic acid	
CAS number	497-59-6
PubChem	5351448
Molecular formula	$C_7H_4O_7$
Molar mass	200.10



Morphine-6-glucuronide	
CAS number	20290-10-2
PubChem	5360621
ChemSpider	4514548
ChEMBL	CHEMBL1330
Molecular formula	$C_{23}H_{27}NO_9$
Molar mass	461.46 g/mol



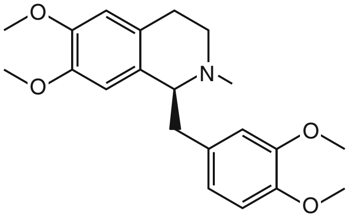
Narcotoline	
CAS number	521-40-4
PubChem	442330
Molecular formula	$C_{21}H_{21}NO_7$
Molar mass	399.39 g mol^{-1}



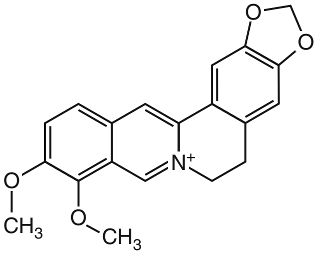
Pseudomorphine	
Abbreviations	2,2′-bimorphine
CAS number	125-24-6
PubChem	234570
ChemSpider	4590027
Molecular formula	$C_{34}H_{36}N_2O_6$
Molar mass	568.66 g mol^{-1}

(*continued*)

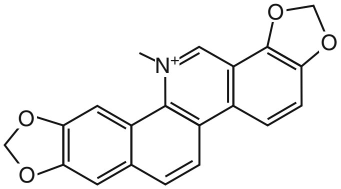
Table 15.8 (continued)



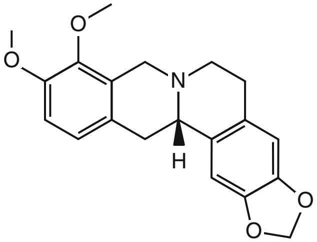
Laudanosine	
CAS number	2688-77-9
PubChem	73397
Molecular formula	$C_{21}H_{27}NO_4$
Molar mass	357.44 g mol^{-1}
Melting point	89 °C



Berberine	
CAS number	633-66-9
PubChem	2353
ChemSpider	2263
DrugBank	DB04115
ChEBI	CHEBI:16118
ChEMBL	CHEMBL12089
Molecular formula	$C_{20}H_{18}NO_4$
Molar mass	336.36122 g/mol



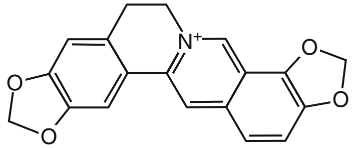
Sanguinarine	
CAS number	2447-54-3
PubChem	CID 5154
ChemSpider	4970
ChEBI	CHEBI:17183
ChEMBL	CHEMBL417799
Formula	$C_{20}H_{14}NO_4$
Mol. mass	332.09



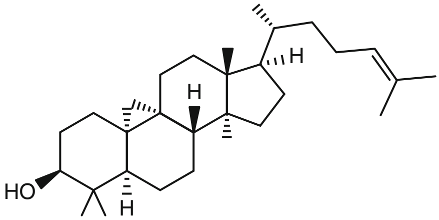
Canadine	
CAS number	522-97-4
PubChem	21171
ChemSpider	19910
ChEMBL	CHEMBL490533
Molecular formula	$C_{20}H_{21}NO_4$
Molar mass	339.38504

(*continued*)

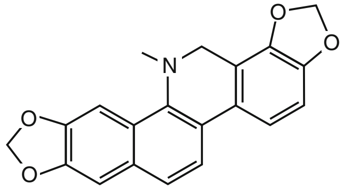
Table 15.8 (continued)



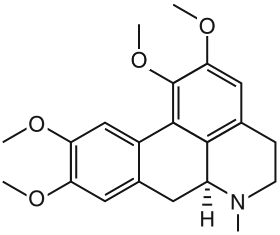
Coptisine	
CAS number	3486-66-6
PubChem	72322
ChemSpider	65268
ChEMBL	CHEMBL362071
Molecular formula	$C_{19}H_{14}NO_4$
Molar mass	320.319



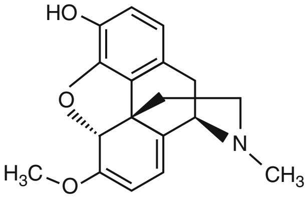
Cycloartenol	
CAS number	469-38-5
PubChem	92110
ChemSpider	16788581
Molecular formula	$C_{30}H_{50}O$
Molar mass	426.72 g/mol



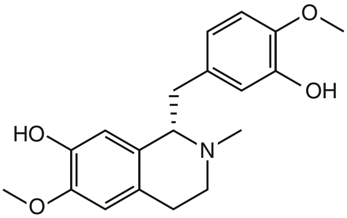
Dihydrosanguinarine	
CAS number	3606-45-9
PubChem	124069
Molecular formula	$C_{20}H_{15}NO_4$
Molar mass	333.34 g mol^{-1}



Glaucine	
CAS number	475-81-0, 5630-11-5
PubChem	CID 16754
ChEMBL	CHEMBL228082
Formula	$C_{21}H_{25}NO_4$
Mol. mass	355.428 g/mol



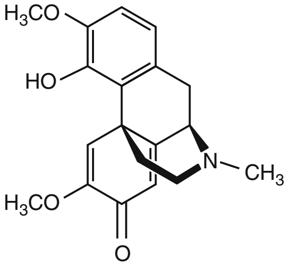
Oripavine	
CAS number	467-04-9
PubChem	5462306
ChemSpider	4575366
KEGG	C06175
ChEMBL	CHEMBL437602
Molecular formula	$C_{18}H_{19}NO_3$
Molar mass	297.35 g mol^{-1}



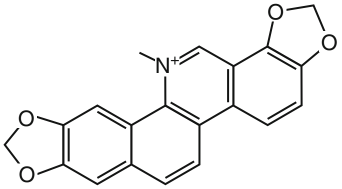
Reticuline	
CAS number	485-19-8
PubChem	439653
ChEMBL	CHEMBL401501
Molecular formula	$C_{19}H_{23}NO_4$
Molar mass	329.39 g/mol

(*continued*)

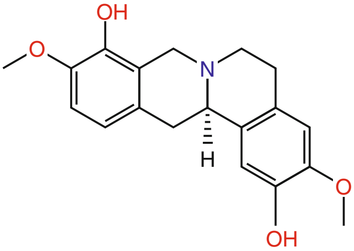
Table 15.8 (continued)



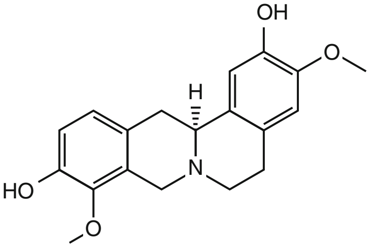
Salutaridine	
CAS number	1936-18-1
PubChem	5408233
ChEMBL	CHEMBL404097
Molecular formula	$C_{19}H_{21}NO_4$
Molar mass	327.37 g mol^{-1}



Sanguinarine	
CAS number	2447-54-3
PubChem	CID 5154
ChemSpider	4970
ChEBI	CHEBI:17183
ChEMBL	CHEMBL417799
Formula	$C_{20}H_{14}NO_4$
Mol. mass	332.09



Scoulerine	
CAS number	605-34-5
PubChem	439654
ChemSpider	388725
ChEBI	CHEBI:17129
ChEMBL	CHEMBL1235966
Molecular formula	$C_{19}H_{21}NO_4$
Molar mass	327.37 g/mol



(−)-Stepholidine	
CAS number	16562-13-3
PubChem	5290
ChEMBL	CHEMBL487387
Molecular formula	$C_{19}H_{21}NO_4$
Molar mass	327.374 g/mol

parallel with agronomical development for more reproducible levels of active compounds could improve the availability of drugs at an acceptable cost and a source of income in malaria endemic countries [21].

8.1 Antimalarial Properties of Alkaloids

Developing countries suffering from various infectious diseases like HIV, tuberculosis, and malaria. Among these diseases, malaria is the major killer of humans, and approximately 300–500 million clinical cases and one million deaths per year caused world wide due to malaria [22]. Four Plasmodium species (*P. vivax, P. ovale, P. malariae, and P. falciparum*) are responsible for human malaria. Out of these, *P. falciparum* species causes most fatal form of malaria. This parasite has a plastid-like organelle called as apicoplast. Resistance to known antimalarial and the lack of an effective vaccine has created an urgent need to discover new biologically active compounds.

Natural compounds as a source for antimalarial drugs. Research on natural compounds has already contributed to the discovery of new antimalarial drugs. Atovaquone, artemisinin, and its semisynthetic derivatives, as well as clindamycin, erythromycin, azithromycine, chlortetracycline, tetracycline, oxytetracycline, and doxycycline, are noteworthy examples of the varied contribution of natural products for the development of effective antimalarial drugs, particularly valuable for the treatment of chloroquine-resistant parasites. Several comprehensive reviews on the antimalarial potency of plant products derived from ethnic medicine were published in the last decade. The quality of the data used in these reviews differs, and in earlier reports the chemical structure of the purified compound was not known and toxicity tests were not performed. However, in many cases, good activity and selectivity were observed. Most importantly, several compounds containing unique structural composition have been isolated and characterized. It is therefore not surprising that natural compounds dominate the recent malaria patent literature [21]. Although many compounds cannot be further developed for reasons mentioned above, the discovered lead compounds provide valuable bioactive scaffolds which could be further adjusted by semisynthetic approaches to obtain effective antimalarials.

Living organisms, especially plants, provide an innumerable number of molecules with potential for the treatment of many serious diseases. The current chapter attempts to give an overview on the potential of such plant-derived natural products as antiprotozoal leads and/or drugs [23]. Many plant species are used in traditional medicines of malarious countries, and a relatively few number of these have been investigated for evaluation of their antimalarial effect. Still lower is the number of those that have had the active natural compounds isolated and the toxicity determined [24]. Indole alkaloids are one of the important class of marine-derived secondary metabolites, with wide occurrence among variety of marine sources such as sponges, tunicates, algae, worms, and microorganisms and have been extensively studied for their biological activities. Among this chemical family, a sponge-derived bis-indole alkaloid fascaplysin (1) exhibited broad range of bioactivities including antibacterial, antifungal, antiviral, anti-HIV-1-RTase, p56 tyrosine kinase inhibition, antimalarial, anti-angiogenic, antiproliferative activity against numerous cancer cell lines, specific inhibition of cyclin-dependent kinase-4 (IC(50) 350 nM) and action as a DNA intercalator [25].

The possible anti-plasmodial compounds from leaf, stem, root, and flower extracts of Ocimum canum (*O. canum*), *Ocimum sanctum* (*O. sanctum*), and *Ocimum basilicum* (*O. basilicum*) have been analyzed by Inbaneson et al. [35]. The leaf extract of O. sanctum showed excellent anti-plasmodial activity ($IC_{(50)}$ 35.58 μg/mL) followed by leaf extract of O. basilicum ($IC_{(50)}$ 43.81 μg/mL).

The in vitro anti-plasmodial activity might be due to the presence of alkaloids, glycosides, flavonoids, phenols, saponins, triterpenoids, proteins, resins, steroids, and tannins in the ethanolic extracts of tested plants. Thirty bioactive compounds belonging to a variety of chemical classes such as spermine and isoquinoline alkaloids, glycosylflavones, phenylethanoid glycosides, ecdysteroids, quercetin arabinofuranosides, clerodane-type diterpenoids, sipandinolid, galloylquercetin derivatives, gallates, oleamide, and mangiferin derivatives [26]. Bioassay-guided fractionation of the MeOH extract from the stem bark of Neonauclea purpurea used in traditional medicine resulted in the isolation of 2 indole alkaloids, cadambine (1) and alpha-dihydrocadambine (2), as well as a quinolic compound, 2,6-dimethoxy-1,4-benzoquinone (3). Antimalarial activity evaluation showed that compounds 2 and 3 exhibited mild in vitro antimalarial activity against *Plasmodium falciparum*, [27].

Two new indole alkaloids, bisnicalaterine D (1), consisting of an eburnane and a corynanthe type of skeletons, and nicalaterine A (2) were isolated from the bark of Hunteria zeylanica. Their structures were elucidated by various spectroscopic data such as NMR and CD spectra. A series of bisnicalaterines and nicalaterine A showed potent anti-plasmodial activity against *P. falciparum* 3D7 [28].

8.2 Anticancer Properties of Alkaloids

Several methods exist for the treatment of cancer in modern medicine. These include chemotherapy, radiotherapy, and surgery; most cancer chemotherapeutants severely affect the host normal cells. Hence, the use of natural products now has been contemplated of exceptional value in the control of cancer. Plant-derived natural products such as flavonoids, terpenes, and alkaloids have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects.

The antioxidant and anticancer evaluation of Scindapsus officinalis (Roxb.) Schott fruits has been attempted to investigate its antitumor activity [29]. The collection and authentication of the plant material, mainly fruits, and their various extractions were done. Identification of plant's active constituents by preliminary phytochemical screening was carried out. An *in vitro* cytotoxic assay using the brine shrimp lethality assay with brine shrimp eggs (Artemia salina) at a dose of 1–10 μg/ml with the fruit extract was performed.

Increasing recurrence of mammalian tumors and severe side effects of chemotherapeutic agents reduce the clinical efficacy of a large variety of anticancer agents that are currently being used. Vinca alkaloid and their derivatives, alone and in combination with therapeutic agents, have been used for a long time for the treatment of various types of cancers. Polyphenols form one of the most important and extensively used classes of plant-derived therapeutics for cancer prevention or chemotherapy. The present chapter highlights a plethora of studies focused on the antineoplastic properties of plant-derived chemicals, such as vinca alkaloids, saponins, and flavonoids [30].

Nature is the main source of compounds for pharmaceutical purposes, either by providing the natural organic chemical compounds of interest or as a source of inspiration for the design of new drugs. The known antiinflammatory and anticancer agents belong to a great diversity of structural skeletons since inflammatory and cancer processes involve many different biological targets. Their origins extend to plants, fungi, bacteria, and marine organisms, besides those produced by semisynthesis and total synthesis. The tasks of the organic chemist are the screening, the structure assignment, and the semi and total syntheses of active molecules. The active compounds are organized by their biosynthetic origins as terpenoids; macrolides, polyketides, and ansamycins; phenolics; alkaloids; peptides; glycoconjugates; other compounds; and food compounds [31]. The isolation of the vinca alkaloids, vinblastine, and vincristine. Paclitaxel (Taxol®, **3**) from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. Camptothecin, isolated from the Chinese ornamental tree *Camptotheca acuminate* Decne (Nyssaceae), Topotecan and irinotecan are semisynthetic derivatives of camptothecin and are used for the treatment of ovarian and small cell lung cancers, and colo-rectal cancers, respectively [32]. Epipodophyllotoxin is an isomer of podophyllotoxin which was isolated as the active antitumor agent from the roots of *Podophyllum* species, *Podophyllum peltatum* Linnaeus and *Podophyllum emodi* Wallich (Berberidaceae) [33]. Etoposide and teniposide are two semisynthetic derivatives of epipodophyllotoxin and are used in the treatment of lymphomas and bronchial and testicular cancers [34]. Homoharringtonine, isolated from the Chinese tree *Cephalotaxus harringtonia* var. *drupacea* (Sieb and Zucc.).

9 Conclusion

In this chapter, we studied and analyzed the various biosynthetic routes involved in biosynthesis of alkaloids especially tyrosine-derived alkaloids. The plant alkaloids have been known to have important medicinal values and provided the medicines for the treatments of cancer, malaria, tuberculosis, etc. Keeping in mind the therapeutic importance, there is an urgent need to investigate their biosynthesis at the level of enzyme and gene.

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Web Resources

Alkaloids. www.friedli.com/herbs/phytochem/alkaloids/alkaloid1.html
KEGG: Kyoto Encyclopedia of Genes and Genomes. www.genome.jp/kegg/
Plant Alkaloids – Wayne's Word. http://waynesword.palomar.edu/ww0703.htm
Wikipedia-Alkaloid. http://en.wikipedia.org/wiki/Alkaloid/

Alkaloids Derived from Tyrosine: Penethylisoquinoline (Autumnaline, Colchicine)

16

Pijush Paul, Mihir Halder, and Sumita Jha

Contents

1 Introduction 462
2 Occurrence 464
3 Chemistry of Colchicine 464
3.1 General Properties 464
3.2 Structure 464
3.3 Tests for Identity 466
4 Biosynthesis 466
5 Biotechnological Approaches 468
5.1 Optimization of Culture Media and Growth Condition 470
5.2 Effect of Precursors 470
5.3 Use of Elicitor Molecules in the Media 471
6 Biological Activities of Colchicine 472
6.1 Mechanism of "Mitotic Poisoning" by Colchicine 472
6.2 Botanical Uses of Colchicine 472
6.3 Therapeutic Uses of Colchicine 474
7 Future Prospects 475
References 476

Abstract

Alkaloids are one of the most important groups of secondary metabolites used since prehistoric age. It has been used as an anti-inflammatory agent for a long time. Besides, as a potential mitotic poison, colchicine is applied to produce polyploid and double haploid plants by the plant breeders. Colchicine is

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a tricyclic proto-alkaloid, which has nitrogen atom in the side chain. Colchicine and its precursor autumnaline belong to phenethylisoquinoline group of alkaloids derived from tyrosine. Colchicine and its derivatives have been found in several genera such as *Colchicum*, *Gloriosa*, *Merendera*, *Androcymbium*, *Sandersonia*, etc. Biotic and abiotic factors are known to influence the production of secondary metabolites like colchicine in their natural ambience. Therefore, several biotechnological strategies like in vitro culture of colchicine-producing plants, media manipulation, precursor feeding, and application of elicitors have been practiced. After FDA approved colchicine as a drug in 2009, the demand of this high-value low-amount secondary metabolite increased significantly. So, to reduce the gap between demand and yield, the existing strategies should be improved, as well as new techniques can be applied to achieve the goal. The socioeconomic importance of colchicine is sure to encourage greater interest in near future.

Keywords

Alkaloid • anti-inflammatory agent • autumnaline • colchicine • *Colchicum* • double haploid • elicitors • in vitro culture • phenethylisoquinoline • polyploid

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
DH	Double haploid
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
IL	Interleukin
mM	Millimolar
MSU	Monosodium urate
NALP3	NACHT-LRR-PYD-containing protein-3
NSAIDs	Nonsteroidal anti-inflammatory drugs
RNAi	RNA interference
SAM	*S*-adenosyl methionine
US FDA	United states food and drug administration

1 Introduction

Plant-derived alkaloids are widely used in many purposes that include analgesics (morphine and codeine), stimulants (caffeine and nicotine), anticancer agents (vincristine, vinblastine, and camptothecin derivatives), gout suppressant (colchicine), muscle relaxant (C-tubocurarine, antiarrhythmic ajmaline), antibiotic (sanguinarine), and sedative (scopolamine) [1]. So, different biotechnological approaches are undertaken for the large-scale production of these compounds.

Biological precursors of most alkaloids are amino acids, such as ornithine, lysine, phenylalanine, tyrosine, tryptophan, histidine, nicotinic acid, anthranilic

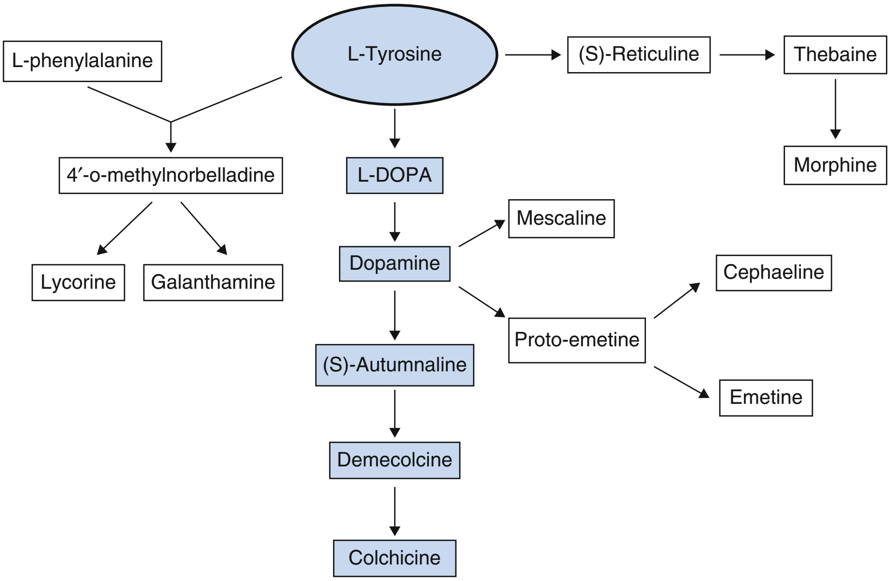


Fig. 16.1 Some important tyrosine-derived alkaloids

acid, and aspartic acid [2]. Numerous pathways are responsible for alkaloid biosynthesis and naturally cannot be easily classified. Tyrosine, as precursor, gives rise to chemically distinct five different groups of alkaloids like phenylethalamine and tetrahydroisoquinoline, benzyl tetrahydroisoquinoline, phenethylisoquinoline, terpenoid tetrahydroisoquinoline, and Amaryllidaceae alkaloids. Autumnaline and colchicine belong to phenethylisoquinoline group of compounds; autumnaline is an intermediate of colchicine biosynthetic pathway. Figure 16.1 shows some important tyrosine-derived alkaloids.

Colchicine was originally extracted from *Colchicum autumnale* (commonly known as autumn crocus or meadow saffron). Besides that, the presence of colchicine was also reported from *C. luteum*, *C. speciosum*, *C. hierosolymitanum*, *C. tunicatum*, *Gloriosa superba*, *Merendera* sp., etc. Colchicine is not a typical alkaloid in true sense, as the nitrogen atom it possesses is not involved in a heterocyclic ring; rather, the nitrogen atom is present in the side chain of tropalone nucleus. Thus, it is termed as non-heterocyclic alkaloids or proto-alkaloids [2]. Colchicine was first isolated in 1820 by Pelletier and Caventon. Since then, it has been used as therapeutic agent for many diseases. Due to its drastic side effects when used in high dose, it never got the approval until recently. After the approval of colchicine as drug for gout in 2009 by Food and Drug Administration (FDA, USA), there has been revival of interest in colchicine research and applications [3]. Colchicine is well known for its anti-inflammatory properties. Thus, it has been successfully used in gouty arthritis, inflammatory dermatitis, and familial Mediterranean fever since the past decade. Colchicine is also well known as “mitosis poison agent” as it binds to tubulin and inhibits its polymerization, which leads to blocking of chromosomal segregation [4]. By exploiting this property, many types of plants are generated commercially.

2 Occurrence

The term "colchicine" originated from the area known as "*Colchis*" near Black Sea. However, *C. autumnale* grows wild in Europe and Africa, while *Gloriosa* sp. is distributed in Africa and Asia including foothills of Himalayas, Burma, Indonesia, Malaya, etc. Table 16.1 gives a summary of the occurrence of colchicine reported since 1946.

3 Chemistry of Colchicine

3.1 General Properties

Pure colchicine is a fine, pale yellow powder. It darkens on exposure to light due to photoisomerization and formation of α-, β-, and γ-lumicolchicines [5, 6]. The optimum storage temperature for colchicine is −15°C to −25°C, in dark-colored bottles.

It is a neutral alkaloid and has been extracted from either acidic or alkaline solution by chloroform ($CHCl_3$). It is readily soluble in alcohol, chloroform, or cold water, but is less soluble in hot water or in cold benzene and is almost insoluble in ether [4].

3.2 Structure

Colchicine is a tricyclic alkaloid; the main features of which include a trimethoxyphenyl ring (A ring), a seven-membered ring (B ring) with an acetamide at the seventh position, and a tropolonic ring (C ring) (Fig. 16.2). Chemically, colchicine is (*S*)-*N*-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo(a)heptalen-7-yl) acetamide [7] with the molecular formula $C_{22}H_{25}NO_6$ (molecular weight 399.43; melting point 151–152°C).

(–)-Colchicine has only one stereogenic center, C_7. The designation of this carbon is *S*, according to the common Cahn-Ingold-Prelog rules. However, colchicine is also asymmetric due to axial chirality. The single bond between the A and C ring is rotationally restricted; this restriction adds a degree of asymmetry to the molecule. In 1933, Kuhn designated this type of stereoisomerism as atropisomerism (from Greek "a" meaning not and "tropos" meaning turn). The designation of this asymmetry is "a*S*" or "a*R*," according to the rules of molecular asymmetry, in which the "a" stands for axial chirality [8]. In colchicine, the C–C bond between the A and C rings is the chiral axis. In light of this molecular asymmetry, colchicine has four stereoisomers (Fig. 16.3). Each pair has either the *R* or *S* configuration at C_7 (–)-(a*S*, 7*S*)-. Colchicine, the natural isomer, can interconvert between the two conformational isomers a*R* and a*S*, given enough energy.

Table 16.1 Natural occurrence of colchicine

	Name of the plants	Occurrence	Source of colchicine
1.	*Androcymbium gramineum* McBr.	South Africa	Corms, seeds
2.	*Androcymbium melanthioides* var. stricta Bak.	Africa	Corms, seeds
3.	*Colchicum vernum* Ker-Gawl	Eastern Europe	Corms, leaves, Flowers
4.	*Colchicum aggripinum* Bak.	Central Europe	Corms
5.	*Colchicum alpinum* Lam et DC	European Alps	Corms
6.	*Colchicum autumnale* var. album Hort	–	Corms
7.	*Colchicum autumnale flore pleno* Hort	–	Corms
8.	*Colchicum autumnale major* Hort	–	Corms
9.	*Colchicum autumnale minor* Hort	–	Corms
10.	*Colchicum bornmuelleri* Freyn	Asia Minor	Corms
11.	*Colchicum cilicicum* Hayek	Asia Minor	Corms
12.	*Colchicum cornigerum* Tackh et Drar	UAR	Corms, seeds
13.	*Colchicum crocifolium* Schott et Kotschy	Southeast Persia	Corms
14.	*Colchicum hierosolymitanum* Feinbr.	Israel	Corms
15.	*Colchicum kesselringii* Rgl	USSR	Aerial parts
16.	*Colchicum lactum* Stev.	Caucasus	Corms
17.	*Colchicum lusitanicum* Brot.	North Africa and Iberia	Corms, flowers
18.	*Colchicum macedonicum* Kos	–	Seeds
19.	*Colchicum montanum* L.	Iberia	Corms
20.	*Colchicum ritchii* R. Br.	Israel	Corms, whole leafy plant
21.	*Colchicum speciosum* Stev.	USSR	Corms, leaves, Flowers, seeds
22.	*Colchicum steveni* Kunth	Israel	Whole plant
23.	*Colchicum tunicatum* Feinbr	Israel	Whole plant
24.	*Colchicum variegatum* L.	Asia Minor	Corms
25.	*Colchicum vernum* Ker-Gawl	Eastern Europe	Corms
26.	*Dipidax triquetra* Bak.	–	Corms
27.	*Gloriosa rothschildiana* O'Brien	Tropical Africa	Corms
28.	*Gloriosa simplex* L.	Africa	Corms, seeds
29.	*Gloriosa superba* L.	India and Africa	Corms, seeds
30.	*Gloriosa virescens* Lindl.	Tropical Africa	Corms, seeds
31.	*Iphigenia indica* A. Gray	India	All parts
32.	*Iphigenia pallida* Bak.	India	All parts
33.	*Littonia modesta* Hook	Africa	Corms, leaves, seeds
34.	*Merendera attica* Boiss et Sprun	Greece and Bulgaria	Corms
35.	*Merendera persica* Boiss et Kotschy	Abyssinia, Pakistan	Corms
36.	*Merendera robusta* Bge	USSR	All parts
37.	*Merendera sobolifera* Fisch et Mey	Persia	Corms
38.	*Merendera trigina* Stapf	Hungary	Seeds

(*continued*)

Table 16.1 (continued)

	Name of the plants	Occurrence	Source of colchicine
39.	*Ornithoglossum glaucum* Salisb. var. *grandiflorum*	South and East Africa	Corms
40.	*Ornithoglossum viride* Dryand	Africa	Corms
41.	*Sandersonia auranticata* Hook	Africa	Corms

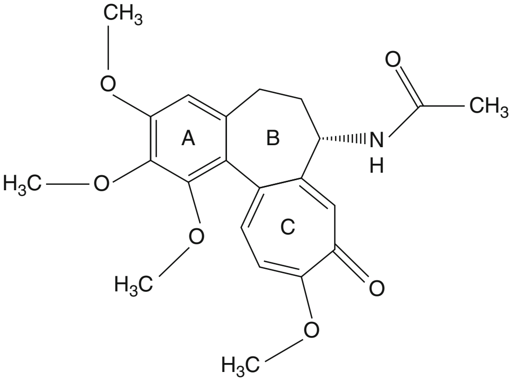


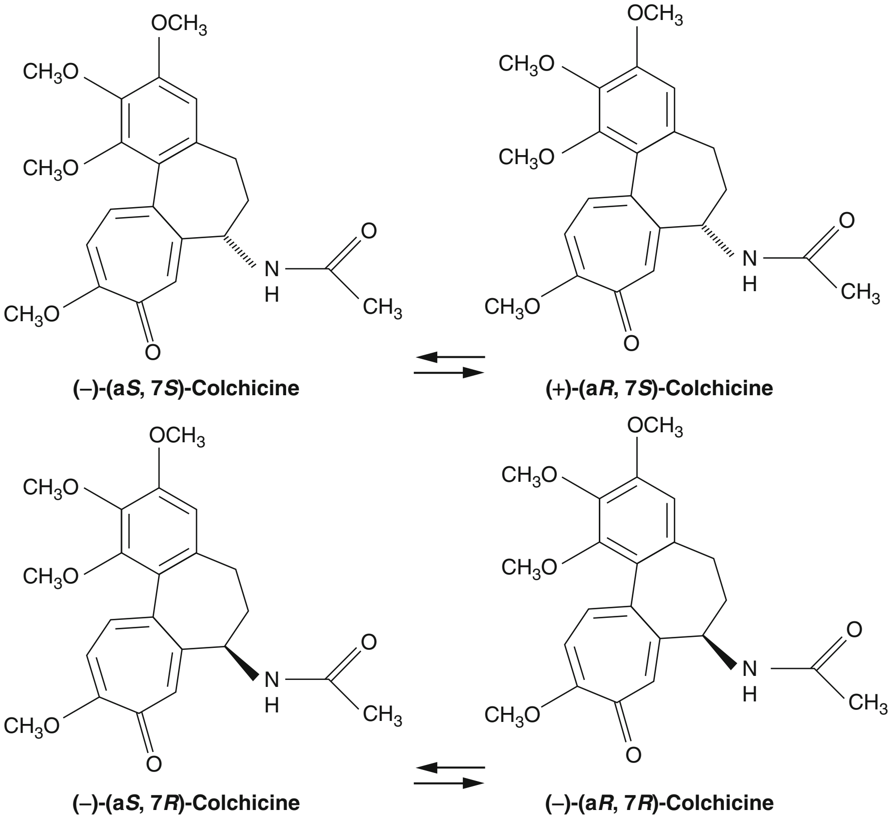
Fig. 16.2 Structure of colchicine

3.3 Tests for Identity

When colchicine solution is treated with a few drops of dilute mineral acid, an intense yellow-colored solution of colchiceine is obtained. However, colchicine can easily be distinguished from colchiceine by treating with ferric chloride ($FeCl_3$). Colchicine gives red color, whereas colchiceine produces olive green coloration (Fig. 16.4).

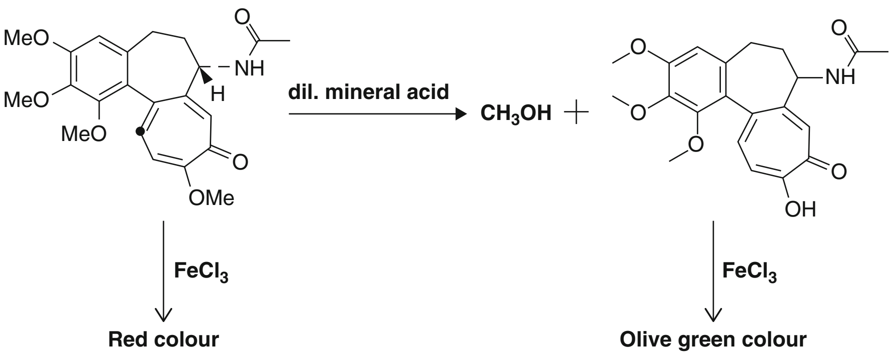
4 Biosynthesis

Several experiments have shown that the biosynthesis of colchicine involves the amino acids phenylalanine and tyrosine as precursors. When *Colchicum autumnale and C. byzantinum* were fed with radioactive tyrosine-2-C^{14} and phenylalanine-2-C^{14}, respectively, incorporation of radioactivity in the ring system of colchicine was observed. These labeling experiments suggested that tyrosine and phenylalanine both are required for colchicine biosynthesis. However, phenylalanine provides a C_6–C_3 unit rather than a C_6–C_2 fragment to form the intermediate (*S*)-autumnaline (Fig. 16.5). Further reports indicate that acetate is the precursor of the *N*-acetyl group; peripheral methyl groups were produced from methionine in *C. byzantium* and from methanol in *C. autumnale* [9]. The tropolone ring was demonstrated to be derived from tyrosine by ring expansion of a C_6–C_1 unit [10].



(–)-(a*S*, 7*S*)-Colchicine
(+)-(a*R*, 7*S*)-Colchicine
(–)-(a*S*, 7*R*)-Colchicine
(–)-(a*R*, 7*R*)-Colchicine

Fig. 16.3 Stereoisomers of colchicine



dil. mineral acid
CH3OH +
FeCl3
FeCl3
Red colour
Olive green colour

Fig. 16.4 Test for identity: $FeCl_3$ test for differentiation between colchicine and colchiceine

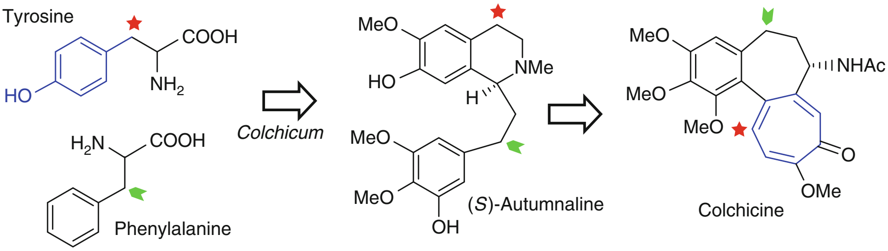


Fig. 16.5 Radioactive tracer experiment showing the formation of colchicine from the precursors

A para-para phenolic coupling reaction of isoquinoline (1*S*)-autumnaline catalyzed by isoandrocymbine cytochrome P-450 reductase produces the intermediate isoandrocymbine [11]. Then, *O*-methylandrocymbine methyltransferase catalyzes *O*-methylation of isoandrocymbine resulting in a key intermediate dienone *O*-methylan-drocymbine, where *S*-adenosyl methionine (SAM) acts as methyl group donor. Cleavage of cyclopropane ring of *O*-methylandrocymbine along with successive oxidation leads to the formation of *N*-formyldemecolcine, containing the seven member tropolone ring [12]. Hydrolysis of *N*-formyldemecolcine generates the demecolcine molecule, which undergoes oxidative demethylation that generates deacetylcolchicine. The molecule of colchicine appears finally after addition of acetyl coenzyme A to deacetylcolchicine by colchicine *N*-acetyltransferase [13, 14]. Figure 16.6 depicts colchicine biosynthesis steps.

5 Biotechnological Approaches

Like other alkaloids, supply of colchicine from traditional sources is limited either due to the gradual decreasing condition of plant sources or due to trace amounts of compounds present in the plants. Therefore, several alternative strategies have been adapted to increase the production of colchicine and colchicine derivatives.

Plant tissue culture represents a sustainable production system, which is constantly improving with respect to reliability and capacity. Commercial importance of the secondary metabolites greatly evolved in recent years, which caused great interest in secondary metabolism and particularly in the possibility to alter the production of bioactive plant metabolites by means of cell culture technology. Plant cell culture technologies were introduced at the end of the 1960s as a possible tool for both studying and producing plant secondary metabolites. The principle advantage of this technology is that it may provide continuous, renewable, reliable source of valuable medicinal compounds (plant pharmaceuticals), flavors, fragrances, and colorants, which cannot be produced by microbial cells or chemical synthesis. This system could be used for the large-scale culture of plant cells from which these metabolites can be extracted. Figure 16.7 shows some potential strategies for bio-production of secondary metabolites.

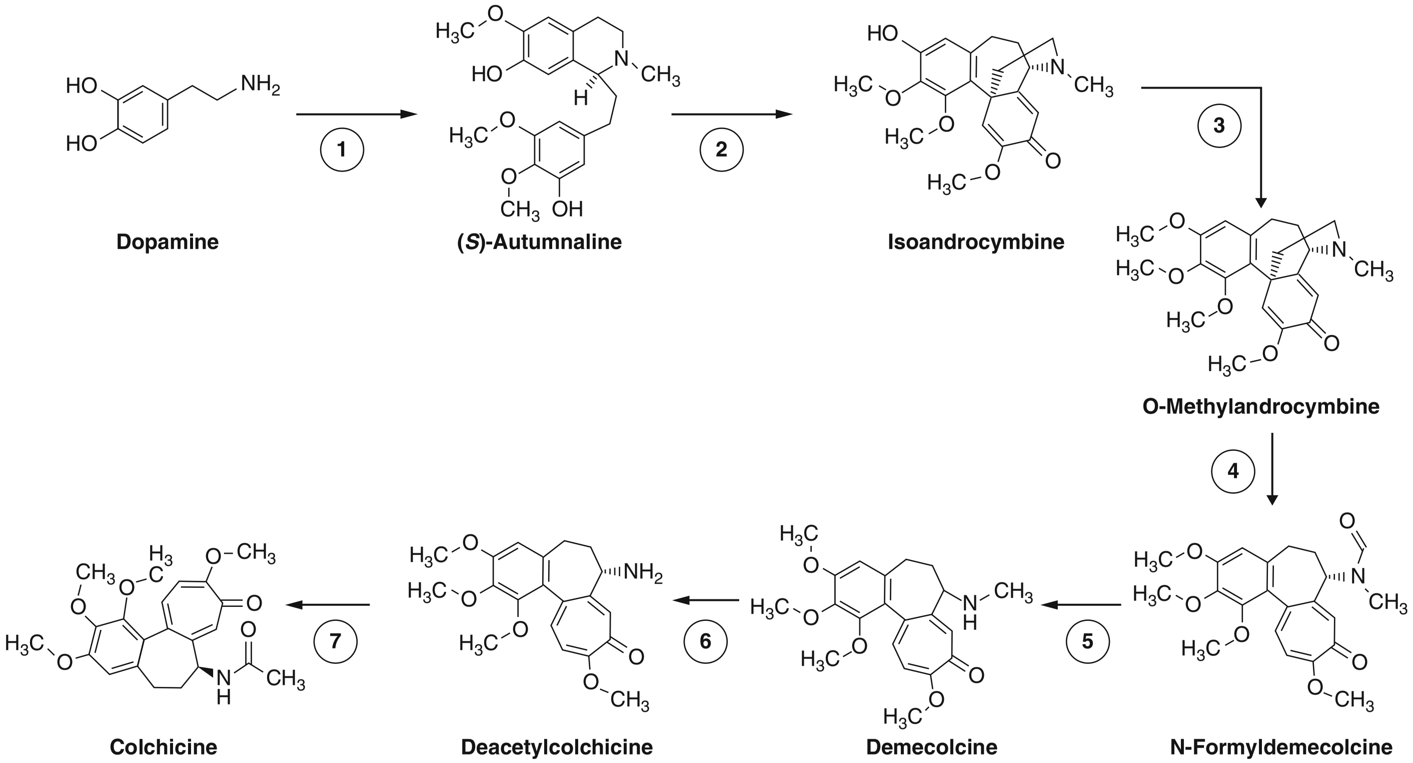

Dopamine
(S)-Autumnaline
Isoandrocymbine
O-Methylandrocymbine
N-Formyldemecolcine
Demecolcine
Deacetylcolchicine
Colchicine
1
2
3
4
5
6
7

Fig. 16.6 Colchicine biosynthesis pathway. Number within the brackets are the enzymes for the respective steps: (2) isoandrocymbine cytochrome P-450 reductase, (3) *O*-methylandrocymbine methyltransferase, and (7) colchicine *N*-acetyltransferase; enzymes for steps (1), (4), and (6) are yet to be discovered

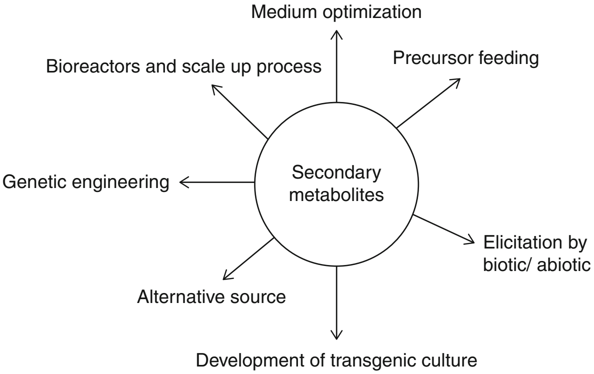

Medium optimization
Bioreactors and scale up process
Precursor feeding
Genetic engineering
Secondary metabolites
Elicitation by biotic/ abiotic
Alternative source
Development of transgenic culture

Fig. 16.7 General strategies for improvement of secondary metabolites production by biotechnological approaches

5.1 Optimization of Culture Media and Growth Condition

Standardization of medium is an important parameter in in vitro tissue culture technology since the composition of the culture media influences both the biomass yield as well as secondary metabolite production. Composition of the medium, culture conditions, and exogenous phytohormone combinations together influence the metabolite accumulation in the cell. Composition of macro- and micronutrients, nature and amount of carbon source, and level of total nitrogen has been found to play a vital role in the production of secondary metabolites.

C. autumnale is the first plant species exploited for the production of colchicine in vitro. Hunault [15] reported successful induction of callus culture of *C. autumnale* on Linsmaier-Skoog medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. Growth and colchicine accumulation were increased in MS media supplemented with 20-mM ammonium and 40-mM nitrate. Addition of sulfate (SO_4^{2-}) also increased the accumulation of colchicine in *C. autumnale* [16].

5.2 Effect of Precursors

It has been observed that addition of precursors or related compounds (which are intermediate at the beginning of a secondary metabolite biosynthetic route) to the culture media sometimes stimulate secondary metabolite production. Phenylalanine and tyrosine play a central role for the production of colchicine. The lack of signal-inducing enzyme activity and biosynthetic precursors may act as a rate-limiting factor for the lower production of colchicine in vitro.

Feeding of some precursors, like phenylalanine and tyrosine, had no effect on in vitro colchicine formation in cell suspension cultures of *Colchicum autumnale*; however, *p*-coumaric acid, tyramine, and demecolcine feeding significantly increased colchicine production [17].

Callus culture of *G. superba* is an alternative source of colchicine. Feeding of tryptophan, phenylalanine, and tyrosine results in enhancement of colchicine content in callus culture of *G. superba* [18]. These results suggest that precursors are an important regulatory factor in colchicine accumulation in vitro. On the other hand, Ghosh et al. [19] reported enhanced colchicine production in root cultures of *G. superba* with the treatment of *p*-coumaric acid and tyramine.

5.3 Use of Elicitor Molecules in the Media

Plants show physiological and morphological responses to a range of physical and chemical factors known as "elicitors." These responses have been considered as defense reactions elicited by the plants' biochemical factory to ensure their survival, persistence, and competitiveness. The addition of trace amounts of elicitors in in vitro culture has been developed as one of the main strategies to improve the yield of secondary metabolites.

Elicitors trigger signals for the formation of secondary metabolites and are classified as abiotic or biotic elicitors. Elicitors of nonbiological origin are called abiotic elicitors, which predominantly consist of physical and chemical stresses such as UV radiation, extreme high or low temperatures, ethylene, fungicides, antibiotics, salts of heavy metals or high salt concentrations, etc. Biotic elicitors are substances with biological origin such as polysaccharides derived from plant cell walls (pectin or cellulose) or microorganism cell wall (chitin or glucans) and glycoproteins or intracellular proteins which activate or hinder a number of enzyme activities or ion channels. Biotic elicitors are categorized into two types based on their origin: exogenous (originated outside the cell) and endogenous elicitors (originated inside the cell). Elicitors reduce the processing time to attain high product concentrations. Application of elicitors to plant cell and organ cultures is useful for enhancing the biotechnological productivity of valuable secondary metabolites in vitro [20, 21]. An added biotechnological benefit is that they also promote release of the metabolites into the medium.

Various biotic and abiotic elicitors have been used to increase colchicine production. Application of methyl jasmonate as a biotic elicitor in the media showed increase in growth of the root cultures of *Gloriosa superba*. Higher concentration of methyl jasmonate decreased growth but enhanced intracellular colchicine content of the root cultures. However, such treatments with methyl jasmonate did not release colchicine into the medium [22].

Abiotic elicitors like cadmium chloride ($CdCl_2$), calcium chloride ($CaCl_2$), and aluminum chloride ($AlCl_3$) promoted many fold increase in biomass accumulation in root cultures of *G. superba*. However, casein hydrolysate, yeast extract, and silver nitrate had no significant effect on growth and colchicine accumulation in root cultures. The increase in $CdCl_2$ concentration in root cultures of *G. superba* facilitates intracellular colchicine accumulation as well as exudation into the medium. However, among the abiotic elicitors, $AlCl_3$ showed the most significant increase in colchicine production in root cultures of *G. superba* [22].

6 Biological Activities of Colchicine

6.1 Mechanism of "Mitotic Poisoning" by Colchicine

Colchicine has long been used as the potent chromosome-doubling agent. It induces chromosome doubling by inhibiting mitotic spindle of dividing cells. Mechanism of "mitotic poisoning" by colchicine has recently been elucidated. Colchicine has direct effect on microtubule assembly.

Microtubule consists of α- and β-tubulin proteins. Tubulin polymerizes end to end with the α-subunit of one tubulin dimer joining with the β-subunit of the next. The protofilament bundles are parallel to one another. Thus, in a microtubule, there is one end with only β-subunits exposed called (+) end, while the other end has only α-subunits exposed called (−) end. That is why a microtubule shows polarity. The (−) end is capped so elongation of the microtubule occurs from the (+) direction.

Microtubules are dynamic structures that lengthen or shrink by elongation at one end and dissolution at the other. Drugs like paclitaxel or docetaxel blocks dynamic instability by stabilizing GDP-bound tubulin in the microtubule. Thus, even when hydrolysis of GTP reaches the tip of the microtubule, there is no depolymerization and the microtubule does not shrink back. Colchicine does not enhance the rate of microtubule dissolution but inhibits the process of microtubule self-assembly in a substoichiometric fashion by binding α-tubulin with the formation of tubulin-colchicine complexes [23]. Addition of tubulin-colchicine complexes at the microtubule ends hampered normal polymerization of microtubulin. Thus, the spindle fiber formation during the cell division is hindered. In a recent study, it was found that colchicine analogues with modifications in the B ring bind to tubulin at the same site as colchicine. Thus, it is suggested that chemical specificity of the colchicine-binding site of tubulin is less stringent for the presence of the B ring than the A and C rings of colchicine [24].

6.2 Botanical Uses of Colchicine

6.2.1 Development of Double Haploid Lines

Anther culture followed by chromosome doubling generally gives rise to double haploid (DH) lines. Chromosome doubling may be spontaneous or induced by chemical agents (Fig. 16.8). The rate of spontaneous chromosome doubling in anther culture-derived haploid plants varies with species and even among varieties of the same species [25]. Charmet et al. [26] reported that about 10% of DH plants ($2n = 6x = 42$) of triticale show spontaneous chromosome doubling. The low rate of spontaneous doubling in many plants emphasizes the need of artificial chromosome doubling in order to enhance the number of DH lines. Probably, endomitosis (endoreduplication) explains the origin of spontaneous 2n and 4n embryos from microspore.

Colchicine is the most efficient and commonly used chromosome-doubling agent. It induces chromosome doubling by the inhibition of mitotic spindle of dividing cells. Inactivation of spindle inhibits the polar migration of chromosomes,

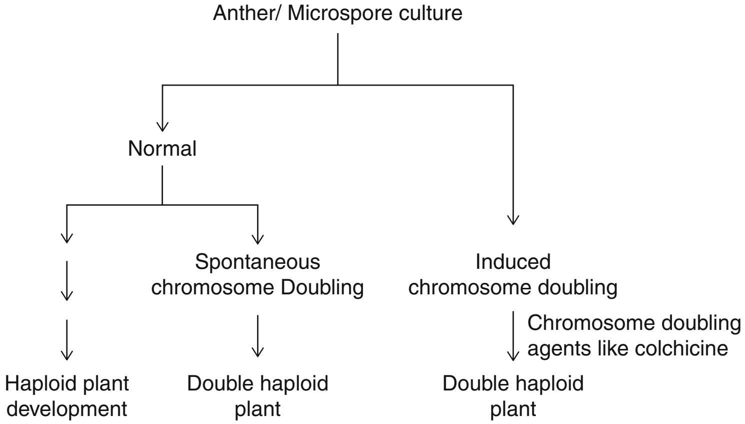

Anther/ Microspore culture
Normal
Spontaneous chromosome Doubling
Induced chromosome doubling
Chromosome doubling agents like colchicine
Haploid plant development
Double haploid plant
Double haploid plant

Fig. 16.8 Schematic representation of double haploid lines

producing "restitution" nuclei, thus resulting in a cell with a doubled chromosome number (i.e., diploid instead of haploid, as gametes usually are). There have been some reports on in vitro colchicine treatment of anthers in wheat and in maize [27, 28] for chromosome doubling.

6.2.2 Production of Polyploid Plants

Generally, polyploidy is fatal to animal system, but in plant cells, it is well tolerated and frequently resulting to plants that differ from the diploid plants for larger size (of leaves, stems or roots, flowers, seeds, and fruits), faster growth, and their greater yield. For this reason, this type of genetic manipulation is frequent in commercial plant breeding. Thus, polyploid plants have enormous importance in agriculture. Colchicine is a well-known chemical agent that is largely used to alter ploidy level frequencies in cell populations, either in vivo or in vitro, to obtain tetraploid plants, starting with diploid material.

In addition, when such a tetraploid plant is crossed with a diploid plant, most of the time, it produces a sterile triploid offspring, which may be commercially useful in itself by requiring growers to buy seed from the supplier. Besides, these triploid offspring produces "seedless" fruits which have immense commercial value.

On the other hand, colchicine's ability to induce polyploidy can solve many important problems of plant breeding. Colchicine renders infertile hybrids fertile. For example, the maximum *Fuchsia* species are diploid or tetraploid. The cross between diploid and tetraploid results often in a triploid, which is mostly sterile because the process of meiosis requires the pairing of similar chromosomes and due to lack of mechanism to allow the alignment of three similar chromosomes. However, colchicine treatment produces fertile hexaploid plants. Breeding of triticale from wheat and rye shows similar problem: wheat is typically tetraploid and rye is diploid, with the triploid hybrid being infertile. Here also, treatment with colchicine results in fertile hexaploid triticale.

Colchicine treatment can also generate mixoploid cells which subsequently give rise to chimera plants consisting of diploid and tetraploid cells or tissues [25].

6.2.3 Callus Induction

Recently, an interesting effect of colchicine has been observed where it induced cell division. Positive effect of colchicine on pollen callus induction and plant regeneration on in vitro culture of *Triticum aestivum* and *Zea mays* is reported [29]. A similar effect has been observed on in vitro anther and isolated microspore cultures of *Brassica napus* where colchicine stimulated cell division and embryogenesis [30].

6.3 Therapeutic Uses of Colchicine

6.3.1 Colchicine for Treatment of Gout

Colchicine has long been known for the treatment of acute flare of gout from as early as 500 B.C. [31]. But surprisingly, it is only in July 2009 that the US FDA approved the use of it as a remedy to reduce pain in patients suffering from acute gout [3].

Gout is generally associated with hyperuricemia and is characterized by buildup of monosodium urate (MSU) crystals in the body, subsequently deposited within the joints. Consequently, it is considered as one of the most painful acute conditions a human being can experience. Gout management therapy mainly based on three aspects: treatment of acute gout flares, prophylaxis against acute gout flares, and urate-lowering therapy, which is considered as the long-term treatment of chronic gout [32]. Colchicine works by stopping inflammation and also prevents further attack. When the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is unavailable, ineffective, or creating immunologic problems, colchicine is the best possible drug to be used for the patients with acute gouty arthritis [33].

Recently, the mechanisms of action of colchicine in crystal-associated arthropathies have been elucidated. It is found that at micromolar concentrations, it suppresses MSU crystal-induced NACHT-LRR-PYD-containing protein-3 (NALP3) inflammasome-driven caspase-1 activation, thereby inhibiting the processing and release of IL-1β [34]. At nanomolar concentrations, colchicine blocks neutrophils from entering the inflammation site. It does so by modulating the distribution of adhesion molecules on the endothelial cells and inhibiting crystal-derived chemotactic factor from neutrophil lysosomes as well as MSU crystal-induced production of superoxide anions [35].

6.3.2 Use of Colchicine in Familial Mediterranean Fever Treatment

Familial Mediterranean fever, an autosomal recessive disease, is characterized by short attacks of serositis (peritonitis, pleuritis, or arthritis) and fever. This genetic disorder is caused by a mutated protein pyrin, or marenostrin, encoded by the gene MEFV expressed in the cytoplasm of mature neutrophils and monocytes. Inflammatory mediators like interferon-γ and tumor necrosis factor are effective stimuli for expression of the gene which further suggest its role in neutrophil-mediated inflammation [36]. Since 1972, colchicine has been the first-line treatment for patients with familial Mediterranean fever. Colchicine does not stop an established attack; eventually, discontinuation may result in an attack within a few days.

In the clinical trials, it was found that colchicine prevents febrile attacks in 60 % of patients and significantly reduces the number of attacks in another 20–30 % [37]. In familial Mediterranean fever patients, death occurs mainly due to amyloidosis. However, treatment with colchicine greatly altered the prognosis by arresting amyloidosis and reversing proteinuria [38].

6.3.3 Colchicine and Dermatology

Colchicine with its microtubule-disrupting properties limits the chemotactic and phagocytic activity of polymorphonuclear lymphocytes. It also induces the release of prostaglandin E, a suppressor of leukocyte function by increasing the level of cyclic adenosine monophosphate [39]. Furthermore, the ability of colchicine in inhibition of IL-1 production and histamine release makes it an excellent drug for a number of dermatitis-related complexities like psoriasis, Behçet's syndrome, recurrent aphthous stomatitis, leukocytoclastic vasculitis and urticarial vasculitis, bullous disease, scleroderma, fibromatosis, Sweet's syndrome, amyloidosis, and many more [40].

6.3.4 Other Therapeutic Uses

Colchicine prevents recurrences of acute pericarditis in adults and children, thereby replacing prolonged administration of corticosteroids [41]. The Australian biotechnology company Giaconda has recently developed a combination therapy to treat constipation-predominant irritable bowel syndrome which combines colchicine with the anti-inflammatory drug olsalazine. The British drug development company Angiogene is developing a prodrug of a colchicine congener, ZD6126 (also known as ANG453), as a treatment for cancer.

6.3.5 Side Effects: The Dark Side of Colchicine

Colchicine was not shown to adversely affect reproductive potential in males or females. It crosses the placenta, but there is no evidence of fetal toxicity. Colchicine is excreted into breast milk and considered compatible with lactation.

The lowest reported lethal doses of oral colchicine are 7–26 mg. Colchicine overdose may lead to diarrhea, mild sore throat, nausea, stomach pain or cramping, vomiting, etc. It is also reported that due to colchicine poisoning, death may result from rapidly progressive multi-organ failure and sepsis. But most of the time, severity does not go that far; rather, recovery typically occurs within a few weeks of ingestion [42].

7 Future Prospects

Although the alkaloid field is a very old one, it is still in its infancy with regard to understanding its biosynthesis pathways: enzymology and their regulation. Exploitation of the biotechnological potential of alkaloid biosynthesis has only just begun.

We are still a long way from understanding how most alkaloids are produced in plants and how their biosynthesis is regulated. We also have much to learn about the chemical ecology of alkaloids so that we can better understand why these sophisticated and diverse structures evolved. It is evident that alkaloids as integral components of medicinal plants have enjoyed a long and important history in traditional medicine.

For the majority of secondary metabolism pathways, the proposed biosynthesis was mostly deduced from chemical considerations and feeding experiments, which need verification by identification of the corresponding enzymatic reactions. Thus, direct manipulations of the pathways are not possible due to the lack of enzymological background. In the past, enzymological knowledge related to secondary metabolism pathways has been drawn from the studies of cell suspension systems, but organ cultures and intact plants can also be excellent sources for study of such enzymes.

Identification and knowledge about the regulatory and rate-limiting enzymes was also very important to alter the flux of the particular secondary metabolites. Manipulation of these enzymes sometimes results in quantitative alteration of final product. It is also necessary to know whether the other biosynthetic enzymes are co-induced with the proposed regulatory enzyme or are present even in nonproducing cell cultures. Such knowledge helps to evaluate the possibility of specific manipulation of these secondary metabolism pathways to increase the yield of the desired product.

Different strategies, using in vitro systems, have been studied extensively with the objective of improving the production of colchicine, like media manipulation; establishment of cell culture, organ culture, and root culture of colchicine-producing plants; elicitation with biotic and abiotic elicitors; etc. Instead of these progresses, production of colchicine at commercial scale is yet not achieved.

Biosynthetic pathways of colchicine in plants as well as in in vitro cultures are not fully understood. The challenge remains to elucidate the complete biosynthetic pathways, thereby exploiting cell cultures more rationally for the production of this compound so much in need by the pharmaceutical industry. Whenever the detailed knowledge of the complete biosynthetic pathways will be available, genes controlling rate-limiting steps in the biosynthetic pathway can be overexpressed or synthesis of unwanted metabolites can be suppressed by various RNAi technologies. Moreover, colchicine has a potential of becoming a promising alkaloid for extensive study by the plant biotechnologists, which may lead to exciting opportunities to engineer colchicine metabolism in plants.

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Amaryllidaceae Alkaloids

17

Zhong Jin and Xiao-Hua Xu

Contents

1 Introduction 480
2 Occurrence 484
2.1 Classification of Amaryllidaceae Alkaloids 484
2.2 Occurrence of Amaryllidaceae Alkaloids 504
3 Biosynthesis 504
3.1 Norbelladine Type 505
3.2 Lycorine and Homolycorine Type 505
3.3 Crinine and Haemanthamine, Tazettine, Narciclasine, and Montanine Types 507
3.4 Galanthamine Type 508
3.5 Miscellaneous 509
4 Biological Activity 510
5 Conclusion 513
References 514

Abstract

Plants of the Amaryllidaceae family, including *ca.* 75 genera and about 1,100 species, are among the top 20 in the most widely considered medicinal plant families. A number of pharmacologically active compounds, such as phenols, alkaloids, lectins, peptides, etc., have been identified and characterized from this family. As primary constituents, up to 500 structurally diverse Amaryllidaceae

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alkaloids have been isolated to date. These biogenetically related alkaloids are basically classified into 12 different skeleton types according to their ring systems. Representative structures for each type of Amaryllidaceae alkaloids are shown in this chapter. Biosynthetic pathways for each type of Amaryllidaceae alkaloids including those newly established subgroups are also discussed. In addition, recent reports on the occurrence and biological activity profiles of Amaryllidaceae alkaloids are summarized.

Keywords

Alkaloid • amaryllidaceae • biological activity • biosynthesis • tyrosine

Abbreviations

Ac	Acetyl
AChE	Acetylcholinesterase
DNA	Deoxyribonucleic acid
Et	Ethyl
Glu	Glucoside
Me	Methyl
Nic	3-Nicotinyl acid
Phe	L-Phenylalanine
Tyr	L-Tyrosine

1 Introduction

The family Amaryllidaceae, its name taken from the genus *Amaryllis*, belongs to the monocot order Asparagales and is a class of herbaceous, perennial, and bulbous flowering plants [1, 2]. This family consisting of *ca.* 75 genera and 1,100 species in the 15 tribes is widely distributed in the tropics and warm temperate regions of the world. Geographically, distribution of the family Amaryllidaceae shows obviously regional endemism, with four main clades including the basically African, the Eurasian, the Australasian, and the American clades (Fig. 17.1). Based on modern molecular biology, phylogenetic evidence has indicated that the family evolved originally in Africa and subsequently dispersed to other continents, such as Eurasia, Australasia, and the New World, and South America is the center of secondary diversification [3].

The native habitats of the wild species of the family Amaryllidaceae varies greatly ranging from lowland to high mountain, including grassland, scrub, woods, river banks, and even rocky cliffs. They usually flower in late winter and spring with occasional exception in autumn. Most species have the large and showy and actinomorphic flowers. Shape of the flowers varies from star-like to trumpet-shaped or tubular, and colors range from red, orange, yellow, and pink to white, whereas bluish flowers are only found in the genera *Griffinia*, *Worsleya*, and *Lycoris*.

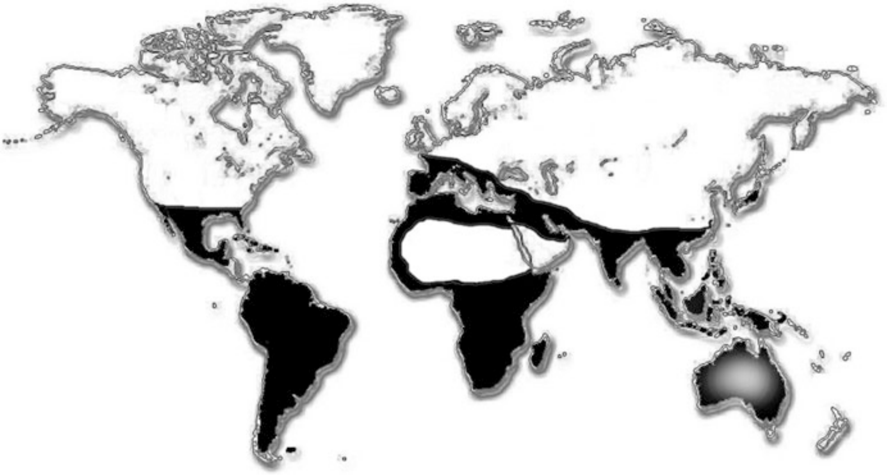


Fig. 17.1 Distribution of plants of the family Amaryllidaceae in the world

Moreover, the flowers often possess highly scented odor. As a result, plants of the family Amaryllidaceae, especially the genus *Narcissus* species (daffodils), have been horticulturally cultivated as ornamental plants for centuries.

For thousands of years, plants of the family Amaryllidaceae have been extensively identified as useful folk herbal remedies against various diseases in many countries and areas. Traditionally, Africans use the bulbs and leaves of this family as poultices and decoctions for treating sores and digestive disorders. With development of modern phytochemistry, a large number of natural chemical substances, such as alkaloids, phenolics, flavonoids, glycosides, etc., have been found to occur in the plants of this family over the past decades. Among them, structurally diverse alkaloids, termed as Amaryllidaceae alkaloids, are frequently associated with the significant pharmacological effects of the plants.

According to the most recent Angiosperm Phylogeny Group (APG) classification, APG III [4], the circumscription of the family Amaryllidaceae has been successively revised to a broader range. Therefore, the family was subdivided into three different subfamilies, one of which is the Amaryllidoideae (the old Amaryllidaceae family), the others being the Allioideae (the former Alliaceae family) and the Agapanthoideae (the former Agapanthaceae family). The subfamily Amaryllidoideae includes *ca.* 60 genera and approximately 800 species with a worldwide distribution (Table 17.1). The subfamily Allioideae was further divided into three tribes in the 16 genera, 13 of which are endemic to the temperate South America. The genus *Agapanthus* is the sole genus in the subfamily Agapanthoideae.

For their unique alkaloid chemistry and morphology, chemistry and biology of Amaryllidaceae alkaloids have been summarized on several occasions [5–19], and

Table 17.1 Wild species distribution of the family Amaryllidaceae

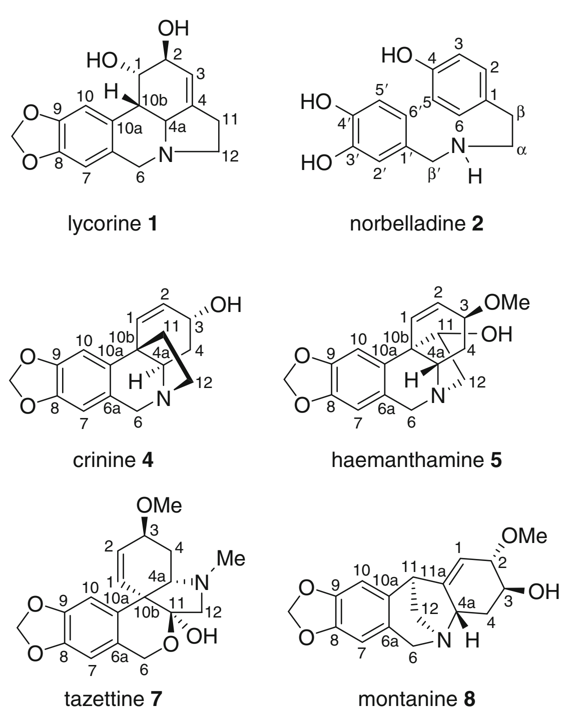
Plant genus	Location
Acis Salisb. (9 species)	Western and central Mediterranean region and Northern Africa
Amaryllis L. (2 species)	South Africa
Ammocharis Herb. (6 species)	Africa
Apodolirion Baker (6 species)	South Africa
Boophone Herb. (2 species)	Tropical and Southern Africa
Brunsvigia Heist. (*ca.* 20 species)	South Africa
Caliphruria Herb. (4 species)	Tropical regions of South America, mainly in Colombia
Calostemma R. Br. (3 species)	Australia
Chlidanthus Herb. (10 species)	Tropical South America, mostly native to the Andes
Clinanthus Herb. (22 species)	Ecuador to northwestern Argentina
Clivia Lindl. (5 species)	Native to Southern Africa
Crinum L. (*ca.* 180 species)	Tropical and subtropical areas worldwide
Crossyne Salisb. (2 species)	Southern Africa
Cryptostephanus Welw. ex Baker (2 species)	Southern Africa
Cyrtanthus Aiton (56 species)	Southern and Eastern Africa
Eucharis Planch & Linden (*ca.* 20 species)	Central America and South America from Guatemala south to Bolivia
Eucrosia Ker Gawl. (8 species)	Ecuador to Peru
Eustephia Cav. (6 species)	Peru
Galanthus L. (20 species)	Europe to Northern Iran
Gethyllis L. (30 species)	South Africa
Griffinia Ker Gawl. (21 species)	South America, Brazil
Griffiniopsis Dutilh & Meerow (1 species)	South America
Habranthus Herb. (74 species)	South America
Haemanthus L. (22 species)	South Africa, Namibia, and the kingdoms of Lesotho and Swaziland
Hannonia Braun-Blanq. & Maire (1 species)	Morocco
Hessea Herb. (13 species)	Southern Africa
Hieronymiella Pax. (8 species)	Southern Bolivia to northwestern Argentina
Hippeastrum Herb. (*ca.* 90 species)	South America from Argentina north to Mexico and the Caribbean
Hymenocallis Salisb. (63 species)	Southeastern USA, Mexico, and West Indies
Ismene Salisb. ex Herb (10 species)	Central Andean region
Lapiedra Lag. (1 species)	Western Mediterranean
Leptochiton Sealy (2 species)	Central Andean region
Leucojum L. (3 species)	Central and Southern Europe, from the Pyrenees to Romania and western Russia

(*continued*)

Table 17.1 (continued)

Plant genus	Location
Lycoris Herb. (22 species)	Eastern and Southern Asia including Japan, southern Korea, Eastern and Southern China, Northern Vietnam, Northern Laos, Northern Thailand, Northern Burma, Nepal, Northern Pakistan, Afghanistan, and Eastern Iran
Mathieua Klotzsch (1 species)	Peru
Namaquanula D.Müll.-Doblies & U.Müll.-Doblies (2 species)	Southern Africa
Narcissus L. (56 species)	Europe, North Africa, and Asia, from Macaronesia to Afghanistan, and Southeastern China to Japan
Nerine Herb. (23 species)	Southern Africa
Pamianthe Stapf (3 species)	Peru, Ecuador, and Bolivia
Pancratium L. (21 species)	South Africa to the Mediterranean and Asia
Paramongaia Velarde (2 species)	Peru, Ecuador, and Bolivia
Phaedranassa Herb. (9 species)	Colombia to Ecuador
Phycella Lyndl. (5 species)	South America
Placea Miers (6 species)	South America
Plagiolirion Baker (1 species)	Central America to Andean South America
Proiphys Herb. (4 species)	Malaysia, Indonesia, the Philippines, and Tropical Australia
Pyrolirion Herb. (6 species)	Peru to Northern Chile
Rauhia Traub (4 species)	Peru
Rhodophiala C. Presl (28 species)	South America
Scadoxus Raf. (9 species)	Tropical Africa
Sprekelia Heist. (2 species)	South America
Stenomesson Herb. (*ca.* 16 species)	Colombia to Northern Peru
Sternbergia Waldst. & kit. (8 species)	Central and Southern Europe to Central Asia
Strumaria Jacq. ex Willd. (24 species)	South Africa
Traubia Moldenke (1 species)	South America
Ungernia Bunge (10 species)	Central Asia
Urceolina Rchb. (5 species)	Colombia to Northern Peru
Vagaria Herb. (2 species)	Morocco to Lebanon and Israel
Worsleya Traub (1 species)	Brazil
Zephyranthes Herb. (94 species)	America

this topic is regularly reviewed by the journal *Natural Product Reports* of the Royal Society of Chemistry [20–26]. Due to the sufficiently structural particularity of Amaryllidaceae alkaloids, only the alkaloids isolated from the subfamily Amaryllidoideae, namely, the old Amaryllidaceae family, will be dealt with in this chapter.


lycorine 1
norbelladine 2
homolycorine 3
crinine 4
haemanthamine 5
narciclasine 6
tazettine 7
montanine 8
galanthamine 9

Scheme 17.1

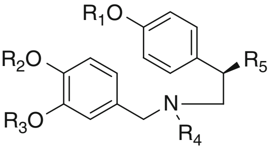
2 Occurrence

A unique characteristic for plants of the family Amaryllidaceae is the production of a group of structurally diverse but biogenetically relative alkaloids. Since lycorine **1** was isolated as the first alkaloid from *Narcissus pseudonarcissus* in 1877, up to 500 alkaloids have been identified from plants of this family to date. Furthermore, the number remains increasing every year. At present, most genera of this family are validated to produce Amaryllidaceae alkaloids.

2.1 Classification of Amaryllidaceae Alkaloids

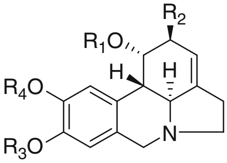
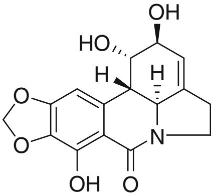
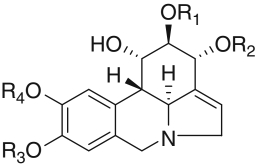
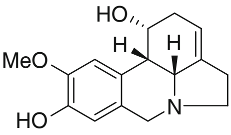
Although the structures of Amaryllidaceae alkaloids are greatly diverse, they are considered to be biogenetically related and have a common precursor alkaloid norbelladine **2**, which originally derived from the natural amino acids L-phenylalanine (Phe) and L-tyrosine (Tyr). Conventionally, according to molecule skeletons of the alkaloids, the large number of Amaryllidaceae alkaloids was classified mainly into nine different types, as represented by lycorine **1**,

Table 17.2 Norbelladine-type Amaryllidaceae alkaloids



$R_1 = R_2 = R_3 = R_4 = R_5$ = H, norbelladine **2**
$R_1 = R_3 = R_4 = R_5$ = H, R_2 = Me, 4′-*O*-methylnorbelladine **10**
$R_1 = R_2 = R_3 = R_4 = R_5$ = Me, belladine **11**
$R_1 = R_2 = R_3 = R_5$ = Me, R_4 = H, *N*-demethylbelladine **12**
$R_1 = R_3$ = H, $R_2 = R_4$ = Me, R_5 = OH, 2*R*-hydroxy-*O*,*N*-dimethylnorbelladine **13**

Table 17.3 Lycorine-type Amaryllidaceae alkaloids

	R_1 = H, R_2 = OH, R_3,R_4 = -CH_2-, lycorine **1**
	$R_1 = R_4$ = H, R_2 = OH, R_3 = Me, pseudolycorine **14**
	$R_1 = R_2$ = H, $R_3 = R_4$ = Me, pluviine **15**
	$R_1 = R_2 = R_4$ = H, R_3 = Me, 9-norpluviine **16**
	R_1 = Ac, $R_2 = R_3$ = H, R_4 = Me, 1-*O*-acetyl-8-norpluviine **17**
	R_1 = H, R_2 = OMe, $R_3 = R_4$ = Me, galanthine **18**
	R_1 = Ac, R_2 = OH, R_3 = H, R_4 = Me, sternbergine **19**
	$R_1 = R_2$ = H, R_3,R_4 = -CH_2-, caranine **20**
	$R_1 = R_4$ = H, R_2 = OMe, R_3 = Me, 10-*O*-demethylgalanthine **21**
	R_1 = H, R_2 = Ac, R_3,R_4 = -CH_2-, 2-*O*-acetyllycorine **22**
	$R_1 = R_2$ = H, R_3,R_4 = -CH_2-, 2-deoxylycorine **23**
	R_1 = H, R_2 = OMe, R_3,R_4 = -CH_2-, hippamine **24**
	8-Hydroxylycorine-7-one **25**
	R_1 = Me, R_2 = H, R_3,R_4 = -CH_2-, ungiminorine **26**
	$R_1 = R_3 = R_4$ = Me, R_2 = Ac, 3-*O*-acetylnarcissidine **27**
	$R_1 = R_3 = R_4$ = Me, R_2 = H, narcissidine **28**
	$R_1 = R_2$ = H, $R_3 = R_4$ = Me, pancrassidine **29**
	Fortucine **30**

(*continued*)

Table 17.3 (continued)

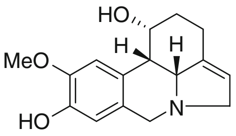
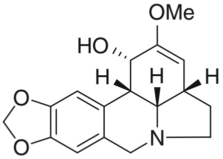
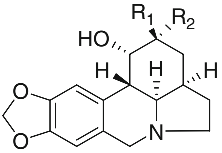
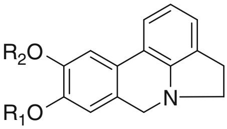
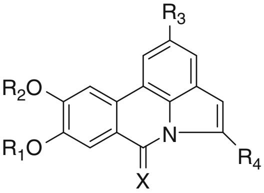
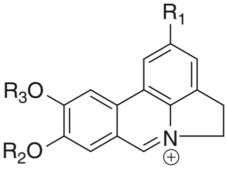
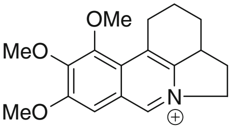
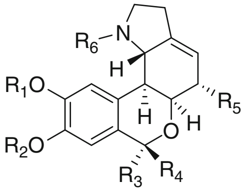
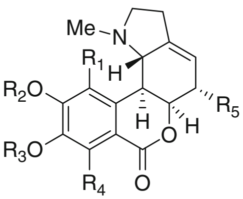
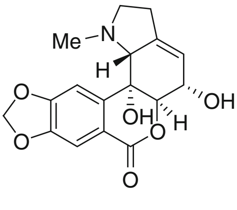
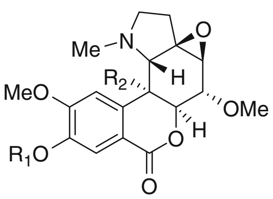
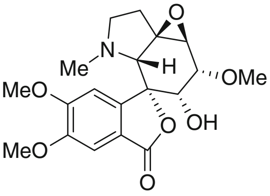
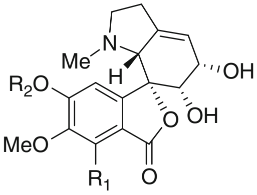
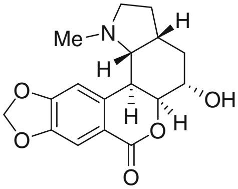
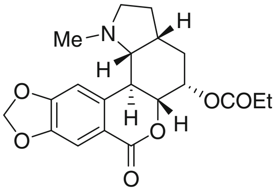
	Kirkine **31**
	Amarbellisine **32**
	R_1 = H, R_2 = OH, zephyranthine **33** R_1 = R_2 = H, dihydrocaranine **34** R_1 = OH, R_2 = H, dihydrolycorine **35**
	R_1,R_2 = -CH_2-, anhydrolycorine **36** R_1 = R_2 = Me, assoanine **37**
	R_1,R_2 = -CH_2-, R_3 = R_4 = H, X = O, hippadine **38** R_1,R_2 = -CH_2-, R_3 = X = H_2, 3,4-dihydroanhydrolycorine **39** R_1 = R_2 = Me, R_3 = R_4 = H, X = O, hippacine/pratosine **40** R_1,R_2 = -CH_2-, R_3 = OMe, R_4 = H, X = O, lycoranine A **41** R_1,R_2 = -CH_2-, R_3 = OMe, R_4 = Me, X = O, lycoranine B **42**
	R_1 = H, R_2,R_3 = -CH_2-, anhydrolycorinium **43** R_1 = O^-, R_2,R_3 = -CH_2-, ungeremine **44** R_1 = O^-, R_2 = Me, R_3 = H, zefbetaine **45** R_1 = H, R_2 = R_3 = Me, vasconine **46** R_1 = OMe, R_2 = R_3 = Me, tortuosine **47**
	Roserine **48**

Table 17.4 Homolycorine-type representative alkaloids

	
	$R_1 = R_2 = R_6 =$ Me, $R_3,R_4 =$ O, $R_5 =$ H, homolycorine **3**
	$R_1,R_2 =$ -CH_2-, $R_3,R_4 =$ O, $R_5 =$ OH, $R_6 =$ Me, hippeastrine **49**
	$R_1 =$ H, $R_2 =$ Me, $R_3,R_4 =$ O, $R_5 =$ H, $R_6 =$ Me, 9-*O*-demethylhomolycorine **50**
	$R_1 = R_6 =$ Me, $R_2 =$ H, $R_3,R_4 =$ O, $R_5 =$ H, 8-*O*-demethylhomolycorine **51**
	$R_1 = R_6 =$ Me, $R_2 =$ Ac, $R_3,R_4 =$ O, $R_5 =$ H, 8-*O*-demethyl8-*O*-acetylhomolycorine **52**
	$R_1 =$ H, $R_2 = R_6 =$ Me, $R_3,R_4 =$ O, $R_5 =$ OH, 9-*O*-demethyl-2α-hydroxyhomolycorine **53**
	$R_1 = R_2 = R_6 =$ Me, $R_3 =$ OH, $R_4 = R_5 =$ H, lycorenine **54**
	$R_1 = R_2 = R_6 =$ Me, $R_3 =$ OMe, $R_4 = R_5 =$ H, *O*-methyllycorenine **55**
	$R_1 = R_6 =$ Me, $R_2 = R_4 = R_5 =$ H, $R_3 =$ OMe, 8-*O*-demethyl-6-*O*-methyllycorenine **56**
	$R_1,R_2 =$ -CH_2-, $R_3,R_4 =$ O, $R_5 =$ H, $R_6 =$ Me, masosine **57**
	$R_1,R_2 =$ -CH_2-, $R_3,R_4 =$ O, $R_5 = R_6 =$ H, normasosine **58**
	$R_1,R_2 =$ -CH_2-, $R_3 =$ OH, $R_4 = R_5 =$ H, $R_6 =$ Me, oduline **59**
	$R_1,R_2 =$ -CH_2-, $R_3 =$ OMe, $R_4 = R_5 =$ H, $R_6 =$ Me, *O*-methyloduline **60**
	$R_1,R_2 =$ -CH_2-, $R_3 =$ OMe, $R_4 =$ H, $R_5 =$ OH, $R_6 =$ Me, 2α-hydroxy-*O*-methyloduline **61**
	$R_1 =$ OCOCH$_2$CH(OH)Me, $R_2 = R_6 =$ Me, $R_3,R_4 =$ O, $R_5 =$ OAc, dubiusine **62**
 Me N R1 H R2O R3O H O H R5 R4 O	$R_1 =$ OMe, $R_2,R_3 =$ -CH_2-, $R_4 =$ H, $R_5 =$ OH, neronine **63** $R_1 =$ OH, $R_2,R_3 =$ -CH_2-, $R_4 =$ H, $R_5 =$ OH, 10-norneronine **64** $R_1 = R_5 =$ H, $R_2 = R_3 =$ Me, $R_4 =$ OMe, albomaculine **65**
	Pancratinine A **66**
	$R_1 =$ Me, $R_2 =$ H, galwesine **67** $R_1 = R_2 =$ H, 9-*O*-demethylgalwesine **68** $R_1 =$ Me, $R_2 =$ OH, 10b-hydroxygalwesine **69** $R_1 =$ H, $R_2 =$ OH, 10b-hydroxy-9-*O*-demethylgalwesine **70**

(*continued*)

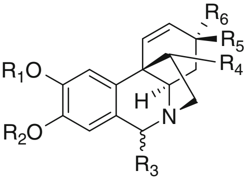
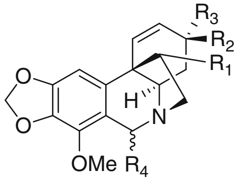
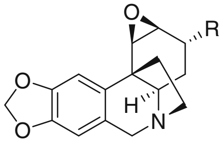
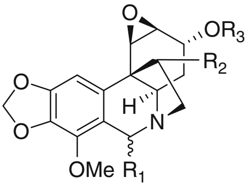
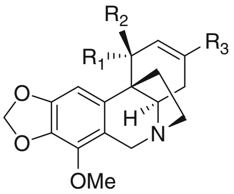
Table 17.4 (continued)

	Galasine **71**
	R_1 = OMe, R_2 = H, hostasine **72** R_1 = R_2 = H, 8-demethoxyhostasine **73** R_1 = H, R_2 = Me, 8-demethoxy-10-*O*-methylhostasine **74** R_1 = OMe, R_2 = Me, 10-*O*-methylhostasine **75**
	Clivonine **76**
	Poetinatine **77**

norbelladine **2**, homolycorine **3**, crinine **4**, haemanthamine **5**, narciclasine **6**, tazettine **7**, montanine **8**, galanthamine **9** (Scheme 17.1) [9]. However, with continuous phytochemistry studies, some alkaloids owning new skeleton types have been isolated from plants of this family in recent years [8, 21, 22], which suggests that the classification of Amaryllidaceae alkaloids should be reconsidered and also their biosynthetic pathways.

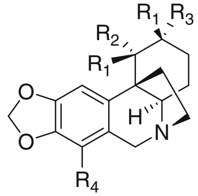
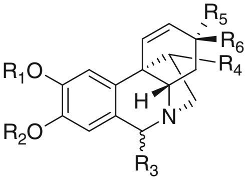
On the basis of recent phytochemistry investigation about Amaryllidaceae alkaloids, a new 12-type classification, including (1) norbelladine type, (2) lycorine type, (3) homolycorine type, (4) crinine and haemanthamine types, (5) tazettine type, (6) montanine type, (7) plicamine type, (8) graciline type, (9) galanthindole type, (10) galanthamine type, (11) phenanthridone and phenanthridine types, and (12) other minor species populations, are deduced according to their ring systems. Representative structures for each type of Amaryllidaceae alkaloids are shown in Tables 17.2–17.13.

Table 17.5 Crinine- and haemanthamine-type Amaryllidaceae alkaloids

Crinine type	
 R$_6$ R$_5$ R$_4$ R$_1$O R$_2$O H N R$_3$	R_1,R_2 = -CH$_2$-, $R_3 = R_4 = R_5$ = H, R_6 = OH, crinine **4**
	R_1,R_2 = -CH$_2$-, $R_3 = R_4$ = H, R_5,R_6 = O, oxocrinine **78**
	R_1,R_2 = -CH$_2$-, $R_3 = R_4 = R_6$ = H, R_5 = OH, *epi*crinine **79**
	R_1,R_2 = -CH$_2$-, $R_3 = R_4$ = H, R_5,R_6 = O, crinan-3-one **80**
	R_1,R_2 = -CH$_2$-, $R_3 = R_4 = R_5$ = H, R_6 = OMe, buphanisine **81**
	R_1,R_2 = -CH$_2$-, $R_3 = R_4 = R_6$ = H, R_5 = OMe, *epi*buphanisine **82**
	$R_1 = R_3 = R_4 = R_5$ = H, R_2 = Me, R_6 = OH, macowine **83**
	R_1,R_2 = -CH$_2$-, $R_3 = R_4$ = OH, R_5 = OMe, R_6 = H,
	3β-Methoxy-6,11-dihydroxycrinane **84**
	R_1,R_2 = -CH$_2$-, $R_3 = R_6$ = H, $R_4 = R_5$ = OH,
	3β,11-Dihydroxycrinane **85**
	R_1,R_2 = -CH$_2$-, $R_3 = R_4 = R_5$ = H, R_6 = OAc, krepowine **86**
	$R_1 = R_2 = R_4$ = H, R_3 = OMe, buphanidrine **87**
	R_1 = Ac, R_2 = H, R_3 = OMe, 11-*O*-acetylambelline **88**
	R_1 = OH, R_2 = H, R_3 = OMe, ambelline **89**
	R_1 = ONic, $R_2 = R_4$ = H, R_3 = OMe, filifoline **90**
	$R_1 = R_2 = R_4$ = H, R_3 = OH, powelline **91**
	$R_1 = R_2$ = H, R_3 = OMe, R_4 = OMe, 6α-methoxybuphanidrine **92**
	$R_1 = R_2$ = H, R_3 = OMe, R_4 = OH, 6α-hydroxbuphanidrine **93**
	R = OH, flexinine **94**
	R = OMe, augustine **95**
	R = H, zephyramine **96**
	$R_1 = R_2$ = H, R_3 = Me, undulatine **97**
	R_1 = H, R_2 = OH, R_3 = Me, 1,2-epoxyambelline **98**
	R_1 = OH, R_2 = H, R_3 = Me, 6α-hydroxyundulatine **99**
	$R_1 = R_2 = R_3$ = H, crinamidine **100**
	R_1 = OH, R_2,R_3 = H, buphanamine **101**
	R_1,R_2 = O, R_3 = OMe, distichamine **102**

(*continued*)

Table 17.5 (continued)

	R_1 = OH, R_2 = R_3 = H, R_4 = OMe, 1-*O*-deacetylbowdensine **103**
	R_1 = OH, R_2 = R_3 = R_4 = H, 4a-dehydroxycrinamabine **104**
	R_1 = R_4 = H, R_2 = R_3 = OH, amabiline **105**
	R_1 = R_4 = H, R_2 = OH, R_3 = OAc, josephinine **106**
Haemanthamine type	
	R_1,R_2 = -CH_2-, R_3 = R_5 = H, R_4 = OH, R_6 = OMe, Haemanthamine **5**
	R_1,R_2 = -CH_2-, R_3 = R_4 = R_5 = H, R_6 = OH, vitattine **107**
	R_1,R_2 = -CH_2-, R_3 = R_5 = H, R_4 = R_6 = OH, 11-hydroxvitattine **108**
	R_1 = R_2 = Me, R_3 = R_4 = R_5 = H, R_6 = OH, maritidine **109**
	R_1 = R_2 = Me, R_3 = R_4 = H, R_5,R_6 = O, oxomaritidine **110**
	R_1,R_2 = -CH_2-, R_3 = R_4 = OH, R_5 = H, R_6 = OMe, Haemanthidine **111**
	R_1,R_2 = -CH_2-, R_3 = OH, R_4 = R_5 = H, R_6 = OMe, *Ent*-6-hydroxybufisine **112**
	R_1 = R_2 = Me, R_3 = R_4 = R_5 = H, R_6 = OMe, 3-*O*-methylmaritidine **113**
	R_1 = Me, R_2 = R_3 = R_4 = R_5 = H, R_6 = OH, 8-*O*-demethylmaritidine **114**
	R_1 = R_3 = R_4 = R_5 = H, R_2 = Me, R_6 = OH, 9-*O*-demethylmaritidine **115**
	R_1,R_2 = -CH_2-, R_3 = R_6 = H, R_4 = OH, R_5 = OMe, crinamine **116**
	R_1,R_2 = -CH_2-, R_3,R_6 = OMe, R_4 = OH, R_5 = H, 6-*O*-methylhaemanthidine **117**
	R_1,R_2 = -CH_2-, R_3 = R_6 = H, R_4 = R_5 = OMe, 11-*O*-methylcrinamine **118**
	R_1,R_2 = -CH_2-, R_3 = R_6 = H, R_4 = OH, R_5 = OAc, 11*R*-yemenine A **119**
	R_1,R_2 = -CH_2-, R_3 = R_6 = H, R_4 = R_5 = OH, 11*R*-yemenine B **120**
	R_1,R_2 = -CH_2-, R_3 = OMe, R_4 = R_6 = OH, yemenine C **121**
	R_1,R_2 = -CH_2-, R_3 = R_6 = H, R_4 = R_5 = OH, hamayne **122**
	R_1,R_2 = -CH_2-, R_3 = R_6 = H, R_4 = OH, R_5 = OAc, 3-*O*-acetylhamayne **123**
	R_1,R_2 = -CH_2-, R_3 = R_4 = OH, R_5 = OMe, R_6 = H, 6-hydroxycrinamine **124**
	R_1 = R_2 = Me, R_3 = OH, R_4 = R_5 = H, R_6 = OMe, papyramine **125**
	R_1,R_2 = -CH_2-, R_3 = R_5 = OMe, R_4 = OH, R_6 = H, 6-*O*-methoxycrinamine **126**
	R_1,R_2 = -CH_2-, R_3 = R_5 = H, R_4 = R_6 = OH, bulbispermine **127**
	R_1 = Me, R_2 = R_3 = R_4 = R_6 = H, R_5 = OH, siculine **128**
	R_1 = Me, R_2 = R_3 = R_5 = H, R_4 = OH, R_6 = OMe, narcidine **129**

(*continued*)

Table 17.5 (continued)

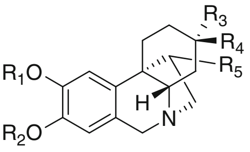
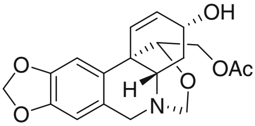
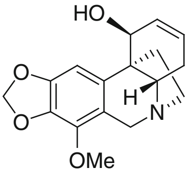
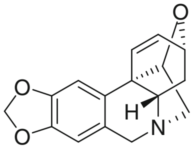
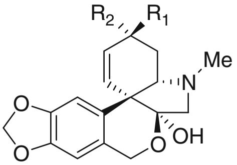
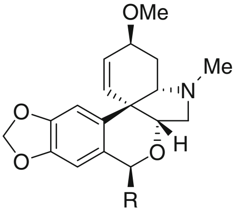
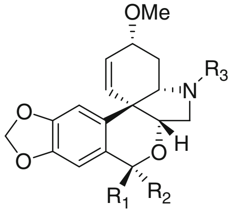
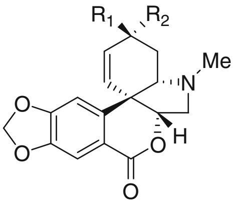
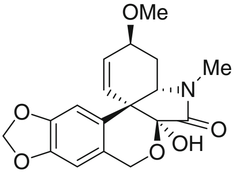
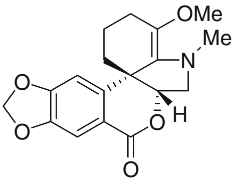
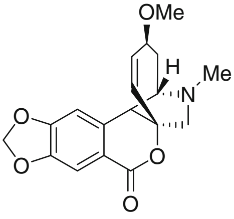
 R3 R4 R5 R1O R2O H N	$R_1 = R_3 = R_5 = H$, $R_2 = Me$, $R_4 = OAc$, cantabricine **130** R_1,R_2 = -CH_2-, $R_3 = OMe$, $R_4 = H$, $R_5 = OCH_2CH(OH)Et$, Narcimarkine **131**
 OH O OAc O H N O	Bujeine **132**
	Phaedranamine **133**
	Apohaemanthamine **134**

Table 17.6 Tazettine-type Amaryllidaceae alkaloids

 R2 R1 Me N O O O OH	$R_1 = OMe$, $R_2 = H$, tazettine **7** $R_1 = H$, $R_2 = OMe$, criwelline **135** $R_1 = OCOCH_2CH(OH)Me$, $R_2 = H$, 3-*O*-(3′-hydroxybutyryl)tazettinol **136** $R_1 = H$, $R_2 = OH$, isotazettinol **137**
 OMe Me N O O O H R	R = H, 6a-deoxytazettine **138** R = OH, pretazettine **139** R = OMe, 6-*O*-methylpretazettine **140**

(*continued*)

Table 17.6 (continued)

 OMe R3 N O O H O R1 R2	R_1 = H, R_2 = OEt, R_3 = H, *N*-demethyl-8α-ethoxypretazettine **141**
	R_1 = OEt, R_2 = H, R_3 = H, *N*-demethyl-8β-ethoxypretazettine **142**
	R_1 = H, R_2 = OEt, R_3 = Me, 8α-ethoxyprecriwelline **143**
 R1 R2 Me N O O H O O	R_1 = OMe, R_2 = H, 3-*epi*macronine **144**
	R_1 = OH, R_2 = H, 3-*O*-demethyl-3-*epi*macronine **145**
	R_1 = H, R_2 = OH, 3-*O*-demethylmacronine **146**
 OMe Me N O O O OH O	6-oxotazettine **147**
 OMe Me N O O H O O	(Unnamed) **148**
 OMe H Me N O O O O	Obesine **149**

2.1.1 Norbelladine Type

This type of Amaryllidaceae alkaloids is derived initially from the condensation of tyramine and protocatechuic aldehyde or its derivatives in plants, and the intermediate Schiff's bases have been found to exist in nature (Table 17.2) [27, 28].

Table 17.7 Montanine-type Amaryllidaceae alkaloids

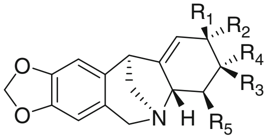
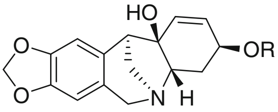
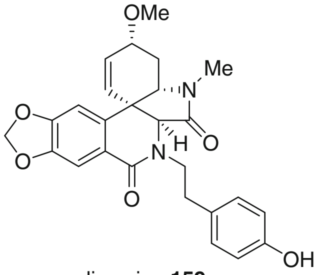
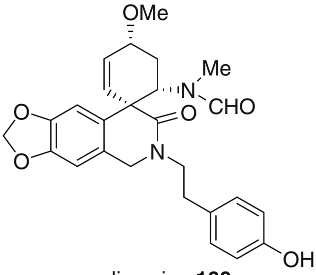
 R1 R2 R4 R3 R5 O O N H	$R_1 = R_4 = R_5 = H, R_2 = OMe, R_3 = OH$, montanine **8**
	$R_1 = R_4 = R_5 = H, R_2 = R_3 = OH$, pancracine **150**
	$R_1 = R_2 = R_3 = H, R_4 = R_5 = OH$, nangustine **151**
	$R_1 = R_3 = R_5 = H, R_2 = R_4 = OH$, brunsvigine **152**
	$R_1 = R_4 = R_5 = H, R_2 = R_3 = OMe$, manthine **153**
	$R_1 = R_3 = R_5 = H, R_2 = OMe, R_4 = OH$, manthidine **154**
	$R_1 = OMe, R_2 = R_4 = R_5 = H, R_3 = OH$, coccinine **155**
	$R_1 = R_2 = R_4 = R_5 = H, R_3 = OH$, 2-demethoxymontanine **156**
	R = Me, pancratinine B **157**
	R = H, pancratinine C/squamigine **158**

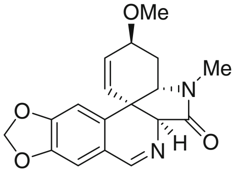
Table 17.8 Plicamine-type Amaryllidaceae alkaloids


OMe
Me
N
O
O
N
H
O
O
OH

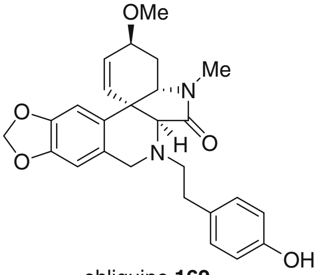
plicamine **159**


OMe
Me
N
CHO
O
O
O
N
OH

secoplicamine **160**


OMe
Me
N
O
O
N
H
O

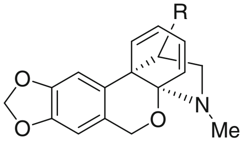
plicane **161**


OMe
Me
N
O
O
N
H
O
OH

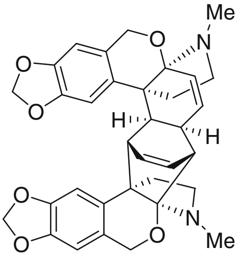
obliquine **162**

Generally, norbelladine **2** and its congeners 4′-*O*-methylnorbelladine **10** are believed to be the biosynthetic precursor in the metabolic pathway of Amaryllidaceae alkaloids [29].

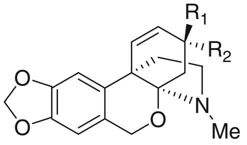
Table 17.9 Graciline-type Amaryllidaceae alkaloids



R = H, graciline **163**
R = OAc, 11-acetoxygraciline **164**

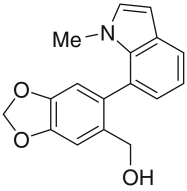

Me
O
N
O
O
H
H
O
O
O
N
Me

digracine **167**

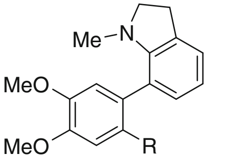

R1
R2
O
O
O
N
Me

R_1 = OH, R_2 = H, 3,4-dihydro-3-hydroxygraciline **165**
R_1 = H, R_2 = OH, 3-epi-3,4-dihydro-3-hydroxygraciline **166**

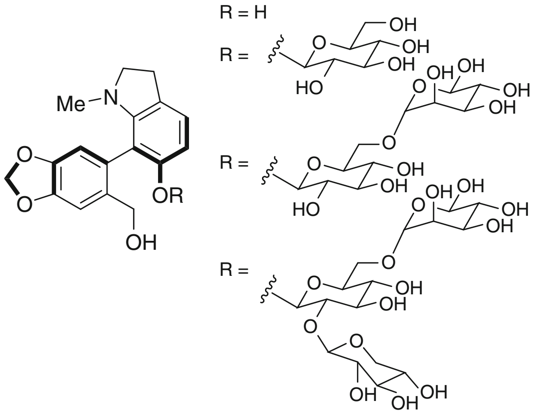
Table 17.10 Galanthindole-type Amaryllidaceae alkaloids



galanthindole **168**

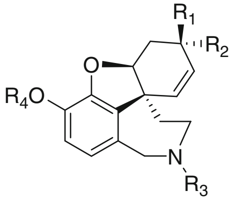
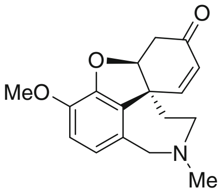
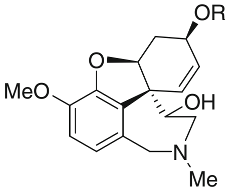
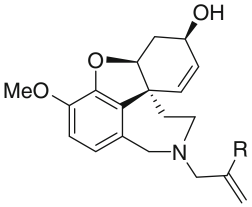
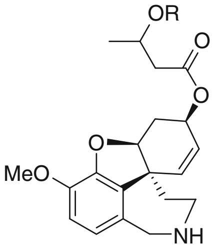

Me
N
MeO
MeO
R

R = CH_2OH, lycosinine A **169**
R = CHO, lycosinine B **170**


Me
N
O
O
OR
OH
R = H
R =
O
OH
OH
OH
HO
R =
O
OH
OH
HO
O
O
OH
OH
OH
OH
R =
O
OH
OH
O
O
OH
OH
OH
OH
O
O
OH
OH
OH

171
172
173
174

Table 17.11 Galanthamine-type Amaryllidaceae alkaloids

 R₁ R₂ O R₄O N R₃	R_1 = OH, R_2 = H, R_3 = R_4 = Me, galanthamine **9**
	R_1 = OH, R_2 = R_3 = H, R_4 = Me, norgalanthamine **175**
	R_1 = H, R_2 = OH, R_3 = R_4 = Me, 3-*epi*galanthamine **176**
	R_1 = OH, R_2 = H, R_3 = CHO, R_4 = Me, *N*-formylnorgalanthamine **177**
	R_1 = OAc, R_2 = H, R_3 = R_4 = Me, *O*-acetylgalanthamine **178**
	R_1 = R_3 = H, R_2 = OH, R_4 = Me, *epi*norgalanthamine **179**
	R_1 = OH, R_2 = R_4 = H, R_3 = Me, sanguinine **180**
	R_1 = OH, R_2 = R_3 = R_4 = H, norsanguinine **181**
	R_1 = OAc, R_2 = R_4 = H, R_3 = Me, 3-*O*-acetylsanguinine **182**
	R_1 = OH, R_2 = H, R_3 = Ac, R_4 = Me, narcisine **183**
	R_1 = OMe, R_2 = R_4 = H, R_3 = Me, chlidantine **184**
	Narwedine **185**
	R = H, habranthine **186**
	R = Ac, 3-*O*-acetylhabranthine **187**
	R = H, *N*-allylnorgalanthamine **188**
	R = Me, *N*-(14-methylallyl)norgalanthamine **189**
	R = H, leucovernine **190**
	R = Ac, acetylleucovernine **191**

(*continued*)

Table 17.11 (continued)

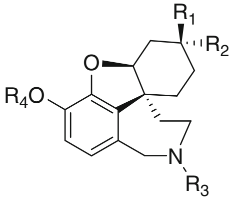
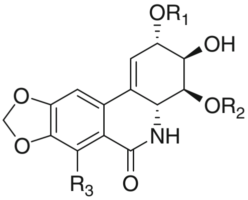
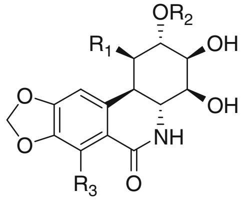
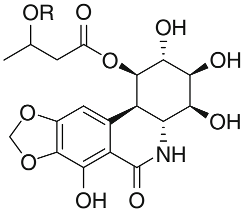
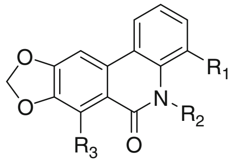
	R_1 = OH, R_2 = H, R_3 = R_4 = Me, lycoramine **192**
	R_1 = OH, R_2 = R_3 = H, R_4 = Me, norlycoramine **193**
	R_1 = OAc, R_2 = H, R_3 = R_4 = Me, 3-*O*-acetyllycoramine **194**
	R_1 = R_3 = H, R_2 = OH, R_4 = Me, *epi*norlycoramine **195**
	R_1 = OH, R_2 = R_4 = H, R_3 = Me, *O*-demethyllycoramine **196**

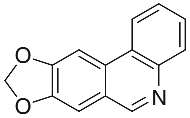
Table 17.12 Phenanthridone- and phenanthridine-type Amaryllidaceae alkaloids

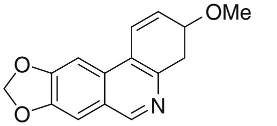
Phenanthridone type	
	R_1 = R_2 = H, R_3 = OH, narciclasine **6**
	R_1 = R_2 = R_3 = H, 7-deoxynarciclasine/lycoricidine **197**
	R_1 = β-D-Glu, R_2 = H, R_3 = OH, kalbreclasine **198**
	R_1 = H, R_2 = β-D-Glu, R_3 = OH, 4-*O*-glucosylnarciclasine **199**
	R_1 = R_2 = H, R_3 = OH, *trans*-dihydronarciclasine **200**
	R_1 = R_2 = R_3 = H, 7-deoxy-*trans*-dihydronarciclasine **201**
	R_1 = R_3 = OH, R_2 = H, pancratistatin **202**
	R_1 = OH, R_2 = R_3 = H, 7-deoxypancratistatin **203**
	R_1 = R_3 = OH, R_2 = β-D-Glu, pancratiside **204**
	R_1 = R_3 = OH, R_2 = 3′-deoxy-3-acetyl-β-D-Glu, telastaside **205**
	R = H, 1-*O*-(3′-hydroxybutyryl)-pancratistatin **206**
	R = β-D-Glu, 1-*O*-(3′-*O*-β-D-glucopyranosylbutyryl)-pancratistatin **207**
	R_1 = R_3 = OH, R_2 = H, narciprimine **208**
	R_1 = R_3 = H, R_2 = H, crinasiadine **209**
	R_1 = R_3 = H, R_2 = Me, *N*-methylcrinasiadine **210**
	R_1 = OH, R_2 = R_3 = H, arolycoricidine **211**

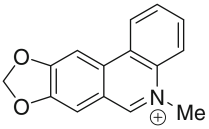
(*continued*)

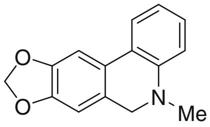
Table 17.12 (continued)

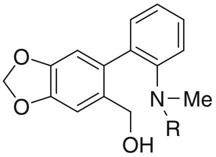
Phenanthridine type


trisphaeridine **212**


3-methoxy-3,4-dihydrotrisphaeridine **213**

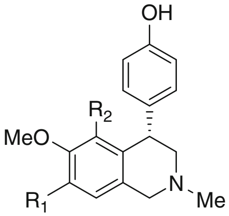
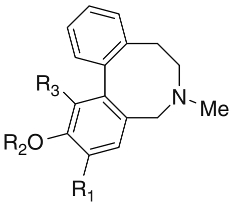
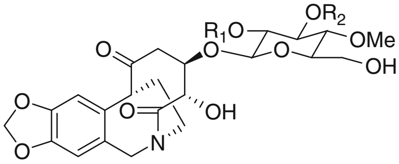

bicolorine **214**


5,6-dihydrobicolorine **215**



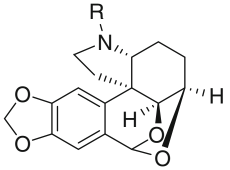
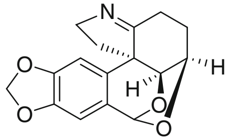
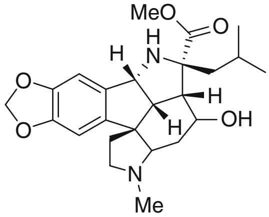
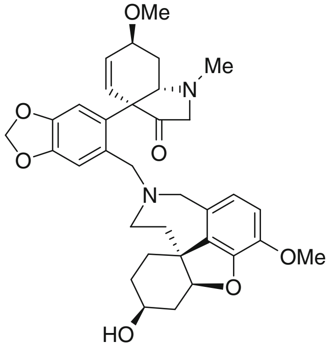
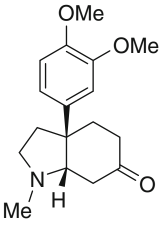
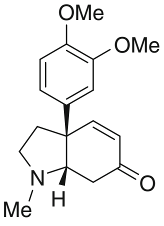
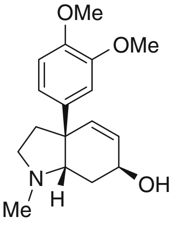
R = H, ismine **216**
R = CHO, *N*-formylismine **217**

Table 17.13 Miscellaneous Amaryllidaceae alkaloids

	$R_1 = OH, R_2 = H$, cherylline **218** $R_1 = H, R_2 = OH$, latifine **219**
	$R_1 = OMe, R_2 = Me$, $R_3 = H$, buflavine **220** $R_1 = R_2 = H, R_3 = OH$, apogalanthamine **221**
	R_1,R_2 = -CH_2OCH_2-, cripowellin A **222** $R_1 = R_2 = Me$, cripowellin B **223**

(*continued*)

Table 17.13 (continued)

 R = Me, augustamine **224** R = H, noraugustamine **225**	 4α,*N*-didehydronoraugustamine **226**	
		Gracilamine **227**
		Pallidiflorine **228**
 mesembrine **229**	 mesembrenone **230**	 mesembrenol **231**

2.1.2 Lycorine Type

This type of Amaryllidaceae alkaloids owns an unique pyrrolo[*d*,*e*]phenanthridine skeleton and is one of the most common alkaloids in plants of the family Amaryllidaceae (Table 17.3). Usually, they have a *trans*-junction in the B/C ring, but a few alkaloids with *cis*-form unions of the B/C ring, such as fortucine **30** [30], amarbellisine **32** [31], etc., have been isolated recently. It is interesting to find that a part of this type of alkaloids has a completely unsaturated aromatic C ring and some possess an unusual quaternary nitrogen [32–35].

2.1.3 Homolycorine Type

The alkaloids of homolycorine type are biogenetically formed through a restructuring of lycorine-type alkaloids and have a common [3,4-*g*] benzopyranone ring due to the oxidation of the hydroxyl group at the C6 (Table 17.4). Almost all alkaloids of this type have a *cis*-form B/C ring junction with only an exception of poetinatine **73**. It is noteworthy that, alkaloids shown an oxygenated function at the C10b, such as pancratinine A **66** [36, 37], 10b-hydroxygalwesine **69**, and 10b-hydroxy-9-*O*-demethylgalwesine **70** [38], are very unusual, which is the probable precursor of alkaloids galasine **71** and hostasine **72**, incorporating a scarce spirolactone moiety.

2.1.4 Crinine and Haemanthamine Types

The crinine- and haemanthamine-type alkaloids, together with lycorine-type alkaloids, are the most abundant alkaloids in the plants of the family Amaryllidaceae. Both crinine-type and haemanthamine-type alkaloids have a 5,10b-ethano bridge moiety in their frameworks, a very significant taxonomic feature, and the configurations of the 5,10b-ethano bridge are opposite to each other (Table 17.5). Scarcely, haemanthamine-type alkaloids bujeine **132** owns an unusually modified bridge with a heteroatom between C11 and C12 and an acetoxymethyl substituent at the 11-*endo* position [39].

2.1.5 Tazettine Type

Alkaloids of this type, derived from the haemanthamine-type alkaloids, are characterized as a linkage between C6 and C11 by an oxygen atom to form a [3,4-*g*]benzopyran framework (Table 17.6) [40, 41]. Alkaloid obesine **149** is an only exception, which has a seven-member ring skeleton. The position on the C12 can be oxidized occasionally to produce an amide group, such as in 6-oxotazettine **147**.

2.1.6 Montanine Type

Montanine-type alkaloids constitute a limited group in Amaryllidaceae alkaloids and show a characteristic 5,11-methanomorphanthridine framework (Table 17.7). There are two classes of $C = C$ double bond substitution patterns in the C ring of alkaloid skeletons. Alkaloid nangustine **151** is the first member in this subgroup with a hydroxyl group at the C4 [42].

2.1.7 Plicamine Type

Alkaloids of this type are the first example of the dinitrogenous-type Amaryllidaceae alkaloids, where the oxygen atom at the C6 in the tazettine-type alkaloids is replaced by a nitrogen atom, substituted with a pendant

4-hydroxyphenethyl unit (Table 17.8) [40, 43]. In addition, all alkaloids of this minor subgroup have an amide group on the C12. So far, only four members **159–162** of this subgroup have been isolated from plants of this family.

2.1.8 Graciline Type

Alkaloids of this type belong to a newly established subgroup of Amaryllidaceae alkaloids and own a 10b,4a-ethanoiminodibenzo[*b*,*d*]pyrane skeleton (Table 17.9). To date, a total of five alkaloids including four monomeric alkaloids **163–166** and one dimeric alkaloid **167** have been isolated from plants of the family Amaryllidaceae [41, 44].

2.1.9 Galanthindole Type

Alkaloids of this type belong to another new subgroup isolated from the Amaryllidaceae family plants and own a 7-arylindole or 7-aryl-2,3-dihydroindole skeleton (Table 17.10). It is interesting that alkaloids of this type **171**–**174** recently isolated from the genus *Narcissus* cultivar show a rare axial chirality [45].

2.1.10 Galanthamine Type

Galanthamine-type alkaloids have a dibenzofuran nucleus and are the only group in the Amaryllidaceae alkaloids showing two *ortho* aromatic protons in ring A - (Table 17.11). Galanthamine **9** and lycoramine **192** are the two most abundant alkaloids in the family Amaryllidaceae plants, especially in the genera *Galanthus* and *Narcissus*.

2.1.11 Phenanthridone and Phenanthridine Types

Phenanthridone-type alkaloids have the highest oxygenated C ring in all Amaryllidaceae alkaloids, and they always show an amide group in ring B. On the contrary, alkaloids of the phenanthridine type often show completely aromatic ring system, occasionally with a methylation quaternary nitrogen atom, such as alkaloid bicolorine **214** (Table 17.12).

2.1.12 Miscellaneous

In addition to the abovementioned types of alkaloids, other minor subgroups have also been identified from plants of the family Amaryllidaceae (Table 17.13), including cherylline type, buflavine type, cripowelline type, augustamine type, gracilamine type, and so on. These scarce populations often show the unique skeleton in their molecular architectures. For instance, cripowellins A **222** and B **223** belong to structurally unique members of the Amaryllidaceae alkaloids with a 10-membered fused [5.3.2]bicyclic lactam core [46]. Furthermore, alkaloid gracilamine **227**, owning a unique pentacyclic core [47], represents the second dinitrogenous type in the Amaryllidaceae alkaloids besides plicamine-type alkaloids. Additionally, pallidiflorine **228** is the only bis-alkaloids formed directly from the junction of two different types of Amaryllidaceae alkaloids [48].

Table 17.14 Occurrence of Amaryllidaceae alkaloids in the different plant genera

Amaryllidaceae alkaloids	Plant genus/species	References
Norbelladine type		
N-demethylbelladine **12**	*Nerine filifolia*	[49]
Belladine **11**		
4′-*O*-methylnorbelladine **10**	*Crinum kirkii* Baker	[50]
2*R*-hydroxy-*O*,*N*-dimethylnorbelladine **13**	*Lycoris squamigera*	[51]
Lycorine type		
Lycoranines A **41**, B **42**	*Lycoris radiata* (L'Her)	[52]
LT1 **232**	*L. traubii* Hayward	[53]
Asiaticumines A **233**, B **234**	*Crinum asiaticum*	[54]
2-*O*-(3′-hydroxybutanoyl)lycorine **235**	*Galanthus elwesii*	[55]
Pancratinine D **236**	*Pancratium canariense*	[36]
2-*O*-(3′-acetoxybutanoyl)lycorine **237**	*Galanthus nivalis*	[56]
Amarbellisine **32**	*Amaryllis belladonna* L.	[31]
8-Hydroxylycorin-7-one **238**	*Crinum bulbispermum*	[57]
2-Deoxylycorine **239**		
Anhydropseudolycorine **240**	*Hymenocallis guianensis*	[58]
	H. lobata	
	H. tubiflora	
4,5-Dehydroanhydropseudolycorine **241**	*H. lobata*	[58]
7-Methoxyoxoassoanine **242**	*Eucharis amazonica*	[59]
Zephyranthine **33**,	*Cyrtanthus elatus*	[60]
1,2-*O*-diacetylzephyranthine **243**		
Mooreine **244**	*Crinum moorei*	[61]
Hippacine **40**	*C. bulbispermum*	[62]
1-*O*-acetyl-9-*O*-demethylpluviine **245**	*Ammocharis coranica*	[63]
Homolycorine type		
Neronine **63**	*Galanthus reginae-olgae*	[64]
9-*O*-demethylhomolycorine **50**	*G. fosteri* Baker	[65]
	G. caucasicus	[66]
	Narcissus angustifolius	[42]
Pancratinine A **66**	*Pancratium canariense*	[36]
Hippeastrine **49**	*G. elwesii* Hook	[67]
8-*O*-demethylhomolycorine **51**	*G. woronowii* A. Los.	[68]
	G. krasnawii Hohrjakov	
	G. caucasicus Bacer A.	
	G. latifolius Wor.	
	Leucojum aestivum L.	
	Sternbergia colchiciflora	
	Pancratium maritimum L.	
	Crinum giganteum L.	
	Narcissus tazetta L.	

(*continued*)

Table 17.14 (continued)

Amaryllidaceae alkaloids	Plant genus/species	References
O-methyllycorenine **55**	*Lycoris aurea*	[69]
Albomaculine **65**	*Haemanthus albiflos*	[70]
Momolycorine **3**	*Narcissus confusus*	[71]
Crinine and haemanthamine types		
8-*O*-demethylmaritidine **114**	*Lycoris radiata*	[72]
11-hydroxvitattine **108**		
Haemanthidine **111**	*L. squamigera*	[51]
Haemanthamine **5**		
Crinamine **246**	*Crinum asiaticum*	[54]
11-*O*-methylcrinamine **118**		
3-*O*-acetylhamayne **123**		
6-methoxycrinamine **247**	*C. augustum* Rox.	[73]
Buphanisine **81**		
3,3′-*O*-(3′,3″-dihydroxybutanoyl)hamayne **248**,	*Galanthus nivalis*	[55]
11,3′-*O*-(3′,3″,-dihydroxybutanoyl)hamayne **249**		
Vittatine **250**	*Pancratium canariense*	[36]
Crinine **4**	*Boophone disticha* L.	[74–76]
Buphanidrine **251**		
Distichamine **102**		
Buphanamine **101**		
Buphanisine **81**		
Powelline **91**		
Bulbispermine **127**	*Habranthus brachyandrus*	[77]
Ambelline **89**	*Nerine bowdenii*	[78]
11-*O*-acetylambelline **88**	*N. filifolia*	[49]
Undulatine **97**	*Crinum moorei*	[79]
Filifoline **90**		
Phaedranamine **133**	*Phaedranassa dubia*	[80, 81]
11-*O*-(3′-hydroxybutanoyl)hamayne **252**,	*Galanthus nivalis*	[56]
3,11-*O*-(3′,3″-dihydroxybutanoyl)hamayne **253**,		
3-*O*-(2″-butenoyl)-11-*O*-(3′-hydroxybutanoyl)hamayne **254**,		
3,11,3″-*O*-(3′,3″,3‴-trihydroxybutanoyl)hamayne **255**		
Hamayne **122**		
Maritidine **109**	*Pancratium tortuosum*	[82]
Yemenines A–C, **119–121**	*Crinum yemense*	[83]
Flexinine **256**	*Crinum kirkii* Baker	[50]
Amabiline **105**	*C. macowanii*	[84]
Macowine **83**		
Apohaemanthamine **134**	*Eucharis amazonica*	[59]

(*continued*)

Table 17.14 (continued)

Amaryllidaceae alkaloids	Plant genus/species	References
8-*O*-demethylmaritidine **114**	*Pancratium sickenbergeri*	[85]
Ent-6α/6β-hydroxybuphanisine **257**		
Zephyramine **96**	*Zephyranthes citrina* Baker	[86]
3-[4′-(8′-Aminoethyl)phenoxy]bulbispermine **258**	*Crinum moorei*	[61]
Oxomaritidine **110**	*Zephyranthes citrina* Baker	[87]
Tazettine type		
Tazettine **7**	*Lycoris squamigera*	[51]
6-*O*-methylpretazettine **140**	*Eucharis amazonica*	[59]
Criwelline **135**	*Crinum asiaticum L. var. sinicum Baker*	[54]
Alkaloid **148**	*Narcissus serotinus* L.	[88]
6a-Deoxytazettine **138**	*Pancratium maritimum*	[89]
3-Epimacronine **144**	*Sprekelia formosissima*	[90]
Pretazettine **139**	*Hymenocallis x festalis*	
3-*O*-(3-hydroxybutyryl) tazettinol **136**	*Galanthus plicatus*	[41]
Montanine type		
Squamigine **158**, montanine **8**	*Lycoris squamigera*	[51]
Pancratinine B **157**, pancracine **150**	*Pancratium canariense*	[36]
Manthidine **154**	*Haemanthus pauculifolius*	[70]
Coccinine **155**	*H.deformis*	
2-Demethoxymontanine **156**	*Galanthus ikariae*	[91]
	Narcissus tazetta	
Nangustine **151**, pancracine **150**	*N. angustifolius*	[42]
Plicamine type		
Plicamine **159**, secoplicamine **160**	*Galanthus plicatus*	[43]
Plicane **161**	*G. plicatus*	[41]
Obliquine **162**	*Cyrtanthus obliquus*	[92]
Graciline type		
Graciline **163**	*Galanthus gracilis*	[93]
3,4-Dihydro-3-hydroxygraciline **165**,	*G.gracilis*	[41]
3-*Epi*-3,4-dihydrograciline **166**		
11-Acetoxygraciline **164**	*G. plicatus*	
Digracine **167**	*G.gracilis*	[93]
Galanthindole type		
Alkaloids **171–174**	*Narcissu* cultivar “Dutch Master”	[45]
Lycosinines A **169**, B **170**	*Lycoris aurea*	[69]
Galanthusin **259**	*Galanthus caucasicus*	[66]
Galanthindole **168**	*G. plicatus*	[94]

(*continued*)

Table 17.14 (continued)

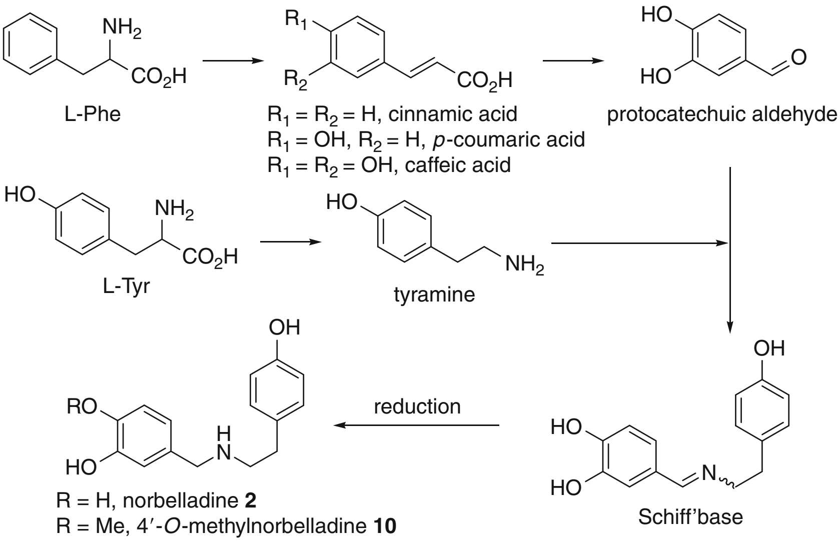
Amaryllidaceae alkaloids	Plant genus/species	References
Galanthamine type		
3-*O*-acetylhabranthine **187**	*Pancratium canariense*	[95]
Alkaloids **188**, **189**	*Leucojum aestivum*	[96]
Galanthamine **9**	*Lycoris guangxiensis*	
Epinorgalanthamine **179**		
Narwedine **185**		
Sanguinine **180**	*Phaedranassa dubia* H.B.&K.	[81]
Leucovernine **190**, acetylleucovernine **191**	*Leucojum vernum*	[97]
O-demethylgalanthamine **260**,	*Lycoris aurea*	[69]
O-demethyllycoramine **196**		
Sanguinine **180**, 3-*O*-acetylsanguinine **182**	*Crinum kirkii* Baker	[50]
N-formylnorgalanthamine **177**	*Pancratium maritimum*	[89]
Phenanthridone and phenanthridine types		
Bicolorine **214**,	*Lycoris radiate*	[72]
Trisphaeridine **212**	*Crinum yemense*	[83]
	Cyrtanthus obliquus	[92]
Narciclasine **6**, lycoricidine **197**	*L. traubii* Hayward	[53]
Pancratistatin **202**, narciclasine **6**, 7-deoxynarciclasine **197**, 7-Deoxy-*trans*-dihydronarciclasine **201**	*Hymenocallis littoralis*	[98, 99]
	Brachystola magna	[100]
3-Methoxy-3,4-dihydrotrisphaeridine **213**	*Hymenocallis x festalis* Hort. ex Schmarse	[101]
Miscellaneous		
Cherylline **218**	*Crinum moorei*	[61, 79]
	C. macowanii	[102]
Cripowellins A **222**, B **223**	*C. powellii*	[46]
Noraugustamine **225**, 4α,*N*-didehydronoraugustamine **226**	*C. kirkii* Baker	[50]
Gracilamine **227**	*Galanthus gracilis*	[47]

2.2 Occurrence of Amaryllidaceae Alkaloids

Recent examples for each type of Amaryllidaceae alkaloids isolated from the wild, cultivar, and intersectional hybrid species are summarized in Table 17.14.

3 Biosynthesis

Studies on the biosynthesis of Amaryllidaceae alkaloids initiated as early as the 1960s. In 1957, Barton and Cohen proposed for the first time that norbelladine **2** or its congener alkaloid was probably the original precursor of other structurally diverse Amaryllidaceae alkaloids [103]. The primary metabolic processes leading to the Amaryllidaceae alkaloids include mainly (a) intramolecular phenol


L-Phe
R1 = R2 = H, cinnamic acid
R1 = OH, R2 = H, p-coumaric acid
R1 = R2 = OH, caffeic acid
protocatechuic aldehyde
L-Tyr
tyramine
reduction
R = H, norbelladine 2
R = Me, 4′-O-methylnorbelladine 10
Schiff'base

Scheme 17.2

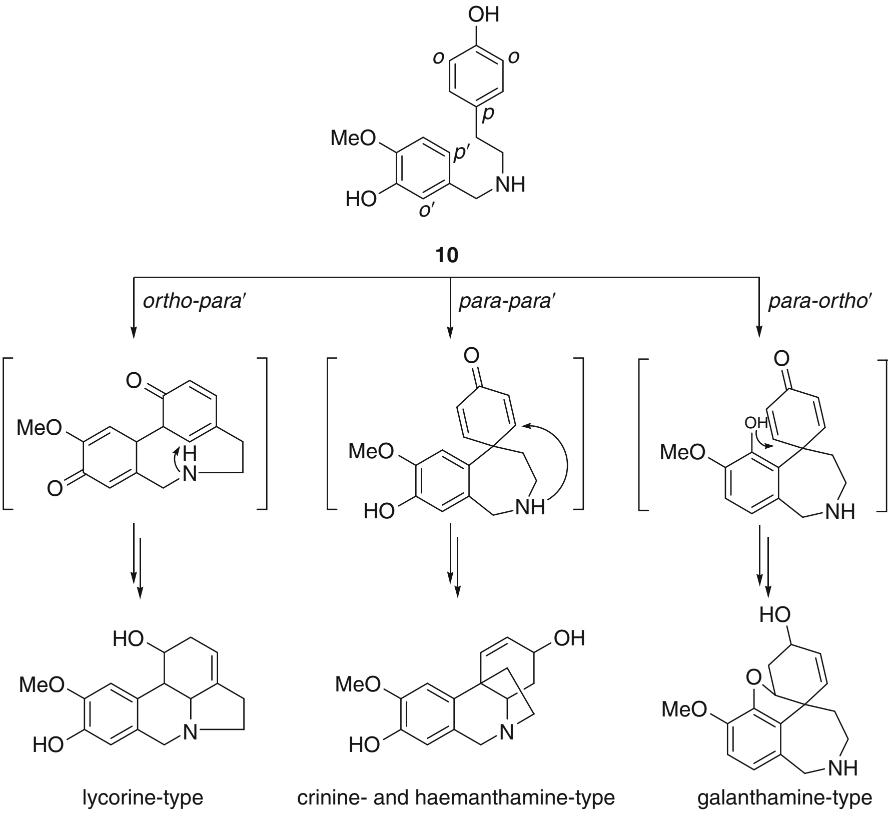
oxidative coupling cyclization, (b) *O*- and *N*-methylation, (c) hydrogenation reduction of $C = O$ and $C = N$ double bonds, (d) oxidation of *C–O* and *C–H* bonds to $C = O$ and *C–OH* bond, and (e) enzyme-catalyzed epoxidation of $C = C$ double bond.

3.1 Norbelladine Type

In plants, norbelladine is produced from the condensation of protocatechuic aldehyde, and tyramine and the latter two metabolites arise from the naturally occurring L-Phe and L-Tyr, respectively (Scheme 17.2). Hydrogenation reduction of the resulting Schiff's base affords alkaloid norbelladine **2**, which is further converted into 4′-methylnorbelladine **10** after methylation on the 4′-*O* position. Generally, alkaloid 4′-methylnorbelladine **10** was considered as the key precursor for alkaloids of this family [104]. Alkaloid 4′-methylnorbelladine **10** can be converted into other structurally complicated alkaloids through three different oxidative coupling approaches: (1) *ortho–para′*, (2) *para–para′*, and (3) *para–ortho′* (Scheme 17.3).

3.2 Lycorine and Homolycorine Type

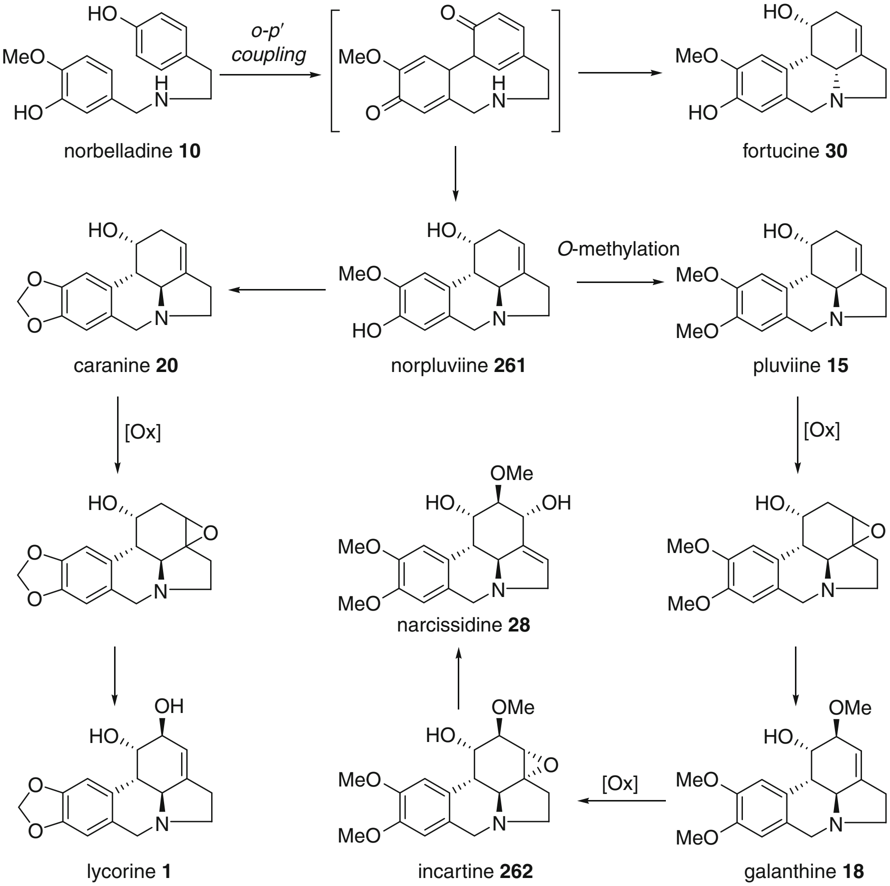
In the biosynthesis of lycorine **1**, alkaloid norpluviine **261** and its congener fortucine **30** were demonstrated as the first intermediate formed via *ortho–para′* phenol oxidative coupling of 4′-methylnorbelladine **10** based on the labeling


OH
o
o
p
MeO
p′
HO
o′
NH
10
ortho-para′
para-para′
para-ortho′
O
MeO
H
N
O
MeO
HO
NH
O
OH
MeO
NH
HO
MeO
HO
N
MeO
HO
N
OH
HO
O
MeO
NH
lycorine-type
crinine- and haemanthamine-type
galanthamine-type

Scheme 17.3

experiments (Scheme 17.4) [105–107]. The hydroxyl group at C2 in lycorine is incorporated via an epoxidation of $C = C$ double bond followed by a sequence of ring opening and allylic rearrangement [108–110]. In a similar manner, norpluviine **261** is converted into alkaloid galanthine **18** after 8-*O*-methylation. In addition, galanthine is considered the probable precursor of alkaloid narcissidine **28**, which is formed through an analogous epoxidation process [111]. The conversion of the *O*-methoxyphenol to the methylenedioxy group may occur late in the biosynthetic pathway [105].

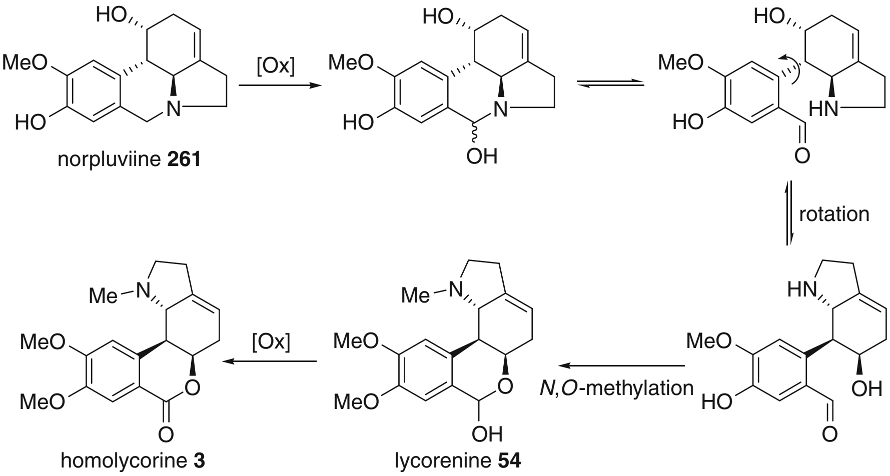
The conversion of norpluviine **261** into homolycorine **3** has been validated via the benzylic oxidation at C6 position followed by B-ring opening to form an amino aldehyde with a free hydroxyl group, which gives alkaloid lycorenine **54** after *N,O*-methylation [112]. Finally, a subsequent oxidation produces homolycorine **3** (Scheme 17.5). Recently, the transformation from lycorine-type skeleton into homolycorine-type alkaloid has been experimentally demonstrated through a total synthesis of alkaloid clivonine **76** [113].


o-p′ coupling
norbelladine 10
fortucine 30
O-methylation
caranine 20
norpluviine 261
pluviine 15
[Ox]
[Ox]
narcissidine 28
[Ox]
lycorine 1
incartine 262
galanthine 18

Scheme 17.4

3.3 Crinine and Haemanthamine, Tazettine, Narciclasine, and Montanine Types

Crinine- and haemanthamine-type alkaloids are derived from norbelladine **10** through an intramolecular "*para–para′*" oxidative coupling sequence as shown in Scheme 17.3 [114, 115]. Additionally, the feeding labeling experiments showed that tazettine-, narciclasine-, and montanine-type alkaloids also originated from the crinine- and haemanthamine-type 5,10b-ethanophenthridine skeletons (Scheme 17.6). The biosynthetic conversion of haemanthamine **5** to tazettine-type alkaloids was demonstrated by feeding tritium-labeled alkaloids to the species *Sprekelia formosissima* [116]. In plant, haemanthamine **5** is converted to haemanthidine **111** by oxidation at C6 and subsequent to pretazettine **139** in

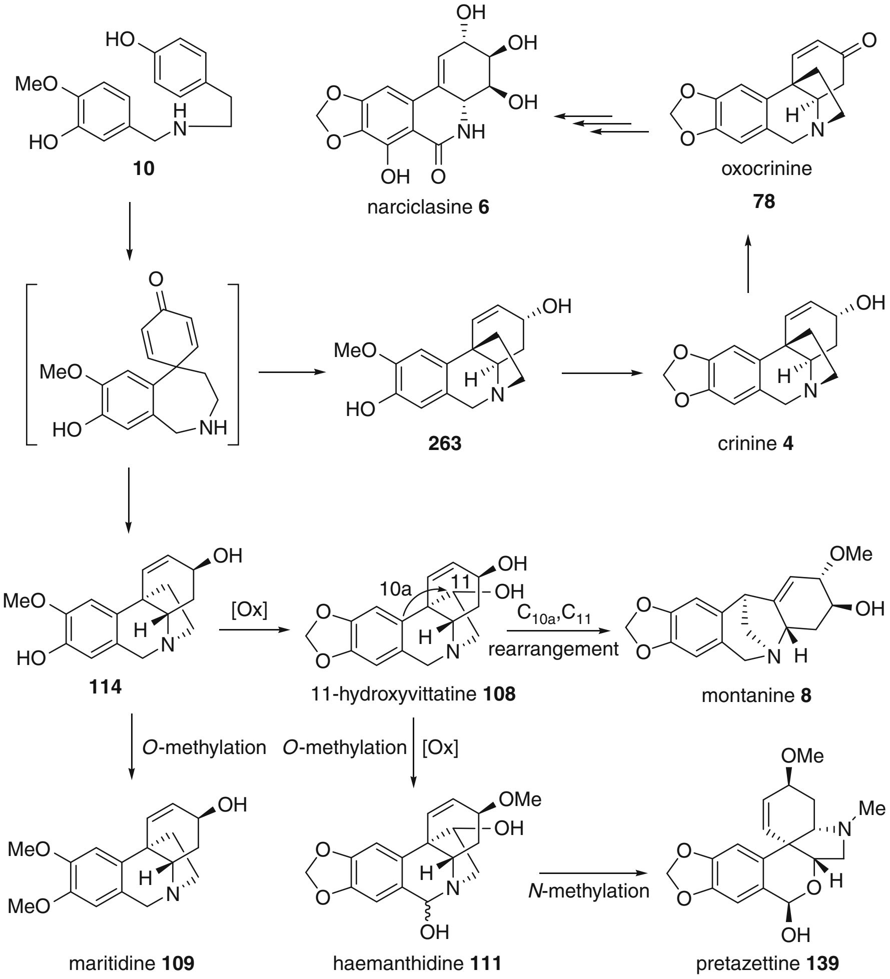


Scheme 17.5

an essentially irreversible manner. Phenanthridine alkaloid narciclasine **6** was proved to be derived from oxocrinine **78** by the loss of the two-carbon ethane bridge [117, 118]. Furthermore, oxocrinine **78** is also the probable precursor of alkaloid ismine **216** [119]. Based on labeling studies in the species *Rhodophiala bifida*, 11-hydroxyvittatine **108** was indicated as the intermediate in the biosynthetic pathway of alkaloid montanine **8** [115].

3.4 Galanthamine Type

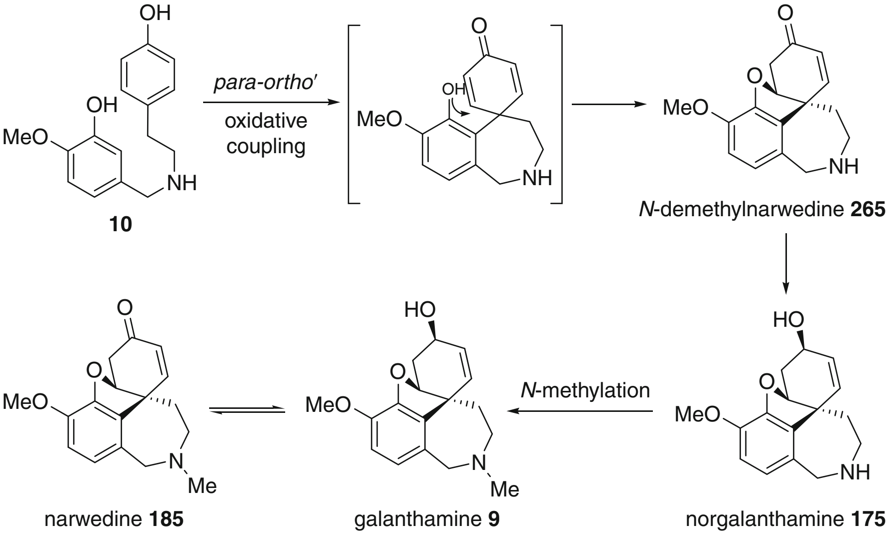
Galanthamine-type alkaloids originated from the intramolecular *para-ortho′* oxidative coupling of 4′-*O*-methylnorbelladine **10** (Scheme 17.3). Initial studies showed that the intramolecular *para-ortho′* oxidative coupling does not proceed from 4′-*O*-methylnorbelladine **10** but from *N,O*-dimethylnorbelladine **264** [120], which has been isolated from the species *Pancratium maritimum* along with galanthamine **9** [121]. However, subsequent ^{13}C-labeled experiments in *Leucojum aestivum* demonstrated that the phenol oxidative coupling does proceed from 4′-*O*-methylnorbelladine **10** to form a postulated dienone, which gives alkaloid norgalanthamine **175** after reduction of $C = O$ double bond at C3 [122]. The final step of biosynthesis involves *N*-methylation of norgalanthamine **175** to galanthamine **9** [Scheme 17.7]. In addition, narwedine 185 is not the direct precursor of galanthamine **9** and could be possibly interconvertible with galanthamine in plants.


10
narciclasine 6
oxocrinine
78
263
crinine 4
114
[Ox]
11-hydroxyvittatine 108
10a
11
C_{10a},C_{11}
rearrangement
montanine 8
O-methylation
O-methylation
[Ox]
maritidine 109
haemanthidine 111
N-methylation
pretazettine 139

Scheme 17.6

3.5 Miscellaneous

With continuous phytochemistry studies, some new skeleton-type alkaloids have been isolated from plants of this family in recent years and the biosynthesis of these alkaloids still remains unexploratory. Notwithstanding, their biosynthesis can be tentatively deduced from crinine- and haemanthamine-type alkaloids according to the structural characteristics (Scheme 17.8). The above-mentioned tentative biogenetic proposals for novel alkaloids should be further verified by feeding experiments with isotopically precursors as well as

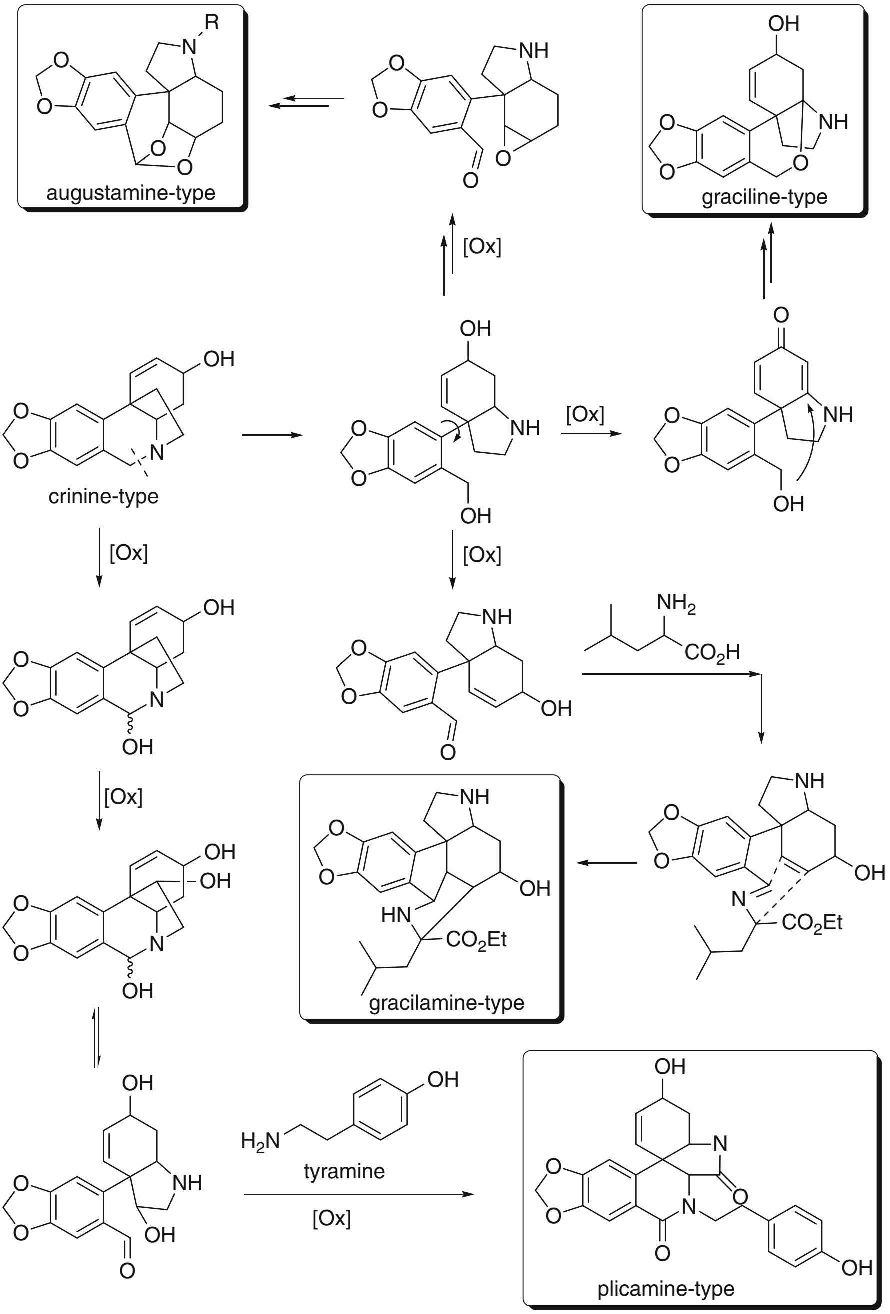

OH
OH
MeO
NH
10
para-ortho'
oxidative
coupling
O
OH
MeO
NH
O
O
MeO
NH
N-demethylnarwedine **265**
O
O
MeO
N
Me
narwedine **185**
HO
O
MeO
N
Me
galanthamine **9**
N-methylation
HO
O
MeO
NH
norgalanthamine **175**

Scheme 17.7

synthetic studies. In fact, some recent synthetic examples have implicated the possible biosynthesis. For example, analogues of norbelladine could be readily converted into the tetrahydroisoquinoline skeleton of the cherylline-type Amaryllidaceae alkaloids via an oxidation–cyclization sequence [123].

4 Biological Activity

Alkaloids isolated from the plants of Amaryllidaceae family show diverse biological activities, such as antitumor, antibacterial, antifungal, antimalarial, antiviral, analgesic, and AChE inhibitory activities. Among them, the well-known galanthamine **9** has been commercialized as a selective and reversible AChE inhibitor for the treatment of Alzheimer's disease. Amaryllidaceae isocarbostyril derivatives, such as narciclasine, pancratistatin, and their congeners, are very promising antitumor agents. Recently, the anticancer potential of Amaryllidaceae alkaloids and their synthetic derivatives has been reviewed [124]. In addition, lycorine and its derivatives as novel lead compounds for anticancer drug design have also been summarized very recently [125]. The biological activities of alkaloids isolated from the genera *Pancratium* [5], *Crinum* [15, 18], and *Narcissus* [9, 14] have also been summarized in the previous reviews [17, 19]. Recent reports about the biological activities of Amaryllidaceae alkaloids are listed in Table 17.15.


augustamine-type
graciline-type
crinine-type
[Ox]
[Ox]
[Ox]
[Ox]
[Ox]
gracilamine-type
tyramine
[Ox]
plicamine-type

Scheme 17.8

Table 17.15 Biological activities of Amaryllidaceae alkaloids

Amaryllidaceae alkaloids	Biological activities	References
Lycorine type		
Amarbellisine **32**	Antiproliferative activity	[126]
2-*O*-acetyllycorine **22**	Apoptosis-inducing effect	[127]
Lycorine **1**	Apoptosis-inducing effect	[128–130]
	Antitumor activity	[131, 132]
	Antiviral activity	[133, 134]
	Anti-inflammatory effect	[135]
	Antifungal, antimicrobial	[136]
	Antimalarial effect	[137]
	AChE inhibitory activity	[138]
	Antiretroviral activity	[139]
Pratorinine **266**	Antimicrobial effect	[140]
1-*O*-acetyllycorine **267**	AChE inhibitory activity	[141, 142]
Ungeremine **44**	AChE inhibitory activity	[143]
3-*O*-acetylnarcissidine **27**	Antifeedant action	[144]
Homolycorine type		
Homolycorine **3**	Antitumor activity	[145]
	Antiretroviral activity	[139]
8-*O*-demethylhomolycorine **51**	Antitumor activity	[145]
	DNA-binding activity	[146]
Dubiusine **62**	Antitumor activity	[145]
	DNA-binding activity	[146]
Lycorenine **54**	Antitumor activity	[145]
	DNA-binding activity	[146]
Hippeastrine **49**	Antiviral activity	[147]
	Antifungal activity	[148]
Crinine and Haemanthamine type		
6-Methoxycrinamine **247**	Antifouling activity	[73]
Crinamine **246**		
Buphanisine **81**		
Buphanamine **101**	Inhibitory activity against the serotonin transporter	[75, 76]
Buphanidrine **251**		
Buphanisine **81**		
Distichamine **102**		
11-*O*-acetylambelline **88**	AChE inhibitory activity	[149]
Haemanthamine **5**	Antimalarial activity	[80, 137]
6-Hydroxyhaemanthamine **267**		
Crinine **4**	Apoptosis-inducing activity	[150–152]
Haemanthamine **5**		
Crinamine **246**		
Ambelline **89**		
Amabiline **105**		
Josephinine **106**		

(*continued*)

Table 17.15 (continued)

Hamayne **122**	AChE inhibitory activity	[138]
Haemanthamine **5**	Antiretroviral activity	[139]
Tazettine type		
Pretazettine **139**	Apoptosis-inducing activity	[127]
8α-Ethoxyprecriwelline **143**	affinity to the serotonin reuptake transport protein	[153]
N-demethyl-8α-ethoxypretazettine **141**		
N-demethyl-8β-ethoxypretazettine **142**		
Tazettine **7**	Weak antimalarial activity	[137]
Montanine type		
Montanine **8**	Antiproliferative activity	[154]
	AChE inhibitory activity	[155]
	Antimicrobial activity	[156]
	psychopharmacological activities including anxiolytic, antidepressive, and anticonvulsive effects	[157]
Galanthamine type		
3-*O*-acetylhabranthine **187**	AChE inhibitory activity	[95]
Norgalanthamine **175**	Promoting proliferation of dermal papilla	[158]
Galanthamine **9**	AChE inhibitory activity	[91, 159]
N-allylnorgalanthamine **188**	AChE inhibitory activity	[96]
N-(14-methylallyl) norgalanthamine **189**		
Phenanthridone and phenanthridine types		
Narciclasine **6**	Pleiotropic cytostatic effect	[160]
	Antiproliferation	[161]
	Antitumor effect	[162]
	Apoptosis-inducing cytotoxicity	[163]
	Antitumor, plant growth regulator activities	[164]
	Inhibitory activity on protein synthesis	[165]
Pancratistatin **202**	Antineoplastic activity	[166, 167]

5 Conclusion

So far, a great deal of the structurally diverse alkaloids have been isolated and identified from plants of the family Amaryllidaceae. However, given large numbers of the unexploratory plant species in this family, one can expect that more new alkaloids will be isolated from the plant species of this family in the future. In addition, although studies about biosynthesis of Amaryllidaceae alkaloids initiated as early as the 1960s, biosynthetic pathways of Amaryllidaceae alkaloids, especially those newly established scarce subgroups, are far away from complete comprehension to date. With continuous occurrence of new skeleton-type Amaryllidaceae alkaloids, considerable efforts aiming to explore the biogenetic origin of these alkaloids are still required.

In view of the significantly biological activities exhibited by Amaryllidaceae alkaloids, development of new phytomedicines derived from these natural products is of great importance. For instance, alkaloid galanthamine **9** has been launched into market for the treatment of Alzheimer's disease in Western countries in 2001 as a selective AChE inhibitor [168]. Besides, Amaryllidaceae isocarbostyril alkaloids, such as narciclasine **6**, pancratistatin **202**, and their hemisynthetic derivatives, are also in the preclinical development as very promising antitumor agents. Due to their superior pharmacological profiles as well as limited availability from nature, chemical synthetic approaches to these plant metabolites and related analogues represent an important vehicle for investigation of biological activity. Meanwhile, biotechnological approaches to these alkaloids also provide an inexpensive, efficient, and practical alternative [169].

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Bioactive Alkaloids from Fungi: Psilocybin 18

Zafar Alam Mahmood

Contents

1 Introduction 524
2 Occurrence 526
3 Phytochemistry 529
4 Biosynthesis 536
5 Biological Acitivies 540
5.1 Pharmacology (Pharmacodynamics and Pharmacokinetics) 541
5.2 Physiological and Psychological Effects 542
5.3 Therapeutic Role (Advanced Stage Cancer, Cluster Headaches, Obsessive-Compulsive Disorders) 543
5.4 Toxicity (Side Effects and Risks) 543
6 Biotechnological Approaches 544
7 Conclusion/Prospects 545
References 545

Abstract

Studies on bioactive alkaloids have gained considerable importance in recent years and have been given prominent position in the field of medicine, both with respect to their biological activity and role played in the introduction of new pharmaceuticals. The biotechnological approach has further enhanced their industrial applications. Some specific groups of bioactive alkaloids have been ignored and not been given much attention because of various reasons, including their legal status. The tryptophan-based indole alkaloids, psilocybin and psilocin, are the best examples in this category which have been identified as most attractive bioactive alkaloids in large number of mushrooms, especially of the genus *Psilocybe*, but waiting since long for more control studies to ascertain

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their therapeutic role in some other conditions, apart from psychiatry. These alkaloids have long history of association with mankind for their use as sacraments in religious ceremonies along with medical and recreational purposes and thus need more attention to explore their therapeutic role.

Keywords
Hallucinogenic mushroom • magic mushroom • neurotropic fungi • psilocin • psilocybe • psilocybin • psychedelic

Abbreviations

5HT	5-Hydroxytryptamine
CNS	Central Nervous System
DMACA	Dimethylaminocinnamaldehyde
DMT	Dimethyltryptamine
GC/MS	Gas chromatography coupled with mass spectrometry
INCB	International Narcotics Control Board
LSD	Lysergic acid diethylamide
OCD	Obsessive-compulsive disorders

1 Introduction

Alkaloids have occupied a unique and prominent position in both science and culture due to their diversified source of availability, biochemical properties, and applications. It represents one of the largest groups of natural products which almost all phyla are capable to synthesize at any stage of their life cycle. Fungi (mushrooms), in particular, are able in common along with the higher plants and bacteria to produce alkaloids. The indoleamines (four-substituted compounds) found in mushroom have a long history of investigation and research topic for various laboratories and institutions all over the world, and as a result, more than five decades back in 1958, Dr. Albert Hofmann, the discoverer of LSD, further reported two most fascinating bioactive alkaloids – Psilocybin and Psilocin – from mushroom *Psilocybe mexicana*. Both alkaloids are derived from L-tryptophan. Later, he also developed a synthesis technique for the production of psilocybin and psilocin [1, 2]. The chemical synthesis of psilocybin opened a new vista which ended with the introduction of first synthetically developed psilocybin under the brand name of "Indocybin" marketed by Sandoz for experimental and psychotherapeutic purposes in the 1960s. Psilocybin, also known as psilocybine, is classified as neurotropic or psychedelic indole alkaloid of the tryptamine family, with mushrooms of the genus *Psilocybe*, commonly known as hallucinogenic mushroom or magic mushroom [3] as the most common source. Various species of genus *Psilocybe* are found all over the world in most terrestrial (land) biomes, and more than 100 species have been so far identified and reported, but their potential to produce psilocybin is still to be determined because in some species the level of psilocybin and psilocin are reported as nondetectable. *Psilocybe cubensis* is considered as the most widely known species capable of producing psilocybin and psilocin in considerable quantity, though originally both alkaloids were isolated from *Psilocybe mexicana* [4]. The capability to produce psilocybin and psilocin varies among species on dry weight basis of mushroom and dependent upon various biochemical factors and the parts (mycelium or fruit bodies) of the mushroom used for processing and extraction. The fruit bodies have more alkaloid content than the mycelium. The rate limiting factors or the critical steps in the analysis of psilocybin and psilocin in mushrooms are the techniques used for the extraction, separation, and purification. The biotechnological approach and its utilization have greatly affected the production capacity of these alkaloids using submerged fermentation technology by manipulating media composition and growth parameters as well as with advanced extraction and purification techniques.

The chemistry of psilocybin and psilocin is not complex. Chemically, psilocybin is 4-phosphoryloxy-*N,N*-dimethyl-tryptamine, whereas psilocin is 4-Hydroxy-*N, N*-dimethyl-tryptamine. Psilocybin is regarded as prodrug which is finally converted into psilocin – the pharmacologically active form of the drug in the body by a dephosphorylation reaction and thus acts as a partial agonist at the serotonin receptor (5-HT$_{\mathbf{2A}}$), with high affinity in the brain where it mimics the effects of serotonin (5-HT). Based on this mechanism of action, psilocin is classified as a 5-HT$_{\mathbf{1A}}$, 5-HT$_{\mathbf{1D}}$, 5-HT$_{\mathbf{2A}}$, and 5-HT$_{\mathbf{2C}}$ agonist, and accordingly its psychotomimetic effect can be blocked by 5-HT$_{\mathbf{2A}}$ antagonists, such as ketanserin and risperidone in a dose-dependent pattern [4].

In view of its nature and classification as a neurotropic or psychedelic, psilocybin is capable of acting on CNS, crossing the blood–brain barrier and consequently altering perception, mood, consciousness, cognition, and behavior in a dose-dependent fashion. The use of *Psilocybe* (mushroom) and its two constituents, psilocybin and psilocin, remains very popular in the USA and Europe till the mid-1960s for its recreational and therapeutic purposes when finally in 1968 all three (mushroom and its two alkaloids) were declared illegal in the USA, and as a result psilocybin and psilocin were added to the new "Drug Abuse Prevention and Control Act of 1970" (commonly known as Controlled Substance Act), which came into force in 1971 and both listed in Schedule I drugs under the United Nations Convention on Psychotropic Substances. Despite all these, research actives continued till mid-1970s, and research activities continued to be halted throughout the 1980s and 1990s due to strict governmental control worldwide. The restrictions imposed worldwide during these periods have greatly affected the research activities on psilocybin and psilocin, preventing researchers to investigate the biological activities to ascertain biochemical and pharmacological properties of these two psychedelic indole alkaloids. So far, psilocybin has not been recognized as drug but has been extensively investigated in the treatment of various psychological disorders. However, findings of various investigations are not only highlighting the possible role and importance of psilocybin but also suggesting and demanding more organized and controlled studies to explore its role in the treatment of various mental conditions, including chronic cluster headaches and obsessive-compulsive disorders (OCD) and OCD-related clinical depression.

2 Occurrence

The archaeological evidences available throughout different cultures indicate history of psilocybin mushrooms as quite ancient. Though it is really difficult to correlate and extend any scientific note on such a diversified and scattered subject, yet the mushroom-shaped statuettes or mushroom-formed stones and several Mesolithic rock paintings reported at different archaeological sites, such as Guatemala, El Salvador, Mexico, and Tassili n'Ajjer (a prehistoric North African site identified with Caspian culture), and anthropological literatures further support the presence and history of traditional use of these mushrooms that dates back as far as 2000 BC [5, 6]. The period between fourteenth and sixteenth century remains much popular with respect to use of psilocybin mushrooms when Aztecs (ruler Moctezuma II) and Chichimecas (Nahua people of Mexico) were also reported as users of such mushrooms which they called "teonanacatl" (good mushrooms or flesh of the gods or wondrous mushroom, etc.). In the Western medical literature, the first scientific report appeared in 1799 when a critical article was published in London Medical and Physical Journal with the title "On a poisonous species of agaric." There is hardly any scientific study or report appeared between 1799 and 1899 on psilocybin mushrooms. Some activity was finally been reported by scientists working on psilocybin mushrooms in Harvard University, USA, during the end of the 1930s and 1940s [7] followed by the introduction of the word "magic mushrooms," appeared for the first time in Western literature in 1957 to describe the hallucinogenic property of psilocybin mushrooms [3]. The subsequent studies resulted finally in the discovery of both psilocybin and psilocin in 1958 [1].

Psilocybin mushrooms are quite diversified in nature and thus geographically have a wide range of distribution worldwide which covers both tropical and subtropical regions. These include Mexico, Cuba, Guatemala, Colombia, Bolivia, Brazil, Chile, Argentina, the USA (Florida, Vancouver, Washington, Oregon, California), Canada, England, Norway, Finland, the Netherlands, Germany, Austria, Spain, Thailand, Vietnam, India, Sri Lanka, Nepal, Cambodia, Indonesia, the Philippines, Japan, and Australia. Some species of Psilocybe are reported exclusively in Mexico and Guatemala. Mexico has the highest number of *Psilocybe* species (44 out of total 76 neurotropic fungal species), representing 39% of the total species reported worldwide [8–10]. Being classified as saprotrophs, psilocybin mushrooms can grow on various kinds of decaying organic matters in most biomes, with the exception of high deserts. Based on extensive work of various researchers and their findings reported elsewhere, presence of psilocybin has been reported in large number of species belonging to about 25 different genera [11–13]. These include *Agrocybe, Amanita, Conocybe, Copelandia, Coprinus, Entoloma,*

Table 18.1 Psilocybin and psilocin content of some major species of *Psilocybe*

	Alkaloidal content (%)[a]			
Species	Psilocybin	Psilocin	Baeocystin	References
P. azurescens	1.70	0.38	0.35	[15]
P. baeocystis	0.85	0.59	0.10	[16, 17]
P. bohemica	1.34	0.11	0.02	[18, 19]
P. cubensis	0.63	0.60	0.025	[19, 20]
P. cyanescens	0.85	0.36	0.03	[16, 21]
P. cyanofibrillosa	0.21	0.04	0.00	[22]
P. hoogshagenii	0.60	0.10	0.00	[23]
P. liniformans	0.16	0.00	0.005	[21]
P. pelliculosa	0.12	0.00	0.00	[24]
P. samuiensis	0.36	0.21	0.02	[25]
P. semilanceata	0.98	0.02	0.36	[19]
P. semperviva	0.30	0.07	0.00	[23]
P. subcubensis	0.80	0.02	0.00	[26]
P. stuntzii	0.36	0.12	0.02	[16, 24]
P. tampanensis	0.68	0.32	0.00	[19]
P. weilii	0.61	0.27	0.05	[6]

[a] Average content and may vary in different regions due to environmental conditions and parts used

Galerina, Gerronema, Gymnopilus, Hygrocybe, Hypholoma, Inocybe, Marasmius, Mycena, Naematoloma, Panaeolina, Panaeolopsis, Panaeolus, Pholiota, Pluteus, Psathyra, Psathyrella, Psilocybe, Rickenella, and Stropharia. Out of 216 known species of neurotropic fungi reported elsewhere, most (116 species) fall in the genus *Psilocybe* [9], and out of 190 different mushrooms tested, 90 species have been identified to contain either psilocybin or psilocin or both with genus *Psilocybe*, showing the highest numbers (41 out of 55 species or varieties) in terms of psilocybin or psilocin content [14]. Few species of genus *Psilocybe* are quite prominent in producing psilocybin and psilocin, such as *P. azurescens, P. baeocystis, P. bohemica, P. cubensis, P. cyanescens, P. cyanofibrillosa, P. hoogshagenii, P. liniformans, P. pelliculosa, P. samuiensis, P. semilanceata, P. semperviva, P. subcubensis, P. stuntzii, P. tampanensis*, and *P. weilii* (Table 18.1).

Mushroom species with high psilocybin and psilocin content belonging to genera other than *Psilocybe* include *Agrocybe praecox, Conocybe cyanopus, Copelandia cambodginiensis, Gymnopilus purpuratus, Inocybe aeruginascens, Panaeolus subbalteatus*, and *Pluteus salicinus* (Table 18.2).

The quantity of psilocybin as mentioned in Tables 18.1 and 18.2 is quite variable (approximately 0.5–2.0% dry weight) and dependent on several factors, including age (mature mycelium contains more than the young one), growing and drying conditions (heat and light can affect alkaloidal content), and specific part of the mushroom used for estimation of psilocybin and other alkaloids. In some cases, variation in psilocybin content may be recorded up to 4x for species grown under controlled condition and as much as 10x for ones that are not.

Table 18.2 Psilocybin and psilocin content of some genera other than *Psilocybe*

	Alkaloidal content (%)[a]			
Species	Psilocybin	Psilocin	Baeocystin	References
Agrocybe praecox	0.80	0.00	0.00	[26]
Conocybe cyanopus	0.93	0.70	0.03	[16, 17, 27]
Copelandia cambodginiensis	0.30	0.13	0.02	[28]
Gymnopilus purpuratus	0.34	0.29	0.05	[19]
Inocybe aeruginascens	0.40	0.11	0.20	[25]
Panaeolus subbalteatus	0.16	0.00	0.08	[17, 21]
Pluteus salicinus	1.20	0.00	0.00	[29]

[a] Average content and may vary in different regions due to environmental conditions and parts used

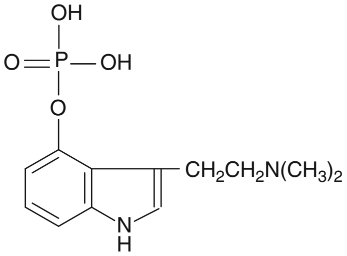
The word *Psilocybe* is derived from Greek roots – *psilos* and kube – which means "bald head" or "bare headed." *Psilocybe* is moderately large genus, with a variety of species. The characteristic macroscopic and microscopic features and taxonomy all are well documented elsewhere comprehensively [5, 6, 8–10, 15, 22, 30] and thus are beyond the scope of the present title for discussion. Precisely, the species of the genus *Psilocybe* are small brown to tan mushrooms with a primary distinguishable feature that they bruise blue when handled or injured. Though *Psilocybe* is placed taxonomically in the agaric (*Agaricales*) family *Strophariaceae* based upon spore and pileipellis morphology, yet the genus is quite complex in terms of classification and thus difficult to separate from genera *Stropharia* and *Hypholoma*, and all three are no doubt closely related. According to some authors, the relationship is close enough to combine these genera into one genus, *Psilocybe* [31]. It has long been suspected that the mushrooms in *Pholiota, Stropharia, Hypholoma, and Psilocybe* share a common ancestor. The current interpretation of DNA has resulted in a different way, suggesting *Psilocybe* comprising of two groups (one producing psilocybin and other does not) that are only distantly related to each other and both are distantly related to *Stropharia*. Presently, both groups are classified under *Psilocybe* but in fact resembling more to *Hypholoma* and *Pholiota* than to *Stropharia* [32, 33]. The DNA studies of mushrooms, no doubt, are revealing some new findings and relationships that were previously unsuspected, but the technology is still unable to answer a number of hidden questions which mycologist and molecular biologists may be interested to know to reach to final conclusion. The overall distribution of psilocybin among various genera has been reported as *Psilocybe* (116 species), *Gymnopilus* (14 species), *Panaeolus* (13 species), *Copelandia* (12 species), *Hypholoma* (6 species), *Pluteus* (6 species), *Inocybe* (6 species), *Conocybe* (4 species), *Panaeolina* (4 species), *Gerronema* (2 species), and *Agrocybe*, *Galerina*, and *Mycena* (1 species each) [9]. The mushroom caps contain more psilocybin or psilocin than the stem, while spores do not contain either psilocybin or psilocin [34, 35]. Most references cited elsewhere [10, 36–39] suggest that the distribution of psilocybin mushrooms may have their origin in southern hemisphere, mainly in South America and Australia and to some extent in South Africa because of the highly diversified geographical and climatic conditions in these regions.

The lower level of industrialization, smaller landmasses, and overall low population densities may have contributed well in the growth and propagation of enormous number of mushroom species in these regions. The historical findings also suggest the traditional users of psilocybin mushrooms among various ethnic groups located in Mexico and Colombia (South America), New Guinea (greater Indo-Australian Archipelago) and in Africa. From South America, it is believed to have further migrated to northern parts (North America, Canada, and Europe), while from Australia, it may have reached to Southeast Asia, South Asia, and other parts of Asia Pacific (Table 18.3).

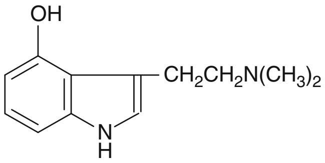
3 Phytochemistry

The primary bioactive alkaloids reported in most *Psilocybe* mushrooms include psilocybin and psilocin and to a lesser extent baeocystin and norbaeocystin **(1–4)**.

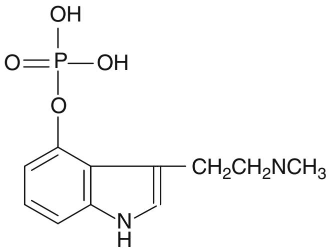
Psilocybin: 4-phosphoryloxy-*N*,*N*-dimethyltryptamine;
3-[2-(dimethylamino)ethyl]-1 H-indol-4-ol-dihydrogen phosphate
Psilocin: 4-hydroxy-*N*,*N*-dimethyltryptamine;
3-[2-(dimethylamino)ethyl]-1-H-indol-4-ol
Baeocystin: 4-phosphoryloxy-N-methyltryptamine
Norbaeocystin: 4-phosphoryloxytryptamine.



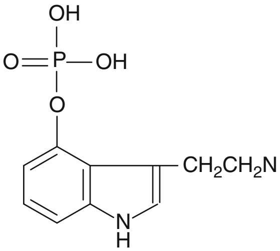
Psilocybin
1



Psilocin
2



Baeocystin
3



Nor-baeocystin
4

Apart from these four bioactive indole alkaloids, *Psilocybe* mushrooms are capable of producing a number of indole derivatives (Table 18.4). Psilocybin has the molecular formula $C_{12}H_{17}N_2O_4P$ (mol. weight = 284.27). It is a prodrug that is

Table 18.3 Occurrence of psilocybin and psilocin in various species of mushrooms

Genus/Species	1	2	Country	References
Agrocybe				
Agrocybe farinacea Hongo	+	–	Japan	[40]
Agrocybe praecox (Pers.) Fayod	+	–	Austria	[26]
Amanita				
Amanita muscaria (L.: Fr.) Hooker	+		Brazil	[20]
Conocybe				
Conocybe cyanopus (Atk.) Kuhner	+	+	Finland, Norway, USA	[24, 41, 42]
Conocybe kuehneriana (Sing.) Kuhner	–	+	Finland	[41]
Conocybe smithii Watling	+	–	USA	[43]
Copelandia				
Copelandia anomala Murrill	+	–	USA (Hawaii)	[28]
Copelandia bispora (Malencon and Bertault) Singer and Weeks	+	–	USA (Hawaii)	[28]
Copelandia cambodginiensis (Ola'h and Haim) Singer and Weeks	+	+	USA (Hawaii)	[28]
Copelandia chlorocystis sp. Nov. Singer and Weeks	+	+	Brazil	[44]
Copelandia cyanescens (Berk. and Br.) Singer	+	+	Italy	[45]
Copelandia cyanescens (Berk. and Br.) Singer	+	–	USA (Hawaii)	[28]
Copelandia tropicalis (Ola'h) Singer	+	–	USA (Hawaii)	[28]
Copelandia sp.	+	+	Japan	[46]
Galerina				
Galerina steglichii Besl spec. nov	+	+	Germany	[47]
Gerronema				
Gerronema fibula (Bull) Singer	+	–	Germany	[48]
Gerronema swartzii (Fr.) Kreisel	+	–	Germany	[48]
Gymnopilus				
Gymnopilus aeruginosus (Peck) Sing	+	–	USA	[49]
Gymnopilus liquintiae (Pers.) P.Karst	+	–	Japan	[40]
Gymnopilus luteus (Peck) Hesler	+	–	USA	[49]
Gymnopilus purpuratus (Cooke and Mass.) Sing.	+	–	Germany	[50]
Gymnopilus purpuratus (Cooke and Mass). Sing	+	–	Thailand	[25]
Gymnopilus sapineus (Fr.) Maire.	+	–	USA	[49]
Gymnopilus spectabilis (Fr.) A.H.Sm.	+	–	USA	[49]
Gymnopilus validipes (Peck) Hesler	+	–	USA	[49]
Gymnopilus viridans Murr.	+	–	USA	[49]
Hygrocybe				
Hygrocybe psittacina (Schff.ex.Fr.) Wunsche (f.optima R. Schultz).	+	+	Germany	[48]

(*continued*)

Table 18.3 (continued)

Genus/Species	1	2	Country	References
Hypholoma				
Hypholoma aurantiaca	+	–	Australia	[50]
Inocybe				
Inocybe aeruginascens Babos	+	–	Germany	[51]
Inocybe aeruginascens Babos	+	–	Germany	[19]
Inocybe aeruginascens Babos	+	–	Germany	[52]
Inocybe aeruginascens Babos	+	–	Germany	[53]
Inocybe aeruginascens Babos	+	–	Germany	[54]
Inocybe aeruginascens Babos	+	–	Germany	[55]
Inocybe aeruginascens Babos	+	–	Hungary	[54]
Inocybe aeruginascens Babos	+	–	Germany	[56]
Inocybe aeruginascens Babos	+	+	Switzerland	[21]
Inocybe calamistrata Gillet	+	+	Germany	[48]
Inocybe corydalina Quel	+	–	Switzerland	[21]
Inocybe corydalina Quel	+	–	Germany	[48]
Inocybe corydalina Quel	+	–	Switzerland	[20]
Inocybe corydalina Quel. var. erinaceomorpha (Stangl and Veselsky)	+	–	Switzerland	[21]
Inocybe coelestium Kuyp	+	–	Switzerland	[21]
Inocybe haemacta (Berk.et Cooke)	+	+	Germany	[48]
Inocybe haemacta (Berk.et Cooke)	+	–	Switzerland	[20, 21]
Inocybe haemacta (Berk.et Cooke)	+	+	Czech Republic	[57]
Panaeolina				
Panaeolina foenisecii (Pers.ex.Fr.)	+	–	Finland	[27]
Panaeolina foenisecii (Pers.ex.Fr.)	+	–	Austria	[51]
Panaeolus				
Panaeolus africanus Ola'h	+	+	Sudan	[58]
Panaeolus antillarum	+	+	Thailand	[59]
Panaeolus ater (J.E. Lange) Bon	+	+	India	[58]
Panaeolus cambodginiensis Ola'h and Heim	+	–	USA	[60]
Panaeolus campanulatus (Fr.) Gillet	+	–	Italy	[45]
Panaeolus castaneifolius (Murr)	+	+	Canada, France	[58]
Panaeolus cyanescens (Berk and Broome) Sacc.	+	+	Germany	[61]
Panaeolus cyanescens (Berk and Broome) Sacc.	+	+	USA (Hawaii), Thailand	[19, 20, 59]
Panaeolus fimicola (Pers.) Gillet	+	+	United Kingdom	[58]
Panaeolus foenisecii (Pers.) J. Schrot	+	+	Canada, France	[58]
Panaeolus foenisecii (Pers.) J. Schrot	+	–	Italy	[45]
Panaeolus olivaceus F.H. Moller	+	–	Finland	[27]
Panaeolus retirugis (Fr.) Gillet	+	–	Italy	[45]
Panaeolus sphinctrinus	+	+	Canada	[58]

(continued)

Table 18.3 (continued)

Genus/Species	1	2	Country	References
Panaeolus subbalteatus (Berk and Br.)	+	+	Finland	[27]
Panaeolus subbalteatus (Berk and Br.)	+	–	Italy, Mexico	[45, 60]
Panaeolus subbalteatus (Berk and Br.)	+	+	Canada, USA	[58]
Panaeolus subbalteatus (Berk and Br.)	+	–	Brazil, Russia	[20, 62]
Pluteus				
Pluteus atricapillus Singer	+	–	Finland	[27]
Pluteus glaucus Singer	+	+	Brazil	[20]
Pluteus nigroviridis Babos	+	–	Switzerland	[63]
Pluteus salicinus (Pers.) P Kumm	+	+	USA, Finland	[27, 64]
Pluteus salicinus (Pers.) P Kumm	+	–	Germany, Switzerland	[21, 29, 63]
Pluteus salicinus (Pers.) P Kumm	+	+	Czech Republic	[57]
Pluteus salicinus (Pers.) P Kumm	+	–	Norway	[65]
Psathyrella				
Psathyrella candolleana (Fr.) Maire	+	+	Finland	[27]
Psathyrella candolleana (Fr.) Maire	+	–	Japan, Germany	[40, 48]
Psilocybe				
Psilocybe argentipes K. Yokohama	+	–	Japan	[40]
Psilocybe argentipes K. Yokohama	+	+	Japan	[66]
Psilocybe arcana Borovicka and Hlavacek	+	+	Czech Republic	[57]
Psilocybe atrobrunnea (Lasch) Gillet	+	–	Norway	[65]
Psilocybe aztecorum Hem var. aztecorum emend. Guzman	+	–	Mexico	[67]
Psilocybe aztecorum var. bonetii Guzman	+	–	Mexico	[60]
Psilocybe azurescens Stamets and Gartz	+	+	USA	[15]
Psilocybe baeocystis Singer and A.H.Sm.	+	+	USA	[17, 24, 68]
Psilocybe bohemica Sebek	+	+	Czech Republic	[18, 19, 57, 69–71]
Psilocybe bohemica Sebek	+	+	Switzerland	[21]
Psilocybe bonetii Guzman	+	–	Mexico	[60]
Psilocybe caeruleoannulata Sing	+	+	Brazil	[20]
Psilocybe caerulescens Murrill	+	+	Brazil	[20]
Psilocybe caerulipes Peck	+	+	USA	[72]
Psilocybe candidipes Singer and A.H.Sm	+	–	Mexico	[60]
Psilocybe copninifacies (Roll). Pouz	+	–	Czech Republic, Slovenia	[73]
Psilocybe cubensis (Earle) Singer	+	+	Japan, Germany, Mexico, Brazil	[20, 46, 61, 74]
Psilocybe cyanescens Wakef	+	+	Czech Republic, Switzerland, USA	[17, 21, 57, 75]
Psilocybe cyanofibrillosa Guzman and Stamets	+	+	USA	[22]
Psilocybe fimetaria (P.D. Orton) Watling	+	–	United Kingdom	[43]
Psilocybe hoogshagenii Heim	+	+	Brazil	[20]
Psilocybe liniformans Guzman and Bas	+	–	USA	[21, 22]

(continued)

Table 18.3 (continued)

Genus/Species	1	2	Country	References
Psilocybe mexicana Heim	+	+	Mexico	[1, 2, 76]
Psilocybe muliericula Singer and AH Sm	+	+	Mexico	[1]
Psilocybe pelliculosa Singer and AH Sm	+	+	USA	[24, 77, 78]
Psilocybe pseudobullacea (Patch)	+	+	Venezuela	[79]
Psilocybe samuiensis Guzman	+	+	Thailand	[19]
Psilocybe semilanceata (Fr.) Kumm	+	+	Czech Republic	[54, 55, 70, 73]
Psilocybe semilanceata (Fr.) Kumm	+	+	Finland, Germany	[27, 54, 80, 81]
Psilocybe semilanceata (Fr.) Kumm	+	–	Germany, Norway	[82–85]
Psilocybe semilanceata (Fr.) Kumm	+	–	Sweden, United Kingdom	[43, 86, 87]
Psilocybe semilanceata (Fr.) Kumm	+	+	Switzerland, USA, Germany, Czech Republic	[57, 61, 77, 88]
Psilocybe semilanceata (Fr.) Kumm	+	–	Switzerland, Netherlands, USA	[57, 89]
Psilocybe semperviva Heim and Cailleux	+	–	Mexico	[23, 90]
Psilocybe serbica Mos. and Horak	+	–	Serbia	[91]
Psilocybe strictipes A.H. Sm.	+	–	USA	[72]
Psilocybe stuntzii Guzman and Ott	+	+	USA	[24, 92]
Psilocybe subaeruginosa Cleland	+	–	Australia	[93, 94]
Psilocybe subaeruginosa Cleland	+	+	Australia	[51]
Psilocybe subcubensis Guzman	+	–	Venezuela	[79]
Psilocybe subcubensis Guzman	+	+	Japan	[66]
Psilocybe subyungenis Guzman	+	+	Brazil	[20]
Psilocybe tampanensis Guzman and Pollock	+	+	Thailand	[19]
Psilocybe tampanensis Guzman and Pollock	+	+	Germany	[61]
Psilocybe thailandensis Guzman and Allen	+	+	Thailand	[20]
Psilocybe uruguayensis Sing	+	+	Brazil	[20]
Psilocybe wasanii Heim	+	+	Mexico	[76]
Psilocybe weilii Guzman	+	+	USA	[6, 22]
Psilocybe zapotecorum Heim	+	+	Mexico	[2, 20, 23]
Stropharia				
Stropharia cubensis Earle	+	+	Mexico	[76]
Stropharia cubensis Earle	+	+	Cambodia	[23, 67]
Stropharia cubensis Earle	+	+	Thailand	[23, 67]

1 Psilocybin, *2* Psilocin, + Detected, – Not detected

converted by the mechanism of dephosphorylation into psilocin (another pharmacologically active compound). Psilocin has the molecular formula $\mathbf{C_{12}H_{16}N_2O}$ (mol. weight = 204.27).

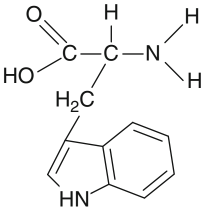
Psilocybin is regarded as tryptamine compound based on its biosynthesis route and precursor. Being a tryptamine compound, the chemical structure is derived from the amino acid tryptophan **(5)** which has a ring configuration, simply known as

Table 18.4 List of indole derivatives reported in various *Psilocybe* mushrooms

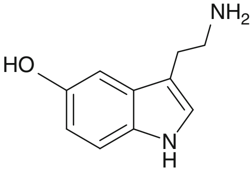
Compound[a]	Compound[a]
Indole	3-Indolelactic acid
2-Methylindole	3-Indoleacetamide
3-Methylindole	3-Indoleacetic acid
5-Methylindole	γ (Indole)-N-butyric acid
5-Methoxyindole	β (Indole-3)-propionic acid
5-Hydroxyindole	3-Indolecarboxylic acid
3-Indoleacetonitrile	β-Indole-3-acrylic acid
3-Hydroxymethyl indole	3-Indolealdehyde
3-Indoleacetic acid ethyl ester	3-Indoleacetaldehyde
5-Hydroxy-3-indole acetic acid	3-Hydroxyethyl indole
N-Methyltryptophan	Indoxylacetate
5-Methoxy-2-carboxyindole	Indoxylbutyrate
5-Benzyloxy-3-indole acetic acid	5-Methyltryptophan
Tryptamine hydrochloride	L-Tryptophane
5-Hydroxytryptamine creatinine sulfate	5-Hydroxytryptophane
N,*N*-Dimethyltryptamine hydrogen-oxalate	Isatin
Bufotenine monooxalate	Gramine

[a]The list indicates addition compounds, apart from well-known alkaloids

indole (C_8H_7N), linked to an ethylamine substituent. Structurally, psilocybin has a close resemblance with a neurotransmitter serotonin or 5-HT (**6**). Tryptamines are one of the four categories of hallucinogenic indoles in more than 20 classes of indole compounds comprising approximately 600 alkaloids.



Tryptophan
5



Serotonin
6

Psilocybin is zwitterionic in nature (containing both positive and negative charges), insoluble in most organic solvents (such as chloroform, benzene, etc.,), slightly soluble in ethanol, moderately soluble in methanol (120 parts of boiling methanol), and soluble in water (20 parts of boiling water). Aqueous solution is highly unstable and rapidly oxidizes. This is an important factor and should be taken into consideration when psilocybin is used as an analytical standard [39]. Physically, psilocybin is white, needle-like crystalline structure with melting point between 185–195 °C [14] and 190–198 °C [95]. A 1% solution of psilocybin dissolved in 50% ethanol has a pH of 5.2 [2]. Scientific work carried out in

Japan with respect to large-scale chemical synthesis of psilocybin without chromatographic purification suggests use of 4-hydroxyindole to produce psilocybin from psilocin with a yield of 85% [95].

For presumptive analysis (color test) of psilocybin, both Ehrlich and Marquis tests can be applied. Care should be taken as some other C_2 unsubstituted indoles (such as DMT and LSD) may give similar colors with test reagent, thus making it mandatory for a confirmatory test before extending any final concluding remarks. With Ehrlich's reagent (1 g of p-dimethylaminobenzaldehyde in 10 ml methanol + 10 ml con. orthophosphoric acid), a violet to gray-violet color indicates the possible presence of psilocybin or psilocin. The detection limit of this method is approximately 1 μg. While with Marquis' reagent (solution A: 8–10 drops of 40% formaldehyde solution to 10 ml glacial acetic acid; solution B: con. H_2SO_4), an orange color indicates the possible presence of psilocybin and a green-brown color the presence of psilocin. The detection limit of this method is about 10 μg. Since the positive color reaction for psilocybin with Marquis' reagent is obscured by vegetable matter and as some other natural products present in mushrooms may develop a yellow color, therefore with sulfuric acid, this color test is not suitable for the detection of psilocybin or psilocin. Mandelin tests can also be applied to detect psilocybin. Application with Mandelin reagent (1 g of ammonium vanadate in 100 ml con. H_2SO_4) results in the development of green color, while DMACA reagent (0.5 g of p-dimethylaminocinnamaldehyde in 50 ml methanol + 10 ml con. HCl) can be sprayed to detect after chromatographic analysis [96, 97], or few drops can directly be added over the suspected material on a spot plate. Psilocybin gives gray-violet to violet color, whereas psilocin turns blue. The DMACA reagent is more sensitive than Ehrlich reagent and also reflects better color stability. The detection limit for DMACA reagent is about 20 ng for psilocybin and 10 ng for psilocin. Other techniques used to detect and quantify psilocybin include gas chromatography coupled to mass spectrometry [98], ion mobility spectrometry [26], capillary zone electrophoresis [99], ultraviolet spectroscopy [39], infrared spectroscopy [55], high-performance liquid chromatography (HPLC) with ultraviolet [39], fluorescence [100], and electrochemical [101] or electrospray mass spectrometric methods [102]. These chromatographic techniques can also be used to detect psilocin in the body fluids.

Solvent system does play very important role in the isolation and extraction of psilocybin. It is quite evident from the early phase of research activities that very polar solvent, such as methanol or mixtures of ethanol and water, is most suitable for the isolation of psilocybin. The polar properties of the phosphate group make psilocybin soluble in water and methanol (polar solvent), and as a result solubility decreases with the decrease in polarity, i.e., less soluble in polar solvent. In comparison, psilocin is less polar and thus readily soluble in polar solvent, such as 1-chlorobutane. For thin layer chromatography, solvent systems comprising of n-butanol + acetic acid + water (20:10:10) or methanol + con. ammonia solution (100:1.5) may be used to develop the plates, and visualization of the blue spots on fluorescent background can be made using ultraviolet (UV) light at 254 and 365 nm. The absorption characteristics of both psilocybin and psilocin in the UV region are quite prominent. Both exhibit native fluorescence and are electrochemically active.

Mushrooms of the genus *Psilocybe* generally have a brownish cap (pileus) when moist in fresh fruiting bodies but fade gradually in color during the process of drying. In addition, some species (typically the hallucinogenic species) have the tendency to change the color (a condition called blue staining reaction) when fruiting body is damaged. This is also described as blueing phenomenon. Some authors do conclude that the blueing phenomenon ultimately reflects and provides a reasonable correlation between psilocybin and other indole derivatives content present in that particular species [43, 103]. However, at the same time, others suggest not to use this phenomenon as a definite method for identification or determining the potency of a mushroom [6, 17]. The phenomenon that psilocybin has the tendency to form blue color in solution can further be linked to its conversion into psilocin (which is relatively unstable in solution due to its phenolic hydroxyl –OH group) by dephosphorylation in presence of enzyme phosphatase followed by oxidation of psilocin by cytochrome oxidase, or copper oxidase or Fe $^{2+}$ [104]. Animal module also supported and suggested a similar mechanism for the development of blue color by these mushrooms when psilocybin was incubated with the homogenates of rat kidney or other mammalian tissues. A rapid liberation of psilocin was observed through the action of alkaline phosphate which further undergoes oxidative degradation and forms a blue-colored product [105]. A mitochondrial enzyme (cytochrome oxidase) was found responsible in the oxidation of psilocin to a dark blue color by the tissue homogenates [106].

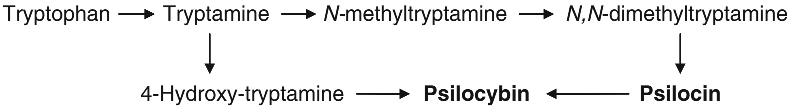
4 Biosynthesis

Studies on biosynthesis mechanism and the routes including enzymes involved in different steps are the best known toll in understanding the conversion or production of various biochemicals in living system. In case of psilocybin biosynthesis, though the experimental evidences are limited, but the overall mechanism can safely be discussed starting from chorismic acid, which is produced through shikimic acid pathway, a well-known pathway present in fungi which regulates the production of various biochemicals, including psilocybin and some other tryptamine-based psychedelic drugs, such as DMT and LSD.

The shikimic acid pathway leading to the production of chorismic acid is regulated in the cytosol of the fungal cells. Cytosol or intracellular fluid (cytoplasmic matrix) is a complex mixture of substances dissolved in water. These include ions (such as calcium, sodium, and potassium), macromolecules, and large complexes of enzymes that act together to carry out metabolic pathways. Production of chorismic acid in the cytosol is ultimately utilized in the synthesis of folate, ubiquinone, and amino acids, the most important of which is tryptophan which plays a major role in the biosynthesis of psilocybin.

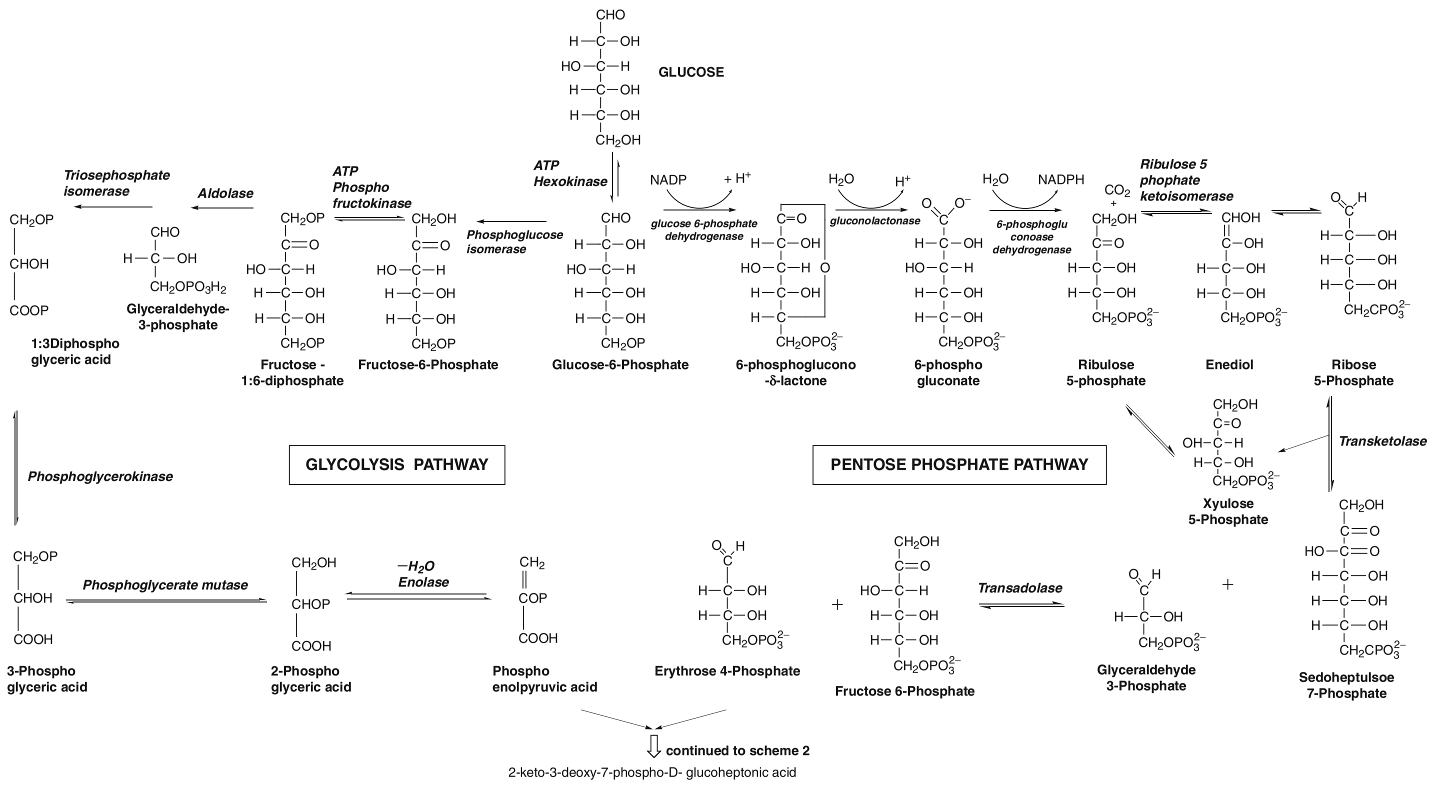
The two starting materials of the shikimic acid pathway, phosphoenolpyruvate (a product of glycolysis) and erythrose 4-phosphate (a product of pentose phosphate pathway), act as phosphate donors. Glucose is phosphorylated by ATP, which loses a phosphoryl group to become ADP. The glucose 6-phosphate thus produced, enter into two different pathways, glycolysis and pentose phosphate pathway, to produce both the starting materials required to regulate shikimic acid pathway with the production of chorismic acid, a substrate to give anthranilic acid which is a precursor of tryptophan (Scheme 18.1). With a few minor exceptions, tryptophan and its decarboxylation product, tryptamine, give rise to the large class of indole alkaloids, including psilocybin [107, 108] (Scheme 18.2).

Earlier findings published in 1961 [107] suggested that psilocybin is finally been synthesize from tryptophan. The hypothesis was based on the results obtained from still cultures of *Psilocybe semperviva*. Subsequent studies on the subject carried out using *Psilocybe cubensis* under submerged cultural condition also supported this hypothesis that psilocybin may biosynthetically derive from tryptophan and tryptamine [108]. More details were then published to clarify the sequence of events which leads from tryptophan to psilocybin by incorporating H^{3}– and C^{14}- labeled hypothetical intermediates (such as DL-Tryptophan-^{3}H, Tryptamine-^{14}C, *N*-methyltryptamine-C^{14}-^{3}H, *N,N*-Dimethyltryptamine-^{14} C-^{3}H, 4-hydroxytryptamine-^{14} C-^{3}H, Psilocin-^{3}H, DL-4-OH-Tryptophan-^{3}H) into psilocybin in submerged cultures of *Psilocybe cubensis* [109, 110]. Other reports [111, 112] indicated no effect or influence of tryptophan on the biosynthesis of psilocybin when added in the culture medium during submerged fermentation of *Psilocybe cubensis* and *Psilocybe baeocystis*. It can be concluded from the results of most findings that tryptophan has to be modified by decarboxylation, methylation of the amino group, hydroxylation of the 4-position of the indole moiety, and phosphorylation of the 4-hydroxyindole moiety for the production of psilocybin with below sequence [108–110, 113].

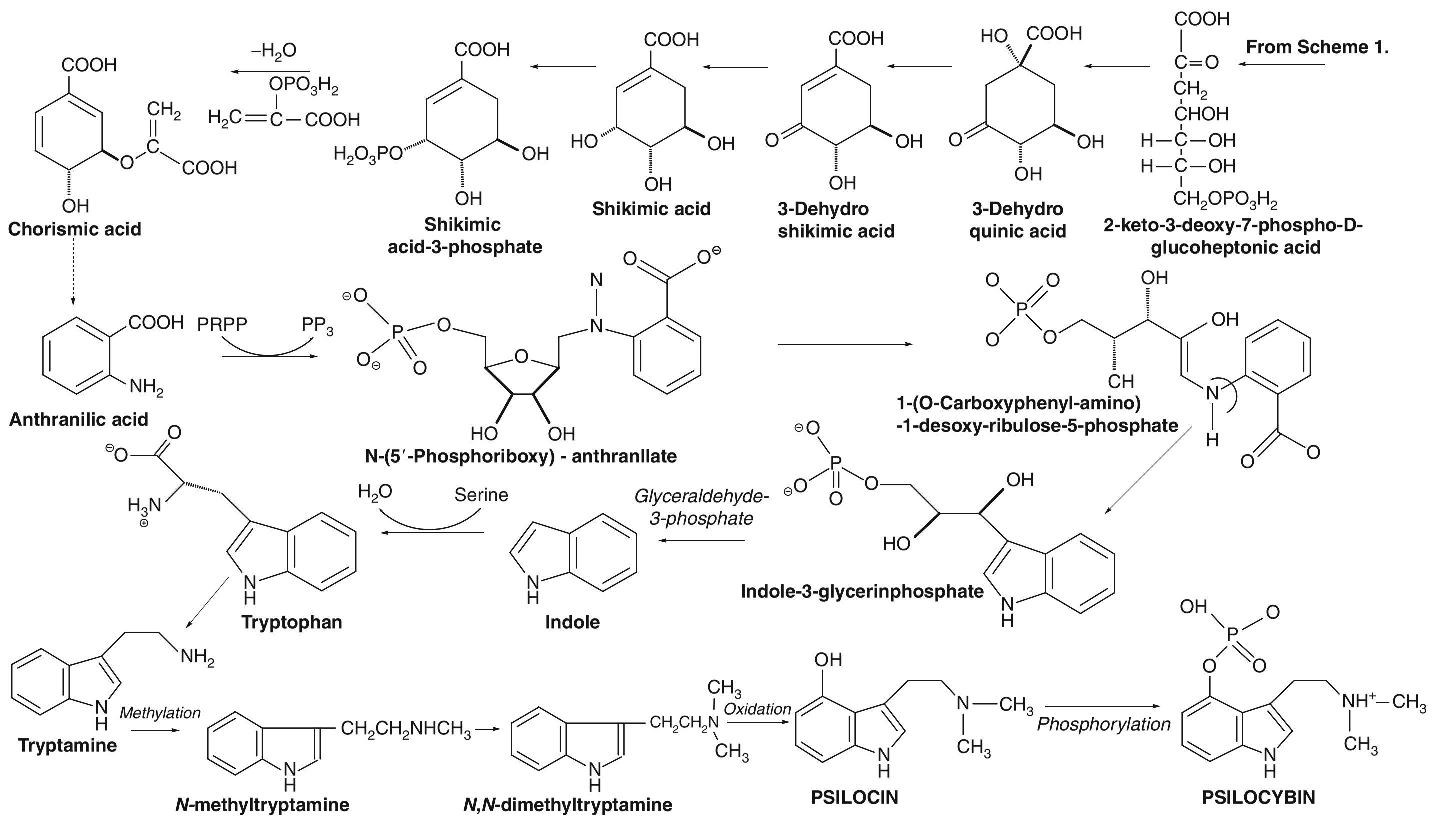


However, this may not be the only route. The fungus still may have an alternate route to convert 4-hydroxytryptamine to psilocybin. This probably because phosphorylated intermediate baeocystin and norbaeocystin are also produced, in addition to psilocybin, which is most likely produced via one phosphorylase enzyme from psilocin.

The radioactive tryptamine is observed to function as a better precursor than the tryptophan and 4-hydroxytryptophan in submerged culture of *Psilocybe cubensis* [113]; it is reasonable to conclude that the decarboxylation of tryptophan to tryptamine is the most crucial step in the biosynthetic pathway. This step is highly sensitive to feedback inhibition due to allosteric enzymes. It is believed that as the concentration of psilocin increases in the culture media, it starts inhibiting further conversion of tryptophan to tryptamine, and as a result tryptophan will not be converted to tryptamine, and thus no psilocin or psilocybin is


GLUCOSE
ATP
Hexokinase
Glucose-6-Phosphate
Phosphoglucose
isomerase
Fructose-6-Phosphate
ATP
Phospho
fructokinase
Fructose -
1:6-diphosphate
Aldolase
Glyceraldehyde-
3-phosphate
Triosephosphate
isomerase
1:3Diphospho
glyceric acid
Phosphoglycerokinase
3-Phospho
glyceric acid
Phosphoglycerate mutase
2-Phospho
glyceric acid
−H2O
Enolase
Phospho
enolpyruvic acid
GLYCOLYSIS PATHWAY
NADP
+ H+
glucose 6-phosphate
dehydrogenase
6-phosphoglucono
-δ-lactone
H2O
H+
gluconolactonase
6-phospho
gluconate
H2O
NADPH
6-phosphoglu
conoase
dehydrogenase
CO2
+
Ribulose
5-phosphate
Ribulose 5
phophate
ketoisomerase
Enediol
Ribose
5-Phosphate
Transketolase
Xyulose
5-Phosphate
Sedoheptulsoe
7-Phosphate
PENTOSE PHOSPHATE PATHWAY
Glyceraldehyde
3-Phosphate
Transadolase
Fructose 6-Phosphate
Erythrose 4-Phosphate
continued to scheme 2
2-keto-3-deoxy-7-phospho-D- glucoheptonic acid

Scheme 18.1 Glycolysis and pentose phosphate pathway


From Scheme 1.
2-keto-3-deoxy-7-phospho-D-glucoheptonic acid
3-Dehydro quinic acid
3-Dehydro shikimic acid
Shikimic acid
Shikimic acid-3-phosphate
−H$_2$O
Chorismic acid
Anthranilic acid
PRPP
PP$_3$
N-(5′-Phosphoriboxy) - anthranllate
1-(O-Carboxyphenyl-amino) -1-desoxy-ribulose-5-phosphate
Indole-3-glycerinphosphate
Glyceraldehyde-3-phosphate
Indole
H$_2$O
Serine
Tryptophan
Tryptamine
Methylation
N-methyltryptamine
N,N-dimethyltryptamine
Oxidation
PSILOCIN
Phosphorylation
PSILOCYBIN

Scheme 18.2 A proposed biosynthesis pathway of psilocybin biological activities formed. Further addition of tryptophan will have no impact on the formation of psilocybin. While conversion of tryptamine to N-methyltryptamine is not as sensitive to feedback inhibition; therefore, addition of tryptamine will increase the production of psilocin. Apart from this, all other steps are also not as sensitive to feedback inhibition either. There still exists some controversy among authors regarding biosynthetic route from tryptamine to psilocybin [114]. When *Psilocybe cubensis* (mini-cultures) grows under deuterium-labeled precursor solution, a wide range of tryptamine can readily be absorbed by mycelia and translocated into developing mushrooms. The deuterated tryptamine can be incorporated more efficiently into psilocin and psilocybin than were monomethyltryptamine and dimethyltryptamine, suggesting that though hydroxylation enzyme operates normally on tryptamine, but may be flexible enough to oxidize dimethyltryptamine or other natural compounds forced on it at a high concentration. As the hydroxylation of dimethyltryptamine to produce psilocin was noted to occur with NIH shift, therefore an intermediate compound (tryptamine-4,5-epoxide) is most probably present between tryptamine and psilocin.

It is really difficult to interpret and match the data obtained through submerged cultural condition with that of fruit bodies grown in wild to ascertain the biosynthesis mechanism. Some observations indicate that surface cultures of *Psilocybe semilanceata* have a high hydroxylation and phosphorylation capacity, although the ability to methylate tryptamine derivatives is low. These findings are in accordance with the earlier mentioned hypothesis that the fungus may have alternate route to convert 4-hydroxytryptamine to psilocybin.

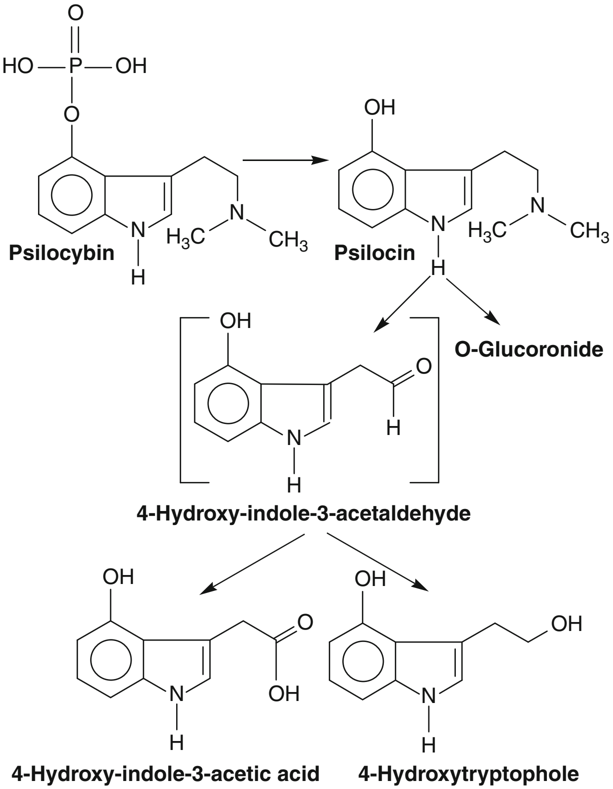
5 Biological Acitivies

Despite the introduction of psilocybin more than five decades back by Sandoz, under the brand name of Indocybin, limited data are available for discussion. The original Indocybin tablets manufactured during 1958–1965 are still available in its original packaging (glass bottle with metal screw on lid, labeled: Sandoz Pharmaceuticals, 50 tablets, Psilocybin, each tablet contains 10 mg) in Bureau of Forensic Services Laboratory, Eureka, California. Traces of psilocin were still detectable by GC/MS in these tablets, but most peaks reflect tablet excipients. During the early mid-1960s (until it is classified as Schedule I in 1970 in the USA), psilocybin was used in psychiatric and psychological research as well as psychotherapy. Psilocybin mushrooms have had numerous medicinal and religious uses in dozens of cultures throughout history and have a drastically lower potential for abuse than other Schedule I drugs. Also, the UN's International Narcotics Control Board (INCB) has made it clear that *Psilocybe* mushrooms are not controlled by the UN [115, 116]. Research into the biological activity of psilocybin resumed in the mid-1990s, and currently a number of research institutes are working on biological activities of psilocybin to ascertain its clinical role in different disorders and syndromes. In view of highly scattered biological activities of psilocybin, it will be appropriate to discuss this topic under subheadings, such as Pharmacology (Pharmacodynamics and Pharmacokinetics); Physiological and Psychological Effects; Therapeutic Role (Advanced Stage Cancer, Cluster Headaches, Obsessive-Compulsive Disorders); and Toxicity (Side Effects and Risks).

5.1 Pharmacology (Pharmacodynamics and Pharmacokinetics)

After oral ingestion, psilocybin is rapidly dephosphorylated into psilocin which is structurally related to serotonin (5-HT) and lysergide (LSD-25). Psilocin has high affinity ($Ki = 6nM$) for 5-HT receptors, especially the 5-HT$_{\mathbf{2A}}$ in the brain (cerebral cortex) where it mimics the effects of serotonin and thus capable of altering perception, mood, consciousness, and cognition. It also has affinity (but binds less tightly, $Ki = 190$ nM) with other serotonergic receptors, such as 5-HT$_{\mathbf{1A}}$ (hippocampus), 5-HT$_{\mathbf{1D}}$ (cranial blood vessels), and 5-HT$_{\mathbf{2C}}$ (choroid plexus) and thus can also contribute to the subjective and behavioral effects. Psilocybin and psilocin have no affinity for dopamine $D_{\mathbf{2}}$ receptors [117]. The mode of action can precisely be explained in a way that psilocybin acts by altering the concentration of indoles, including serotonin, in the central nervous system and thus interfering with the transmission and processing of external stimuli. The psychotomimetic effects of psilocybin can be blocked in a dose-dependent manner by the 5-HT$_{\mathbf{2A}}$ antagonist drugs, such as ketanserin and risperidone [118].

Psilocybin and psilocin are stoichiometrically equivalent in potency. After dephosphorylation to psilocin, presumably via a first pass effect by hepatic metabolism, it is easily taken up by tissues. More than 50% of the psilocybin is absorbed through the lining of mouth, stomach, and intestine and is detectable as psilocin in the blood within 20–40 min. Distribution throughout the body is quite uniform, including the brain [119, 120]. The absolute bioavailability of psilocin after oral administration of psilocybin is recorded as 52.7 ± 20%. The average peak concentration of 8.2 ± 2.8 ng ml^{-1} ($C_{\mathbf{max}}$ ranging from 4 to 21 ng ml^{-1}) is usually achieved within 2 h (105 ± 37 min.) after oral administration of 10–20 mg psilocybin (0.224 ± 0.02 mg kg$^{\mathbf{-1}}$ body weight) and decline over the next 3–4 h. While after intravenous injection of 1 mg psilocybin, peak plasma concentration of 12.9 ± 5.6 ng ml^{-1} reaches within 1.9 ± 1.0 min. The average half-life ranges between 2.5 and 3 h [121–123], while quantification of psilocin can be made within 6–7 h after oral administration [121, 124]. Psilocin is excreted mainly as glucuronide and to some extent as unchanged psilocin as well in urine. According to one report, 65% of the absorbed psilocybin is excreted into urine and 15–20% into bile and feces within 24 h [125]. Other metabolites detected in quantities include 4-hydroxyindole-3-yl-acetaldehyde, 4-hydroxyindole-3-yl-acetic acid, and 4-hydroxytryptophol [121, 126–129]. A proposed metabolic route for psilocybin is suggested in Scheme 18.3.


Psilocybin
Psilocin
O-Glucoronide
4-Hydroxy-indole-3-acetaldehyde
4-Hydroxy-indole-3-acetic acid
4-Hydroxytryptophole

Scheme 18.3 Metabolism of psilocybin

5.2 Physiological and Psychological Effects

The effects of psilocybin are quite variable and usually dependent upon dose and duration of intake. This may also be linked with the individual brain chemistry and physiology which may have significant role in determining the response to psilocybin. Though the recreational dose ranges from 10 to 50 mg, yet only 4–10 mg is enough to induce hallucination [5, 6]. Some people may require relatively high dose to gain a low-dose effects of psilocybin. The mental and physical tolerance to psilocybin may build but dissipates quickly. So dose adjustment (especially for frequent users) may be required to avoid side effects. Cross-tolerance is also possible between psilocybin and LSD, as well as between psilocybin and phenylethylamine hallucinogens, such as mescaline and 2,5-dimethoxy-4-methylamphetamine [130–132].

The general features which are characteristic of the psychedelic reaction are same for psilocybin as well. The most common features include changes in perception (perceptual changes such as illusion, pseudohallucination, and hallucination) and changes in vision (objects, pictures, or patterns seem to come alive, shift, ripple, or become wavy). The psychological symptoms are greatly affected after psilocybin ingestions. Various trial reports published elsewhere [133–135] highlight these effects when psilocybin was given to healthy volunteers in decreasing order. The emotional alterations noted in 100% of the volunteers include disorders/alterations of consciousness in 91%, depersonalization in 84%, perceptual alterations in 75%, disorders/alteration of volition and psychomotor behavior in 34%, body image distortions in 25%, disorders/alteration of attention in 22%, disturbances in thought process in 22%, and disorders of memory in 19% of the volunteers.

5.3 Therapeutic Role (Advanced Stage Cancer, Cluster Headaches, Obsessive-Compulsive Disorders)

Results of various double-blind, placebo-controlled pilot studies carried out with psilocybin in the management of these conditions or reducing the severity of the various associated symptoms are highly encouraging. To reduce anxiety and improved quality of life in people with advanced stage of cancer, psilocybin has been observed as good alternate [136, 137]. In case of cluster headaches which has been recognized as one of the worst pain syndromes and difficult to control through presently available drugs, psilocybin has been reported to have promising effect in reducing the severity of the attack as well as to increase the attack-free periods in such patients [138–140]. A substantial reduction in symptoms in patients associated with obsessive-compulsive disorders (OCD) has also been noted with psilocybin therapy. The proposed mechanism and reason for this is the reduction in serotonin levels at 5-HT$_{2A}$ receptors due to psilocybin which ultimately suppresses the responsiveness to serotonin [141, 142]. In most cases, only sub-hallucinogenic dose of psilocybin is required to achieve the desired therapeutic effect without any major side effects.

5.4 Toxicity (Side Effects and Risks)

The therapeutic index of psilocybin as given by “The Registry of Toxic Effects of Chemical Substances” is quite high (therapeutic index = 641), in comparison to aspirin and nicotine for which the values are quite low (therapeutic index = 199 and 21, respectively), representing better a better safety profile [143]. Therapeutic index is the ratio of the dose that produces toxicity (TD$_{50}$) to the dose that produces a clinically desired or effective response (ED$_{50}$) in a population. The therapeutic index is a measure of a drug’s safety, because a larger value indicates a wide margin between doses that are effective and doses that are toxic. LD$_{50}$ in rats by oral route is 280 mg kg^{-1} while that of intravenous route in rabbit is 12.50 mg kg^{-1} [144]. The common side effects are very similar to those reported for other hallucinogens, such as ergoline and LSD. These include perception of time and space, derealization, depersonalization, dilated pupils, dizziness, fatigue, impaired concentration, unusual body sensations and thoughts, transient increase in blood pressure or heart rate, transient psychosis, change in mood, nausea, etc. These effects are mostly noted as acute which usually last for about 4–6 h [117, 145–148]. Oral intake of psilocybin appears to be at the lowest risk out of all other psychoactive drugs, while long-term toxicity studies in mice indicated no teratogenicity or mutagenicity [149, 150].

6 Biotechnological Approaches

The biotechnological approach has now occupied a prominent place while discussing topics in applied biology. However, the features and objects may vary based on scope of the study. In search of new drug molecules or to evaluate the existing therapeutically active molecules, especially from higher fungi, is increasing day by day. The higher fungi have tremendous capability to synthesize biologically active compounds, especially the alkaloids with diversified chemistry and pharmacological activity. However, unfortunately less attention has so far been given by the researchers to utilize biotechnological approach to produce tryptamines (e.g., psilocybin and psilocin), perhaps because of the legal status and their classification under Schedule I drug category in the USA which restricted such studies and funding as well. The Schedule I drugs deemed to have a high potential for abuse and are not recognized for medical use. In Canada, both psilocybin and psilocin are classified as restricted drugs and are listed in Schedule III of Canada's Food and Drugs Act [151]. However, with the change in scenario and after having some prominent results of psilocybin in the management of various disorders, the situation is thus demanding substantial quantity of such molecules for pilot study and commercialization. So the improvement in strains capability, media, and culture conditions as well as regulation of enzyme activity involved in different steps of the biosynthetic pathway of psilocybin require more attention. The function of enzymes is to effect the integration of overall metabolic pathway. The formation alkaloid relates to endogenous metabolism. Thus, the energy released during the process is utilized to maintain the viability and integrity of the cells. In the biosynthesis of psilocybin, tryptophan plays important role, both as a regulatory and a precursor. It will be worth to utilize biotechnological approach to study the enzyme tryptophan decarboxylase (responsible for the decarboxylation of tryptophan to form tryptamine) and its regulatory mechanism. This is the last major step in the pathway that is significantly inhibited by a self-feedback mechanism. Due to this, tryptophan decarboxylase stops converting tryptophan to tryptamine, and as a result less psilocybin is produced. No report available showing role of biotechnology in this direction or subject. The only reference available describes molecular cloning of enzymes (enzyme coded by cDNA) from the psilocybin biosynthesis pathway in *Psilocybe tampanensis* to determine enzymatic activities [152].

DNA-based approach (phylogenetic approach) has been reported by Canadian investigators for various species of psilocybin mushrooms which are quite helpful in the identification of species that contain the psychoactive compounds [153]. The method includes DNA amplification and sequencing of the internal transcribed spacer region of the rDNA (ITS-1) and a $5^{\prime}$portion of the nuclear large ribosomal subunit of rRNA (nLSU rRNA or 28 S). The method is quite useful in the identification of unknown fungal species with regard to presence or absence of psilocybin and psilocin. Genetic techniques based on polymerase chain reactions (PCR) of specific regions of the genomes can also be used to identify and differentiate between psilocybin and non-psilocybin-producing species and strains of various mushrooms. The submerged culture technique for the production psilocybin is still believed to be the best biotechnological approach. Though improvement can also be done through field cultivation of mushrooms, yet it will be quite laborious, expensive, time consuming, and will also depend upon several environmental factors not easily manageable.

7 Conclusion/Prospects

With the increasing number of psychotic patients worldwide, there is a definite need to explore and find new pathways of treatment by both discovering new molecules as well as utilizing known drugs by modifying the dimension of the research activities and protocols. The introduction of biotechnology will have significant impact to this approach. Both psilocybin and psilocin are excellent examples such actives to initiate research in a broader way to explore these two bioactive indole alkaloids not only to see the possibility of their use in psychiatry but to widen their application for the treatment of other disorders as well. Thus, further research studies on these psychedelic to examine their overall effect on cerebral nervous system are proposed. Apart from this, the clinical role of these novel alkaloids can further be explored by modifying the dosage form from immediate to modified (extended) release dosage form to establish more convenience in terms of dosage and to reduce side effects. Prospects are quite open but seem to be challenging.

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Alkaloids Derived from Tryptophan: Harmine and Related Alkaloids

19

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Contents

1 Synonyms 554
2 Introduction 554
3 Occurrence 555
4 Phytochemistry 555
4.1 Physicochemical Characteristic 555
4.2 Chemical Structures 555
4.3 Extraction, Isolation, and Analysis 555
5 Biosynthesis 562
6 Chemical Synthesis 563
7 Biological Activities 563
7.1 Interaction with DNA and RNA 565
7.2 Interaction with Enzymatic Systems and Receptors 565
7.3 Neurotoxic Effects 566
7.4 Antitumor Activities 566
7.5 Antiviral Activities 566
7.6 Antimicrobial Activities 567
7.7 Antiparasitic Activity 567
7.8 Antithrombotic Activity 567
7.9 Antioxidant Activity 567
7.10 Antiplasmodial Activity 568
7.11 Other Activities 568
8 In Vitro Production 568
9 Conclusion 568
References 569

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Abstract

Over 140 beta-carboline (Harmala) alkaloids are detected in bacteria, algae, fungi, various plant groups, food products, alcoholic beverages, tobacco smoke, marine bryozoa, insects, and animal tissue including human. Some of these alkaloids and their derivatives are synthesized chemically. Several natural and chemically formed alkaloids are biologically active, and they can be used as potent pharmaceutical drug for anticancer therapy, angiogenesis, Alzheimer's, free radical scavenger, *Leishmania*, and viruses Herpes, Influenza, Polio, and HIV.

Keywords

Angiogenic • antitumor • β-carboline • harmala alkaloids • harmine

Abbreviations

CNS	Central nervous system
CNTs–GCE	Carbon nanotubes–modified glassy carbon electrodes
GABA	Gamma-Aminobutyric acid
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
LC	Liquid chromatography
MAO	Monoamine oxidase
MS	Mass spectroscopy
TLC	Thin-layer chromatography

1 Synonyms

Harman: aribine, colloturine, loturine, passiflorin, 2-methyl-β-carboline, 3-methyl-4-carboline; Harmaline: harmidine, Harmalol, methyl ether, *O*-methyl-harmalol, 3,4-dihydroharmine; Harmine: telepathine, leucoharmine, yageine, banisterine; Tetrahydroharmine: elaeagnine, calligonine, leptaflorin

2 Introduction

β-carboline alkaloids are heterocyclic amines with a 9-*H*-pyrido[3,4,b] indole structure derived from amino acid tryptophan. Initially, they have been isolated from plant *Peganum harmala* L. (Syrian Rue) and are also known as harmala alkaloids. They are active constituents in hallucinogenic plants and have a long tradition in ethnopharmacology. Since then, they have been reported in variety of plant groups, fungi, microorganisms, and in animal tissue including human beings [1–3]. More than 140 different types of β-carbolines are reported so far in plant and animal system [4, 5]. Norharman, harmane, and harmine are also known as mammalian indole alkaloids because they are endogenously produced in human and animal tissues as a product of secondary metabolism [6]. These compounds are also found in some medicinal plants [7]. During food production, processing, and storage, the chemical condensation between indoleamines and aldehydes or keto acids occurs naturally and results in formation of β-carbolines. Their presence is noted in well-cooked meat and fish and also in alcoholic beverages, tobacco smoke, and marijuana smoke [8]. They possess diverse biological properties due to their capability to bind to benzodiazepine or imidazoline receptors, such as hallucinogenic, tremorogenic, hypotensive or cardiovascular actions, and psychotropic properties.

3 Occurrence

β-carboline alkaloids are found mostly in plant system, but they also showed their presence in some animal tissue. In man, tetrahydro-beta-carboline (tetrahydronorharman), formed from tryptamine condensed with formaldehyde, occurs normally in plasma and is highly concentrated in platelets. After alcohol intake, its concentration is usually greatest at the time of hangover. These alkaloids may be found in particularly high concentrations (ng/g) in animal protein (i.e., meat). It is assumed that dietary sources were 50 times greater than endogenous sources [9]. Some β-carbolines, notably tryptoline and pinoline, are formed naturally in the human body [10].

β-carbolines are detected in bacteria, fungi, algae, and in plants belonging to bryophytes, pteridophytes, gymnosperm, angiosperms, and some specific marine organism (Table 19.1).

4 Phytochemistry

4.1 Physicochemical Characteristic

See Table 19.2.

4.2 Chemical Structures

See Fig. 19.1.

4.3 Extraction, Isolation, and Analysis

4.3.1 Extraction

Hundred grams of dried and powdered natural material (fruits, stem, or leaf) macerated with 250 ml of methanol at 50 °C in a water bath for 1 h., filter the extract with Whatman filter paper No. 1, and repeat the maceration of the material for four times. Combine the extract and evaporate to dryness. The residue was

Table 19.1 β-carboline alkaloids from some microorganism, plants, and animals

Sr. no.	Binomial name, family, and group	Common name	Part used	Type of β-carboline alkaloid	Reference
1	*Peganum harmala* L. Zygophyllaceae Dicotyledons	Harmal, Syrian rue	Seeds	Harman, harmine, harmaline, harmalol, harmidine, harmalidine	[11–13]
2	*Passiflora incarnata* L. Passifloraceae Dicotyledons	Maypop, passion flower	Aerial parts	Harmine, harman, norharman, harmol	[14, 15]
3	*Symplocos racemosa* Roxb. Symplocaceae Dicotyledons	Lodh tree, Lodh pathani	Bark	Harman	[16]
4	*Simira rubra* (Mart.) Steyerm. Rubiaceae Dicotyledons		Bark	Harman, harmine	[16]
5	*Banisteriopsis caapi* (Spr. ex Briesb.) Malpighiaceae Dicotyledons	Ayahuasca, caapi, yage	Aerial parts	Tetrahydroharmine, harmaline, harmine	[17]
6	*Elaeagnus angustifolia* L. Elaeagnaceae Dicotyledons	Russian silverberry	Bark	Tetrahydroharmine, harman	[18]
7	*Leptactina densiflora* Hook. f. Rubiaceae Dicotyledons	Leptactinia	Entire plant	Tetrahydroharmine	[19]
8	*Zygophyllum fabago* L. Zygophyllaceae Dicotyledons	Syrian bean-caper	Entire plant	Harmine	[20]
9	*Strychnos barnhartiana* Krukoff. Loganiaceae Dicotyledons		Leaves	Norharman	[21]

10	*Calligonum minimum* Lipski Polygonaceae Dicotyledons		Roots	Tetrahydroharmine	[7]
11	*Tribulus terrestris* L. Zygophyllaceae Dicotyledons	Puncture vine	Leaves	Harmaline, harmine, norharman, harman,	[22, 23]
12	*Grewia bicolor* Juss. Malvaceae Dicotyledons	White raisin	Aerial parts	Harman	[24]
13	*Uncaria attenuata* Korth.; *U. orientalis* Guill.; *U. canescens* Korth. Rubiaceae Dicotyledons		Leaves	Harman	[25]
14	*Oxalis tuberosa* Molina Oxalidaceae Dicotyledons	Oca	Tubers	Harmine, harmaline	[26]
15	*Festuca arundinacea* Schreb. Poaceae Monocotyledons	Tall fescue	Aerial parts	Norharman	[7, 16]
16	*Lolium perenne* L. Poaceae Monocotyledons	Ryegrass	Aerial parts	Norharman	[7, 16]
17	*Hypodematium squamuloso-pilosum* Ching Hypodematiaceae Pteridophyta	Fern	Aerial parts	1-acetyl-8-hydroxy-β-carboline; 1-acetyl-β-carboline	[27]
18	*Dichothrix baueriana* (Grun.) Bornet & Flahault Rivulariaceae Algae	Cyanobacteria	Biomass	7-chloro-9-mehtyl-β-carboline (bauerine A); 7,8-dichloro-9-mehtyl-β-carboline (bauerine B); 7,8-dichloro-1hydroxy-9-mehtyl-β-carboline (bauerine C)	[28]

(*continued*)

Table 19.1 (continued)

Sr. no.	Binomial name, family, and group	Common name	Part used	Type of β-carboline alkaloid	Reference
19	*Callophycus oppositifolius* (C. Agardh) P. C. Silva. Rhodophyceae Algae	red alga	Biomass	Tetrahydro-β-carbolines, 3-benzylamino-β-carboline (callophycin A)	[29]
20	*Nostoc* 78-12A Algae	blue green alga	Biomass	6-chloro-2-methyl-9 H-pyrido[3,4-b]indol-2-ium (nostocarboline)	[30]
21	*Nodularia harveyana* Thuret ex Bornet et Flahault; *Anabaena cylindrica* Lemmermann; *A. inaequalis* (Kützing) Bornet & Flahault; *Anabaenopsis siamensis* (Antarikanonda) Komárek & Anagnostidis; *Nostoc carneum* C. Agardh ex Bornet & Flahaul; *N. commune* Vaucher ex Bornet & Flahault Nostocaceae; *Phormidium foveolarum* Montagne ex Gomont Phormidiaceae; *Chroococcus Minutes* (Kützing) Nägeli Chroococaceae Chroococaceae	blue green algae	Biomass	Norharman	[31, 32]
22	*Streptomyces* spp. B1848; *Streptomyces* spp. B6005; *Flavobacterium* Bio215 Bacteria		Biomass	1-acetyle-β-carboline; perlolyrin; 1-[5-(hydroxymethyl)furan-2-yl]-9H-pyrido[3,4-b]indole-3-carboxylic acid (Flazin); 1-(9*H*-*β*-carbolin-1-yl)-3-hydroxy-propan-1-one	[5]
23	*Cribiceflina cribaria*	Marine bryozoa Ectoprocta	Biomass	1-vinyl-8-hydroxy-β-carboline; 1-Ethyl-4-methylsulfone-β-carboline; harman; I-ethyl-β-carboline	[33]
24	*Pterocella wesiculosa* Catenicellidae Ectoprocta	Marine bryozoa	Biomass	5-Bromo-8-methoxy-1-methyl-β-carboline	[34]

25	*Dragmacidon* sp. Axinellidae Demospongiae	Sea sponge	Body	Dragmacidonamine A; dragmacidonamine B	[35]
26	*Lignopsis spongiosum* Briareidae Anthozoa	Soft coral	Body	2-methyl-9H-pyrido [3,4*b*]-indole-3-carboxylic acid	[36]
27	*Nephila calviceps* Linn. Anthozoa Arthropoda	Golden orb web spider	Web of spider	1-(2-guanidinoethyl)-1,2,3,4-tetrahydro-6-hydroxymethyl)-β-carboline	[37]
28	7 species of butterflies Nymphalidae Insecta		Insect body	Harmine	[37]
29	*Rattus* sp. Muriade Mammalia	Rat		5-hydroxytryptamine; 5-methoxytryptamine	[38]
30	*Homo sapiens* Linn. Hominidae Mammalia	Human	Plasma and platelets	Tetrahydronorharman; harman	[38]

Table 19.2 Some characteristic features of selected β-carboline alkaloids

Alkaloid type	IUPAC name	Crystal	M. P.	Fluorescence in UV light	UV_{max} (nm)	Solubility
Harman $C_{12}H_{10}N_2$	1-methyl-9H-pyrido[3,4,b] indole	Orthorhombic	237–238 °C	Bright blue	234, 287, 347	Dilute acids
Harmaline $C_{13}H_{14}ON_2$	4,9-dihydro-7-methoxy-1-methyl-3 H-pyrido[3,4-b]indole	Orthorhombic bipyramidal prisms	229–231 °C	Blue	218, 260, 376	Dilute acids and hot ethanol
Harmine $C_{13}H_{12}ON_2$	7-methoxy-1-methyl-9 H-pyrido [3,4-b]-indole	Slender, orthorhombic prisms	261 °C	Blue	241, 301, 336	Slightly soluble in water, ethanol, ether, and chloroform
Norharman $C_{11}H_8N_2$	9 H-pyrido[3,4-b]indole	Orthorhombic	199–201 °C		281, 288, 339, 350	Ethanol, dilute acid
Harmol $C_{12}H_{12}ON_2$	1-methyl-2,9-dihydropyrido[3,4-b] indol-7-one	Slender needles	231 °C	Violet	303, 326, 338	Ethanol, dilute acid
Harmalol $C_{12}H_{12}N_2O$	1-Methyl-4,9-dihydro-3*H*-pyrido [3,4-*b*]indol-7-ol	Red needlelike crystals	211–212 °C	Green	330	Water, acetone, chloroform
Tetrahydroharmine $C_{13}H_{16}N_2O$	(1R)-7-methoxy-1-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole	Slender colorless needles	232–234 °C	Blue	225, 269, 296	Chloroform, ethyl acetate, ethanol, methanol

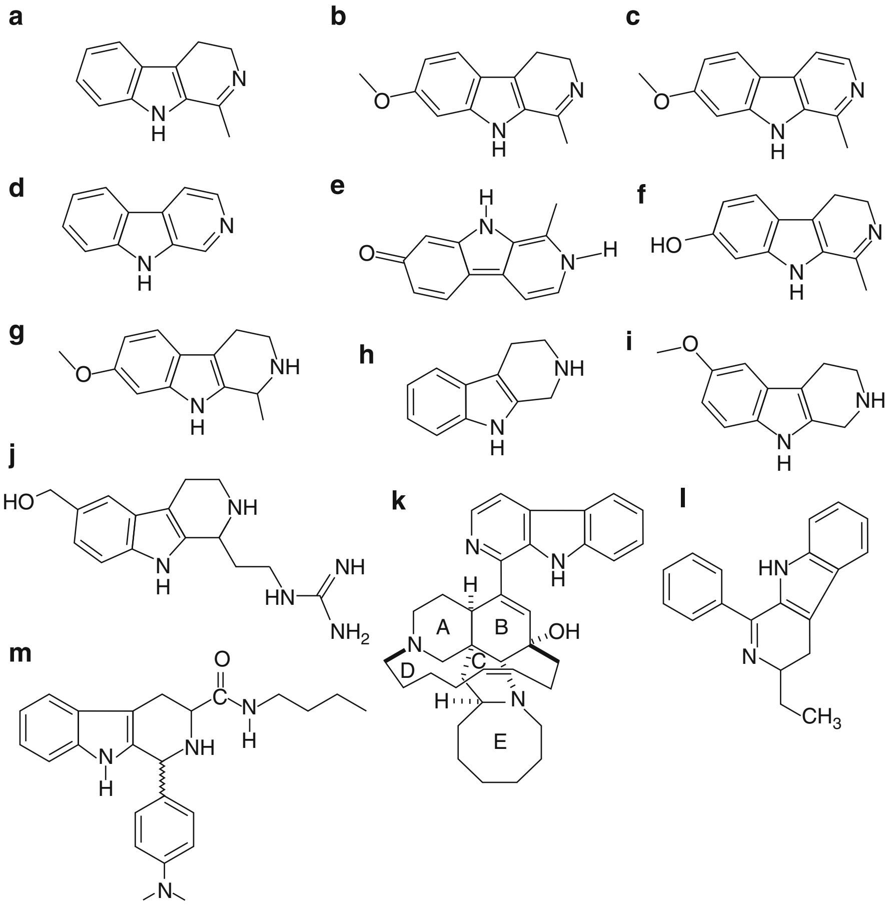

Fig. 19.1 Structure of some β-carbolines synthesized biologically and chemically: (1) From plant (**a**, **b**, **c**, **d**, **e**, **f**, and **g**); (2) From animals (**a**, **d**, **g**,**h**, **i**, **j**, and **k**); (3) Synthesized chemically (**l** and **m**). (**a**) Harman; (**b**) harmaline; (**c**) harmine; (**d**) norharman; (**e**) harmol; (**f**) harmalol; (**g**) tetrahydroharmine; (**h**) tryptoline; (**i**) pinoline; (**j**) 1-(2-guanidinoethyl)-1,2,3,4-tetrahydro-6-hydroxymethyl)-β-carboline [37]; (**k**) manzamine A [39]; (**l**) 3-ethyl-1-phenyl-4,9-dihydro-3*H*-beta-carboline; (**m**) *N*-butyl-1-(4-dimethylamino)phenyl-1,2,3,4-tetrahydro-β-carboline-3-carboxamide [40]

dissolved in 20 ml HCl (2%) and filtered through 0.45 μ filter. Wash the residue with HCl (2%) till colorless filtrate is obtained. Combine the filtrate and extract three times with equal volume of petroleum ether. The aqueous acid layer is basified (pH > 10) with NH_4OH or NaOH and then extracts with equal volume of chloroform and repeat the extraction four times with chloroform. Wash the combine organic layer with water, dry the organic layer over anhydrous sodium sulfate, and remove the solvent under reduced pressure to get the residue. The residue can be used after dissolution in methanol for detection and quantification using TLC, HPTLC, or HPLC method or adsorb the entire residue on a column grade silica gel. Place it on a column of grade silica gel. Carry out the gradient elution with increasing polarities of chloroform–methanol mixture. Monitor the column fractions with TLC/HPLC/HPTLC.

4.3.2 Isolation

For purification and separation, the final residue obtained by above-mentioned method is mixed with 50 ml of 3% v/v acetic acid and allowed to stand for 24 h with occasional stirring. Repeat it for at least three times. Combine the acid extract containing acetates of alkaloids and treat with NaCl (10 gm/100 ml extract), allow to cool and conversion of alkaloidal acetates to alkaloidal hydrochlorides precipitate (ppt). Discard the supernatant. Repeat the process, dissolve the ppt in warm water (50–60 °C), and add ammonia carefully till harmine begins to crystallize, filter, and separate harmine crystals. Add ammonia to filtrate and allow to precipitate harmaline. Wash the crystals and subject to purification or recrystallization [41].

4.3.3 Analysis

Selective and sensitive detection of β-carbolines is possible using HPLC methods in combination with UV, chemiluminescence, and fluorometry. Besides this, LC-MS and GC-MS are the techniques predominantly used for identification, separation, and quantitation of β-carbolines and tetrahydro-β-carbolines [13, 42]. These alkaloids are detected in foods and beverages by HPLC with electrochemical detection at carbon nanotubes–modified glassy carbon electrodes (CNTs–GCE) [43]. In the seeds of *Peganum harmala* L., the alkaloids harmol, harmalol, harmine, and harmaline were separated using a Metasil ODS column by isocratic elution with isopropyl alcohol:acetonitrile:water: formic acid (100:100:300:0.3) (v/v/v/v pH adjusted 8.6 with triethylamine) and detected at 330 nm [13]. These alkaloids can be detected by HPTLC method [44].

Simultaneous quantification of 11 compounds found in ayahuasca (A popular Amazonian botanical medicine and religious sacrament) has been achieved using direct injection/liquid chromatography- electrospray ionization (ESI)-selected reaction monitoring (SRM) - tandem mass spectrometry procedure [45, 46].

5 Biosynthesis

The β-carboline alkaloids are heterocyclic amines, biosynthesized from combination of five- and six-ringed (i.e., cyclic) carbon structures, containing an amine group. β-carboline is made up of planar tricyclic ring structures derived from L-tryptophan (i.e., α-aminoindole-3-propinoic acid), a neutral heterocyclic amino acid containing essentially an indole ring system. The shikimic acid pathway converts the simple carbohydrate precursors phosphoenolpyruvate and erythrose-4-phosphate derived from glycolysis and pentose phosphate pathway, respectively, to aromatic amino acids (L-tryptophan). Decarboxylation of L-tryptophan yields tryptamine. The C-2 of indole nucleus is nucleophilic due to adjacent nitrogen atom which allows it to participate in a Mannich/Pictet–Spengler type reaction. These rearrangements enable a Schiff base derived from tryptamine to interact either with aldehyde or keto acid to yield β-carboline carboxylic acid which on oxidative decarboxylation gives rise to 1-methyl β-carboline. In formation of simple structures of β-carboline, keto acids are used, e.g., harman, harmaline, and elaeagnine. For formation of complex structures, the specific pathways used the aldehyde, e.g., ajmaline (terpenoid indole alkaloids). Hydroxylation followed by methylation of 1-methyl-β-carboline yields harmaline, while on reduction it yields elaeagnine, and upon mild oxidation produces harman. Oxidation of harmaline with loss of water molecule generates harmine while on reduction yield tetrahydroharmine. The demethylation of harmine gives rise to the alkaloid harmalol. Harmaline on condensation, oxidation, and decarboxylation yields to norharmine (Fig. 19.2).

6 Chemical Synthesis

Several beta-carboline alkaloids and their derivatives are chemically synthesized through the Pictet–Spengler reaction using tryptophan, its derivatives, and oxidation of $K_2Cr_2O_7$ by a sequential one-pot synthesis method [47]. There are some other methods reported for the chemical synthesis of β-carboline alkaloids [48–51].

$$\begin{array}{r}\beta\text{-phenyltryptamine} + \text{HCOOH} \rightarrow \text{N-formyl-}\beta\text{-phenyltryptamine}\\ \downarrow +\text{POCl}_3\\ \text{4-Phenyl-3, 4-dihydro-}\beta\text{-carboline}(5)(\text{monohydrate})\end{array}$$

7 Biological Activities

β-carboline alkaloids are widespread in plants and animals. Numerous reports investigated the effects of β-carboline alkaloids on the central nervous system (CNS). However, recent interest in this alkaloid and its derivatives has been focused on their potent antitumor, anticancer, antiviral, antimicrobial, antioxidant, antiparasitic, and several other biological activities [52]. The chemical analysis of plants *Banisteriopsis caapi* used in preparation of ancient drug "ayahuasca" showed that the active chemical constituent named telepathine was found to be identical to a chemical already isolated from *Peganum harmala* and was given the name harmaline. Later, it is well established that the β-carbolines are component of

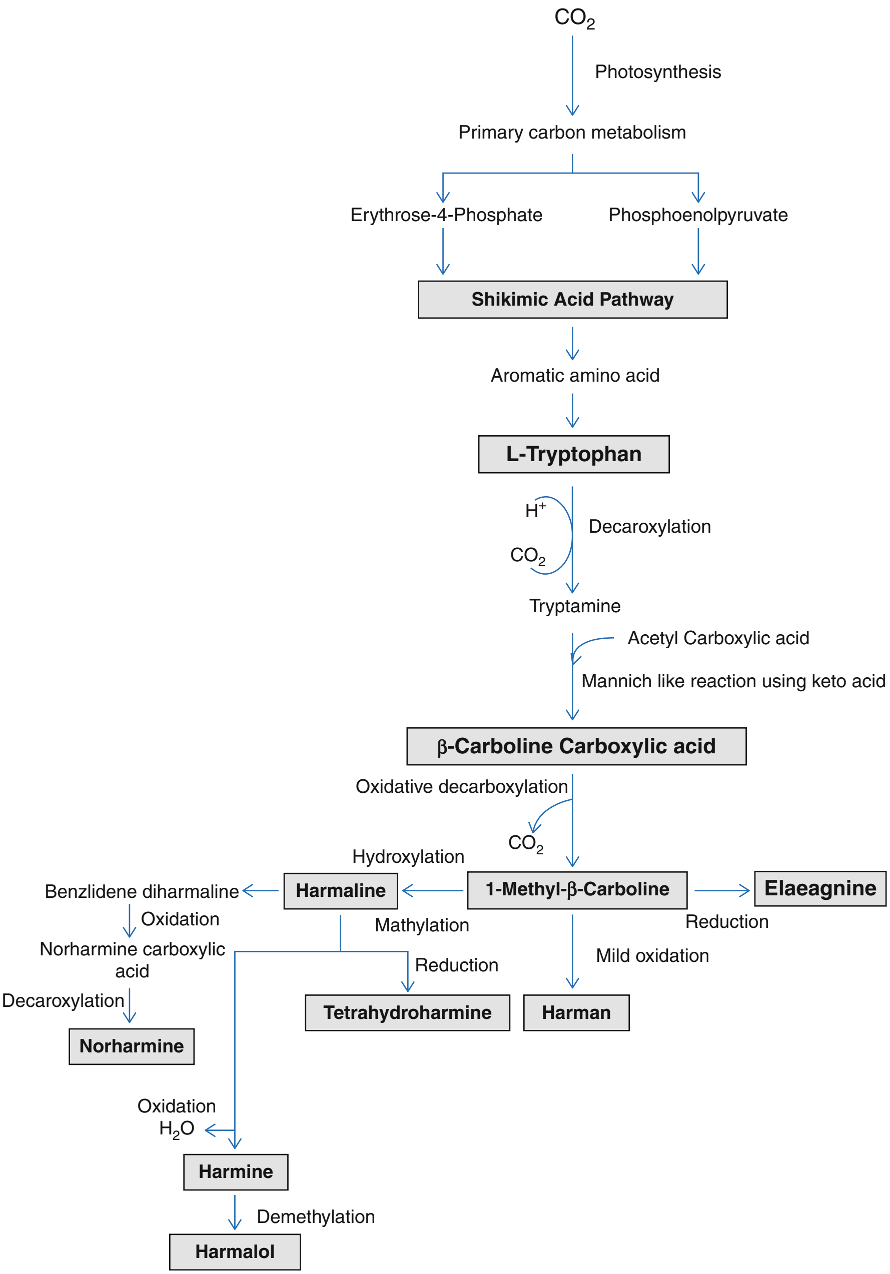
CO2
Photosynthesis
Primary carbon metabolism
Erythrose-4-Phosphate
Phosphoenolpyruvate
Shikimic Acid Pathway
Aromatic amino acid
L-Tryptophan
H+
CO2
Decaroxylation
Tryptamine
Acetyl Carboxylic acid
Mannich like reaction using keto acid
β-Carboline Carboxylic acid
Oxidative decarboxylation
CO2
Hydroxylation
Benzlidene diharmaline
Harmaline
1-Methyl-β-Carboline
Elaeagnine
Oxidation
Mathylation
Reduction
Norharmine carboxylic acid
Reduction
Mild oxidation
Decaroxylation
Tetrahydroharmine
Harman
Norharmine
Oxidation
H2O
Harmine
Demethylation
Harmalol

Fig. 19.2 Outline of harmala alkaloid biosynthesis

some medicinal plants, such as *Peganum harmala* (Zygophyllaceae), *Passiflora incarnata* (Passifloraceae), and *Banisteriopsis caapi* (Malpighiaceae) (details are given in Table 19.1).

Beta carboline has biochemical, neurophysiological and the pharmacological effects in animals and man. They participate in several actions, including inhibition of MAO-A, 5-HT uptake, general inhibiton of Na^+ dependent transports, act as neuromodulators and some may have an endocrinological function [53].

7.1 Interaction with DNA and RNA

Some of the mutagenic and carcinogenic effects of β-carboline alkaloids have been studied in prokaryotic and eukaryotic cells related to their ability to intercalate into DNA [54, 55]. The β-carboline derivatives inhibit DNA topoisomerases and interfere with DNA synthesis. Harmine-, harmaline-, harman-, and norharman-inhibited DNA excisions repair directly or indirectly and lead to altered DNA replication fidelity and enzymatic activities in DNA-repair processes [56, 57]. These alkaloids consequently enhance UV or chemically induced mutagenesis [58]. Harman- and harmine-induced chromosome aberrations in Chinese hamster ovary cells after treatment with the clastogens mitomycin C and UV light [59] while in human neuroblastoma SH-SY5Y cells harman and norharman induced apoptosis as well as necrosis [60]. Aminophenylnorharman, a newly identified beta-carboline alkaloid induced sister chromatid exchange and chromosome aberrations in cultured Chinese hamster lung cells. The different types of β-carboline alkaloids showed variation in affinity to bind DNA, and it is in the order of harmine > harmalol > harmaline > harmane > tryptoline [61]. These alkaloids also bind with yeast RNA [62].

7.2 Interaction with Enzymatic Systems and Receptors

The activity of certain enzymes is inhibited in the presence of beta-carboline alkaloids. The alkaloids interact with neurotransmitter systems such as opiate, GABA, muscarinic, and cholinergic [59]. Inhibition of human DNA Topoisomerase I activity was observed with the seed extract of *Peganum harmala* L. in which harmine, harman, and harmaline are the dominating components [63]. Harmines were found as potent inhibitors of cyclin-dependent kinases [64, 65] and IkappaB kinase. Norharman acts as an inhibitor of the heme containing cytochrome (P450 CYP) related enzymes [66]. Different β-carboline alkaloids show bindings to several receptors like serotonin, benzodiazepines, 5-hydroxytryptamine, dopamine, and imidazoline [67, 68].

Recent results suggest that β-carboline alkaloids may exhibit antidepressant effects [69, 70], probably linked to its inhibitory actions on MAO [71, 72]. This might be the reason for psychopharmacological and toxicological effects of seed and root extracts of *P. harmala* containing β-carbolines and could be the basis for its purported antidepressant actions [73].

7.3 Neurotoxic Effects

Administration of β-carboline alkaloids to human being and a wide variety of laboratory animals produces an intense and generalized action tremor [74]. Harmane is a potent, tremor-producing β-carboline alkaloid and is used in epidemiological studies. It is one of the most abundant of all dietary heterocyclic amines, and human exposure to harmane through diet is greater than that of other heterocyclic amines [9, 67]. Due to its high-lipid solubility, harmane accumulates in brain tissue [75]. It provides the rationale for an in-depth scrutiny of their potential role in the etiology of essential tremor (ET), the most common tremor disorder [76]. β-carboline alkaloids are highly neurotoxic.

7.4 Antitumor Activities

β-carboline alkaloids synthesizing plants such as *P. harmala*, *Banisteriopsis caapi*, and *Passiflora incarnata* have been used as folk medicine in anticancer therapy. Recently, they have drawn attention because of their antitumor and anti-angiogenic activity. Angiogenesis is the physiological process responsible for vasculogenesis and formation of new blood vessels [77]. However, it is also a fundamental step in the transition of tumors from a dormant to a malignant state.

In vivo, anti-angiogenic activity on B16F-10 melanoma cells showed that harmine acts as strong angiogenic inhibitor which significantly decreased pro-angiogenic factors such as vascular endothelial growth factor, nitric oxide, and pro-inflammatory cytokines. At the same time, it increased antitumor factors [78]. β-carboline alkaloids (Callophycin A) show promising antitumor activity against a panel of mammalian cell lines [52, 79, 80]. Several β-carboline derivatives designed and chemically synthesized with various substituents on harmine to produce lower toxicity drug but effective against tumor. However, the structure–activity relationships (SAR) showed that the antitumor activity and acute toxicity as well as neurotoxic effect of β-carboline derivatives are substituent-dependant. Chemically synthesized 1-phenyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid and methyl 1-phenyl-β-carboline-3-carboxylate are potent compounds with better cytotoxicity against insect cultured Sf9 cell line [81].

7.5 Antiviral Activities

The β-carboline alkaloids and their derivatives are emerging as potent antiviral agents. Eudistomins C, D, E, H, I, K, L, N, and Q, manzamine A, and 8-hydroxymanzamine

A were effective against herpes simplex virus-1 (HSV-1and II) [82, 83]. Eudistomin K sulfoxide and eudistomin K have high activities against polio vaccine type-1 virus. Platinum (II) and palladium (II) complexes of harmaline, harmalol, harmine, and harman and (−)-Debromoeudistomin K were also observed to exhibit antiviral activities against influenza virus (A and B) and herpes virus [84]. Recently, harman and its derivatives were found to possess anti-HIV activities against human peripheral blood mononuclear (PBM) cells [85].

7.6 Antimicrobial Activities

Currently, Eudistomins and its derivatives showed antimicrobial activity against *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans* [86, 87]. β-carboline alkaloids from marine bryozoan *Cribricellina cribraria* showed antimicrobial activities against *Pseudomonas aeruginosa* and *Escherichia coli*, *Bacillus subtilis* and fungi *Candida albicans*, *Trichophyton mentagrophytes*, and *Cladisporum resinae* [33]. The antimicrobial activity increased on application of β-carboline alkaloids as binary mixtures against *Proteus vulgaris*, *Bacillus subtilis*, and *Candida albicans* [88]. The antibacterial activity was higher with harmane as compared to harmaline, harmalol, and harmine [89].

7.7 Antiparasitic Activity

The most common β-carboline alkaloids harmine, harmane, and harmaline possess antiparasitic activities against *Leishmania mexicana* [90] and displayed antileishmanial activity toward the intracellular amastigote form of *Leishmania* [91]. Some β-carboline alkaloids are active against epimastigotes of *Trypanosoma* species [56, 92].

7.8 Antithrombotic Activity

The substituent on the β-carboline alkaloids changes the polarity, charge, molecular size, and spatial arrangement which might be the key factors in influencing their biological activity. Antithrombotic activity was detected in phenolic tetrahydro-β-carboline conjugates, perlolyrine, and its analogue [93, 94].

7.9 Antioxidant Activity

It has been reported that β-carbolines and tetrahydro-β-carbolines are potent antioxidants and may be useful for the prevention of diseases associated with oxidative damage [95]. Harmane, harmaline, harmalol, and their peptide conjugates designed and synthesized possess free radical scavenging activity [95, 96].

7.10 Antiplasmodial Activity

Harmine and harmaline showed a moderate in vitro antiplasmodial activity against *Plasmodium falciparum* [97].

7.11 Other Activities

β-carbolines also help in improving object recognition memory and stimulating insulin secretion [98]. They are useful in treatment of hypopigmentation-related disorders such as vitiligo as they induce cellular melanin biosynthesis [99]. Some of the chemically synthesized β-carboline showed better insecticidal activity. Harmine, harmaline, harmol, and harman showed good inhibitory activities against acetylcholinesterase inhibitors (AChE), therefore, they can form the basis of the newest drugs for the treatment of Alzheimer's disease [100]. Recently, it was reported that β-carbolines induce apoptosis by caspase-8 activation in carcinoma cells and function as an anti-inflammatory compound [101]. Pinoline formed naturally in the human body is implicated along with melatonin in the role of the pineal gland in regulating the sleep–wake cycle.

8 In Vitro Production

The alkaloids harmine, harmaline, harman, and harmalol have been detected in callus and cell culture of *Peganum harmala* [102] and *Tribulus terrestris* [23]. The hairy root induction was achieved in *P. harmala* with the help of *Agrobacterium rhizogene*. Harmine is the major alkaloid found in the normal root and hairy root culture. The alkaloids content were enhanced in hairy root culture of *P. harmala* by using the elicitor H_2O_2 and biosynthetic precursor, tryptophan [103]. The somatic embryoids of *Tribulus terrestris* are one of the best sources of Harmala alkaloids [23].

9 Conclusion

Certain β-carbolines are very toxic or precursors of mutagens while others are necessary to maintain normal metabolism or to recover from disorder. Human beings and animals are frequently exposed to beta-carboline alkaloids as they produced in well-cooked food, tobacco smoke, and hallucinogenic beverages. These metabolites are also synthesized endogenously in animal tissue. Some of the medicinal plants particularly *Peganum harmala*, *Passiflora incarnata*, and *Banisteriopsis caapi* are very rich sources of harmala alkaloids and are used in folk medicine for their antispasmodic, anti-jaundice, anti-lumbago, anti-inflammation agent, anticancer, antimalarial, and sedative properties and in the treatment of asthma. The world famous ayahuasca, a narcotic drug, is prepared using *Banisteriopsis* sp. and contains harmala alkaloids. These alkaloids are widely distributed in plants and animals. They are biosynthesized from tryptophan and pyruvate or acetate precursors. Some of these alkaloids and their derivatives are synthesized chemically. Enhanced production of some alkaloids occurs in cell and hairy root culture of *Peganum harmala* and somatic embryoids of *Tribulus terrestris*. Literature survey indicates that the beta-carboline alkaloid had extensive biochemical activities and multiple pharmacological effects. Taking all those reports together, it is revealed that some beta-carbolines and their derivatives from natural source and are chemically synthesized can be an important basis for the design and synthesis of potent pharmaceutical drug.

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Alkaloids Derived from Tryptophan: Terpenoid Indole Alkaloids

20

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Chelliah Jayabaskaran

Contents

1 Introduction: Tryptophan-Derived Alkaloids 576
2 Tryptophan-Derived *Catharanthus* Alkaloids 578
  2.1 Occurrence 578
  2.2 Phytochemistry 580
  2.3 Biosynthesis 581
  2.4 Commercial Aspects 587
  2.5 Biological Activities 588
  2.6 Biotechnological Approaches 589
3 Other Tryptophan-Derived Alkaloids 589
  3.1 Occurrence 589
  3.2 Phytochemistry 596
  3.3 Biosynthesis 596
  3.4 Commercial Aspects 596
  3.5 Biological Activities 598
  3.6 Biotechnological Approaches 600
4 Conclusion and Prospects 600
References 600

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Abstract

L-Tryptophan is an indole ring containing aromatic amino acid derived via the shikimate pathway. The tryptophan-derived alkaloids are found in eight families, of which, Apocynaceae, Loganiaceae, Rubiaceae, and Nyssaceae are the best sources. The alkaloids under discussion are the *Catharanthus* alkaloids, namely, ajmalicine, tabersonine, catharanthine, vindoline, vinblastine, vincristine and vincamine as well as terpenoid alkaloids derived from other families, namely, yohimbine, reserpine, strychnine, brucine, and ellipticine. The above-mentioned alkaloids are pharmacologically very important and hence extremely valuable. This chapter describes various aspects of the tryptophan-derived alkaloids like occurrence, biological activity, phytochemistry, and commercial and biotechnological aspects.

Keywords

Catharanthus • TIA • Tryptophan-derived alkaloids

Abbreviations

16OMT	16-Hydroxytabersonine 16-O-methyltransferase
D4H	Desacetoxyvindoline-4-hydroxylase
DAT	Deacetylvindoline-4-O-acetyltransferase
STR	Strictosidine synthase
T16H	Tabersonine 16-hydroxylase
TDC	Tryptophan decarboxylase
TIA	Terpenoid indole alkaloids

1 Introduction: Tryptophan-Derived Alkaloids

L-Tryptophan is an aromatic amino acid derived via the shikimate pathway. Tryptophan is a precursor for well-known simple alkaloids, namely, melatonin, sumatriptan, eletriptan, and harmine, as well as complex alkaloids such as vinblastine, ajmalicine and serpentine [1] (Fig. 20.1).

Tryptophan decarboxylase (TDC; EC 4.1.1.27) catalyzes the formation of tryptamine from L-tryptophan. Tryptamine is a key precursor of a wide range of terpenoid-derived indole alkaloids in plants. The decarboxylation of L-tryptophan may be viewed as a branching point from primary into secondary metabolism. Tryptamine (indole moiety) condenses with terpenoid moiety provided by the secoiridoid secologanin, which is derived via the mevalonate/ isopentenyl diphosphate pathway. The alkaloids so derived are classified as terpenoid indole alkaloids (TIA) [2]. These pathways are regarded as the limiting branch for TIA biosynthesis in *C. roseus* cell and tissue cultures. Strictosidine synthase (STR: EC 4.3.3.2) is the first enzyme of the TIA pathway catalyzing the condensation of the amino acid-derived tryptamine and the terpenoid secologanin to form strictosidine. STR is the important enzyme leading to the production of either indole or ipecac alkaloids. Strictosidine is the universal

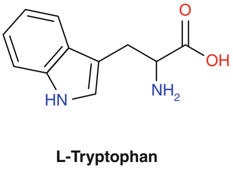

Fig. 20.1 Structure of L-tryptophan, the source of TIAs (Adapted from Ref. [153])

precursor for the therapeutically valuable TIAs, which are produced by four families, namely, Apocynaceae, Loganiaceae, Rubiaceae, and Nyssaceae [3]. Strictosidine occupies a central role in the biosynthesis of all major classes of monoterpenoid indole alkaloids of *C. roseus* as well as in the other members that produce these compounds [4]. Both TDC and STR could be viewed as the important regulatory enzymes, which regulate influx of metabolites into the TIA pathway.

Terpenoid indole alkaloids (TIA) produced via the TIA pathway are one of the largest groups of alkaloids with more than 3,000 representatives. The TIAs are classified as Corynanthe type (e.g., ajmalicine), the Aspidosperma type (e.g., tabersonine), and the Iboga type (e.g., catharanthine) (Fig. 20.2). Tryptophan is a precursor for a wide range of indole alkaloids which are subdivided into simple indole, simple β-carboline, terpenoid indole, quinoline, pyrroloindole, and ergot alkaloids. These pharmacologically important alkaloids are low in occurrence, without synthetic substitutes and therefore have an exorbitant market cost. Research is therefore focused to understand the basic architecture and regulation of biosynthesis of these TIAs in plant and its cultured tissues. *Catharanthus roseus* is a valuable and exclusive source of a number of terpenoid indole alkaloids therefore deserves a special mention in this chapter (Fig. 20.3).

Catharanthus roseus or periwinkle is a perennial subshrub indigenous to Madagascar and belongs to the family Apocynaceae (Fig. 20.2). *Catharanthus* has been used from time immemorial by traditional healers all over the world to treat many ailments. Till date, more than 130 bioactive terpenoid indole alkaloids (TIAs) have been identified. The production and storage of these alkaloids in plants is quite complex and multicellular in organization (at least four cell types involved: epidermis, internal phloem-associated parenchyma, laticifers, and idioblasts) and also involve spatiotemporal organization and is influenced by environmental factors [2, 5]. The TIA pathway is regulated mainly at four levels, inter- and intracellular compartmentation (chloroplast, vacuole, nucleus, endoplasmic reticulum and cytosol) of enzymes and substrates and interorganellar coordination and transport; transcriptional regulation of TIA biosynthetic genes; posttranslational regulation of enzymes and regulation by environment and culture conditions [2, 5] (Fig. 20.4).

The current research on the TIAs focuses on understanding six important aspects, namely, precise localization and expression of pathway enzymes (using modern in situ RNA hybridization tools), mechanisms of trafficking of pathway intermediates (both inter and intracellular), cloning and functional validation of genes belonging to the known or hitherto unknown pathway enzymes, global regulation mechanism of the pathway by transcription factors, control of metabolite flux and diversion at crucial branch points and strategized metabolic engineering approaches to improve the productivity of the desired TIAs in plant or corresponding cultured tissues [5].

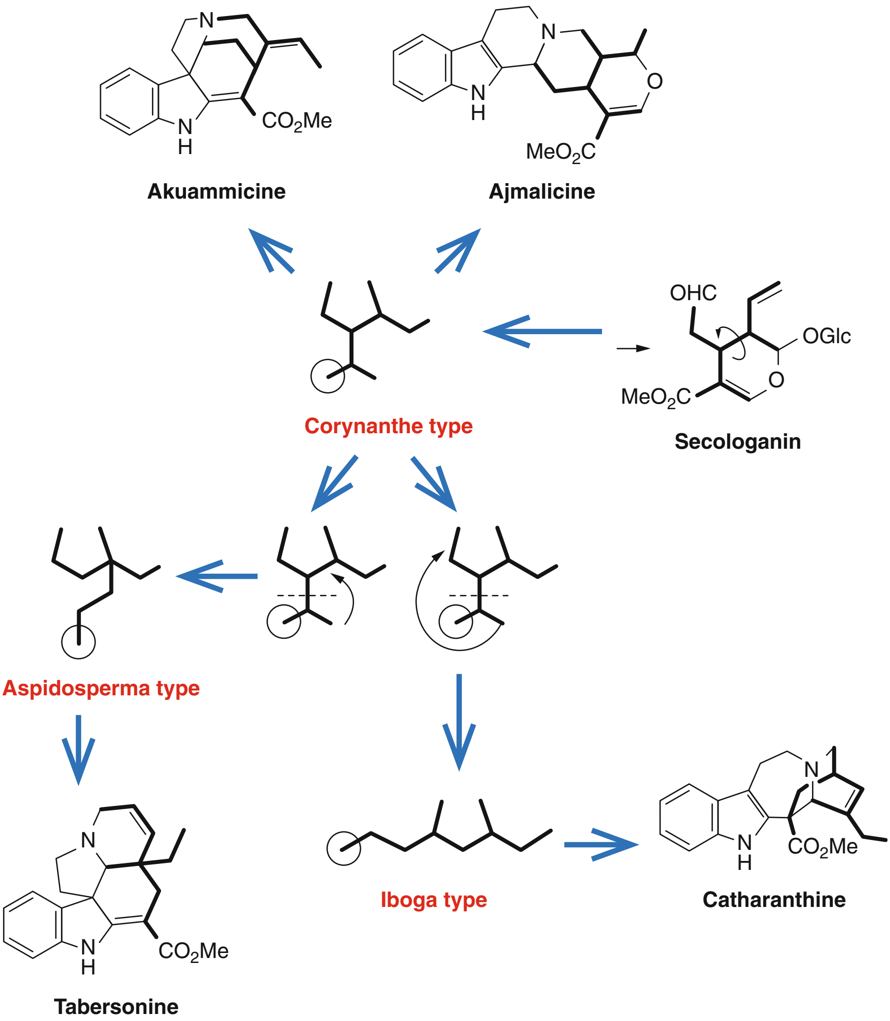
Akuammicine
CO$_2$Me
N
H
Ajmalicine
MeO$_2$C
O
Corynanthe type
OHC
OGlc
MeO$_2$C
Secologanin
Aspidosperma type
Tabersonine
Iboga type
Catharanthine

Fig. 20.2 Types of skeleton in tryptophan-derived alkaloids (Adapted from Ref. [15])

2 Tryptophan-Derived *Catharanthus* Alkaloids

2.1 Occurrence

2.1.1 Ajmalicine

Ajmalicine is found in plants belonging to Apocynaceae, namely, *Catharanthus roseus*, *Rauwolfia serpentina*, and *Rauwolfia vomitoria*. Ajmalicine was first isolated from yohimbe bark and is one of the principal alkaloids of *Catharanthus roseus* and widely used to treat circulatory disorders [6] (Fig. 20.3). Ajmalicine is

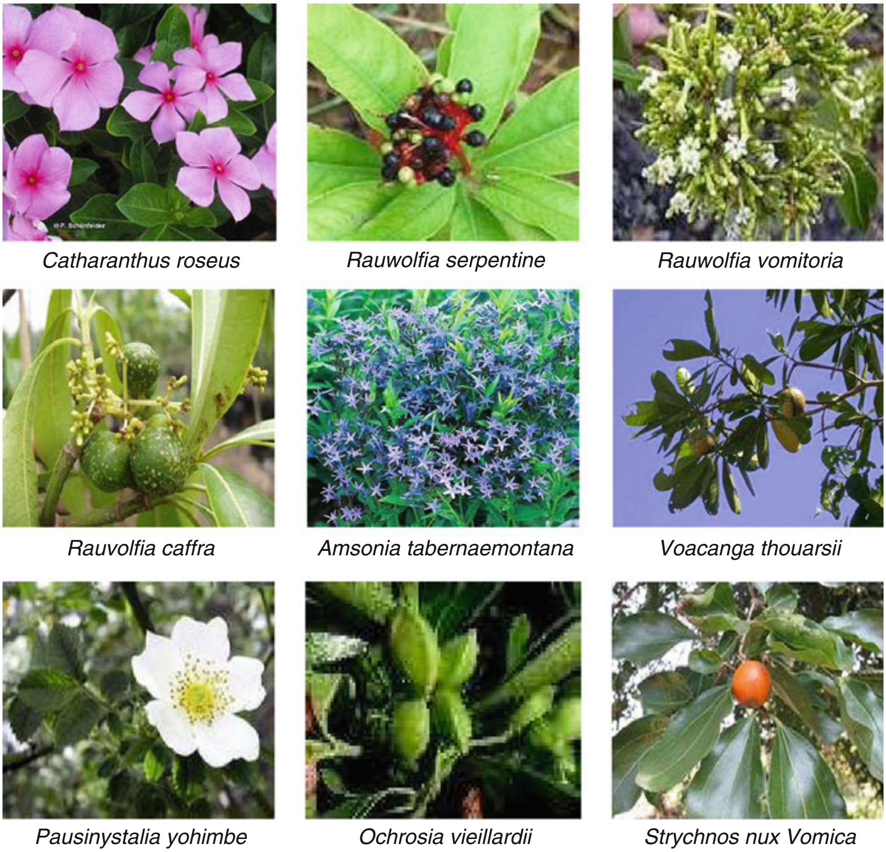
Catharanthus roseus
Rauwolfia serpentine
Rauwolfia vomitoria
Rauvolfia caffra
Amsonia tabernaemontana
Voacanga thouarsii
Pausinystalia yohimbe
Ochrosia vieillardii
Strychnos nux Vomica

Fig. 20.3 TIA producing plants (Courtesy Refs. [154–162])

extracted from the roots of *C. roseus*. Other sources for commercial extraction of ajmalicine are the roots of *Rauwolfia yunnanensis* Tsiang. I. [7], *C. trichophyllus* roots [8], and *Rauwolfia caffra* [9] (Fig. 20.5a).

2.1.2 Tabersonine

Tabersonine has been reportedly found in plants belonging to Apocynaceae, e.g., *Amsonia tabernaemontana*, *Amsonia sinensis*, *Voacanga thouarsii*, and *Melodinus reticulatus*, apart from *C. roseus*. Tabersonine is extracted from voacanga seeds for commercial purposes [10–12] (Fig. 20.5b).

2.1.3 Catharanthine

Catharanthine is produced in young developing leaves of *Catharanthus*. It accumulates entirely in the leaf wax exudates outside the leaf epidermis spatially separating it from vindoline [13] (Fig. 20.5c).

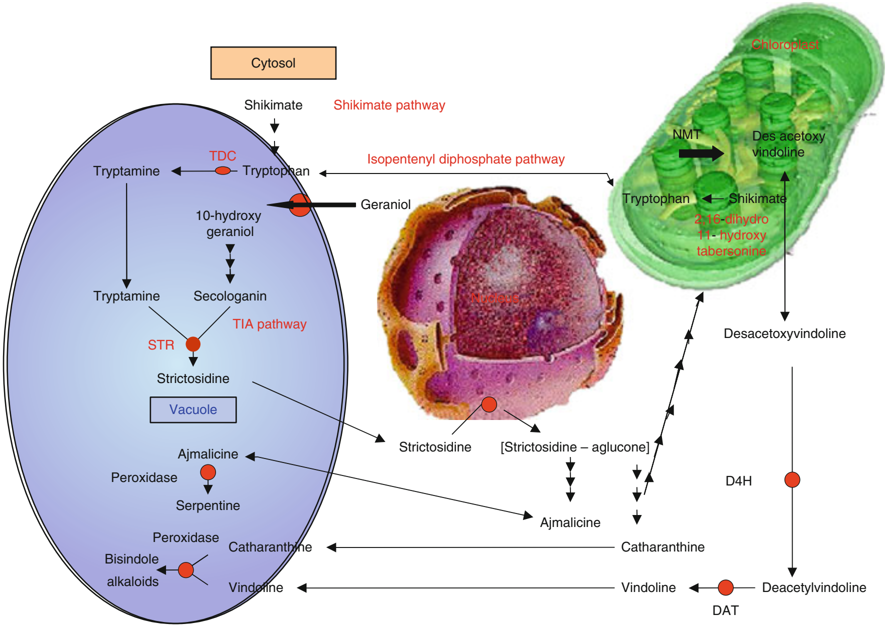
Cytosol
Shikimate
Shikimate pathway
TDC
Tryptamine
Tryptophan
Isopentenyl diphosphate pathway
Geraniol
10-hydroxy geraniol
Tryptamine
Secologanin
TIA pathway
STR
Strictosidine
Vacuole
Ajmalicine
Peroxidase
Serpentine
Peroxidase
Catharanthine
Bisindole alkaloids
Vindoline
Nucleus
Strictosidine
[Strictosidine – aglucone]
Ajmalicine
Catharanthine
Vindoline
DAT
Chloroplast
NMT
Des acetoxy vindoline
Tryptophan
Shikimate
2,16-dihydro 11- hydroxy tabersonine
Desacetoxyvindoline
D4H
Deacetylvindoline

Fig. 20.4 Regulation of TIA pathway (Adapted from Ref. [2])

2.1.4 Vindoline

Vindoline is the main terpenoid indole alkaloid accumulated in leaves of *Catharanthus roseus* [14] (Fig. 20.5d).

2.1.5 Vinblastine and Vincristine

Vinblastine and vincristine are bisindole alkaloids obtained by the condensation of catharanthine and vindoline. The alkaloids are found exclusively in *Catharanthus* leaves and have great economic value [15] (Fig. 20.5e).

2.1.6 Vincamine

Vincamine is extracted from the leaves of *Vinca minor* belonging to Apocynaceae family, known as lesser periwinkle native to central and southern Europe. A closely related semisynthetic derivative of vincamine most widely used as medicine is known as ethyl-apovincaminate or vinpocetine [16–19] (Fig. 20.5f).

2.2 Phytochemistry

The alkaloid-related chemistry is summarized in Table 20.1.

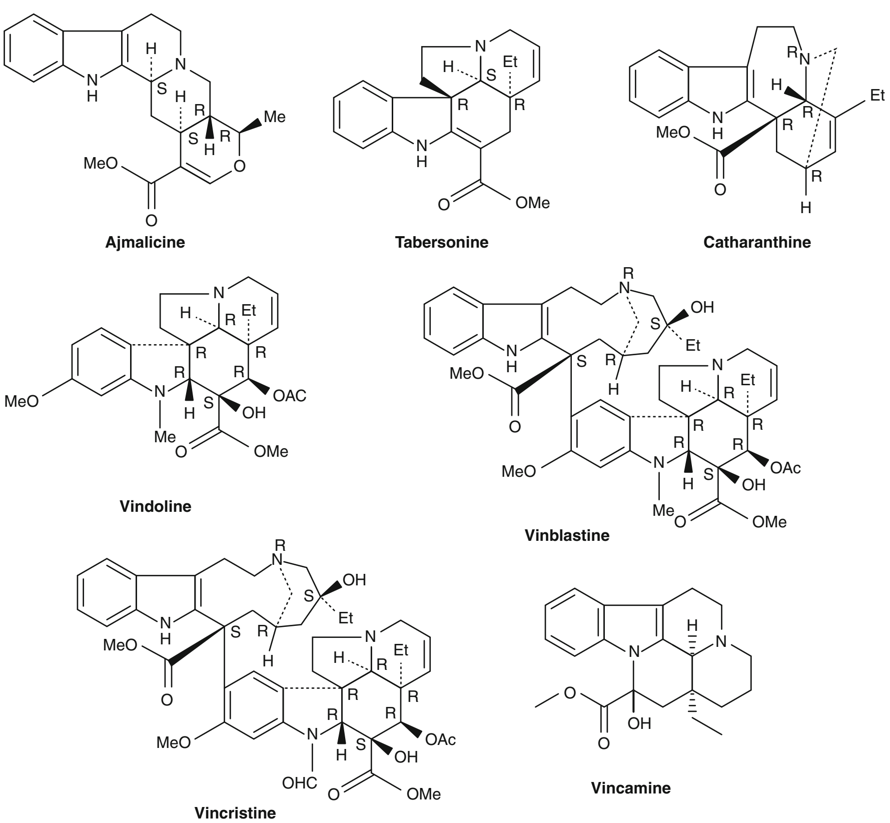

Fig. 20.5 *Catharanthus* alkaloids derived from tryptophan

2.3 Biosynthesis

Tryptophan is converted to tryptamine by tryptophan decarboxylase, encoded by a single gene in *Catharanthus roseus* which is regulated both developmental and inducible regulation. Secologanin biosynthesis is conversion of geraniol to 10-hydroxygeraniol mediated by geraniol 10-hydroxylase (G10H) which is a membrane-bound P450 monooxygenase, dependent on NADPH and O_2, and displays light-reversible CO inhibition. The conversion is also catalyzed by a P450-dependent enzyme. The level of G10H activity positively correlates with alkaloid accumulation indicating that the production of terpenoid precursors might play a regulatory role in TIA biosynthesis since the addition of secologanin or loganin to *C. roseus* cell cultures increases alkaloid accumulation. Strictosidine is produced by the condensation of tryptamine and secologanin which is the common precursor to all TIAs. STR is encoded by a single gene with

Table 20.1 Phytochemistry of catharanthus alkaloids

Alkaloid	Chemical name/synonym	Molecular weight	Molecular formula	Appearance	Identification UV (max) specific optical rotation	References
Ajmalicine	(19α)-16,17-Didehydro-19-methyloxayohimban-16-carboxylic acid methyl ester	352.4	$C_{21}H_{24}N_2O_3$	White to slightly yellow powder	227, 292 nm −60° (chloroform)	[25]
Catharanthine	(+)-3,4-Didehydrocoronaridine; catharanthin ; ibogamine-18-carboxylic acid,3,4-didehydro-,methyl ester,(2-alpha,5-beta,6-a ; methyl(2-alpha,5-beta,6-alpha,18-beta)-3,4-didehydroibogamine-18-carboxylate ; catharanthine; catharanthine base ; methyl (2alpha,5beta,6alpha)-3,4-didehydroibogamine-18beta-carboxylate	336.42	$C_{21}H_{24}N_2O_2$	White to light yellow crystalline	UV max (ethanol): 226, 284, 292 nm; [a]D27 +29.8° (CHCl3)	[26]
Tabersonine	Aspidospermidine-3-carboxylic acid, 2, 3, 6, 7-tetradehydro-, methyl ester	336.42	$C_{21}H_{24}N_2O_2$	White fine powder		[27]
Vindoline	Aspidospermidine-3-carboxylic acid, 4-(acetyloxy)-6,7-didehydro-3-hydroxy-16-methoxy-1-methyl-, methyl ester, (2b,3b,4b,5a,12b,19a)	456.54	$C_{25}H_{32}N_2O_6$	White crystalline powder	UV max (ethanol): 212, 250, 304 nm; [a]D20 −18° (chloroform); [a]D27 +42° (chloroform)	[28]
Vinblastine	Rozevin; vincoblastine; vincaleucoblastin dimethyl (2β,3β,4β,5α,12β,19α)-15-[(5 S,9 S)- 5-ethyl- 5-hydroxy-9-(methoxycarbonyl)]	810.97	$C_{46}H_{58}N_4O_9$	White crystalline fine powder	UV max (methanol): 212, 262, 284, 292 nm; [a] D26 −28°	[29]

Vincristine	VCR; oncovin; oncovin; nsc67574; vincrisul; kyocristine (3aR,3a1R,4R,5 S,5aR,10bR)-methyl 4-acetoxy-3a-ethyl-9-((5 S,7 S,9 S)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-2,4,5,6,7,8,9,10-octahydro-1 H-3,7-methano[1]azacycloundecino[5,4-b]indol-9-yl)-6-formyl-5-hydroxy-8-methoxy-3a,3a1,4,5,5a,6,11,12-octahydro-1 H-indolizino[8,1-cd]carbazole-5-carboxylate	923.04	$C_{46}H_{56}N_4O_{10}{\bullet}H_2SO_4$	White powder	UV max (methanol): 218, 252, 285, 293 nm; [a]D26 +8.5°	[30]
Vincamine	(3α,14β,16α)-14,15-dihydro-14-hydroxyeburnamenine-14-carboxylic acid methyl ester or methyl (15R,17 S,19R)-15-ethyl-17-hydroxy-1,11-diazapentacyclo [9.6.2.02,7.08,18.015,19]nonadeca-2(7),3,5,8(18)-tetraene-17-carboxylate	354.44	$C_{21}H_{26}N_2O_3$	White microcrystalline powder	E1% 1 cm (268 nm): between 231 and 255; +39.0 and +43.5 (dry basis)	[31]

multiple isoforms due to glucosylations. STR cDNAs have been isolated from *Rauwolfia serpentina* and *C. roseus*. Strictosidine is deglycosylated by strictosidine-D-glucosidase (SGD) and reportedly exhibits high molecular mass, suggesting aggregation. The deglycosylated strictosidine is converted via several unstable intermediates to 4,21-dehydrogeissoschizine which is the source of many TIAs. The pathway enzymes for the TIAs so derived still remain obscure or uncharacterized. The conversion to catharanthine is largely unknown, and missing links to the synthesis of vindoline have to be yet resolved. Vindoline is ultimately coupled to catharanthine by a nonspecific peroxidase to yield vinblastine. The first of six steps involved in the conversion of tabersonine to vindoline involves hydroxylation at the C-16 position, 16-*O*-methylation, hydration of the 2,3-double bond, and *N*-methylation of the indole-ring nitrogen. The second-to-last step in vindoline biosynthesis is catalyzed by a 2-oxoglutarate-dependent dioxygenase that hydroxylates the C-4 position of desacetoxyvindoline. The last step in vindoline biosynthesis is catalyzed by acetylcoenzyme A: deacetylvindoline 4-*O*-acetyltransferase (Fig. 20.6a, b, c).

2.3.1 Ajmalicine

Ajmalicine is derived from tryptamine (partly from geraniol) via secologanin, strictosidine, and cathenamine. It has a Corynanthe skeleton from deglycosylated strictosidine [28]. Ajmalicine is synthesized in the cytosol by the action of cathenamine reductase on cathenamine facilitated by NADPH activity [32]. The biosynthesis of ajmalicine has been studied with reference to improving the content by various biotechnological approaches (Fig. 20.4). The regulation per se of ajmalicine production has not been explored in larger detail.

2.3.2 Tabersonine

Tabersonine is derived from strictosidine through a series of yet uncharacterized pathway via an intermediate cathenamine. The genes related to tabersonine biosynthesis as well have been an unexplored area [33].

2.3.3 Catharanthine

Catharanthine is synthesized via an uncharacterized pathway from strictosidine via intermediates, strictosidine aglycone, 4,21-didehydrogeissoschizine, and stemmedine [5].

2.3.4 Vindoline

Four genes and six steps are involved in the synthesis of vindoline in the leaves of *C. roseus*. Tabersonine 16-hydroxylase (T16H) anchored to the ER catalyzes the first step of vindoline biosynthesis. 16-Hydroxytabersonine 16-O-methyltransferase (16OMT) homodimers (excluded from nucleus) in the cytoplasm of aerial organ epidermis catalyzes the second step. Desacetoxyvindoline-4-hydroxylase (D4H) and deacetylvindoline-4-O-acetyltransferase (DAT), located in the nucleocytoplasmic compartment,

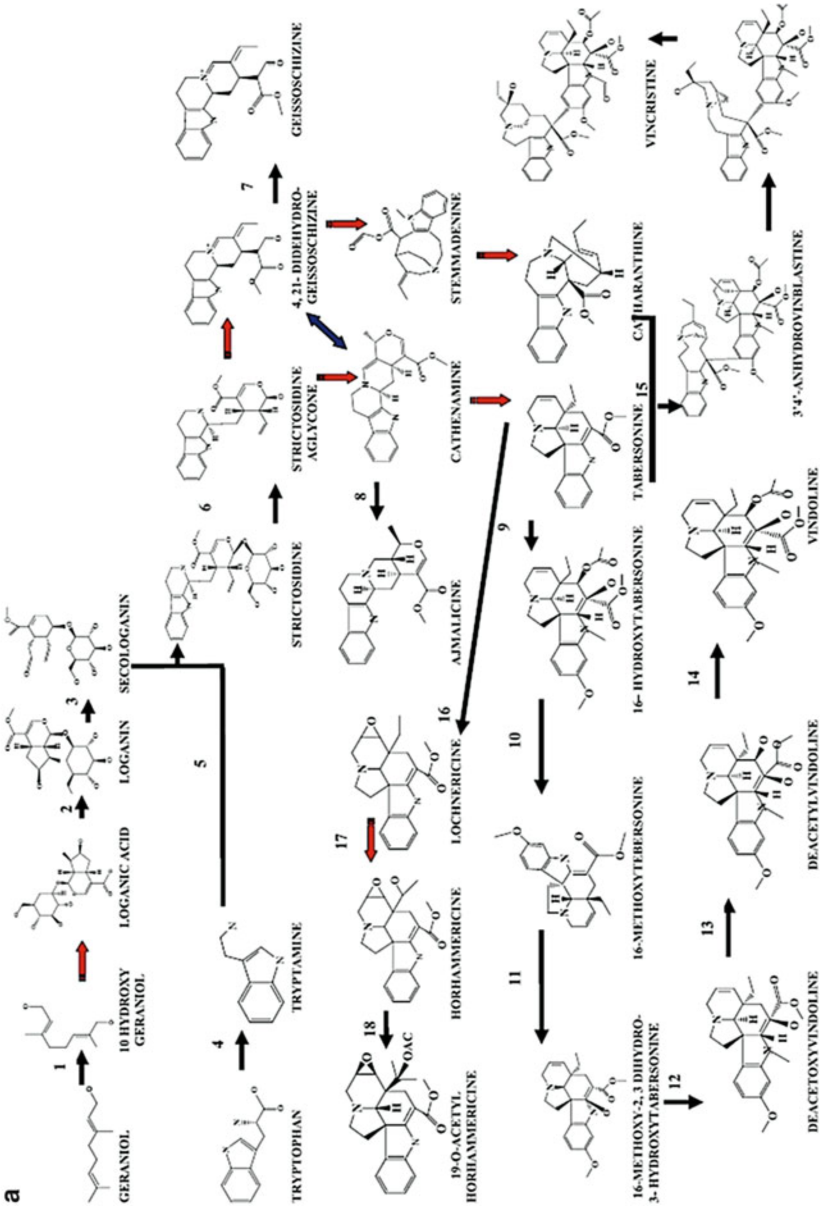
a
GERANIOL
1
10 HYDROXY GERANIOL
LOGANIC ACID
2
LOGANIN
3
SECOLOGANIN
TRYPTOPHAN
4
TRYPTAMINE
5
STRICTOSIDINE
6
STRICTOSIDINE AGLYCONE
4, 21- DIDEHYDRO-GEISSOSCHIZINE
7
GEISSOSCHIZINE
8
AJMALICINE
CATHENAMINE
STEMMADENINE
9
TABERSONINE
CATHARANTHINE
15
16
LOCHNERICINE
17
HORHAMMERICINE
18
OAC
19-O-ACETYL HORHAMMERICINE
16- HYDROXYTABERSONINE
10
16-METHOXYTEBERSONINE
11
16-METHOXY-2,3 DIHYDRO-3- HYDROXYTABERSONINE
12
DEACETOXYVINDOLINE
13
DEACETYLVINDOLINE
14
VINDOLINE
3'4'-ANHYDROVINBLASTINE
VINCRISTINE

Fig. 20.6 (continued)

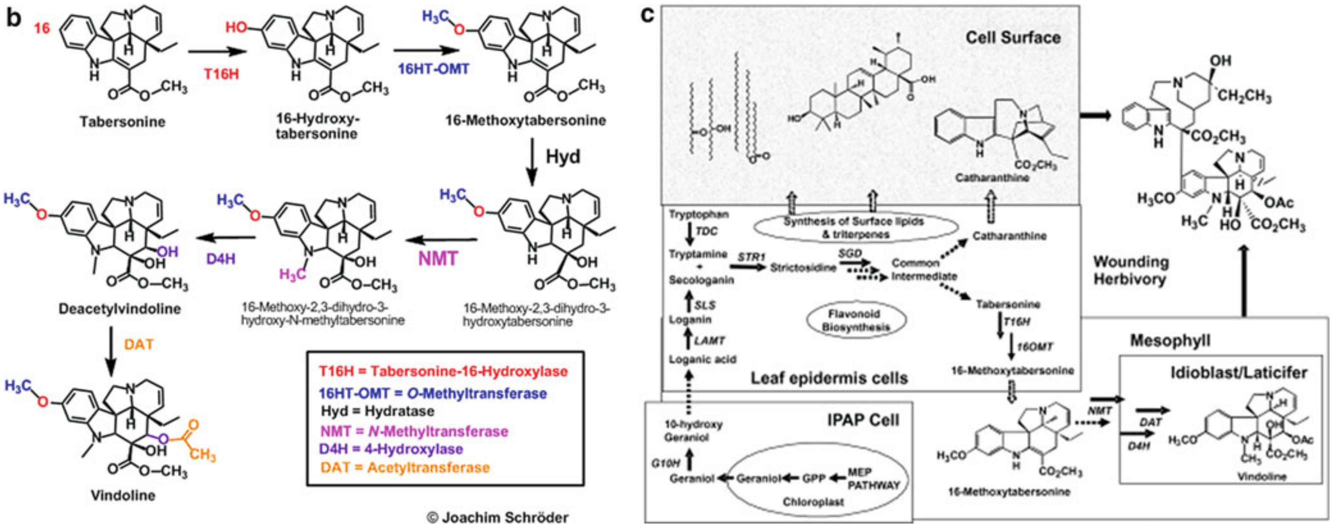
b
16
Tabersonine
T16H
16-Hydroxy-tabersonine
16HT-OMT
16-Methoxytabersonine
Hyd
16-Methoxy-2,3-dihydro-3-hydroxytabersonine
NMT
16-Methoxy-2,3-dihydro-3-hydroxy-N-methyltabersonine
D4H
Deacetylvindoline
DAT
Vindoline
T16H = Tabersonine-16-Hydroxylase
16HT-OMT = O-Methyltransferase
Hyd = Hydratase
NMT = N-Methyltransferase
D4H = 4-Hydroxylase
DAT = Acetyltransferase
c
Cell Surface
Catharanthine
Synthesis of Surface lipids & triterpenes
Tryptophan
TDC
Tryptamine
+
Secologanin
SLS
Loganin
LAMT
Loganic acid
STR1
Strictosidine
SGD
Common Intermediate
Tabersonine
T16H
16OMT
16-Methoxytabersonine
Flavonoid Biosynthesis
Leaf epidermis cells
IPAP Cell
10-hydroxy Geraniol
G10H
Geraniol
GPP
MEP PATHWAY
Chloroplast
Wounding Herbivory
Mesophyll
Idioblast/Laticifer
NMT
DAT
D4H
Vindoline
OH
CH₂CH₃
CO₂CH₃
H₃CO
OAc

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Fig. 20.6 (**a**) Terpenoid indole alkaloid biosynthesis in *C. roseus* (Courtesy Ref. [5]). 1 Geraniol-10-hydroxylase (G10H), 2 loganic acid methyltransferase (LAMT), 3 secologanin synthase (SLS), 4 tryptophan decarboxylase (TDC), 5 strictosidine synthase (STR), 6 strictosidine β-glucosidase (SGD), 7 geissoschizine dehydrogenase (GDH), 8 cathenamine reductase (CR), 9 tabersonine 16-hydroxylase (T16H), 10 16-hydroxytabersonine-16-O-methyltransferase (16OMT), 11 uncharacterized enzyme, 12 16-methoxy-2,3-dihydro-3-hydroxy-tabersonine N-methyltransferase (NMT), 13 deacetoxyvindoline 4-hydroxylase (D4H), 14 6-17-O-deacetylvindoline O-acetyltransferase (DAT), 15 class III basic peroxidase (PRX1), 16 tabersonine 6,7-epoxidase (T6,7E), 17 tabersonine 19 hydroxylase (T19H), 18 minovincinine-19-O-acetyltransferase (MAT) (**b** and **c**) Vindoline biosynthesis and its spatial and temporal regulation (Courtesy Refs. [13, 33])

are monomers following diffuses into nucleus. However, no interaction and therefore channeling of vindoline are detected between D4H and DAT. The understanding of spatial regulation in vindoline biosynthesis is still not complete [5]. Vindoline production in in vitro cultures like cell suspension culture is very less due to absence of chloroplasts.

2.3.5 Vinblastine and Vincristine

Vinblastine and vincristine are synthesized from monomers catharanthine and vindoline. Vinblastine and vincristine differ in the nature of the substituent group on the dihydroindole moiety, which is either a methyl group (vinblastine) or a formyl group (vincristine) [20].

2.3.6 Vincamine

Vincamine is an Aspidosperma type of alkaloid supposedly produced through two chemical reactions beginning with the catalytic reduction of tabersonine, an alkaloid extract of voacanga seeds. The resulting product, vincadiformine is oxidized forming vincamine [34].

2.4 Commercial Aspects

2.4.1 Ajmalicine

Each year, approximately 3,600 kg of ajmalicine is produced at a market price of about $2,000/kg [20]. Technically, naturally grown *C. roseus* has 50 % lower concentration of ajmalicine but would cost ca. $0.70/lb dry biomass ($619/kg ajmalicine). Alternatively, in vitro culture of *C. roseus* as cell culture yields far higher yields of ajmalicine, but the cost of production is estimated to be approximately $7.30/lb dry biomass ($3,215/kg ajmalicine). This is attributed to the slow specific product accumulation rate (0.26 mg/g/day) which has to be improved by a factor of 40 to make the process competitive [9].

2.4.2 Tabersonine

Tabersonine is available commercially at US$ 1,700 per kg [21]. One hundred grams of voacanga beans yields 1.7 g of pure tabersonine hydrochloride [22].

2.4.3 Catharanthine and Vindoline

Catharanthine and vindoline are per se not used as drugs but as precursors for vinblastine and vincristine. Biotechnological approaches at improving the yield in in vitro cultures have been largely varied.

2.4.4 Vinblastine and Vincristine

The yield of the dimeric alkaloids from *Catharanthus* plants is 0.0005 %. The dosage required per adult per injection is 1.4 mg/m^2 body surface. Currently, a single kilogram of vincristine costs around $US 20 million, making it one of the costliest drugs around [23]. Vinblastine, another anticancer drug from the same plant, is present at levels a thousand times higher than vincristine, and the cost is a third of vincristine.

2.4.5 Vincamine

Vincamine constitutes about 25–65 % of indole alkaloids extracted from *Vinca minor* [24].

2.5 Biological Activities

2.5.1 Ajmalicine

Ajmalicine is used to treat peripheral and cerebral vascular disorders. Ajmalicine acts as a selective alpha sympatholytic drug. It acts by depleting peripheral noradrenaline stores resulting in decrease of peripheral resistance and blood pressure. It is also known to deplete catecholamine and serotonin stores in the brain, heart, and many other organs. Many commercial formulations of ajmalicine are available in the market under the trade names, namely, Card-Lamuran^{TM}, Circolene^{TM}, Cristanyl^{TM}, Duxil^{TM}, Duxor^{TM}, Hydrosarpon^{TM}, Iskedyl^{TM}, Isosarpan^{TM}, Isquebral^{TM}, Lamuran^{TM}, Melanex^{TM}, Saltucin Co^{TM}, Salvalion^{TM}, and Srapan^{TM} [32].

2.5.2 Tabersonine

Tabersonine has wide applications as hypotensive, antitumor, hypoglycemic, and diuretic agent. It is used against the consequences of stroke, ischemic hypertensive encephalopathy, cerebrovascular disease caused by depression, anxiety, mood swings, and premature aging and is suitable to eliminate the symptoms of brain degeneration, such as dizziness, headache, memory loss, attention, aphasia, Meniere's syndrome, etc. It is also used during retinal hemorrhage, tachycardia, and other autonomic nerve dysfunction [21].

2.5.3 Catharanthine

Catharanthine is reported to stimulate the release of amylase from pancreatic fragments by causing an increase in cytoplasmic Ca^{2+} and cause extensive degranulation of pancreatic acinar cells with accumulation of membrane material in the Golgi region. Catharanthine is also reported to be the indole part of vinblastine and vincristine required for interaction to tubulin [35, 36].

2.5.4 Vindoline

Vindoline acts as an anchor for mediating the effects evident using vinblastine and vincristine. Catharanthine and vindoline are three times less effective than the dimeric alkaloids in bringing about the inhibition of tubulin self-assembly into microtubules [36].

2.5.5 Vinblastine and Vincristine

Vinblastine is used in treatment of Hodgkin's lymphoma, non-Hodgkin's lymphoma, breast cancer, uterine and cervical cancer, small cell bronchial cancer, rhabdomyosarcoma, and various sarcomas. Vincristine is used in the treatment of acute lymphoblastic leukemia (ALL) especially in children. Vinblastine and vincristine inhibit microtubule formation during mitosis and therefore prevent cells from reproducing which is more pronounced in cancer cells [15, 37].

2.5.6 Vincamine

Vincamine is effective in treating ailments related to central nervous system, cardiovascular system, and on the vessels of the brain. It is used to treat hypertension, vertigo, transient ischemic deficits, headache, and age-related neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's diseases, and improve memory, mental productivity, and concentration [38–45].

2.6 Biotechnological Approaches

The biotechnological approaches attempted have been summarized in Table 20.4 below. In brief, various approaches to improve the extraction from plants and increase the yield using various types of in vitro cultures like hairy root culture, cell culture, and callus culture have been attempted. The in vitro cultures have been optimized with respect to medium composition and culture conditions including the use of various biotic and abiotic elicitors. Strategic approaches to improve yield, namely, feeding of precursors with and without optimized culture conditions and elicitors, have been elaborately explored along with overexpression of homologous and heterologous genes. Genomic, proteomic, and metabolomic approaches have provided valuable insights. The current focus is on understanding the spatial and temporal regulation of the biosynthetic enzymes and products.

3 Other Tryptophan-Derived Alkaloids

3.1 Occurrence

3.1.1 Reserpine

The alkaloid reserpine is obtained naturally from the rhizome and roots of *Rauwolfia* spp. *Rauwolfia serpentina* is a small shrub belonging to Apocynaceae family and found in India, Burma, Java, and Thailand. *Rauwolfia vomitoria* is widely grown in Congo, Africa, and contains more than twice quantity of reserpine. Ninety percent of reserpine is present in the roots and only 10 % in the stem and leaves [107] (Fig. 20.7a).

3.1.2 Yohimbine

Yohimbine is obtained naturally in *Pausinystalia yohimbe, Rauwolfia serpentina,* and *Alchomia floribanda*. The tree is native to the coastal forests of Central Africa

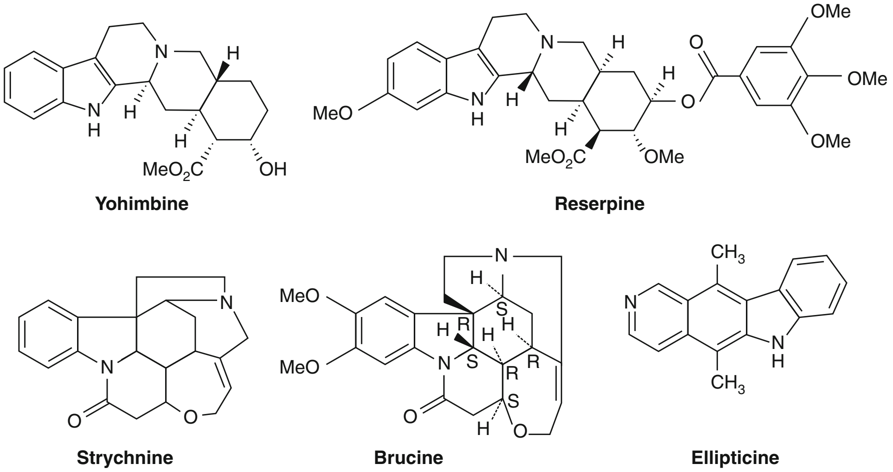

Fig. 20.7 Other tryptophan-derived alkaloids

and distributed from Southeast Nigeria to the Congolese Mayombe region. The commercial product is obtained from the dried bark of *Pausinystalia yohimbe* [108–111] (Fig. 20.7b).

3.1.3 Ellipticine

Ellipticine is isolated from species of the Apocynaceae family, including *Bleekeria vitensis, Aspidosperma subicanum, and Ochrosia* species. Ellipticine was first isolated from the leaf material of *Ochrosia elliptica* Labill by Goodwin et al. in 1959 [112]. It is a tropical evergreen tree belonging to the Apocynaceae family also contained several other alkaloids, including 9-methoxyellipticine. Ellipticine has since been isolated from several other plants of the Apocynaceae family (*Ochrosia vieillardii, Ochrosia acuminata, Ochrosia oppositifolia, and Ochrosia moorei*) [113–115] and from *Strychnos dinkagei* of the Loganiaceae family [115, 116]. *Ochrosia oppositifolia* is widely distributed on the coasts of the islands throughout Southeast Asia and the Pacific. In Africa, it is restricted to the Seychelles. Extracts of the roots of *Ochrosia acuminata* Val. (Apocynaceae) have been used by native people in Savu, Indonesia, to treat tumors and ectopic pregnancy (Fig. 20.7c).

3.1.4 Strychnine and Brucine

Strychnine and brucine are exclusively found abundantly in seeds of *Strychnos nux-vomica* and in varying quantities in roots, fruits, pulp, wood, leaves, and bark. *Strychnos nux-vomica* (Loganiaceae) is a tree 10–13 m high with a distribution including Ceylon, India, East Bengal, Burma, Thailand, Laos, Cambodia, and South Vietnam. These two alkaloids are present together or separately in other *Strychnos* species. This genus consists of around 196 species spread across the globe [117] (Fig. 20.7d, e) (Table 20.2).

Table 20.2 Biotechnological approaches to improve TIA content in *C. roseus* (**a**); vincamine content in Catharanthus (**b**)

	Aspect	Condition/Treatment	Culture type	Ajmalicine	Tabersonine	Catharanthine	Vindoline	Vinblastine	Vincristine	References
Elicitors	Biotic	*M. isabellina* fungal culture filtrate for 3 d in CSC	CSC	400 mg/L	NA	600 mg/L	NA	NA	NA	[46]
		Fungal culture filterates	CSC	2–5-fold increase	NA	2–5-fold increase	NA	NA	NA	[47]
		Fungal homogenates	Plant	90% liberation into medium	NA	NA	NA	NA	NA	[48]
		Phytophthora cactorum	Seedling	60% increase	NA	NA	NA	NA	NA	[49]
		Phytoplastomas	Hairy root	Increase detected	NA	NA	NA	NA	NA	[50]
		Pseudomonas fluorescens	Cell culture	Increase detected	NA	NA	NA	NA	NA	[51]
		Jasmonic acid	Hairy root	40% increase (10.2 mg/L)	Increased yield	NA	NA	NA	NA	[52, 53]
		Ethylene	Hairy root	Improved yield	NA	NA	NA	NA	NA	[54]
		Salicylic acid	Hairy root	Improved yield	NA	NA	NA	NA	NA	[54]
		Abscissic acid	Hairy root	Improved yield	NA	85.25 mg/L after 10 days of cultivation	NA	NA	NA	[54, 55]
		Gibberllic acid	Plant	Improved yield	NA	NA	NA	NA	NA	[56]
		Wounding/1 ppm ethylene		Improved yield	NA	NA	NA	NA	NA	[57, 58]

(*continued*)

Table 20.2 (continued)

Aspect	Condition/Treatment	Culture type	Ajmalicine	Tabersonine	Catharanthine	Vindoline	Vinblastine	Vincristine	References
	Pectinase			NA	150%	NA	NA	NA	[53]
	MeJ		Increase yield	NA	Increase yield	NA	NA	NA	[59]
Abiotic	Light	Cell culture	Improved yield	NA	NA	NA	NA	NA	[60, 61]
	UV-B	Hairy root	Improved yield	NA	NA	NA	NA	NA	[62]
		CSC		NA	3 fold	5 fold	NA	NA	[95–97]
	Ketoconazole	Cell culture	Improved yield	NA	NA	NA	NA	NA	[63]
	Betaine, malic acid, tetramethylammonium bromide and rare-earth elements	Cell culture	5–6-fold	NA	NA	NA	NA	NA	[64]
	Cadmium	Cell culture	Improved yield	NA	NA	NA	NA	NA	[65]
	1,1-dimethylpiperidine	Cell culture	Improved yield	NA	NA	NA	NA	NA	[66]
	Acetyl slaicylic acid	Cell culture	Improved yield	NA	NA	NA	NA	NA	[67]
	Sodium alginate, vanadyl sulphate (10–500 mg/l), rare-earth elements, mannitol, PVP	Cell culture	Improved yield	NA	NA	NA	NA	NA	[64, 68, 69]

Ca+MeJ(3mM +100uM)	Cell culture	4.75 mg/L	NA	NA	NA	NA	NA	[70]
Fungicide triadimefon	Plant	Improved yield	NA	NA	NA	NA	NA	[71, 72]
Cyclodextrins and methyljasmonate, + short exposure to UV	Cell culture	1,040 ± 26.6 mg/L	NA	NA	NA	NA	NA	[73]
Change in redox status		Improved yield	NA	NA	NA	NA	NA	[74–76]
Tetramethyl amminium bromide and Aspergillum niger mycelial homogenate		63 mg/L	NA	17 mg/L	NA	NA	NA	[77]
Malate and sodium alginate		41 mg/L	NA	26 mg/L	NA	NA	NA	[77]
Sodium nitroprusside		Increased yield	Increased yield	Decreases	NA	NA	NA	[77, 81]
Vanadyl sulphate	CSC	131.0 μg/g dry weight ajmalicine	NA	500 μg/g dry weight catharanthine	NA	NA	NA	[79]
7 % glucose + light in CSC	CSC	Switch from ajmalicine to serpentine accumulation	NA	Catharanthine accumulation	NA	NA	NA	[80]
Light and methyl jasmonate	CSC	NA	NA	No increase	Increase in C20hi cells			[82]

(*continued*)

Table 20.2 (continued)

Aspect	Condition/Treatment	Culture type	Ajmalicine	Tabersonine	Catharanthine	Vindoline	Vinblastine	Vincristine	References
Medium composition or conditions	50 mmol x L(-1) NaCl stress	Hairy root	NA	NA	3.56 mg × g/L	4.61 mg × g/L	2.95 mg × g/L	1.19 mg × g/L	[83]
	pH modification to 3.5	Hairy root	400-fold increase	NA	NA	NA	NA	NA	[84, 85]
	Temperature 20 deg C	Hairy root	Improved yield	NA	NA	NA	NA	NA	[86]
	50% O_2 and 0.03% CO_2	Cell culture	Improved yield	NA	NA	NA	NA	NA	[87]
	Cell-inoculum size and the addition of conditioned medium in immobilized cells		2-fold increase in combination	NA	Only conditioned medium increased yield 5-fold				[92]
Feeding	Feeding loganin	Hairy root	85%	NA	225%	NA	NA	NA	[88, 92]
	Feeding of either geraniol, 10-hydroxygeraniol, or loganin at 21 days			increases yield	NA	NA	NA	NA	[90]
	Feeding stemmadenine (intermediate in bs of catahranthine and tabersonine			Increase yield	NA	NA	NA	NA	[91]
Ploidy level	Tetraploids	Callus culture	Improved yield	NA	NA	NA	NA	NA	[93]

Cell Size	Cell length/breadth >2.8	Cell culture	Improved yield	NA	NA	NA	NA	NA	[94]
Heterologous expression	Rabbit P450 2E1hamster 3-hydroxy-3-methylglutaryl coenzyme A (CoA) reductase	Hairy root	Improved yield	NA	NA	NA	NA	NA	[97, 98, 103]
	Str ictosidine synthase gene overexpression	Hairy root		NA	NA	NA	NA	NA	[101, 102]
	Geranyl 10 Hydrolase/ ORCA3	Hairy root	67%	NA	NA	NA	NA	NA	[99]
	1-deoxy-D-xylulose synthase (DXS)	Hairy root	Improved yield	NA	NA	NA	NA	NA	[100]
	CjMDR1, ABC transporter from Coptis japonica		Improved	NA	NA	NA	NA	NA	[89]
	Overexpression of AS alpha and beta subunits + feeding loganin		8 4%	225%	26%	NA	NA	NA	[88, 89]
	Bax overexpression		Improves yield of TIA						

Compound	Condition /treatment	Plant part used	References
Vincamine	MS +2, 4-D 1.0 mg × L(-1) +6-BA 0.5 mg × L(-1) + NAA 0.5 mg × L(-1)	Callus from leaf	[105]
	B5+2.2uM benzyladenine	Hairy root culture	[106]
	WMP salts+MS vitamins +7.21 mg /l+ BAP+0.0186 mg/ l NAA.	Direct shoot from Single node	[107]

3.2 Phytochemistry

It is noteworthy to mention that the synthesis of strychnine by Robert B. Woodward is one of the most famous syntheses in the history of organic chemistry. The structure of strychnine was first determined in 1946 by Sir Robert Robinson. Both chemists won the Nobel Prize (Robinson in 1947 and Woodward in 1965) [123]. Both brucine and strychnine are commonly used as agents for chiral resolution. Strychnine has developed to become a standard reference to NMR spectroscopy in organic chemistry as in this compound many typical structural features are present which cause spectrum to spread over entire chemical (Table 20.3).

3.3 Biosynthesis

The alkaloids under discussion share a common Corynanthe-type nucleus derived from secologanin. Yohimbine is a carbocyclic variant of ajmalicine, and the enzymes that convert deglycosylated strictosidine to yohimbine have not been identified [124, 125]. Strychnine and brucine are synthesized from the preakuammicine structure by hydrolysis, decarboxylation, and condensation reactions to aldehyde (Wieland-Gumlich), and subsequently reaction with acetyl-CoA to make a hemiacetal form of aldehyde (Wieland-Gumlich) and strychnine and brucine. Brucine is a dimethoxy form of strychnine. Ellipticine is a representative member of pyrido[4,3-b] carbozole alkaloid, and the formation of ellipticine is from ajmalicine (corynanthean skeleton) [126] (Fig. 20.8).

3.4 Commercial Aspects

3.4.1 Reserpine

Reserpine content was estimated 0.1 % of dry roots; the major problem in field grown plant is the variation in the compound yield. In 1976, the volume of reserpine sold in the USA for medical use was 440,000 lbs [127]. In 2009, reserpine was produced by seven companies, all in India [128]. Even though the chemical synthesis of reserpine is possible, it costs more than extracting it from natural resources [129].

3.4.2 Yohimbine

Bark may contain up to 6 % total alkaloids, of which 10–15 % is yohimbine [120]. Concentrations of yohimbine in commercial products ranged from less than 0.1 to 489 parts per million (ppm) compared with 7,089 ppm in the authentic material. The alkaloid yohimbine also is obtained from *Aspidosperma quebracho-blanco* and *Rauwolfia serpentina* [121]. Its interest outside the African continent was first recorded in Germany, where yohimbine, the main alkaloid of the drug and also known as aphrodine, quebrachine, or corynine, was isolated for the first time by Spiegel in 1896 [130]. Recent advancements in the development of yohimbe-based

Table 20.3 Phytochemistry of apocyanaceae alkaloids

Alkaloid	Chemical name/synonym	Molecular weight	Molecular formula	Appearance	Identification UV (max) specific optical rotation	References
Reserpine	(Methyl-11,17 –dimethoxy-18b-[(3,4,5-trimethoxybenzoyl) oxy] -3b,20q=yohimban-16b-carboxylate)	608.68 g/mol	$C_{33}H_{40}N_2O_9$	To off-white crystalline powder	220, 267, 292 nm (−)116-(−) 124° (Chloroform)	[119]
Yohimbine	17α-Hydroxy-yohimban-16α-carboxylic acid methyl ester	354.44 g/mol(base) 390.90 g/mol (hydrochloride)	$C_{21}H_{26}N_2O_3$	White crystalline powder; odorless	226, 280, 291 nm	[120, 121]
Ellipticine	5,11-Dimethyl-6 H-pyrido[4,3-b] carbazole	246.3	$C_{17}H_{14}N_2$	Bright yellow solid	239, 277, 286, 294, 332, 382, 400 nm	[122]
Strychnine		334.41 g mol^{-1}	$C_{21}H_{22}N_2O_2$	White, odorless, bitter crystalline powder	255, 280, 290 nm -139oc (Chloroform)	[118, 123]
Brucine		394.46 g mol^{-1}	$C_{23}H_{26}N_2O_4$		(Ethanol): 263, 301 nm D −127° (chloroform)	[118, 123]

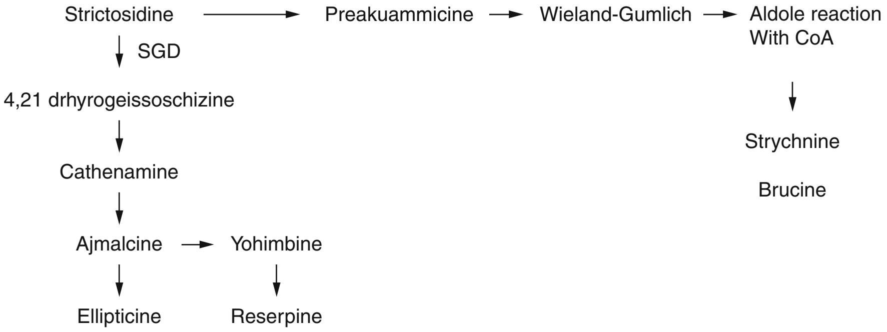

Fig. 20.8 Biosynthetic pathway for the synthesis of yohimbine, reserpine, ellipticine, strychnine, and brucine

remedies have led to an increased demand for the export market. Around 100 t of bark is annually supplied by Plantecam (Cameroon) to Europe [131]. The yohimbe tree presently grows abundantly, and there does not seem to be a problem of regeneration.

3.4.3 Ellipticine

Ellipticine isolated from *Ochrosia elliptica* costs US$ 240,000/kg [132].

3.4.4 Strychnine and Brucine

Strychnine and brucine are sourced from the seeds of the nux-vomica, a tree native to India. The seeds are powdered, blended with alkali, and extracted at ambient temperature with a hydrocarbon solvent. Seeds containing 2.5 % total alkaloids would yield on an average 7 kg each of strychnine and brucine per ton of seeds [151].

3.5 Biological Activities

3.5.1 Reserpine

For centuries, the natives of the Himalayan foothills have used the root of *Rauwolfia* for healing afflictions ranging from snake bites to insanity [133]. Reserpine is used for treatment of mild to moderate hypertension [134] as well as a tranquilizer and sedative in animal feed [127]. It has also been used as a radioprotective agent and contraceptive. Reserpine interferes with the Mg^{2+}– and ATP-dependent uptake of biogenic amines, thereby depleting norepinephrine, dopamine, and serotonin. The effect is universal and irreversible. Reserpine decreases both cardiac output and peripheral vessel resistance [133] by inhibiting normal sympathetic activity by decreasing the storage of catecholamines at the presynaptic, CNS, and peripheral neuron by binding to the storage vesicles which leads to leakage of catecholamine into synapse. Catecholamine when presynaptic neuron is stimulated and this affects

Table 20.4 Biotechnological approaches to improve other TIA content in Apocyanaceae

Compound	Condition /Treatment	Plant part used	References
Reserpine	MS+PABA(3 mg/l) +NAA(3 mg/l)	Callus from leaf	[145]
	MS + IBA (0.2 mg/ l) + NAA (0.2 mg/l)	Callus from shoot	[146]
	MS + IAA(2 mg/l) + Cu_2(5 uM)	Callus from leaf	[147]
	MS + 2,4-D(9 uM) + Tryptophan(50 mg/l)	Callus from leaf	[148]
	MS+2,4-D (9 uM)+NAA(3 uM)	Callus from leaf	[149]
	MS+1mg/l 2,4-D+200 μM MJ	Suspension culture from somatic embryo	[150]
Yohimbine	Ms+BA(1.0 ppm)+ NAA (0–1 ppm)	Callus	[151]

serotonin. This results in a reduction in both cardiac output and peripheral vascular resistance with long-term therapy [135].

3.5.2 Yohimbine

Yohimbine is an α2-adrenoceptor antagonist with potential clinical applications in erectile dysfunction [136]. Yohimbine reportedly may act by adrenergic blockade by increasing cholinergic activity. Yohimbine has a mild antidiuretic action, probably via stimulation of hypothalamic centers and release of posterior pituitary hormone. A more recent open-label study found yohimbine to be effective in managing anorgasmia (orgasmic dysfunction) [137]. Yohimbine is also used for weight loss (body fat) and xerostomia (dry mouth) and has been used in studies investigating autonomic failure and orthostatic hypotension [138].

3.5.3 Ellipticine

Ellipticine and its derivatives exhibit significant antitumor and anti-HIV activities, characterized by high efficiencies against several types of cancer and rather limited toxic side effects [139, 140]. Several mechanisms of their antitumor, mutagenic, and cytotoxic activities are (1) intercalation into DNA, (2) inhibition of DNA topoisomerase II activity, (3) selective inhibition of p53 protein phosphorylation, and (4) disruption of the energy balance of cells by coupling mitochondrial oxidative phosphorylation [141]. Recently, it has been shown that ellipticine also binds covalently to DNA in vitro and in vivo after being enzymatically activated with cytochrome P450 (CYP) or peroxidase, suggesting possible mechanism of action [142]. Ellipticine along with different and safer drug delivery using nanotechnology and gene therapies could significantly contribute to medical practice [143].

3.5.4 Strychnine

Strychnine isolated from *Strychnos nux-vomica* is a potent toxin affecting the nervous system and muscular system bringing about an extremely painful stop to the functioning of the above systems. Strychnine binds itself to receptor sites in the spinal cord and accommodates glycine. It has interesting pharmacological effects on several neurotransmitter receptors, including some members of the superfamily of ligand-gated ion channels. Strychnine today is used primarily as a pesticide, particularly to kill rats [143].

3.5.5 Brucine

Brucine is a dimethoxy form of strychnine and reported to possess antitumor and anti-angiogenic activities in vitro and in vivo [144]. Brucine causes paralysis of peripheral motor nerve without mediating the convulsive action characteristic of strychnine [118]. It is reportedly less toxic than strychnine.

3.6 Biotechnological Approaches

The rate of plant propagation and improvement of yield of tryptophan indole alkaloids through biotechnological technique is important for commercial cultivation to meet the pharmaceutical demand of the said alkaloids. There are scanty references or literature in this direction. Few of the literature surveys are highlighted in Table 20.4.

4 Conclusion and Prospects

Understanding the value and importance of the plant-derived TIAs has steered research in the direction of improving overall yield of the alkaloids. Though many attempts have improved yield of a specific alkaloid significantly, the overall understanding of the pathway, regulation, enzymes, and substrates is still far from complete. The understanding so far emphasizes the fact that TIA production should be viewed and understood from the plant-angle to bring about a revolution in the yield of the same.

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Chemistry and Biology of Cinchona Alkaloids

21

Karol Michał Kacprzak

Contents

1 Introduction 606
2 History 608
3 Occurrence and Isolation 609
4 Chemistry of Cinchona Alkaloids 615
5 Biosynthesis 623
6 Biological Activity 626
7 Biotechnological Approaches 632
8 Perspectives 634
References 634

Abstract

Cinchona alkaloids comprising quinine, quinidine, cinchonidine, and cinchonine as the major members constitute a unique class of quinoline alkaloids with tremendous impact on human civilization. The odyssey of Cinchona alkaloids began with the discovery of their antimalarial properties followed by the very successful application in stereochemistry and in asymmetric synthesis. Currently, the portfolio of applications of Cinchona alkaloids is much broader, involving chiral stationary phases for enantioselective chromatography, novel biological activities, and several useful transformation converting them into other modular and chiral building blocks, such as, for example, quincorine or quincoridine. Current pressure on a more intense exploration of sustainable products and easy access to diverse molecular architectures make Cinchona alkaloids of primary importance for synthetic catalytic and medicinal chemistry.

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The aim of this chapter, which covers almost 300 references, is to summarize all aspects of Cinchona alkaloids chemistry and biology with the special emphasis on new developments.

Keywords

Alkaloids • cinchona alkaloids • cinchonidine • cinchonine • natural products • quinidine • quinine

Abbreviations

AZT	Azidothymidine
Bz	Benzoil
Et	Ethyl
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin layer chromatography
IC_{50}	Half maximal inhibitory concentration
LC-MS	Liquid chromatography-mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
Me	Methyl
Ms	Mesyl (methanesulfonyl)
P	Protection group
Ph	Phenyl
TLC	Thin layer chromatography
tBu	*tert*-Butyl
X	Halogen

1 Introduction

Among the many thousands of natural products isolated and characterized so far, Cinchona alkaloids with quinine as a major member occupy an exceptional position in human civilization [1–3]. *Cinchona* bark was originally used by South American Indian as anti-fever agent and has been introduced to Europe as early as in ca. 1640. The bark dominated in malaria therapy in Europe until 1820 when pure quinine was isolated and largely replaced the natural material. In this context, quinine can be regarded as the first, pure and genuinely active chemotherapeutic. Even today, despite small therapeutic window of quinine as well as increasing resistance of malaria vector *Plasmodium falciparum*, it can compete with newer antimalarial agents, remaining still an important drug for the severe malaria cases (see infra). Considering that quinine is perhaps the oldest drug known, it is not surprising that of all drugs introduced to the therapy, it has saved the largest number of human lives, according to some estimations [4]. However, besides pharmacology of antimalarial quinine and antiarrhythmic quinidine, a natural polygon of the wide exploration of Cinchona alkaloids was organic chemistry. Attempts to obtain quinine by chemical synthesis stimulated an intense research, both on chemical structure elucidation (initially by chemical methods) as well as on the total synthesis, engaging many famous chemists, such as Pasteur, Rabe, Woodward, and Prelog, to list only a few. Despite relative simplicity of the Cinchona alkaloid structure, the first total synthesis of quinine has not been completed until 2001 when Stork has demonstrated the first stereoselective variant, 150 years after quinine isolation [5]!

Perhaps even more important is the fact that Cinchona alkaloids and their derivatives gave foundations of the stereochemistry and consequently of the modern chiral technology. Cinchona alkaloids and their derivatives became popular resolving agents [6–8], catalysts and ligands [8–13], and recently also chiral selectors for enantioselective analytics [14, 15]. Again, it is worth to record the first resolution of a racemate carried out by L. Pasteur as early as in 1853 using cinchotoxine [16] and first examples of catalytic, stereoselective syntheses mediated by Cinchona alkaloids demonstrated by Bredig [17] at the beginning of the twentieth century and by Pracejus in the 1960s [18]. Currently, more than 50 types of highly enantioselective transformations can be efficiently promoted using Cinchona alkaloids or their simple derivatives, including oxidation and reduction and many C–C and C-heteroatom bond formation. The spectacular catalytic capabilities of Cinchona alkaloids and their derivatives resulted in classifying them as "*privileged class*" of chiral catalysts [19].

Last but not least, quinine has also two unique properties: a bitter taste and a strong fluorescence. The first is widely used in beverage and confectionary industry, which consumes ca. 25% of the world production of quinine [20], whereas fluorescence of quinine sulfate is routinely used in fluorescence spectroscopy as quantum yield standard [21, 22].

The highest demand for quinine (and to a lesser extent for other Cinchona alkaloids) has been observed in the nineteenth and in the first half of the twentieth century when quinine remained the only antimalarial drug available. In more recent times, introduction of the other alternative antimalarials resulted in the reduced but relatively stable annual production of quinine at the level of 500–700 t (perhaps the only alkaloid produced in a multiton scale!). Due to their relatively high abundance in the plant resources (*Cinchona* bark) and the well-developed isolation and purification technologies based on the extraction and crystallization, they are inexpensive natural products (ca. 200 € per 1 kg of quinine) with a high potential to be even more extensively explored in the future as green, sustainable, and chiral substrate.

The aim of this chapter is to provide a brief and up-to-date information and bibliography on all aspects of the chemistry and biology of Cinchona alkaloids with a special emphasis on their recent chemistry, applications, and perspectives. Former comprehensive reviews on this topic have been published mainly in the series of *The Alkaloids*, in 1989 by Verpoorte et al. in 1973 [23] by Uskokovic et al. and in 1953 [24] by Woodward et al. [25]. Specific subject such as the use of Cinchona alkaloids in stereoselective synthesis has been recently published in the monograph edited by Song [8] and in reviews by Hiemstra [9], Gawroński [10], Deng [11], Song [12], and Wynberg [13]. The efforts summarizing the progress of the total synthesis of quinine were subjects of the excellent accounts by Seeman [26], Kaufman [27, 28], and Smith [29]. The chemistry of Cinchona alkaloids with the emphasis on the mechanisms and unique reactivity of Cinchona moiety was a subject of authoritative reviews by Hoffmann et al. [30, 31]. Antimalarial activity of quinine has also been extensively reviewed [4, 32–35]. These well-reviewed subjects will be only briefly mentioned here. Similarly, it is not intended to provide the specific chemical and physical properties of Cinchona alkaloids; these data can be conveniently found in the electronic databases such as Reaxys or SciFinder. A good source of such characteristics is also the Dictionary of Alkaloids [36] and a review [23].

2 History

Quinine is deeply rooted in human civilization. This very desired good in the past has provoked a significant economical, political, and scientific ferment [1–3, 7, 37–39], and the science behind quinine had an enormous impact on the rapid development of the medicinal and organic chemistry of natural products.

Cinchona bark has been used as a medicine against fever by South American Quechua Indians who populated Peru long before the Europeans came to America [1, 37–39]. It is however difficult to assess precisely how the bark become popular in Europe. The legend (now historically disproved) [40] tells that this happened when the countess of Chinchon, the wife of a Peruvian viceroy, has been successfully cured in 1638 of malarial fever with the *Cinchona* bark. It is also believed that the name of the tree species and then name of the alkaloid class were derived from the misspelled name of Chinchon. The name quinine is probably derived from the original Quechua Indian word describing *Cinchona* tree bark – "*quina*" or "*quina-quina*" – which roughly means "*bark of bark*" or "*holy bark.*"

Cinchona bark was first become popular in England and has been officially introduced to the British Pharmacopoeia in 1677. Initially, its wider use was restricted in Europe, despite the widespread occurrence of malaria. Several years later, "*Jesuit's powder*" was popular also in Spain, again due to the Jesuits missions in South America. On the other hand, Jesuits activity as well as the successful therapies of aristocracy over Europe brought *Cinchona* bark popularity to the European countries.

The plant used for bark production was botanically indentified and characterized almost a hundred years later in 1735 by Joseph de Jussieu, a French botanist who accompanied the first non-Spanish expedition to South America. In the period ca. 1650–1850, *Cinchona* bark was the primary drug for a successful treatment of malaria. Monopoly of Spain and insufficient sources of *Cinchona* bark in South America and an increasing demand of quinine imposed pressure to break the Spanish hegemony by smuggling of *Cinchona* seeds and establishing of competitive plantations. The first plantations created by the British in India and Ceylon (*Cinchona pubescens*) and by the Dutch in Java (*Cinchona calisaya*) did not provide enough quinine. The situation changed dramatically when *Cinchona* *officinalis* "*ledgeriana*" seeds were smuggled from Bolivia and used by the Dutch in the plantations in Dutch East Indies (currently Indonesia). Extensive cultivation of these *Cinchona* species brought quickly domination of the Dutch in the quinine world market. At the beginning of the twentieth century, the majority of the world's supply of quinine was under the control of the Dutch "*kina bureau*" (quinine bureau) in Amsterdam. The Second World War did significantly change the quinine market. Java was occupied by the Japanese in 1942, resulting in the embargo for the supply of quinine to the Allies. As a partial solution of this problem, new plantations in South Africa have been established by the British, and the less important South American supplies were again more intensively explored. However, these plantations did not immediately satisfy the increasing demand, provoking the intensive research on the total synthesis of quinine. In 1944 Woodward and Doering have demonstrated a synthetic route to quinine, although critical studies recently published confirm that they did not prepare this alkaloid (showing rather a formal synthesis) [26–28, 41]. Although the synthetic quinine was too expensive to compete with the quinine isolated from *Cinchona* bark, the research resulted finally in the development of active and synthetically easily available antimalarials, for example, chloroquine, mefloquine, and primaquine.

3 Occurrence and Isolation

Cinchona alkaloids are characteristic and widely present in the genus *Cinchona* consisting of ca. 40 species which belong to the family *Rubiaceae* [42]. These plants are 15–30-m-high evergreen trees native to the eastern slopes of the Amazonian area of the Andes, growing from 1,500 to 3,000 m from Colombia to Bolivia (Costa Rica, Ecuador, Guatemala, Panama, Peru, Venezuela). They can also be found in the northern part of the Andes (on the eastern slopes of the central and western ranges).

Today, however, the most important *Cinchona* tree cultivators are Indonesia and India, together with Africa (in Zaire, Tanzania, and Kenya). South American countries such as Peru, Bolivia, and Ecuador where the *Cinchona* trees originated from become less important producers. Others plantations are also cultivated in Australia, Guinea, Papua New Guinea, and the Philippines [1–3].

For commercial purposes, three main *Cinchona* species, namely, *Cinchona succirubra* Pav. ex Klotsch (or *C. pubescens* Vahl, red *Cinchona*), *C. officinalis* "*ledgeriana*" (or *C. ledgeriana* Moens ex Trimen, yellow *Cinchona*), and *Cinchona calisaya* Wedd. together with their many varieties, hybrids, and grafts are usually cultivated due to the highest alkaloid content in the bark. For example, "red" bark of *C. succirubra* contains 5–7% of alkaloids, *C. calisaya* gives bark with an alkaloid content of 4–7%, whereas bark of *C. officinalis* "*ledgeriana*" may contain 5–14% of alkaloids. Selected *Cinchona* hybrids under the favorable cultivation condition could yield even more alkaloids, up to 17% in the bark. Analysis of the different types of *Cinchona* species bark shows that quinine is the most abundant alkaloid comprising usually 50–90% of the alkaloids sum [43–45].

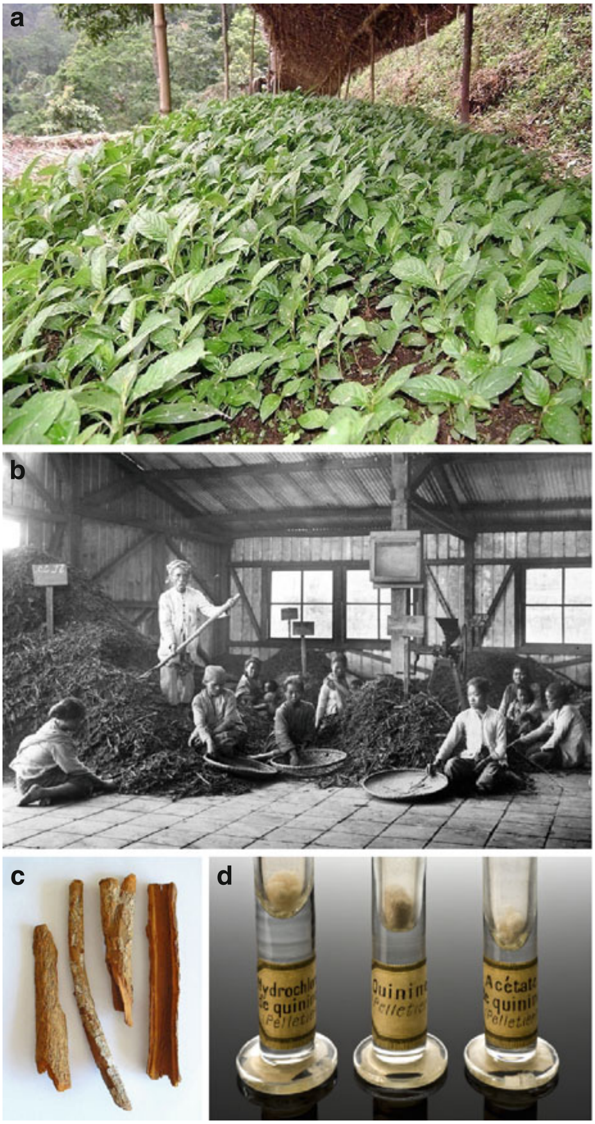


Fig. 21.1 (**a**) Current plantation of Cinchona tree in India (**b**) Workers sorting cinchona bark in Tjinjiroean (West Java, form the period 1915–1930, collection of Tropenmuseum of the Royal Tropical Institute (KIT), (**c**) cinchona bark samples, (**d**) authentic samples of cinchona alkaloids isolated by Pelletier and Caventou in 1834 roku (from www.sciencemuseum.org.uk)

Commercial product of *Cinchona* tree is *Cinchona* bark which is a dried bark from the stem and roots. The trees achieve harvestable bark usually within 6–10 years. The bark is then stripped and collected. The bark partially regenerates on the trees; therefore, a single tree can be explored a few times (depending on the species and cultivation technique) before it is uprooted and replaced by the new plants (Fig. 21.1).

Crude mixture of crystalline alkaloids from the bark has been first isolated by Gomes [46] in Portugal in 1810, and 10 years later, Pelletier and Caventou isolated

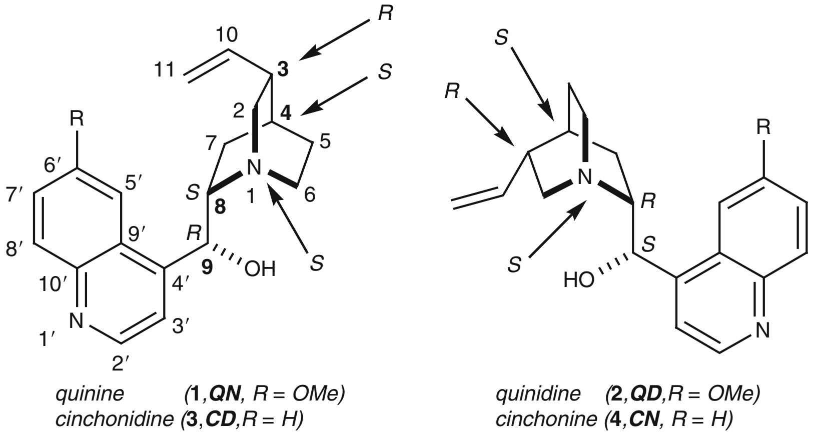


Fig. 21.2 Structure, common atom numbering and absolute configuration of four major Cinchona alkaloids

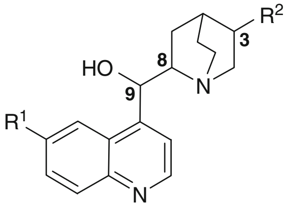
pure quinine and cinchonine [47–49]. In 1826 the researchers opened the first quinine factory processing annually ca. 150,000 kg of *Cinchona* bark and purifying ca. 3,500 kg of quinine. The two other major Cinchona alkaloids quinidine and cinchonidine were isolated by Delondre and Henry [50, 51] and Winckler [52], respectively. The structures of these four major Cinchona alkaloids which usually account for 30–90% of alkaloid content of the bark are given in Fig. 21.2.

Besides quinine (**1**), quinidine (**2**), cinchonidine (**3**), and cinchonine (**4**), a considerable number of their close derivatives have been found in the bark or in the leaves of *Cinchona* species (occasionally also in other species, see Table 21.2), differing mainly with the type of substituents (vinyl or ethyl group) at C-3 of quinuclidine moiety, methoxyl, hydroxyl, or hydrogen in the quinoline ring or in configuration at C-9 (Table 21.1) [23, 36].

Other minor quinoline alkaloids are those of quinicine (quinotoxine) class (**5**–**6**) [58, 59, 64, 67, 71, 72]. Besides quinoline alkaloids produced by *Cinchona* species, there are also a number of indole-type minor alkaloids classified as cinchonamines (**7**–**9**) [67, 72–76] or dimeric cinchophyllamine type (alternative name cinchophylline) (**10**) [76–81]. Other structures include quinamines (**11**) [61–63, 73, 76, 79, 82, 83], tetracyclic 3-isocorynantheol (**12**) [54, 84], or less typical for *Cinchona* species (*C. ledgeriana* and *C. pelleteriana*) pentacyclic aricine (**13**) [54, 79, 85, 86]. Their structures are given in Fig. 21.3.

A fraction of Cinchona alkaloids in the bark are present as the salts of quinic, cinchotannic, or other acids. Enzymatic oxidation of cinchotannic acid occurring during bark processing yields a red pigment, which gives a typical color especially of the “red” bark. Other minor, non-alkaloidal compounds found in *Cinchona* and *Guettarda* species are phenolic acids such as caffeic, chlorogenic, protocatechic and *p*-coumaric acid, epicatechin [87–89], phenylpropane-substituted flavane-3-oles – cinchonaines [87, 90], triterpene

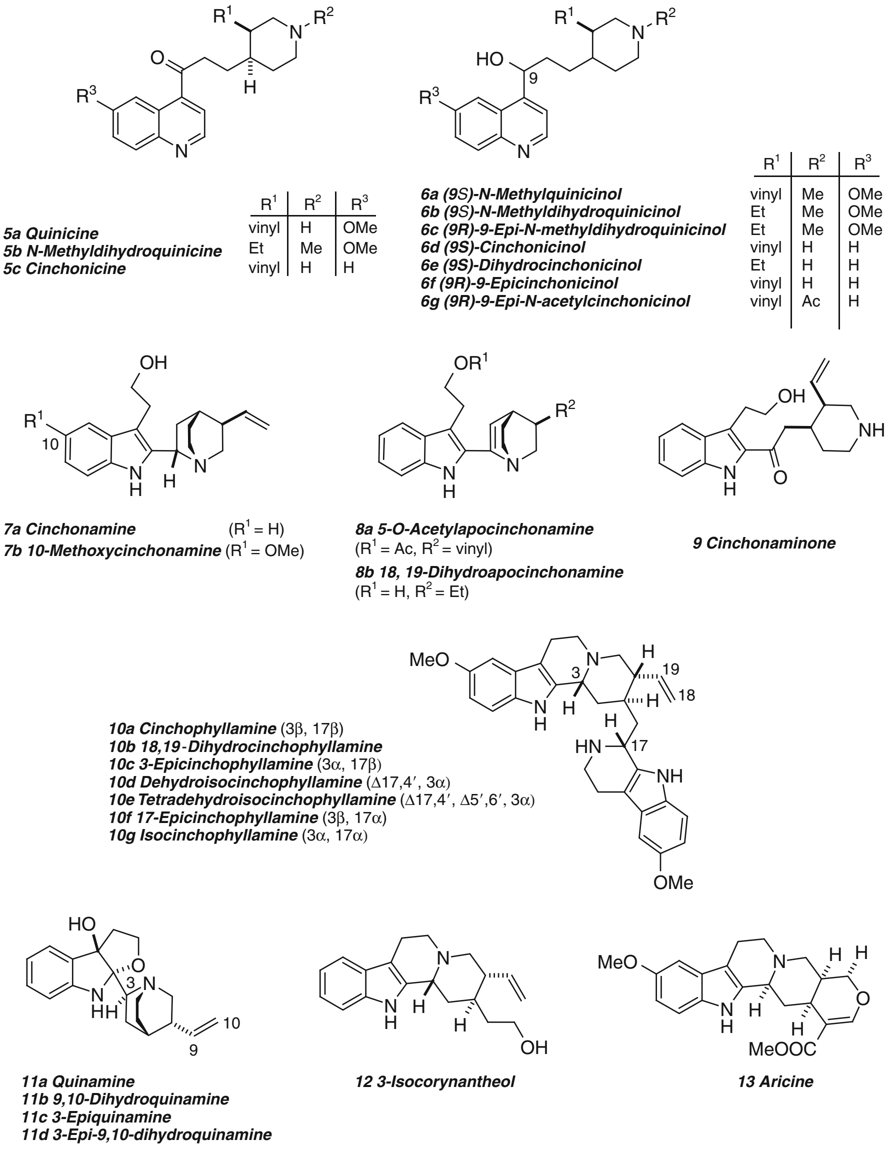
Table 21.1 Cinchona alkaloids occurring in Rubiaceae (mainly in Cinchona species)



No	Name	R^1	R^2	Absolute configuration at		
				C-8	C-9	C-3
1	Quinine	OMe	Vinyl	*S*	*R*	*R*
a	Dihydroquinine	OMe	Ethyl	*S*	*R*	*R*
b	Epiquinine	OMe	Vinyl	*S*	*S*	*R*
c	Epidihydroquinine	OMe	Ethyl	*S*	*S*	*R*
d	Epivinyl quinine	OMe	Vinyl	*S*	*R*	*S*
e	Epivinyl epiquinine	OMe	Vinyl	*S*	*S*	*S*
f	Cupreine	OH	Vinyl	*S*	*R*	*R*
g	Dihydrocupreine	OH	Ethyl	*S*	*R*	*R*
2	Quinidine	OMe	Vinyl	*R*	*S*	*R*
a	Dihydroquinidine	OMe	Ethyl	*R*	*S*	*R*
b	Epiquinidine	OMe	Vinyl	*R*	*R*	*R*
c	Epidihydroquinidine	OMe	Ethyl	*R*	*R*	*R*
d	Epivinyl quinidine	OMe	Vinyl	*R*	*S*	*S*
e	Epivinyl epiquinidine	OMe	Vinyl	*R*	*R*	*S*
f	Cupreidine	OH	Vinyl	*R*	*S*	*R*
g	Dihydrocupreidine	OH	Ethyl	*R*	*S*	*R*
3	Cinchonidine	H	Vinyl	*S*	*R*	*R*
a	Dihydrocinchonidine	H	Ethyl	*S*	*R*	*R*
b	Epicinchonidine	H	Vinyl	*S*	*S*	*R*
c	Epidihydrocinchonidine	H	Ethyl	*S*	*S*	*R*
4	Cinchonine	H	Vinyl	*R*	*S*	*R*
a	Dihydrocinchonine	H	Ethyl	*R*	*S*	*R*
b	Epicinchonine	H	Vinyl	*R*	*R*	*R*
c	Epidihydrocinchonine	H	Ethyl	*R*	*R*	*R*

quinovic acid and its glycoside quinovin [89, 91], and cincholic acids [89] as well as anthocyanosides (cyanidol-3-glucoside and cyanidol-3-rhamnoglucoside) [92], flavonoids (kaempferol, quercetin, avicularin, reynoutrin, delphinin) [87, 88, 93], and relatively high concentration of anthraquinones [23, 75, 94–96].

Investigation of the leaves of various *Cinchona* tree species showed that they differ remarkably in the alkaloid composition. For example, *C*. "*ledgeriana*" contains mainly indole type alkaloids (e.g., cinchophyllamines **10**, quinamine **11**a, and 3-epiquinamine **11**c) [76–79], in contrast to the samples of *C*. *succirubra*



	R1	R2	R3
5a Quinicine	vinyl	H	OMe
5b N-Methyldihydroquinicine	Et	Me	OMe
5c Cinchonicine	vinyl	H	H

	R1	R2	R3
6a (9S)-N-Methylquinicinol	vinyl	Me	OMe
6b (9S)-N-Methyldihydroquinicinol	Et	Me	OMe
6c (9R)-9-Epi-N-methyldihydroquinicinol	Et	Me	OMe
6d (9S)-Cinchonicinol	vinyl	H	H
6e (9S)-Dihydrocinchonicinol	Et	H	H
6f (9R)-9-Epicinchonicinol	vinyl	H	H
6g (9R)-9-Epi-N-acetylcinchonicinol	vinyl	Ac	H

7a Cinchonamine (R^1 = H)
7b 10-Methoxycinchonamine (R^1 = OMe)

8a 5-O-Acetylapocinchonamine
(R^1 = Ac, R^2 = vinyl)
8b 18, 19-Dihydroapocinchonamine
(R^1 = H, R^2 = Et)

9 Cinchonaminone

10a Cinchophyllamine (3β, 17β)
10b 18,19-Dihydrocinchophyllamine
10c 3-Epicinchophyllamine (3α, 17β)
10d Dehydroisocinchophyllamine (Δ17,4′, 3α)
10e Tetradehydroisocinchophyllamine (Δ17,4′, Δ5′,6′, 3α)
10f 17-Epicinchophyllamine (3β, 17α)
10g Isocinchophyllamine (3α, 17α)

11a Quinamine
11b 9,10-Dihydroquinamine
11c 3-Epiquinamine
11d 3-Epi-9,10-dihydroquinamine

12 3-Isocorynantheol

13 Aricine

Fig. 21.3 Structure of minor quinoline alkaloids

from Thailand and *C. succirubra* × *C. ledgeriana* from Guatemala where quinoline alkaloids **1**–**4** dominate [97].

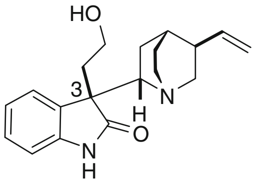
Besides *Cinchona* species, there are many plants producing Cinchona alkaloids of the quinoline and indole type, mainly belonging to the *Rubiaceae* genus. For example, trees from the species *Remijia* which have also been used for medical

Table 21.2 Non-Cinchona species producing alkaloids of Cinchona type

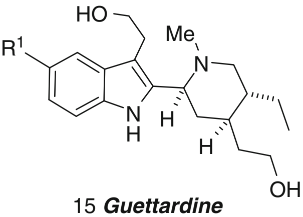
Species	Alkaloid(s)	Ref.
Anthocephalus chinensis L.	Cinchonine, dihydrocinchonine	[53]
Aspidosperma marcgravianum L.	Quinidine, dihydroquinidine, (methoxy) dihydrocorynantheol, aricine	[54, 55, 56]
Guettarda heterosepala	Guettardine	[57]
Guettarda trimera L.	*N*-methyldihydroquinicine, *N*-methyldihydroquinicinol, 2-epi-*N*-methyldihydroquinicinol	[58]
Guettarda noumeana (b)[a]	Cupreine, dihydrocupreine, *N*-methylquinicinol, *N*-methyldihydroquinicinol	[59]
Isertia haenkeana	Apodihydrocinchonamine	[60]
Isertia hypoleuca	dihydroquinamine, epidihydroquinamine	[61–63]
Ladenbergia oblongifolia (b)	Epicinchonicinol, cinchonidicinol, dihydrocinchonicinol, dihydrocinchonidicinol	[64]
Ligustrum vulgare L.	Cinchonidine, cinchonine	[65][b]
Olea europea L.	Cinchonine, dihydrocinchonine, cinchonidine	[65, 66]
Remijia peruviana	Quinine (l + b), cupreine (b), cinchonine (l + b), acetylcupreine (b), *N*-ethylquinine (b) remijinine, epiremijinine, 5-acetyl-apocinchonamine, *N*-acetyl-deoxycinchonicinol, *N*-acetylcinchonicinol, quinamine, epiquinamine (l)	[67, 68]
Remijia pedunculata	Quinidine, cupreine, dihydrocupreine	[69]
Remijia purdieana	Cinchonamine	[70]

[a] (*b*) – found in bark, (*l*) – found in leaves
[b] Only a single not confirmed report

purposes in South America produce a number of typical quinoline alkaloids (Table 21.2) as well as more characteristic for this species – remijinine (**14**) [67, 68]. Guettardine (**15**) which is closely related to the dihydrocorynantheol is another example of the indole alkaloid present in the genus *Guettarda* [57]. Examples of the other species from which Cinchona-type alkaloids (**1**–**15**) have been isolated are given in Table 21.2.



14a ***Remijinine***
14a ***Epiremijinine***



15 ***Guettardine***

Some of the minor Cinchona alkaloids isolated from *Cinchona* or *Remijia* species reported in an old literature, for example, chairamidine, chairamine, cuscamidine, cusconidine, cusconine, javanine, and paricine [36], are up to now of unknown structure, and their identity needs confirmation.

Broad application of Cinchona alkaloids in medicine, food industry, as well as in academic research has stimulated very early the development of both qualitative and quantitative methods for their analysis. Currently, chromatographic TLC [98, 99], HPTLC [99, 100], HPLC [76, 101–104], and LC-MS [105, 106] and electrochromatographic methods [107] are preferred for *Cinchona* alkaloid separation, identification, and quantitative analysis. Older analytics from this field is the subject of a review [23].

4 Chemistry of Cinchona Alkaloids

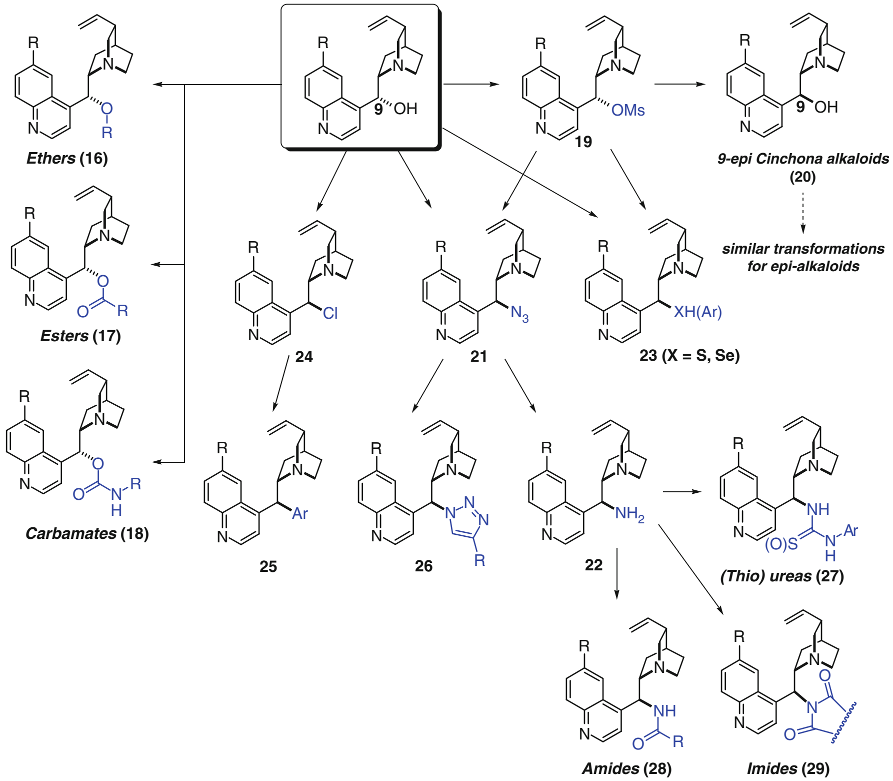
Chemistry of Cinchona alkaloids has a very long history considering that purified quinine was available in Europe as early as in 1820. The seminal transformations of the Cinchona alkaloids, including, for example, the Pasteur's chinotoxine preparation by the treatment of quinine with a weak acid [108], were mainly directed toward the structure elucidation and remained very intuitive until the beginning of the twentieth century when P. Rabe solved the problem of quinine constitution [5, 26, 27, 29, 109, 110]. Nevertheless, a detailed stereochemistry of the Cinchona alkaloids was determined finally by Prelog et al. in 1950 [111]. The X-ray structures of major Cinchona alkaloids **1–4** have been published [112–116] as well, and their conformation was determined in past two decades by a combined NMR and/or computational approach [117–121].

Quinoline-type Cinchona alkaloids (**1–4**) consist of an aromatic quinoline (or 6′-methoxyquinoline) ring joined to the bulky bicyclic quinuclidine moiety by a carbinol linker C-9. Each Cinchona alkaloid contains five stereogenic centers at C-9, C-8, C-4, C-3, and N-1 (Fig. 21.2).

Although quinine and quinidine as well as cinchonidine and cinchonine are diastereoisomers, they are called often pseudoenantiomers because the crucial for the catalytic or chiral discrimination 1,2-aminoalcohol functionality remains enantiomeric in these pairs (however, diastereomeric rather than enantiomeric character is sometimes observed in various applications [8]).

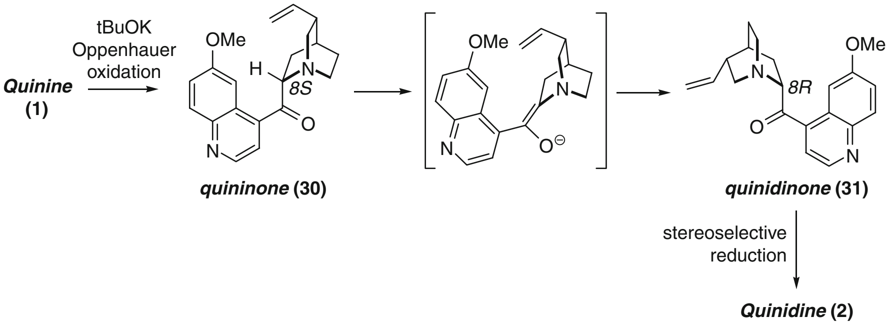
The chemistry of the Cinchona alkaloids involves a typical reactivity of their fragments such as hydroxyl group at C-9 or vinyl group at C-3 and two basic nitrogen atoms incorporated into quinuclidine and quinoline as well as more specific transformation occurring with a rearrangement of their carbon skeleton.

The most popular way to modify alkaloids **1–4** is a derivatization of the alcohol or a replacement of hydroxyl group with retention or inversion of the absolute configuration at C-9 (Scheme 21.1; note quinine is given as a representative example, but most of the reaction could be also carried out with alkaloids **2–4** and the 9-epi series). Alkaloids could be easily transformed into the corresponding 9-*O*-ethers **16** [122–125], esters **17** [126, 127], and carbamates **18** [128, 129]


9
OH
OMs
19
9
OH
Ethers (16)
Esters (17)
Carbamates (18)
9-epi Cinchona alkaloids (20)
similar transformations for epi-alkaloids
Cl
24
N_3
21
XH(Ar)
23 (X = S, Se)
Ar
25
26
NH_2
22
(Thio) ureas (27)
Amides (28)
Imides (29)

Scheme 21.1 Most common transformations of Cinchona alkaloids

without affecting the configuration at C-9. Many of these derivatives are frequently used as catalysts in asymmetric synthesis or as chiral discriminating agents [8–12, 14, 130]. Substitution of the 9-hydroxyl group typically in Mitsunobu reaction or by intermediate 9-*O*-mesylates **19** of **1–4** [131] provides access to many useful derivatives, usually with a complete inversion of the configuration at C-9 (9-epimers are formed), such as 9-azides **21** [132] and the corresponding amines **22** [133]. Using this strategy, Skarżewski et al. obtained a library of Cinchona 9-thiols **23** and bis-cinchona disulphides, phenylsulphides, and phenylselenides as well as the corresponding sulfoxides [134–137]. Similarly, 9-epialkaloids **20** can be conveniently prepared in one step by aqueous hydrolysis of the corresponding 9-*O*-cinchona tosylates [138] or preferably 9-*O*-cinchona mesylates, as optimized by Hoffmann [139], or alternatively by Mitsunobu esterification/hydrolysis sequence [140]. 9-Epicinchona alkaloids **20** undergo many of the mentioned reactions giving in most cases access to 9-naturally configurated derivatives **21–23**. Cinchona 9-chlorides **24** can be favorably prepared by the treatment of alkaloids with $SOCl_2$ with inversion of configuration at C-9 [141, 142]. Cinchona derivatives **21–24** can further be transformed into a number of diverse compounds including

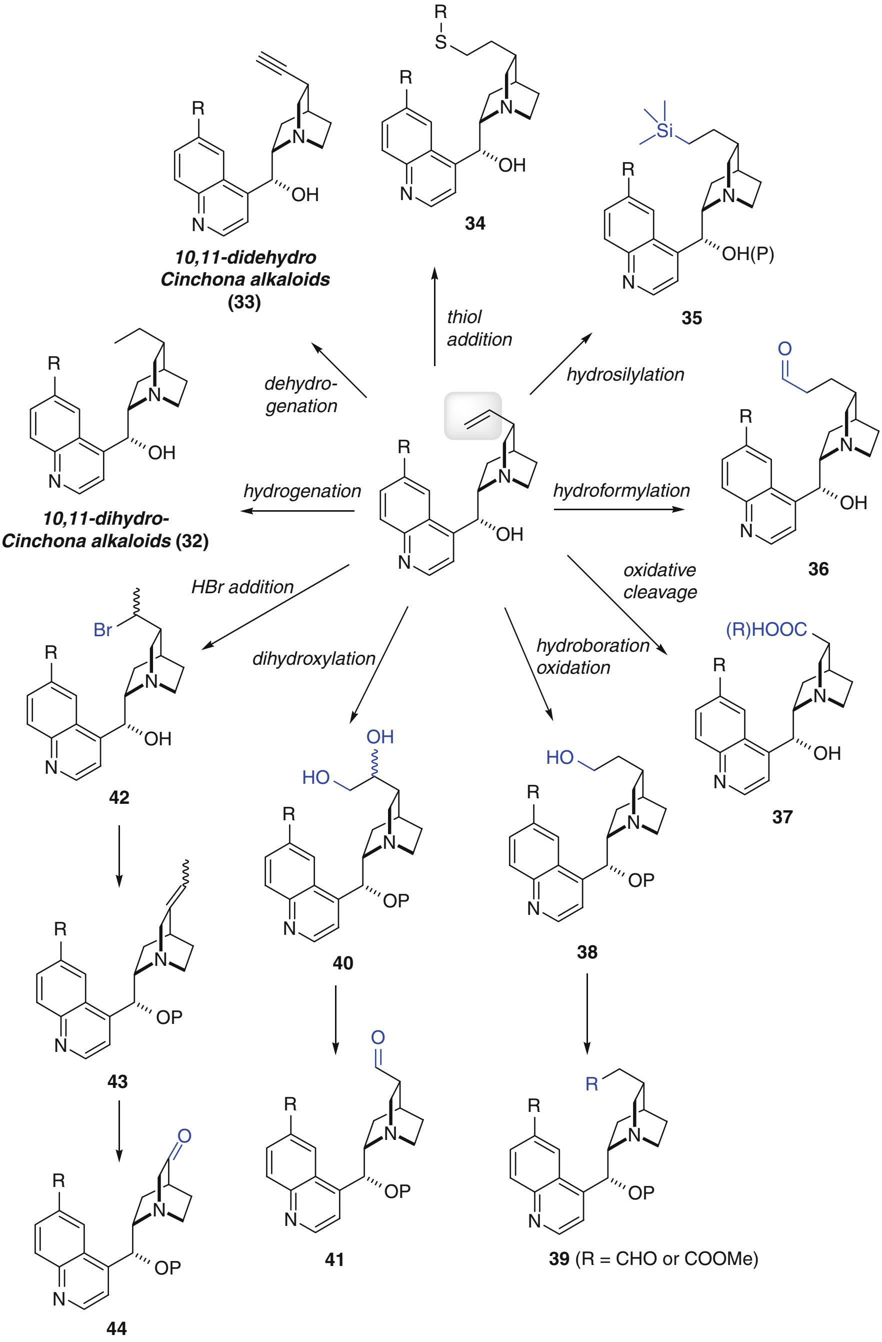


Scheme 21.2

9-aryl or vinyl substituted alkaloids **25** [143–145] as well as to 1,2,3-triazolo derivatives **26** synthesized by *click chemistry* [146]. Particularly important as powerful organocatalysts [147, 148] are currently 9-amino-9-epicinchona alkaloids **22** obtained by a one-pot procedure involving Mitsunobu azidation followed by Staudinger reduction [133]. They serve also as intermediates for the synthesis of thioureas **27** [149–151], amides **28** [152, 153], and imides **29** [154–156] which have found numerous applications in the asymmetric catalysis or chiral discrimination (for comprehensive reviews, see [8, 9]).

A reaction of commercial importance used for a large-scale manufacturing of quinidine (**2**) is the oxidation of hydroxyl group of quinine (**1**) leading to quininone **30** [157–159] (Scheme 21.2). Its epimerization via enol to quinidinone **31** [160] and subsequent reduction yield quinidine and quinine as well as their 9-epimers as unwanted by-products. The most convenient oxidation procedure was developed by Woodward who employed a modified Oppenauer-type oxidation and which was further optimized by Warnhoff [159]. Reduction of quinidinone by diisobutylaluminum hydride shows high stereoselectivity operating via complex formed with the aid of quinuclidine nitrogen. An elegant approach providing near-quantitative yield of quinidine by the reduction of quininone/quinidinone has been patented by Gignier and Bourelly. The strategy comprises of reduction of ketones **30–31** with diisobutylaluminum hydride in the presence of a stereoorienting agent such as pyridine and greatly avoids the formation of undesirable by-products such as quinine, epiquinine, and epiquinidine [161].

The vinyl group attached to the quinuclidine moiety is yet another site frequently used for a modification or immobilization of Cinchona alkaloids (Scheme 21.3). Diverse reactivity of the vinyl group of Cinchona alkaloids has been studied in details and is summarized by Hoffmann [30, 31]. Here the most typical or recent transformation is highlighted. The catalytic hydrogenation of the vinyl group leads to the corresponding 10,11-dihydroalkaloids **32** (often present up to ca. 10% in the isolated alkaloids) and can be completed both by using gaseous hydrogen in the presence of a heterogeneous catalyst, for example, palladium, platinum, or rhodium [162], or under transfer hydrogenation [163]. On the other hand, 10,11-didehydroalkaloids **33** bearing a terminal acetylene group instead of the


R
OH
N
10,11-didehydro Cinchona alkaloids (33)
R
S
34
Si
OH(P)
35
thiol addition
dehydro-genation
hydrosilylation
10,11-dihydro-Cinchona alkaloids (32)
hydrogenation
hydroformylation
O
36
HBr addition
oxidative cleavage
dihydroxylation
hydroboration oxidation
(R)HOOC
37
Br
42
HO
OP
40
38
43
41
39 (R = CHO or COOMe)
44

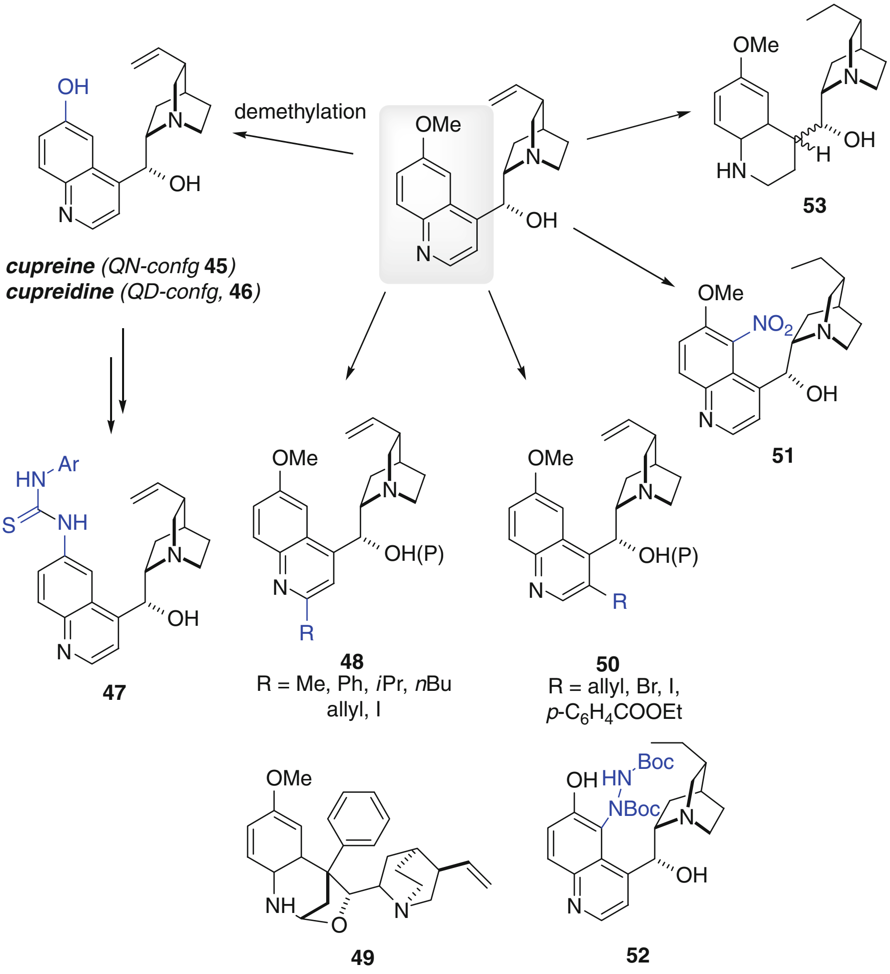
Scheme 21.3 Transformations of vinyl group of Cinchona alkaloids vinyl can be prepared by the bromination of the double bond followed by subsequent double elimination [164, 165]. These alkyne derivatives are more basic, as compared to the native Cinchona alkaloids, and are versatile substrates for a further elaboration. These include *click chemistry* approach for the synthesis of 11-(1,2,3-triazolo)-Cinchona alkaloids [166, 167] and a new Cinchona immobilization method via 1,2,3-triazole linker [168–170] as well as a number of diverse cross-coupling reactions [164, 171].

Radical addition of thiol or thiol-modified support to the vinyl group gives the respective thioether linkage **34** and represents one of the most convenient ways to immobilize Cinchona alkaloids [163, 172]. There are also few examples of platinum-catalyzed hydrosilylation of Cinchona alkaloids toward 11-silyl-substituted derivatives **35** with the use of monomeric silanes or polysiloxane polymers [173–175]. De Vries reported rhodium-catalyzed hydroformylation of the four main members of Cinchona alkaloids carried out on a hundred gram scale. Under optimized condition, linear aldehydes **36** were selectively obtained with the yield over 80% [176].

Oxidative cleavage of the vinyl group of quinine by $KMnO_4$ leads to the Cinchona C-10 acid (quitenine) or ester **37** with low yield [177, 178], whereas more efficient although longer route via hydroboration and subsequent oxidation gives primary alcohol **38** which can be oxidized to C-11 ester or aldehyde **39** [179, 180]. 10-Carbaldehyde **41** can be obtained from the easily accessible 10,11-cinchona diols **40** [180]. Last but not least, addition of HBr to the Cinchona alkaloids gives the corresponding diastereomeric bromides **42** [181] which could be eliminated to give a mixture of *E*/*Z* $\Delta^{3,10}$-isoalkaloids **43** (the so-called apocinchona alkaloids) [181, 182] (direct migration of the double bond has also been reported [182]). Apocinchona alkaloids **43** undergo an oxidative cleavage via the corresponding 1,2-diol, providing access to the ketones **44** (so-called rubanones), as demonstrated by Hoffmann [183].

Perhaps one of the most important transformations of the aromatic ring of quinine or quinidine is demethylation of the quinoline hydroxyl group, leading to cupreine **45** or cupreidine **46**, respectively. These alkaloids as well as their thiourea derivatives, for example, **47**, received recently wide attention as effective organocatalysts in the asymmetric synthesis [8, 9, 184, 185]. Their newer, more selective synthesis from quinine or quinidine involves deprotection mediated by thiolates [185]. The alkylation of cupreine to introduce a bulky alkyl group on the phenolic hydroxyl group in **45** or **46** leads to the corresponding ethers which after further elaboration can be used as efficient chiral selectors in enantioselective chromatography [14, 15, 186] (Scheme 21.4).

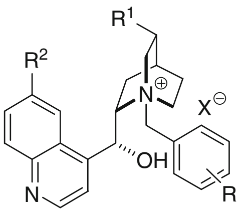
There are only a few reports on the nucleophilic substitution of the aromatic quinoline ring of Cinchona alkaloids. Typically, 2′-substituted products **48** are observed [187–190], but the sterically less demanding Grignard reagents add selectively to 4′-carbon. This leads to the loss of aromaticity and to unexpected formation of bicyclic *N*,*O*-acetals, for example, **49** [190]. An interesting variant of a selective functionalization of quinoline ring of quinine either in 2′ or in 3′ position


demethylation
cupreine (QN-confg 45)
cupreidine (QD-confg, 46)
47
48
R = Me, Ph, iPr, nBu
allyl, I
49
50
R = allyl, Br, I,
p-C6H4COOEt
51
52
53

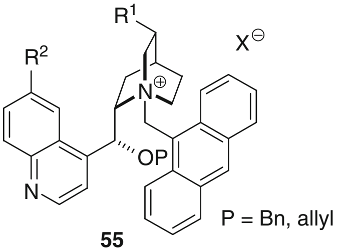
Scheme 21.4 Quinoline ring transformation in quinine

by metalation has recently been reported by the Knochel group. Typically, the metalation occurs at position 3′, but increasing the bulkiness of the C-9 hydroxyl group by formation of silyl ether selectively directs the metalation to position 2′. Quenching of the metalated quinine intermediate by various electrophiles gave access to the number of new quinine derivatives **50** with satisfactory yields [191]. Modification of the quinoline at 5′ involves a rare example of the direct nitration by HNO_3 yielding >90% of corresponding 5′-nitroquinine **51** which has been further converted into amino- and substituted urea derivatives, successfully employed in asymmetric catalysis [192]. Another specific example of the modification of 5′-position of cupreidine involves a direct amination, providing the atropisomeric derivatives **52** [193]. Quinoline ring can also be partially reduced, providing hexahydro derivatives **53** of unknown absolute configuration at the newly formed stereogenic center at C-4′ [194, 195]. These transformations are summarized in Scheme 21.4.

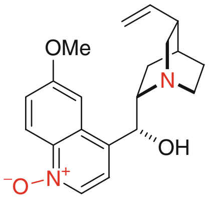
Last but not least, Cinchona alkaloids can be modified by using reactivity of their one or two basic nitrogen atoms. The quinuclidine moiety is a very strong base (pK_b 7.25) and could be preferentially quaternized by benzyl halides, as compared to the less basic quinoline nitrogen (pK_b 2.81). The comparison of the nitrogen reactivity was recently discussed on the basis of experiments and molecular modeling [196]. The simple quaternary salts of Cinchona alkaloids **54** were developed as efficient chiral phase-transfer catalysts in the 1980s, and their catalytic performance was constantly improved by a proper selection of the benzylic halide and the protecting group of 9-hydroxyl substituent (third-generation Cinchona quats **55** [197–199]). Their diversity and the application in asymmetric synthesis were reviewed in detail in [8–10]. Both Cinchona alkaloid nitrogen atoms can also be oxidized to the respective mono- (**56**-**57**) or *bis*-*N*,*N*-oxides **58** [200] (Scheme 21.5). *N*-Oxides of Cinchona alkaloids were not found in the plant sources but have been isolated as the metabolites of pathogenic fungi-colonized *Cinchona* species as well as Cinchona alkaloid metabolites in human and animals (*vide infra*).



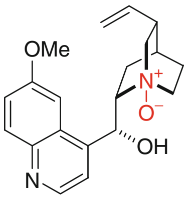
54



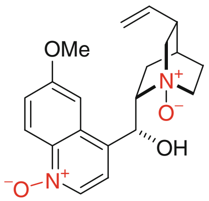
55



56

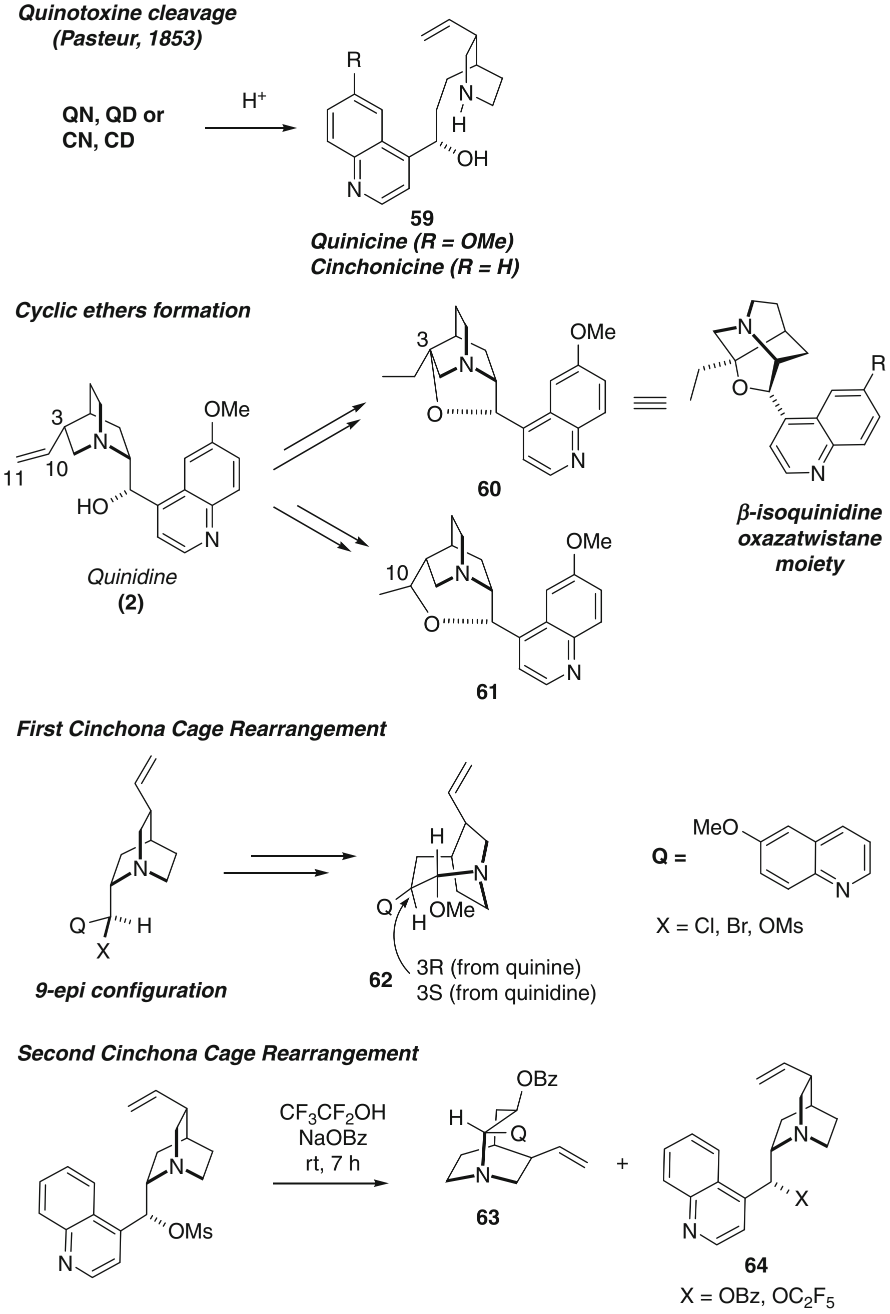


57



58

The multifunctional nature of Cinchona alkaloids makes them especially prone for a complex reactivity that occurs with a degradation or rearrangement of the carbon moiety, often controlled by the stereochemistry of the particular alkaloid. These characteristic for Cinchona alkaloid transformations include an acid-catalyzed formation of cinchotoxines **59** described as early as in 1853 by Pasteur [108] or synthesis of diverse cyclic ethers, for example, *β*-isoquinidine **60** or *β*-isocupreidine **61**, bearing oxazatwistane moiety [180, 201, 202] (Scheme 21.5). This can be used as an efficient catalyst of a stereoselective Baylis-Hillman reaction [8–10, 203, 204]. Hoffmann studied in detail the two distinct cage rearrangements


Quinotoxine cleavage (Pasteur, 1853)
QN, QD or CN, CD
H+
R
N
H
OH
59
Quinicine (R = OMe)
Cinchonicine (R = H)
Cyclic ethers formation
3
OMe
N
11
10
HO
Quinidine
(2)
60
R
β-isoquinidine oxazatwistane moiety
O
61
First Cinchona Cage Rearrangement
Q
H
X
9-epi configuration
OMe
62
3R (from quinine)
3S (from quinidine)
Q =
MeO
X = Cl, Br, OMs
Second Cinchona Cage Rearrangement
OMs
CF3CF2OH
NaOBz
rt, 7 h
OBz
63
+
64
X = OBz, OC2F5

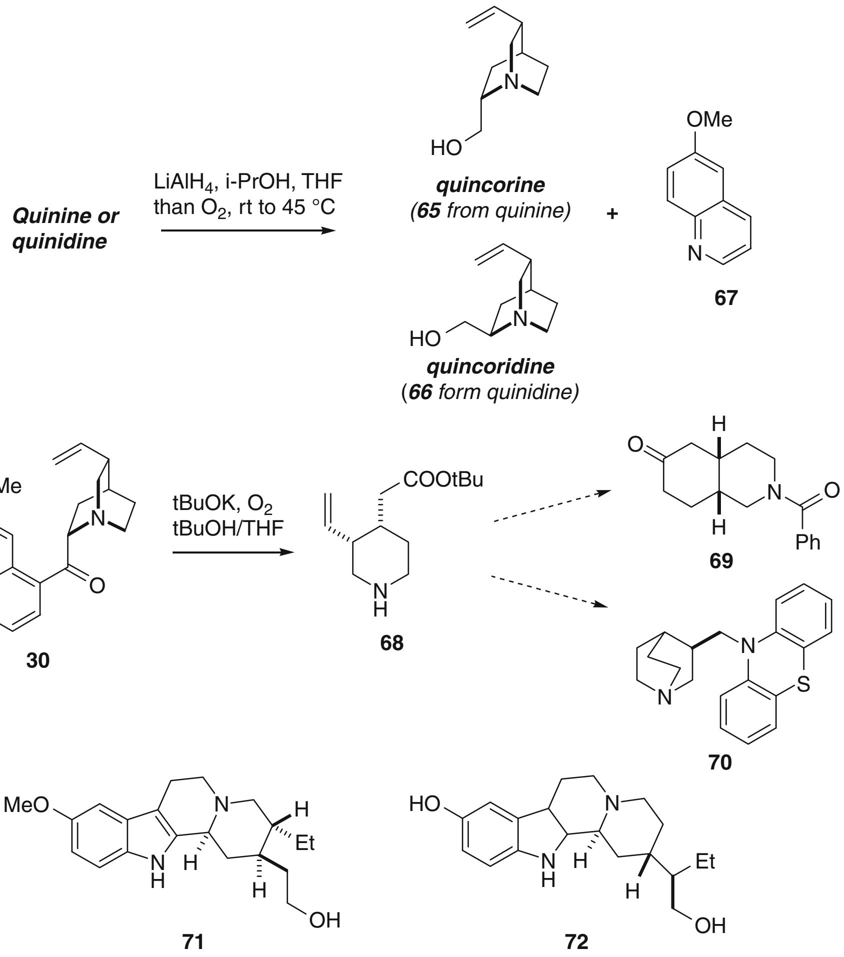
Scheme 21.5 Specific reactivity of Cinchona alkaloids of Cinchona 9-*O*-mesylates (**19**) or 9-halides (**24**) which provide an array of products, for example, **62-64** (depending on the condition and the alkaloid used) [30, 31]. The first "Cinchona rearrangement" leads to the cage-expanded [3.2.2] azabicyclic *α*-aminoethers **62**. The "second Cinchona rearrangement" affords *β*-functionalized [3.2.2] azabicyclic derivatives **63** and solvolysis products **64**. The latter reaction is favored for the so-called *cinch* bases (cinchonine and cinchonidine), and the rearranged products **63** can further be easily elaborated into several functionalized derivatives [205] (Scheme 21.5). Detailed explanations of the mechanisms of these rearrangements were discussed by Hoffmann [30, 31, 205].

An oxidative degradation of quinine or quinidine by a cleavage of C-9-4′ bond leads to quincorine **65** or quincoridine **66**, respectively, preserving the configuration at C-8 chiral center (methoxyquinoline **67** is a by-product of this reaction). This process has been developed by Hoffmann [30, 31, 206] and was further commercialized by Buchler GmbH, giving access to the large quantities of these versatile building blocks [207]. Similarly, degradation of quininone **30** provides meroquinene ester **68** utilized in the stereoselective synthesis of *cis*-isoquinolinone and *cis*-isoquinolinone derivatives **69** [208–210] or antihistaminic (+) mequitazine **70** [210] (Scheme 21.6). The older application of quinine in the synthesis of a number of indole alkaloids, for example, 10-mehoxydihydrocorynantheol **71** and dihydrohunterburnine **72**, has been reported by Sawa et al. [211–213].

5 Biosynthesis

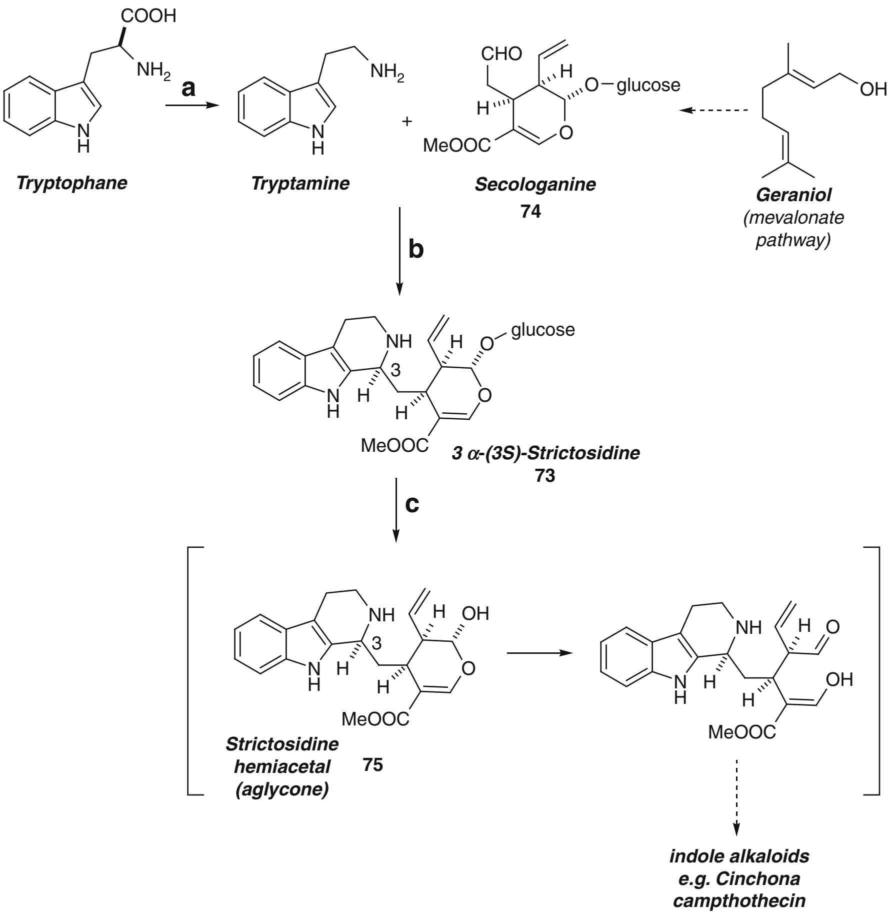
Biogenetically, Cinchona alkaloids are terpenoid indole alkaloids synthesized from tryptophan (quinoline ring) and geraniol (quinuclidine moiety), as it was postulated by Goutarel et al. shortly after their full structure elucidation in 1950 [73]. This was supported later by a series of radioactive feeding experiments of Leete et al. [214] (reviewed in detail in [23, 24, 215]). The key intermediate in Cinchona alkaloid biosynthesis is 3α-(*S*)-strictosidine **73** (general precursor of indole alkaloids) forming in the Pictet-Spengler reaction of tryptamine and secologanin **74** [216]. A detailed mechanism of strictosidine synthase (EC 4.3.3.2) catalysis has been recently proposed, on the basis of both experimental and theoretical studies [217]. The early steps leading to the active hemiacetal form of strictosidine **75** are shown in Scheme 21.7.

Interesting correlation between distribution of the strictosidine synthase activity and alkaloid content in the 6-month *C*. "*ledgeriana*" plants was found by Aerts et al. The enzyme was active in the top of stems, in young leaflets, and in the roots, and these parts also contained the highest concentration of the alkaloids. Quinoline alkaloids were accumulated in the roots, whereas cinchophyllines in aerial part of plants [218]. Similarly high activity of strictosidine synthase in the germinating Cinchona seeds and cell cultures has been reported [219]. Four isoforms of strictosidine synthase have been isolated and purified from the suspension culture of *C. robusta* [220].


Quinine or quinidine
LiAlH4, i-PrOH, THF than O2, rt to 45 °C
HO
N
quincorine
(65 from quinine)
+
OMe
N
67
HO
N
quincoridine
(66 form quinidine)
OMe
N
O
30
tBuOK, O2
tBuOH/THF
COOtBu
N
H
68
H
O
N
O
H
Ph
69
N
S
N
70
MeO
N
H
Et
N
H
H
H
OH
71
HO
N
N
H
H
H
Et
OH
72

Scheme 21.6 Preparatively useful oxidative degradation of Cinchona alkaloids

Strictosidine after hydrolysis, subsequent decarboxylation step and removing one of the carbon atoms from the iridoid moiety is transforming to corynantheal **76**. This intermediate was shown to be effectively incorporated by plants in the feeding experiments giving alkaloids. The next steps involving the formation of the quinoline ring of alkaloids from the indole moiety are complex and not clear. It is generally accepted that cinchonaminal **75** formed from **76** by cleavage of the indole N-C bond is the key precursor; however, there is no experimental evidence until now. Another problem deals the hydroxylation of the aromatic quinoline ring and subsequent methylation of phenolic hydroxyl group. 5-Methoxytryptamine is not incorporated by *C. robusta* cells neither into the alkaloids nor into their precursors, as demonstrated by Verpoorte, giving the evidence that methoxylation is carried out


COOH
NH2
a
N
H
Tryptophane
NH2
N
H
Tryptamine
+
CHO
H
O–glucose
H
MeOOC
O
Secologanine
74
OH
Geraniol
(mevalonate
pathway)
b
NH
3
N
H
H
H
O
glucose
O
MeOOC
3 α-(3S)-Strictosidine
73
c
NH
3
H
OH
O
N
H
H
H
MeOOC
Strictosidine
hemiacetal
(aglycone)
75
NH
H
O
OH
N
H
H
H
MeOOC
indole alkaloids
e.g. Cinchona
campthothecin

a) tryptophane decarboxylase; b) strictosidine synthase c) strictosidine glucosidase

Scheme 21.7 Early steps of Cinchona alkaloids biosynthesis

in the latter steps of biosynthesis [106]. Not confirmed observation suggests that this may occur at or before formation of corynantheal **76** [23, 221]. The middle steps transforming the indole-type cinchonaminal **77** into the quinoline-type cinchonidinone (or quininone) **79** probably via aminoaldehyde **78** is a plausible mechanism again without experimental support. The final steps involving stereoselective reduction of ketones **79–80** to alkaloids **1–4** are catalyzed by specific oxidoreductases [221] which, on the basis of feeding experiments, were shown to be fully reversible [222]. NADPH-dependent cinchoninone oxidoreductase isolated from *C. ledgeriana* is present in two isoforms: I and II. Isoenzyme I is more specific toward cinchoninone (quinoline ring), whereas form II shows broader specificity acting on all of the Cinchona alkaloids bearing quinoline, 6′-methoxy, or

6′-hydroxyquinoline rings [221]. Scheme 21.8 shows the commonly accepted biosynthesis of Cinchona quinoline and indole alkaloids. It must be pointed out that this proposal is only partially verified experimentally (the early and the final steps) as only three specific enzymes of this pathway has been isolated and characterized so far.

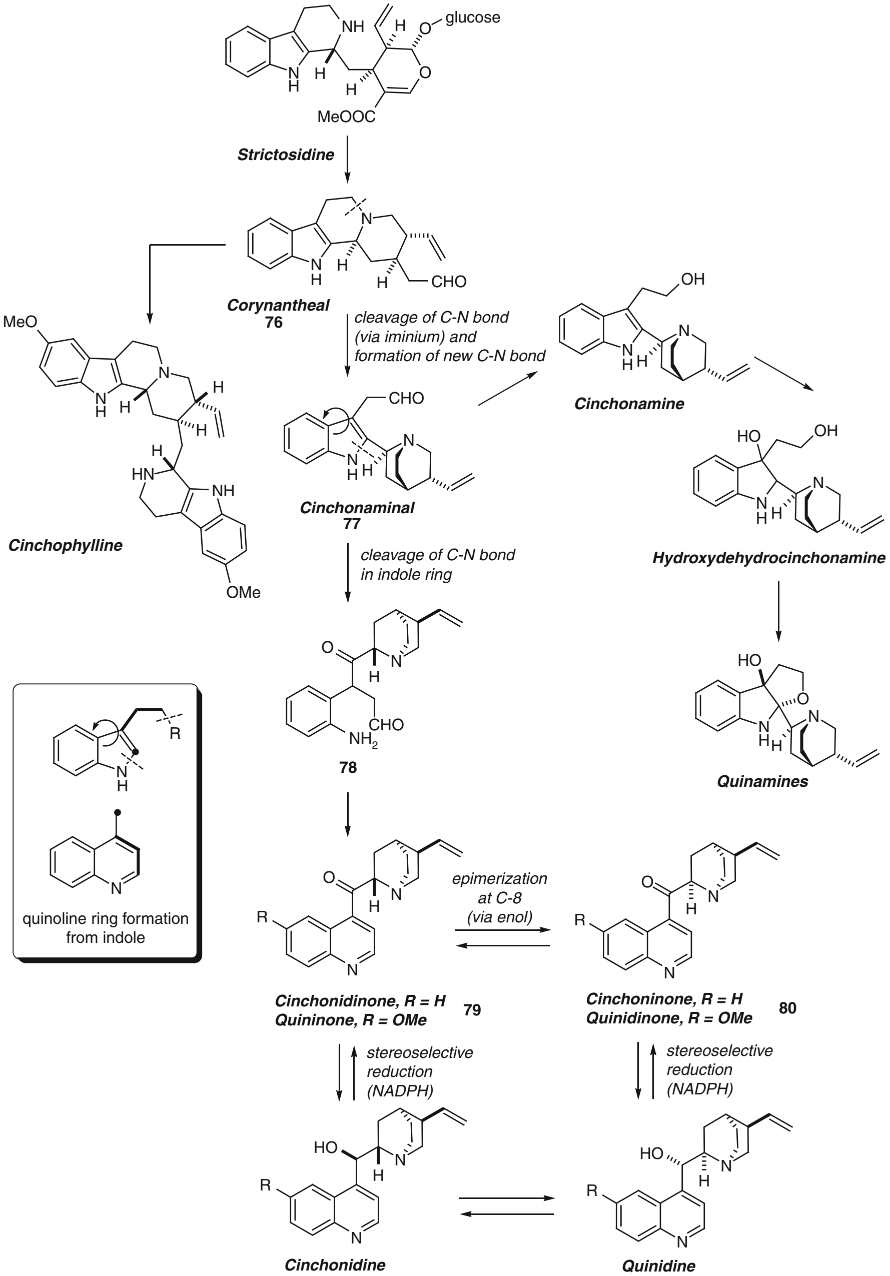
6 Biological Activity

Most important activity of Cinchona alkaloids is antiprotozoal action of quinine used for more than 400 years for the treatment of malaria [1–4, 32–35]. Malaria is a vector-transmitted infectious disease caused by parasites of the genus *Plasmodium*. The life cycle of parasite requires two host organisms: female mosquitoes from the species *Anopheles* and humans (rarely other primates or mammals). Severe malaria is largely caused by *Plasmodium falciparum* (responsible for ca. 90% of deaths), while *P. vivax* or *P. ovale curtisi*, *P. ovale wallikeri*, and *P. malariae* give generally a milder disease with low fatality. Malaria remains a serious public problem in tropical and subtropical regions of Africa, Asia, and America involving more than 90 countries inhabited by approximately 2,400 million people (about 40% of the world population). Annual incidence of malaria is estimated at 250–500 million cases, causing between 1.5 and 2.7 million deaths per year (the highest mortality is observed in children under 5 years of age) mostly in sub-Saharan Africa, but also in Asia, Latin America, and the Middle East.

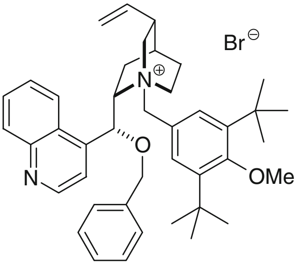
The economic burden of malaria is extremely high, according to the various estimations accounting for ca. 1.3% reduction of the annual economic growth rate in countries where the disease is endemic and costs as high as ca. 40% of whole public health expenditures. The problem of malaria is increasing yet due to the development of the resistance of plasmodia toward most of the synthetic antimalarial drugs used over last decades [4].

The mechanism of malaria is relatively well understood and involves infection of humans by biting by female mosquitoes who inject sporozoites (distinct morphological forms of parasites at mosquito stages) subcutaneously. They enter the bloodstream and migrate to the hepatocytes where they differentiate into merozoites. This form invades further erythrocytes to establish a blood-stage infection, where they appear initially as a ring stage, followed by a growing trophozoite, which develops into a dividing a sexual schizont. Some merozoites may also differentiate into male and female gametocytes (sexual forms) which, if taken up by a mosquito, will infect the insect and continue the life cycle. After complex process of the differentiation of parasite within the mosquito, new sporozoites migrate to the salivary glands and are ready for the infection of the next host organism. The detailed life cycle of *Plasmodium* is given [4, 223].

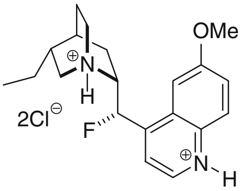
Among four major Cinchona alkaloids, quinidine and cinchonine were most active in the malaria therapy followed by quinine and cinchonidine. For example, quinidine is two- to threefold more active than quinine in both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* [224]. Due to its cardiac


glucose
MeOOC
Strictosidine
Corynantheal
76
cleavage of C-N bond
(via iminium) and
formation of new C-N bond
Cinchonamine
Cinchophylline
Cinchonaminal
77
cleavage of C-N bond
in indole ring
Hydroxydehydrocinchonamine
78
Quinamines
quinoline ring formation
from indole
epimerization
at C-8
(via enol)
Cinchonidinone, R = H
Quininone, R = OMe
79
Cinchoninone, R = H
Quinidinone, R = OMe
80
stereoselective
reduction
(NADPH)
stereoselective
reduction
(NADPH)
Cinchonidine
Quinidine

Scheme 21.8 Biosynthesis of Cinchona alkaloids activity, it cannot, however, be safely used as antimalarial; therefore, most abundant quinine historically continued to be a drug. 10,11-Dihydroanalogues of these alkaloids exhibit similar activity as compared with the parent compounds. Stereochemistry of 1,2-aminoalcohol moiety in alkaloids is crucial for their antiprotozoal action; e*rythro*-configurated natural bases are active, while 9-*epi*-configurated analogues (*threo* configuration) have no activity [224–226]. Detailed analysis of geometry and certain electronic properties of eight major Cinchona alkaloids were used for defining stereoelectronic features responsible for their antimalarial activity. This involves sufficiently acidic hydroxyl proton and an electronic feature of molecule pointing from quinuclidine nitrogen toward the quinoline ring [227]. Interestingly, quaternization of the four major Cinchona alkaloids by 4-methoxy-3,5-di-*tert*-butylbenzyl bromide and their further 9-*O*-etherification lead to antimalarial derivatives **81** comparable to parent alkaloids in in vitro tests [228]. Similarly, C-9-fluorinated quinine **82** showed high antiplasmodial activity reaching IC_{50} value as low as 267 nM for best compound (but below activity of parent alkaloids) [229]. This data may suggest that there is no single pharmacophore of Cinchona moiety responsible for its antimalarial activity.



81 *(from cinchonidine)*



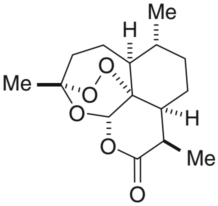
82 *(quinidine confg.)*

Despite newer antimalarial drugs, quinine continues to be important drug used for treatment of the multidrug-resistant malaria and severe malaria. It is also recommended as the only drug for treatment of malaria in the first trimester of pregnancy. Detailed recommendations, dosages, and comparison with other antimalarial drugs for the current medical practice are summarized in many works [4, 32–34]. Although there are some reports on the increasing local resistance of *P. falciparum* toward quinine, for example, in Southeast Asia, South America, and Africa, most of other studies confirm its high activity. In this context, efforts to find the genetic markers of *Plasmodium* indicative for its resistance against quinine have been undertaken [230].

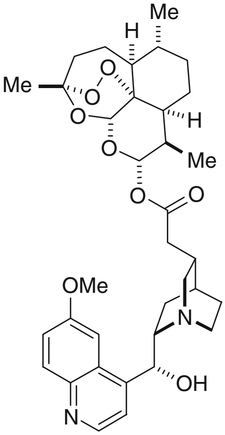
The mechanism of action of quinine is not perfectly understood. It is generally assumed, on the basis of several model studies [231, 232], that Cinchona alkaloids prevent the polymerization of the toxic hematin ([aquaFe(III)protoporphyrin IX]) formed by the degradation of hemoglobin in erythrocytes to hemozoin (β-hematin).

Blocking of this nonenzymatic detoxification process, developed by the parasites, results in a toxicity of the non-polymerized hematin [232, 233]. Other hypothetical scenarios explaining activity of quinine are discussed [232, 233].

Although quinine was the first efficient antimalarial drug, its limited supply, political instability in the past, as well as its narrow therapeutic window stimulated an intense research toward the synthetic quinine and other antimalarials. These efforts did not bring the economically viable synthesis of quinine but provided several synthetically more accessible analogues with antimalarial activity. Among them chloroquine, mefloquine, and primaquine were the most important compounds. These early-generation drugs are the subject of comprehensive reviews [223, 234]. Unfortunately, plasmodia strains acquired resistance to the most of these synthetic drugs (especially to the most popular chloroquine, used widely as an antimalarial prophylaxis) during the years of their constant use, resulting in refreshed interest in the quinine therapy. Newer class of drugs introduced to the antimalarial therapy in recent years include natural product artemisinin **83** and its semisynthetic derivatives artesunate, artemether, and arteether as well as synthetic 1,4-naphtoquinine-based atovaquone with combination of proguanil (Malarone) [4]. Artemisinin-based pharmaceuticals remain currently the first choice for the malaria treatment. Interestingly, synthetic quinine-artemisinin conjugate **84** obtained by Walsh exhibits high activity against the 3D7 and FcB1 (drug-resistant) strains of *P. falciparum* in culture. The synergic effect on the activity was observed, as compared with that of artemisinin or quinine alone and a 1:1 mixture of artemisinin and quinine [235].



83



84

The cardiological effects of Cinchona bark alkaloids have been recognized in an academic medicine at the end of the seventeenth century. Quinine was used at the beginning, but its pseudoenantiomer quinidine has been found to have more beneficial antiarrhythmic properties, thus becoming a standard medication until newer drugs were developed in the mid-twentieth century [236]. Quinidine inhibits fibrillation (uncoordinated contraction of muscle fibers in the heart) by blocking potassium channels [237, 238]. The major problem with the use of this alkaloid is its quick absorption by the gastrointestinal tract giving risk of overdosing. This may lead to diastolic arrest (ventricular arrhythmia) and death. Safety concerns resulted in the significant reduction of quinidine use in a therapy over years. However, recently renewed interest of the medical use of quinidine is observed; in particular, quinidine combination with verapamil has been reported to be safe and efficacious in the treatment of atrial fibrillation. Quinidine has also been used successfully to treat idiopathic ventricular fibrillation, Brugada, and short QT syndromes. Quinidine applications in the modern cardiology are subject of reviews [239, 240].

Muscle cramp is a recurrent and painful condition and a common complaint among elderly subjects and patients treated with hemodialysis. No specific cause of this syndrome was identified; therefore, therapy is mostly symptomatic. Over the years, quinine sulfate was claimed to be a medication relieving the muscle cramp symptoms, but safety issues declined its current use. Several detailed studies have been reported to clarify the controversy of quinine efficacy in this line [241, 242]. Meta-analysis of the different 23 trials with a total of 1,586 participants has been published with conclusion that quinine at the dosage typically 300 mg/day (range 200–500 mg), compared to placebo, significantly reduced cramp number over 2 weeks by 28%, cramp intensity by 10%, and cramp days by 20% but without affecting the cramp duration [241]. In another report, moderate quality evidence has been found confirming that quinine reduces cramp at similar dosage [242]. Typically, only minor side effects of quinine use were observed in patients, leading to the conclusion that this is a relatively safe drug. There are studies which claim beneficial effect of quinine for leg cramps treatment [243].

Quinine and cinchonine since two decades were also investigated in reversing of multidrug resistance (MDR) in cancer patients [24]. Phase I/II of clinical trials conducted for quinine shows that it could be used safely with a number of anticancer agents, for example, mitoxantrone, cytarabine, cyclophosphamide, or paclitaxel, improving the treatment of clinically resistant acute leukemias, breast cancer, or non-Hodgkin's lymphomas [244–246]. Continuation of trials in Phase III in patients with acute leukemia resulted unfortunately only in the modest success [247]. Other preclinical and Phase I studies in this line have shown that cinchonine is superior MDR agent as compared with quinine [248, 249]. Cinchonine combined with cyclophosphamide, doxorubicin, methylprednisolone, and vinblastine may be safely administrated in patients with malignant lymphoid disease, giving MDR reversing activity in every case [250]. Recently, dimeric quinine **85** linked by ester bond was shown to be highly active in MDR that completely reversed the *P*-glycoprotein (P-gp)-mediated paclitaxel resistance phenotype as well as inhibiting its transport in MCF-7/DX1 cell in vitro studies [251]. There are also reports on inhibitory action of quinine for microbial pathogen invasion on human skin [252].

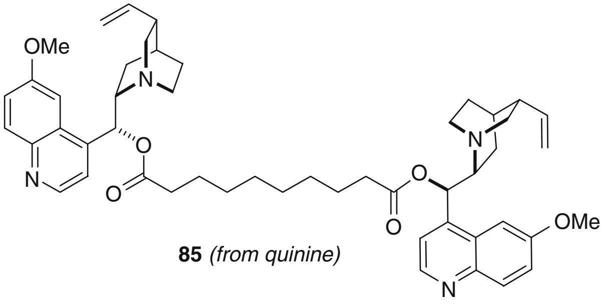
Quinine bark is still popular as a herbal medicine. For example, in South America, besides being antimalarial drug, it is considered a tonic, digestive stimulant, and anti-fever. The bark is also used for anemia, indigestion, gastrointestinal disorders, general fatigue, fevers, and as an appetite stimulant. It is claimed that

Cinchona bark could also be used as a remedy for some types of cancer, amebic infections, heart problems, colds, diarrhea, lumbago, and other problems. These indications of the bark (besides malaria) have no until now strong evidence in the medical literature [253].

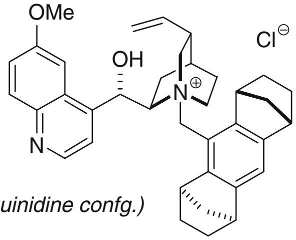
An interesting property of quinine (and quinidine) is their strong bitter taste (gin was originally added for the administration of quinine to make its bitter taste more acceptable) [20]. Thanks this quinine (in a form of water-soluble salts) is a valuable taste modifier in beverage (vermouth and tonic water) and occasionally also in confectionery industry. The typical concentration of quinine in beverages is up to 80 mg/L and could be considered as safe for consumers (however do not provide an effective antimalarial prophylaxis). Recent studies confirmed also that in contrast to some doubts, beverage level of quinine does not inhibit the cytochrome P450 (CYP2D6) which is involved in the metabolism of many drugs [254]. Although the receptors of quinine bitter taste have not been isolated until now, genetic studies carried out by Reed suggest that there is a gene cluster located on chromosome 12 responsible for coding of bitter receptors in the taste receptor cells and salivary proline-rich proteins. Significant genetic variation in this region is responsible also for an individual perception of bitterness of quinine [255].

There are also reports showing specific biological activity of minor Cinchona alkaloids, for example, cinchophyllamine **10** is active against Gram-positive bacteria [81] and cinchonaminone **9** is a monoaminoxidase inhibitor [72] (some older works regarding activity of Cinchona indole alkaloids are summarized in [23]). Dihydrocorynantheol has found to have antiparasitic and cytotoxic activity [256].

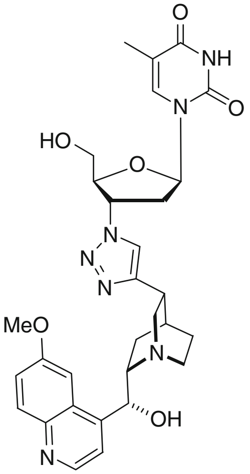
Recently, medicinal chemistry considers synthetic derivatives of Cinchona alkaloids as pool of diverse biological activity. For example, large library of Cinchona alkaloids and their quaternary salts have been tested toward cardiomyogenesis stimulators. Two members of screening set **86–87** exhibited very good activity in the test of cardiomyogenesis of transgenic murine embryonic stem [257]. Other quaternary salts derived from quinine and cinchonidine such as **88** were also shown to be inhibitors of butyrylcholinesterase exhibiting 250-fold selectivity increase as compared with acetylcholinesterase [258]. Cinchona alkaloid-AZT conjugates such as **89** linked by 1,2,3-triazole developed by Celewicz et al. showed marked cytotoxic activity in vitro [167].

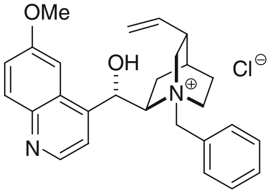


85 *(from quinine)*

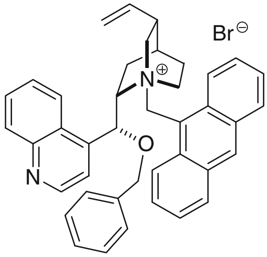


86 *(quinidine confg.)*





87 *(quinidine confg.)*



88 *(from cinchonidine)*

89 *(quinine confg.)*

Last but not least, the ecological role of Cinchona alkaloids should be mentioned. The subject remains fuzzy because relatively few reports are available and most of them do not bring clear answer to the question why *Cinchona* species produce so much alkaloids. The seed germination and the level of the alkaloids in *Cinchona* seedlings were studied by Verpoorte et al. who suggested that initially high productivity of alkaloids has a deterrence of feeding of slugs [259]. In a laboratory experiment, the germination of *Ocimum*, *Spermacoce*, *Catharanthus*, and *Cinchona* itself was strongly inhibited by the alkaloids applied at concentrations higher than 0.3 mM. However, soil experiments with the living *Cinchona* plants showed that despite high concentration of the alkaloids in root (ca. 10 mM), their soil concentration was very low (ca. 0.02 mM); thus, no allelopathic plant-plant interaction was observed and probably they do not occur in the natural environment [260]. Another study by Verpoorte et al. has shown the toxic effect of the indole-type alkaloids (present in young leaves) on larvae *Spodoptera exigua* in contrast to quinoline alkaloids (mainly present in roots) [261]. Alkaloids from *Remijia peruviana* were found to have antifeedant activity on *Leptinotarsa decemlineata* [68].

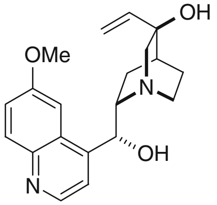
7 Biotechnological Approaches

Biotechnological production of Cinchona alkaloids was considered as an attractive alternative to their isolation more than two decades ago. The major motivation for research in this field were difficulties with *Cinchona* plant harvesting, including high variation of alkaloid content in the bark, long time needed for achieving of tree maturity, and difficult access to the high-placed cultivating areas. Pathogen infection, for example, mold *Phytophthora cinnamomi* often destroying *Cinchona* plantations, is another problem. Several studies on the use of cell cultures for the bioproduction of alkaloids were carried using different strategies of cell cultivation and feeding. Unfortunately, despite intense research, generally low productivity (typically few mg/g of dry weight) was observed. Similarly, large-scale cultures, immobilization of the *Cinchona* cell, and microbial or genetic transformations did not bring the expected level of bioproduction of alkaloids [262–265]. Best results so far were achieved by the use of compact globular structures (CGS) which show a differentiation similar to that found in the *Cinchona* bark. In this case, however, higher levels of alkaloids exceeding 1% of dry weight were compromised by slow growth of CGS system that hampered the overall economy of the process [266]. The more detailed view of the biotechnology of *Cinchona* species was published [267, 268]. Currently, rather stable Cinchona alkaloid world market and relatively low prices of quinine make the biotechnological approach not competitive, at least until as the mentioned problems are solved. This is expected when the genetics of biosynthesis and its regulation in the cell lines become known and fully controllable.

Bioconversion of Cinchona alkaloids is relatively less explored as compared to other natural products. The reason for this is the fact that none of the Cinchona alkaloid precursors are available at lower prices than the final alkaloids. The only exception is tryptophan; however, it has no straightforward influence on the increasing production of alkaloids in cell cultures [267, 268].

A potentially attractive bioconversion involves a stereoselective enzymatic reduction of quinidinone to quinidine (this process is now carried out chemically, vide supra). Among about 450 microorganisms screened by Ray et al., yeast *Hansenula anomala* var. *schneggii* was found to provide quinidine (of unknown stereochemical purity) with 50% yield after 7 days of fermentation [269]. High-yielding transformation of Cinchona alkaloids **1–4** into the *N*-oxides by oxidation with endophytic fungi of *Xylaria* sp. isolated from young stems of *C. pubescens* Wahl. was published by Shibuya et al. [270]. Kieslich identified several fungal species metabolized Cinchona alkaloids **1–4** to quinine 1-*N*-oxide, 1’-*N*-oxide, and 3-hydroxyl derivative as well as 1-*N*-oxides of quinidine, dihydroquinidine, and cinchonidine. This latter alkaloid yielded 3-hydroxyl derivative when *Rhizopus arrhizus* ATTC 10260 was employed [271]. Fungal transformation of quinidine has been studied by Eckenrode who found that 3-hydroxyquinidine is a typical metabolite [272].

Metabolism and toxicology of Cinchona alkaloids in human received much attention since analytical methods became available. It was found that quinine is mainly transformed into (3 *S*)-3-hydroxyquinine **90** [273], but minor metabolites including 2′-oxoquininone **92** and (10 *S*/*R*)-11-dihydroxydihydroquinine **91** have also been identified [103, 274, 275]. Quinidine undergoes similar metabolic pathway giving (3 *S*)-3-hydroxyquinidine as a major metabolite and minor amounts of quinidine-*N*-oxide [276]. Important way to eliminate of quinine and quinidine is the formation of respective glucuronides. Stereoselective hydroxylation of quinine and quinidine is catalyzed by cytochrome type CYP3A4 [277, 278]. More detailed information regarding Cinchona alkaloids metabolism and toxicity in human is a subject of reviews [279, 280].



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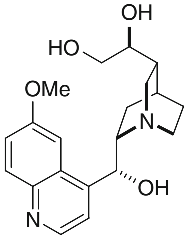

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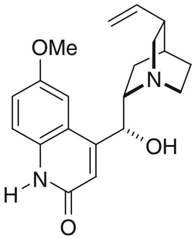

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8 Perspectives

Among many thousands of natural products identified so far, Cinchona alkaloids provide a unique combination of properties, serving as a drugs, chiral catalysts and selectors, indicators, or bitter-taste agents. These multi-faced molecules remain still in a focus of life sciences despite their long history and intense research done over the last century. Currently, their use in catalytic asymmetric synthesis is the most explored area; nevertheless, renewed interest in seeking of novel biological activities is recently observed. This trend is supported by the development of reliable Cinchona alkaloid chemistry which provides diverse molecular architectures for screening.

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Chemistry and Biology of Camptothecin and its Derivatives

22

Karol Michał Kacprzak

Contents

1 Introduction 644
2 Occurrence and Isolation 647
3 Chemistry of Camptothecin 650
3.1 Total Synthesis of CPT and Semisynthesis of CPT-Related Drugs 651
3.2 SAR Studies 654
4 Biosynthesis of Camptothecin 659
5 Biological Activity of Camptothecins 661
5.1 Irinotecan 664
5.2 Topotecan 665
5.3 Other CPT-Related Anticancer Drugs 665
6 Biotechnological Approaches 671
7 Perspectives 672
References 672

Abstract

Quinoline alkaloid camptothecin (CPT) isolated in 1958 from Chinese tree *Camptotheca acuminata* is an important natural product serving as a lead compound for designing many more active and clinically useful anticancer drugs, such as approved topotecan and irinotecan. All camptothecins exhibit a unique mechanism of action involving inhibition of topoisomerase I which leads to the interruption of cell division processes. Current efforts in CPT research focus on construction of better drugs, prodrugs, and new forms of administration. Important area of clinical work involves testing of multidrug regimens of CPTs with conjunction of other anticancer drugs. This often resulted in an improved activity toward malignancies. Beside anticancer

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activity, camptothecin and related compounds display also other activities such as antiviral or antiprotozoal. Insufficient natural supply of camptothecin led to significant improvement of camptothecins total synthesis as well as to attempts of its biotechnological production. The present chapter covering over 300 references is intended to provide a complete but condensed overview on camptothecins, with emphasis on the newest work in the field.

Keywords

Alkaloids • anticancer drugs • camptothecin • irinotecan • topoisomerase I • topotecan

Abbreviations

5-FU	5-Fluorouracil
CPT	Camptothecin
Et	Ethyl
HPLC	High pressure liquid chromatography
HSA	Human serum albumin
IC_{50}	Inhibitory concentration on 50% of investigated sample
Me	Methyl
NMR	Nuclear magnetic resonance
NSCLC	Non-small-cell lung carcinoma
OR	Overall response ratio
Ph	Phenyl
SCLC	Small-cell lung cancer
X	Halogen

1 Introduction

Camptothecin (CPT, **1**, Fig. 22.1) is a quinoline pentacyclic alkaloid (classified as pyrrolo[3,4-*b*]quinoline) originally isolated from the Chinese tree *Camptotheca acuminata* Decne (*Nyssaceae*) in 1958 by Wall and Wani in the frame of NCI anticancer candidates screening program. In 1966 structure of camptothecin was determined by using combination of NMR and X-ray approach [1]. Camptothecin has shown to be very active antitumor compound both in vitro and in animals and quickly entered the clinical investigation. The wider use of camptothecin in the therapy was hampered by its low solubility in water and rapid inactivation due to the lactone ring opening at physiological pH (open hydroxyacid form **2** is produced) as well as considerable toxicity. Sodium salt of camptothecin **3** has been proposed as more soluble form of the drug. Unfortunately, further studies of Phase I and II showed its lesser efficacy and unpredictable severe side effects such as hemorrhagic cystitis and myelotoxicity. This eventually led to the discontinuation of trials in 1974 [2–4].

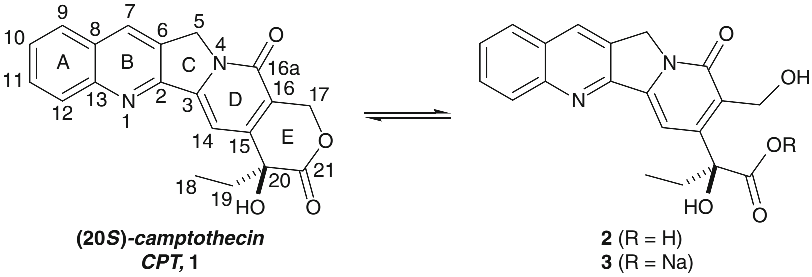


Fig. 22.1 Structure and atom numbering of camptothecin (CPT) and its open hydroxyacid form

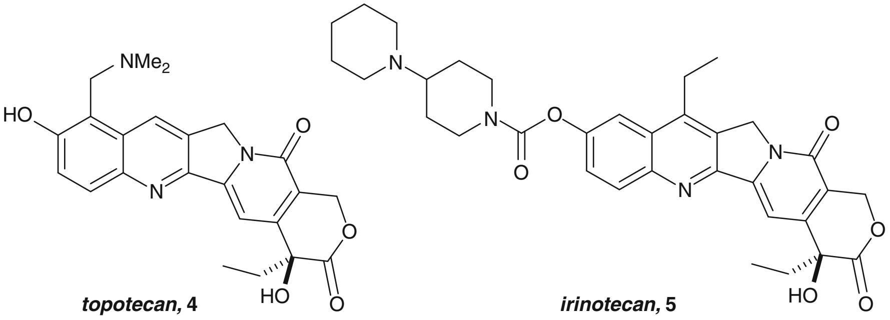


Fig. 22.2 Structure of topotecan and irinotecan

The renewed interest in camptothecin in the mid-1980s appeared after elucidation of its unique molecular mechanism of action which involves the inhibition of DNA topoisomerase I (topo I) [5, 6]. This discovery has stimulated a significant progress in camptothecin chemistry leading finally to the development of several more active and water-soluble derivatives in 1990s. Two of them have been already commercialized as topotecan (**4**, Hycamtin) [4] and irinotecan (**5**, Camptosar, CPT-11) [4] by GlaxoSmithKline and Pharmacia (currently Pfizer), respectively (Fig. 22.2). These drugs with combined annual sale over $600 million in 2010 [evaluatepharma.com] are currently important anticancer medicines in the treatment of metastatic colorectal, primary colon, and metastatic ovarian cancers [4, 7].

A number of other newer-generation camptothecin derivatives, such as belotecan **6** [8–11] gimatecan **7** [12–15], karenitecin **8** [16–19], rubitecan **9** [20–23], silatecan **10** [24, 25], SN-38 **11** [26], exatecan **12** [27], lurtotecan **13**, [28–30], homocamptothecin **14** [31–33], and diflomotecan **15** [34] (Fig. 22.3), are at various stages of clinical trials. Some of them exhibited high activity toward particular cancer types or better formulation/usage characteristics, but none left the clinical trial stage at present [4, 7] (vide infra). Another important area of the camptothecins research involves the design of new formulation of camptothecin and

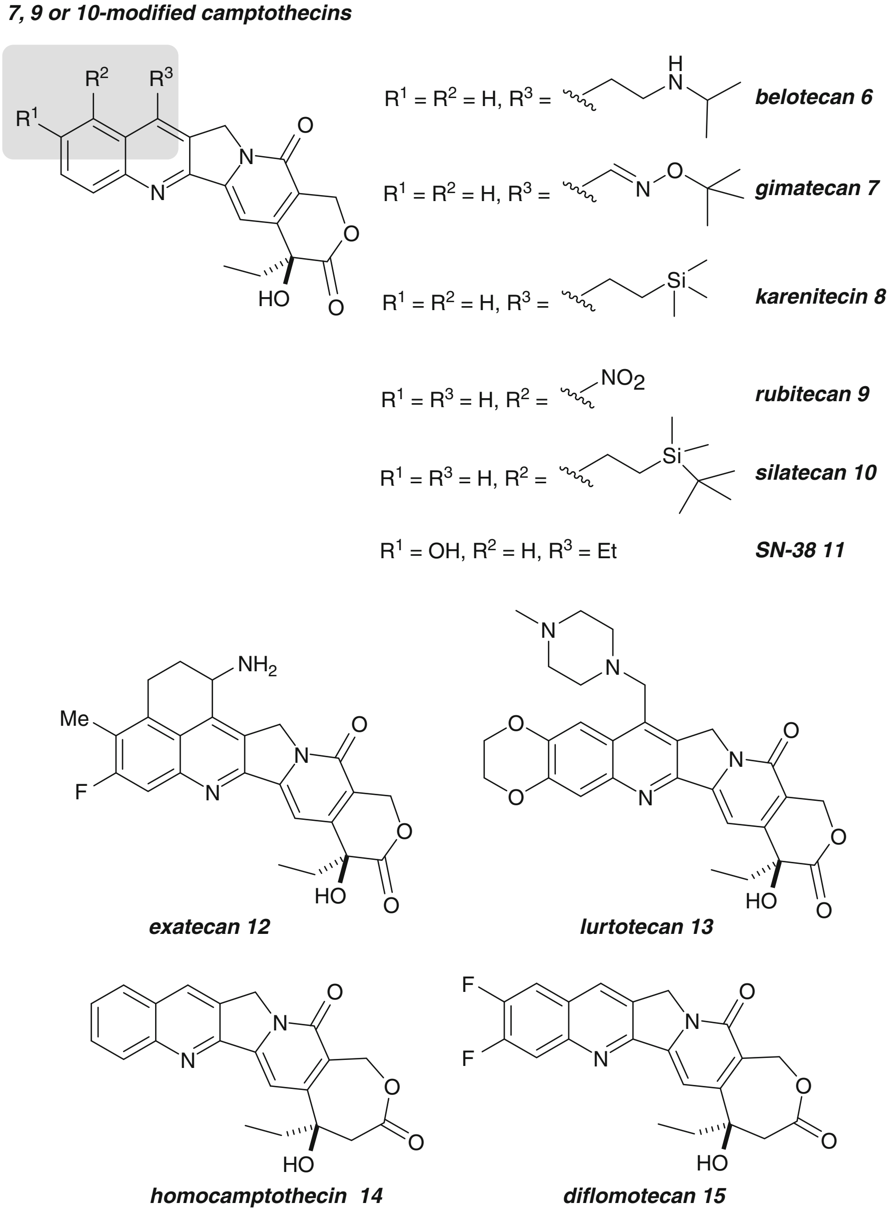

7, 9 or 10-modified camptothecins
R1
R2
R3
N
N
O
O
HO
O
R1 = R2 = H, R3 =
belotecan 6
R1 = R2 = H, R3 =
gimatecan 7
R1 = R2 = H, R3 =
karenitecin 8
R1 = R3 = H, R2 =
NO2
rubitecan 9
R1 = R3 = H, R2 =
silatecan 10
R1 = OH, R2 = H, R3 = Et
SN-38 11
NH2
Me
F
exatecan 12
lurtotecan 13
homocamptothecin 14
F
F
diflomotecan 15

Fig. 22.3 Other anticancer camptothecins

its derivatives, providing the drugs with beneficial therapeutic profile, e.g., extended stability or higher solubility, better pharmacokinetics, or reduced side effects. Typical strategies employed to meet these goals include the use of the dedicated camptothecin carriers such as liposomes or microemulsion [28–30, 35, 36] or nano-sized biohybrids [37], particles [38–40], or drugs nanocrystals [41, 42]. Sophisticated prodrugs of camptothecin with drug covalently bound to the polymeric matrix resulted in the development few active systems, from which some entered into clinical investigation [43–46].

The early developments of CPT research are the subject of authoritative reviews by Wall and Wani [2, 47–51], Hutchinson [3, 52], and Oberlies et al. [53], whereas newer research, including total synthesis and chemical modifications is covered in reviews by Yang [54], Nessler [55], Hecht [56], Zu [57], Du [58], and Dallavalle et al. [59]. Clinical use of camptothecins is a subject of excellent reviews of Verweij [4], Simanek [60], Zunino [61], and a dedicated monograph [7]. Valuable source of chemical data of naturally occurring camptothecins is *Encyclopedia of Alkaloids* [62].

2 Occurrence and Isolation

CPT was initially isolated from the tree *Camptotheca acuminata* (Nyssaceae, common name *happy tree*) which is native of populated warm areas of southwestern provinces of China such as Szechwan, Yunnan, and Kwangsi. This plant is also cultivated in Taiwan, India, and California [50, 62–65]. CPT and related alkaloids comprising mainly simple hydroxylated or methoxylated derivatives or glucosides (Table 22.1) have been found in 12 other plant species belonging to unrelated orders of angiosperms. Among them only Indian *Nothapodytes foetida* Wight, Sleumer (formerly *Mappia foetida*, Miers) [66–71, 85], and *Nothapodytes nimmoniana* [72] constitute another important source of the alkaloid. Other species include *Ervatamia heyneana* (Apocynaceae) [73, 74], *Merrilliodendron megacarpum* [75], *Mostuea brunonis* Didr (Gelsemiaceae) [76], *Ophiorrhiza filistipula* [77], *O. liukiuensis* [78], *O. mungos* [79, 85]*, O. pumila* [80–82], *O. trichocarpon* [83] (Rubiaceae), and *Pyrenacantha klaineana* (Icacinaceae) [84]. Representative examples of camptothecins producing plants and their characteristics are given in Table 22.1.

Besides camptothecines, structurally related (having the same tetracyclic 11*H*-indolizino[1,2-b]quinoline-9-one core) alkaloids such as 18,19-dehydro-CPT **16**, mappicine **17** [86], nothapodytine, A and mappicine ketone **18** [87] (Fig. 22.4) and their hydroxylated or methoxylated derivatives and glucosides were isolated from *Nothapodytes foetida* (Icacinaceae) [65, 71, 76, 78, 81, 82].

Interestingly, CPT and its derivatives have recently been identified as metabolites of various entophytic fungi, e.g., *Fusarium solani* (from *Apodytes dimidiata*, E. Mey. ex Arn, Icacinaceae) [88], *Entrophospora infrequens* (from *Nothapodytes foetida*) [89], and others [90], providing an alternative to the plant cultures for their biotechnological production.

The isolation of CPT from plant material remains the major source of this alkaloid for the pharmaceutical industry (with estimated production ca. 1,000 kg annually) [91]. For this purpose *Camptotheca acuminata* is still in use. Typically,

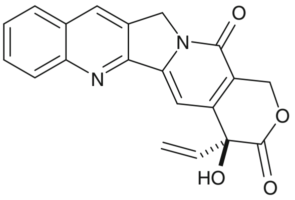
Table 22.1 Occurrence of camptothecin and its derivatives in plant species

Species (origin)	Alkaloid(s)	Tissue concentration mg/g dry weight	Refs.
Camptotheca acuminata Decaisne (Texas, USA)	CPT	Young leaves: 2.4–5.0	[62–65]
		Old leaves: 0.4	
		Seeds: 3.0	
		Bark: 1.8–2.0	
		Roots: 0.4	
	10-Hydroxy-CPT	Young leaves: 0.02–0.03	
		Seeds: 0.025	
		Bark, roots: <0.1	
	10-Methoxy-CPT	Seeds	
	10-Hydroxy-20-deoxy-CPT	Fruit	
	11-Hydroxy-CPT	Seeds	
	18-Hydroxy-CPT	Root bark	
	18-Hydroxycamptothecin-20-*O*-β-glucopyranoside		
	20-Hexanoyl-CPT	Bark	
	20-*O*-Acetyl-10-methoxy-CPT	Root bark	
	20-*O*-Hexanoyl-10-methoxy-CPT	Bark	
Camptotheca lowreyana Li, (Texas, USA)	CPT	Young leaves: 3.9–5.5	[64]
		Old leaves: 0.9–1.1	
Camptotheca yunnanensis Dode (Texas, USA)	CPT	Young leaves: 2.5–4.5	[64]
		Old leaves: 0.6	
Nothapodytes foetida (Wight) Sleumer	CPT	Stem wood: 1.4–2.4	[66–71]
	18,19-Dehydro-CPT		
	20-*O*-Acetyl-CPT		
	9-Methoxy-CPT		
	20-*O*-acetyl-9-methoxy-CPT		
Nothapodytes nimmoniana	CPT	Stem, root bark $\sim$10	[72]
Ervatamia heyneana (Wall) T. Cooke (India)	CPT	Wood, bark: 1.3	[73, 74]
	9-Methoxy-CPT		
Merrilidendron megacarpum (Hemsl.) Sleumer (Guam)	CPT	Leaves, stem 0.53	[75]
	9-Methoxy-CPT		

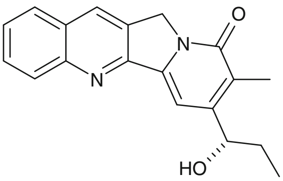
(*continued*)

Table 22.1 (continued)

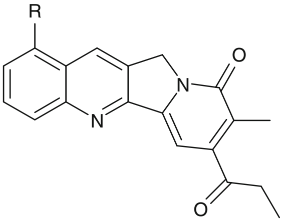
Species (origin)	Alkaloid(s)	Tissue concentration mg/g dry weight	Refs.
Mostuea brunonis Didr.	Camptothecin-20-*O*-*β*-glucopyranoside	Entire plant: 0.1 0.1 0.6	[76]
	Deoxypumiloside		
	Strictosamide		
	2′-O-acetylstrictosamide		
Ophiorrhiza fillistipula	7-Methoxy-CPT		[77]
O. liukiuensis	CPT		[78]
	9-Methoxy-CPT		
	10-Methoxy-CPT		
Ophiorrhiza mungos Linn.	CPT		[79]
	10-MethoxyCPT		
Ophiorrhiza pumila Champ. (Japan)	CPT	Leaves: 0.3–0.4 Young roots: 1.0	[80–82]
	9-Methoxy-CPT	Hairy roots: 1.0	
	9-Methoxy-10-hydroxy-CPT-10-*O*-*β*-D-glucopyranoside (Chaboside)		
Ophiorrhiza trichocarpon	9,10-Methylendioxy-CPT		[83]
Pyrenacantha klaineana	CPT, 9-methoxy-CPT		[84]



18,19-dehydrocampthothecin
16



mappicine
17



nothapodytine A (R = OMe)
mappicine ketone (R = H)
18

Fig. 22.4 Minor camptothecin-type alkaloids

seeds of this tree contain ca. 0.3% and bark 0.2%, whereas leaves yield up to 0.4% of CPT. The bark of *C. acuminata* contains also ca. 0.05% of 10-hydroxycamptothecin. Another important plant source of CPT is the bark and stem wood of *Nothapodytes foetida* (typical content of alkaloid 0.1–0.3% of dry plant material). Isolation of CPT involves the use of classical solvent extraction with nonpolar solvent for removal of fat material, followed by more polar solvent (ethyl acetate, acetone, methanol, or ethanol) for isolation of alkaloids. Typical yield of the solvent extraction lies in the range of 0.1% [92]. In order to improve the efficiency of the isolation (higher yield, lower solvent consumption, and time reduction), several other isolation techniques have been tested and critically compared by Fulzele [93]. Extraction of CPT and 9-methoxy-CPT from *Nothapodytes foetida* by employing either stirring or Soxhlet extraction, ultrasonic extraction, or microwave-assisted extraction (MAE) showed that the MAE gave the highest yield in the shortest time (typical time applied 3 min) [93]. Similarly, favorable results were reported by Yang et al. by using homogenate extraction technology for isolation of CPT and 10-hydroxy-CPT **32** from *Camptotheca acuminata* leaves [94]. Recently, imidazolium-based ionic liquids as extracting material in combination with MAE techniques were used for efficient and rapid isolation of CPT and 10-hydroxy-CPT from *Camptotheca acuminata* [95]. Analytical chemistry, isolation, and chromatographic determination of CPT and the corresponding semisynthetic drugs is a subject of a review by Aldaz et al. [96]. Chromatographic profiling of CPTs and their metabolites by combined techniques such as HPLC-ESI-MS/MS, UPLC-MS or HPLC-SPE-NMR have been reported recently [97, 98].

3 Chemistry of Camptothecin

Despite considerable activity and a unique mechanism of action, CPT exhibits several undesired properties which hampered its use in therapy. Firstly, CPT is very low water soluble complicating administration of the drug. Another problem associated with parent CPT is the α-hydroxylactone ring (ring E) opening under physiological condition, leading to the carboxylate open form **2** of CPT. The carboxylic acid **2** or its sodium salt **3**, although soluble, has much lower anticancer potency as compared with CPT. Moreover, this ionic form preferentially binds to the human serum albumin (HSA), lowering the concentration of the accessible drug. High toxicity of CPT and its sodium salt (which resulted in suspension of its trials in the 1970s) adds to the unfavorable profile of CPT [4, 7]. All of these drawbacks were addressed in an intense research activity for the last 30 years. This led eventually to deeper understanding of the structure–activity relationship (SAR), resulting in a development of two very active and soluble, semisynthetic anticancer drugs – topotecan (**4**) and irinotecan (**5**) – as well as several other being currently investigated (**6**–**15**). In line with SAR studies, chemistry of CPT, especially its transformation into related drugs **6**–**15** and the total synthesis, received much attention. These two aspects are briefly described here with the emphasis on the recent progress in the field. For more detailed view, see the reviews [56, 58, 59].

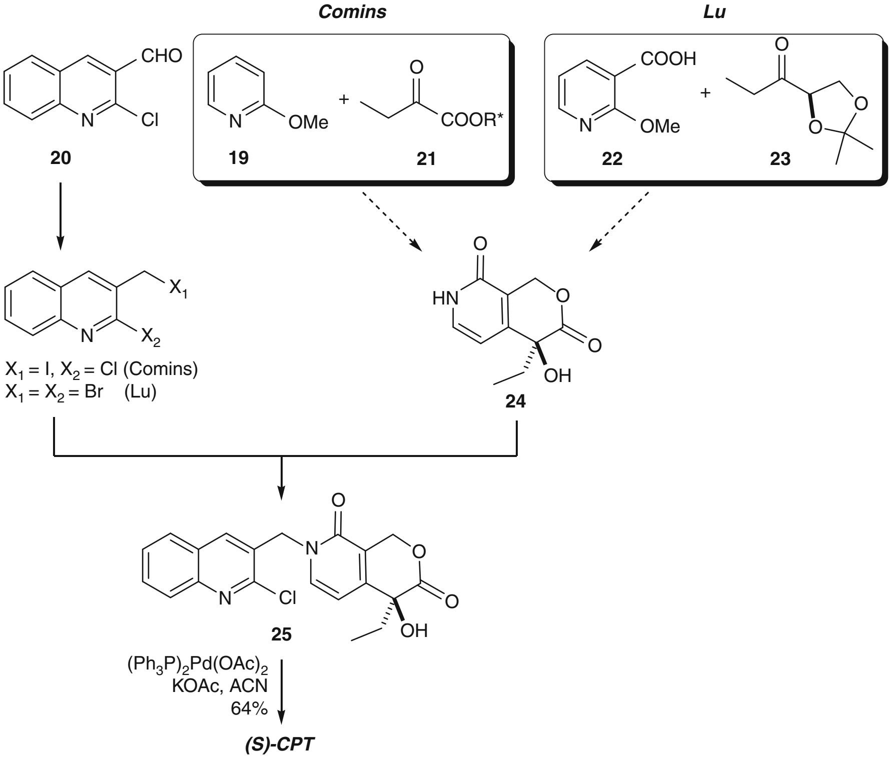
3.1 Total Synthesis of CPT and Semisynthesis of CPT-Related Drugs

Although isolation from plants still remains the major source of camptothecin, limited natural supply and environmental issues led to an enormous progress in its total synthesis since 1971 when the first syntheses of racemic CPT had been reported by Stork and Schultz [99] and Danishevsky [100]. Currently, this alkaloid as well as its derivatives can be efficiently synthesized in a highly stereoselective fashion from simple starting materials in the synthetic sequences typically not exceeding ten steps [58]. Generally, the most popular synthetic strategies are based on C-ring construction, providing CPT from various AB- and DE-ring precursors. The ring closure step usually involves the most broadly applied Friedlander condensation, cascade radical cyclization, Michael addition, or Diels–Alder cycloaddition, although palladium-catalyzed Heck-type reactions become recently very popular [101, 103].

Comins and Nolan adopted their six-step synthesis of racemic CPT [102] to the asymmetric synthesis of **1**, starting from the two commercially available heterocycles, namely, 2-methoxypyridine (**19**) and 2-chloroquinoline-3-carbaldehyde (**20**). The chirality is introduced in the addition of chiral ketoester **21** (esterified by commercially available *trans*-2-(α-cumyl)cyclohexanol which served as a chiral auxillary) to the lithiated pyridine **19**. Further one pot elaboration of the addition product gave key lactone **24** (DE-ring fragment) in 60% yield and 93% ee after recrystallization. Palladium-catalyzed (Heck reaction) intramolecular C-ring formation in **25** has been employed to complete the synthesis (Scheme 22.1). The yield of CPT starting from 2-methoxypyridine was ca. 12% [101]. Similarly, convenient asymmetric synthesis of CPT and SN-38 (**11**, 7-ethyl-10-hydroxy-CPT – active form of irinotecan metabolic activation in vivo) has been recently reported by Luo and Lu. In this case, however, nicotinic acid **22** and chiral glyceraldehyde (**23**) have been used as building blocks for the preparation of DE-ring synthon **24**. Again, Heck reaction has been used for the final C-ring closure of **25**. This synthesis furnished CPT in 14% yield (over 11 steps) and very high 99.9% ee (Scheme 22.1). Non-chromatographic purification of all intermediates is an additional advantage of this procedure [103].

More practical, catalytic asymmetric synthesis of key chiral DE-ring lactone **24** has been reported by Fang and coworkers from Glaxo by applying Sharpless asymmetric dihydroxylation reaction [104]. Other examples of catalytic enantioselective syntheses of CPTs are reviewed by Du in [58], whereas older approaches to racemic CPT are the subject of review by Hutchinson [52].

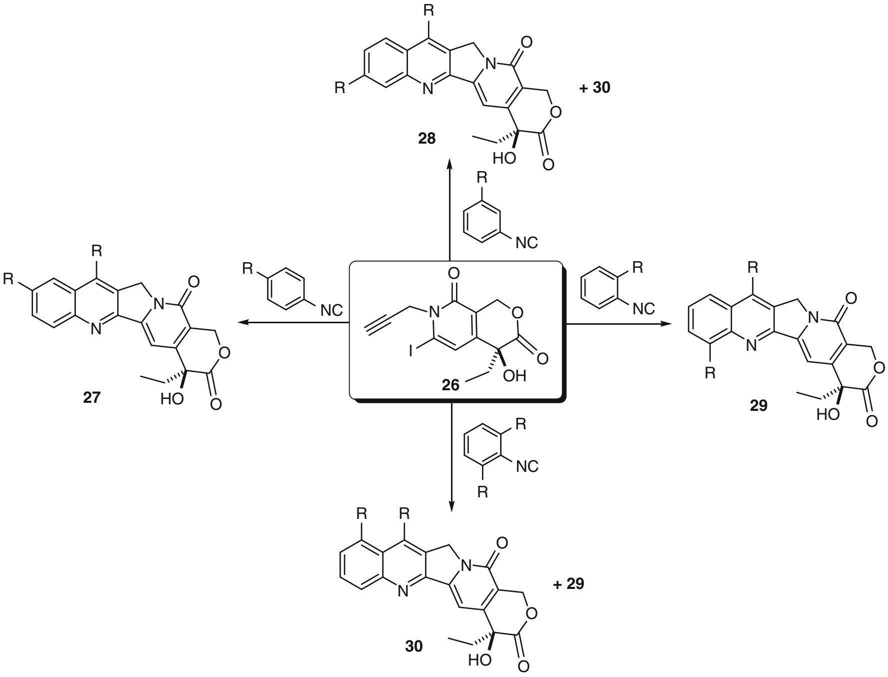
One of the most robust and versatile strategies for the synthesis of CPT and related alkaloids as well as their unnatural analogues has been developed by Curran et al. [105–107]. The synthesis is based on a cascade radical cyclization leading to the C and B rings through 4 + 1 radical annulation reaction of isonitriles (precursors of A ring) with D- or DE-ring precursors (preferentially *N*-propargyl-iodopyridone **26**, Scheme 22.2). This cascade radical annulation



Scheme 22.1 Shortest approach to asymmetric total synthesis of camptothecin by Comins and Lu

approach was applied initially to the synthesis of racemic CPT, followed by asymmetric total synthesis of CPT, (*S*)-mappicine **17**, and mappicine ketone **18** [106]. Furthermore, its generality has been demonstrated in the synthesis of a number of CPT analogues **27–30** difficult to synthesize by other method [107]. This strategy has also been extended by Curran to the parallel, combinatorial synthesis of 115-membered library of homosilatecans [108] and 560-membered library of mappicine ketone analogues [109]. One of homosilatecan library member – DB-67 (**10**) – has recently entered Phase I clinical trial for cancer chemotherapy [24].

Although total asymmetric syntheses of topotecan **4** and irinotecan **5** as well as homocamptothecin **14** have been reported [58], these drugs can be preferentially prepared by short semisynthetic routes starting from natural CPT. For example, SmithKline Beecham developed a synthesis of topotecan **4** from 10-hydroxy-CPT **32** [110]. This intermediate is obtained with 91% yield in two-step reduction–oxidation process involving an initial Pt-catalyzed partial hydrogenation to tetrahydroquinoline intermediate **31** and its subsequent oxidation with the use of PhI(OAc)$_2$ [110]. Condensation of 10-hydroxy-CPT with formaldehyde and



28

27

26

29

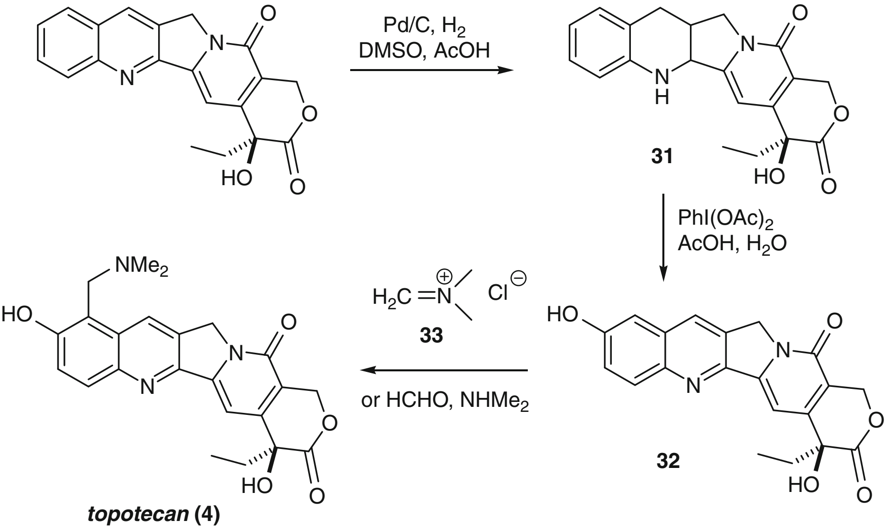
30

Scheme 22.2 Curran's modular strategy of CPTs synthesis

dimethylamine or more efficiently with *N,N*-dimetylmethyleneiminium chloride **33** (minimal formation of undesired 10-hydroxymethyl by-product) [111] gave topotecan **4** with good yield (Scheme 22.3).

Semisynthetic route to irinotecan **5** was developed by Sawada group in 1991 [112, 113]. The key step of this route is based on the addition of ethyl radical (generated in situ by the reaction of propanal, $FeSO_4$, and H_2O_2 in aqueous acidic medium) selectively in the 7-position of CPT. This represents a Minisci-type reaction [114] involving carbon radical addition to electron-deficient heteroaromatics such as pyridine and quinoline, which is especially useful in modification of position 7 in CPT. Subsequent oxidation of 7-ethyl-CPT **34** to the corresponding *N*-oxide **35** followed by photochemical hydroxylation led to 7-ethyl-10-hydroxyCPT **11** (SN-38) with overall yield 49%. Acylation of **11** with [1,4′] bipiperidinyl-1′-carbonyl chloride in pyridine or by 1,4′-bipiperidine/$COCl_2$ gave irinotecan **5** with 80% yield (Scheme 22.4) [112, 113, 115].

Other active derivatives of CPT such as silatecan (**10**), rubitecan (**9**), gimatecan (**7**), belotecan (**6**), and 9-aminocamptothecin (**36**) were also synthesized from CPT with the aid of rather simple chemistry which is summarized in Scheme 22.5. In the case of 7-position modification Minisci type reaction is typically applied with subsequent functional group modification such as in the synthesis of belotecan **6** or gimatecan **7** [116, 117]. A unique addition a silyl radical



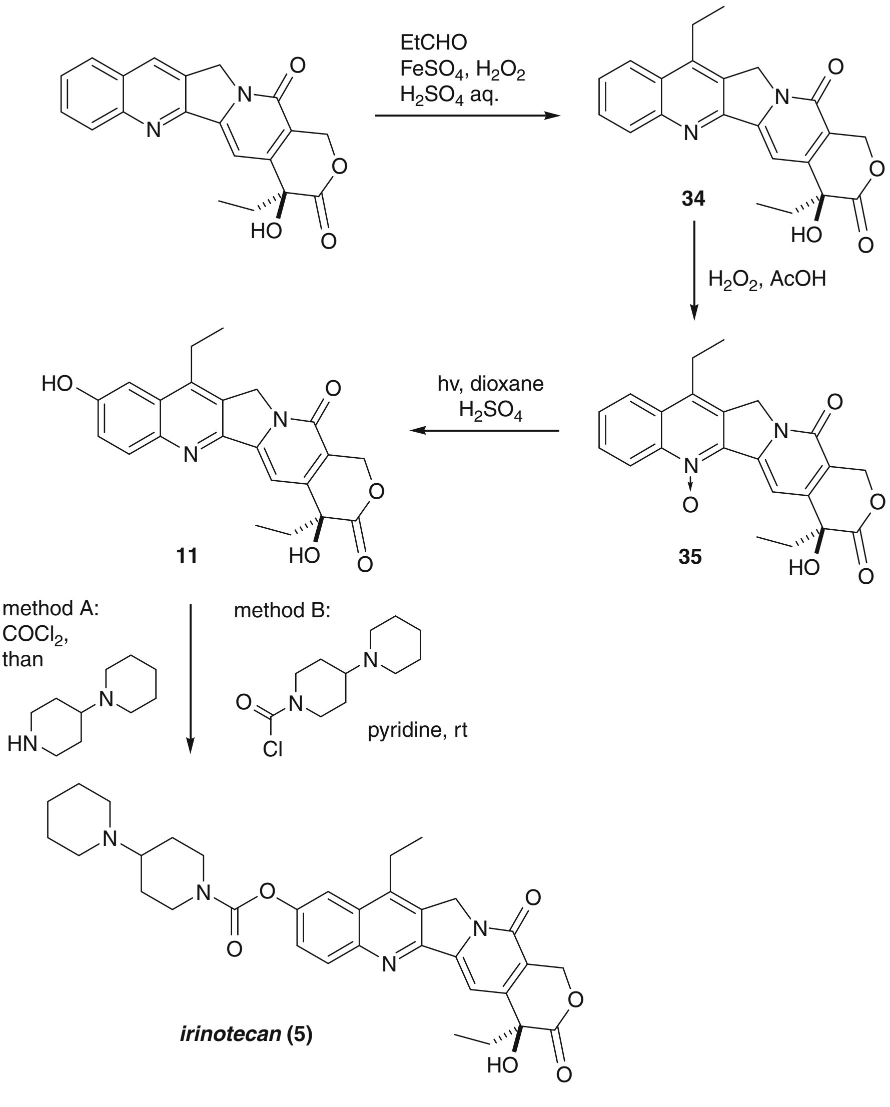
Scheme 22.3 Semisynthesis of topotecan

to heteroaromatic ring of CPT served as a direct but rather low-yielding route to silatecan (**10**, yield 20%, in addition to 50–60% unreacted CPT) [118]. Rubitecan **9** and the corresponding 10-amino-CPT **37** as well as 10-hydroxy-9-nitro-CPT were prepared by nitration and reduction of the parent alkaloid or 10-hydroxy-CPT [119–123]. Semisynthetic approach to homocamptothecin **14** has also been reported as well as its total asymmetric synthesis [58, 124].

3.2 SAR Studies

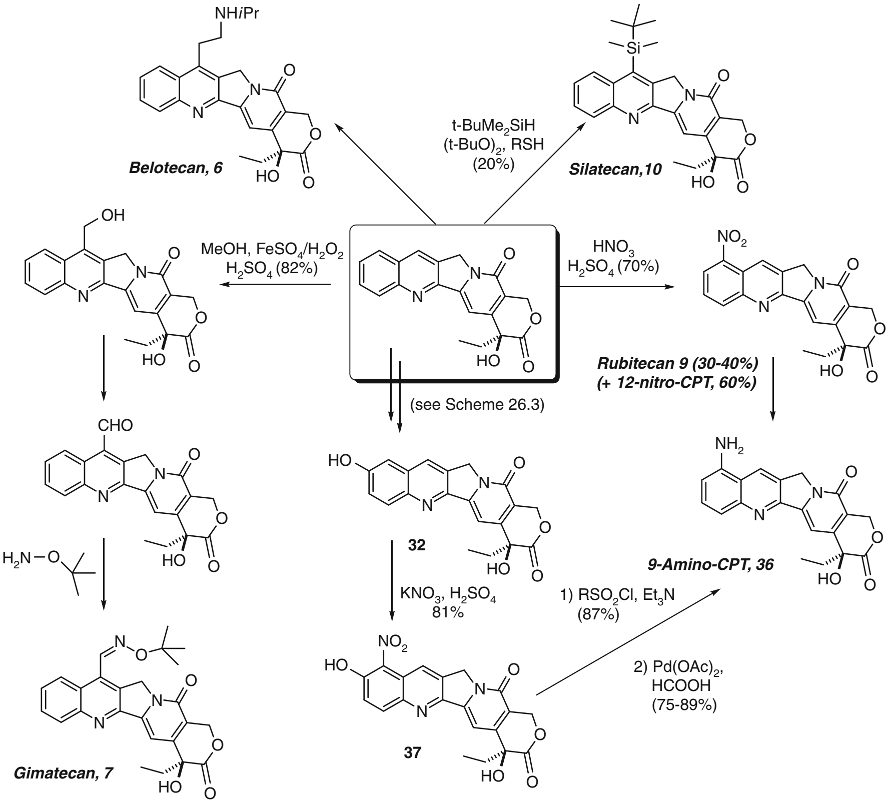
Low solubility of camptothecin as well as instability of the hydroxylactone ring (which undergoes opening under physiological condition to the hydroxyacid **2**) [125, 126] was the main motivation for undertaking an extensive research focusing on the development of more soluble, stable and more active CPT derivatives. Large number of CPT derivatives and biological screening data allowed to identify the detailed SAR map of CPT (Fig. 22.5). This eventually led to the development of a number of clinically useful derivatives **4**–**15** mentioned above.

Generally, modification of A and B rings (quinoline moiety) at the C-7, C-9, and C-10 enhances anticancer activity. Depending of the character of substituents, water-soluble derivatives can be obtained by introduction of polar groups such as in topotecan **4**, irinotecan **5**, or lurtotecan **13**. On the other hand, the introduction of highly nonpolar group at C-7 such as alkylsilyl (e.g., silatecan **10**) resulted in retaining the desired biological activity and enhanced stability toward hydrolysis. These lipophilic derivatives can be preferentially incorporated into lipid bilayer vesicles, offering an additional way of administration. Substituents at C-11 and


EtCHO
$FeSO_4$, H_2O_2
H_2SO_4 aq.
34
H_2O_2, AcOH
hv, dioxane
H_2SO_4
35
11
method A:
$COCl_2$,
than
method B:
pyridine, rt
irinotecan (5)

Scheme 22.4 Semisynthesis of irinotecan

C-12 are only rarely well tolerated [119, 127–130]. For example, seminal work of Wani et al. reported that among many modifications at these positions, only 11-fluoro and 11-cyano derivatives retained considerable activity. Interestingly, introduction of 10,11-(methylenedioxy) or 10,11-(ethylenedioxy) moiety gave derivatives displaying much higher activity compared to the parent CPT. In contrast, installation of two separate methoxy groups at C-10 and C-11 led to lowering of the activity. Similarly, linking positions 7 and 9 led to new hexacyclic ring which


NH*i*Pr
Belotecan, 6
t-BuMe$_2$SiH
(t-BuO)$_2$, RSH
(20%)
Silatecan,10
OH
MeOH, FeSO$_4$/H$_2$O$_2$
H$_2$SO$_4$ (82%)
HNO$_3$
H$_2$SO$_4$ (70%)
NO$_2$
Rubitecan 9 (30-40%)
(+ 12-nitro-CPT, 60%)
(see Scheme 26.3)
CHO
HO
32
NH$_2$
9-Amino-CPT, 36
H$_2$N−O
KNO$_3$, H$_2$SO$_4$
81%
1) RSO$_2$Cl, Et$_3$N
(87%)
2) Pd(OAc)$_2$,
HCOOH
(75-89%)
37
Gimatecan, 7

Scheme 22.5 Camptothecin transformations toward other bioactive CPTs

in combination with small substituents at C-10 and C-11 (such as in exatecan **12** with Me and F at C-10 and C-11, respectively) resulted in superior antitumor activity exceeding the potency of topotecan **4** in some cancer types. Very favorable modification of CPT involves simultaneous introduction of two different groups in the positions C-7 or C-9 and C-10, such as in topotecan **4** or irinotecan **5** [131].

Biochemical studies provided an evidence that stabilization of topoisomerase I–DNA binary complex by CPT derivatives depends on the size of substituent at C-10 in CPT analogue. Small group shows little effect, whereas derivatives with larger group cannot efficiently stabilize the binary complex [130]. This observation contrasted with high activity of irinotecan having a large 4-(1-piperidyl)-1-piperidincarbamate group at C-10. Further studies led to the discovery that irinotecan serves only as a prodrug of 7-ethyl-10-hydroxy-CPT which is the actual active form of the drug [132].

Modifications of C and D ring of camptothecin by introduction of substituents in position C-5 (C ring) or C-14 (D ring) are limited due to difficult synthesis of these analogues. Nevertheless, existing studies clearly show the lack of useful activity of

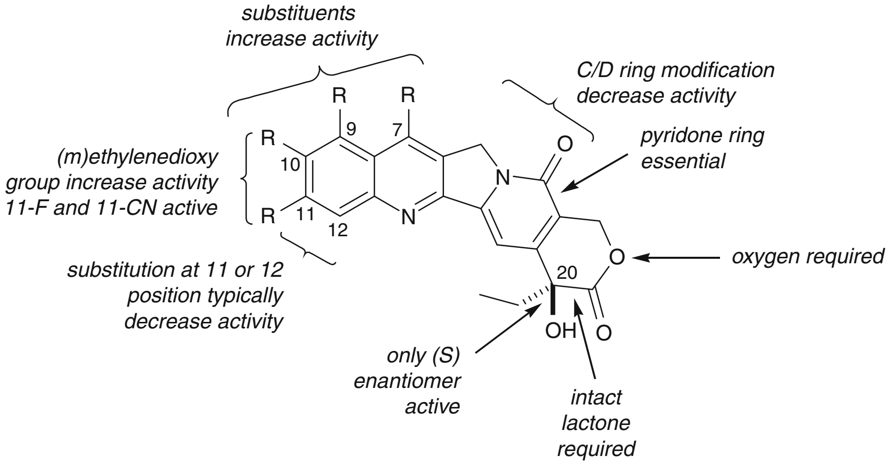

substituents
increase activity
C/D ring modification
decrease activity
pyridone ring
essential
(m)ethylenedioxy
group increase activity
11-F and 11-CN active
oxygen required
substitution at 11 or 12
position typically
decrease activity
only (S)
enantiomer
active
intact
lactone
required
R
R
R
R
9
7
10
11
12
20
N
N
O
O
OH
O

Fig. 22.5 *Structure–activity relationship (SAR)* of camptothecin

such modified derivatives [133–136]. Some exceptions were reported when modification at C-5 was combined with introduction of hydroxy or nitro group in A ring [137]. There are only two reports demonstrating equal to CPT potency of 5-ethylidene-CPT **38** against P388 cells [138] and good in vitro activity of 2-fluoroethyl esters of CPT **39** [139]. Similarly, homologated C ring as well as C-nor-4,6-CPT (lack of C ring) is inactive, presumably due to the loss of planarity of such compounds [140, 141]. 4-Deaza analogue of CPT was found to be approximately 60-fold less efficient as topoisomerase I inhibitor [142]. Contrary, reasonable activity exhibited 14-azacamptothecin **40** [143].

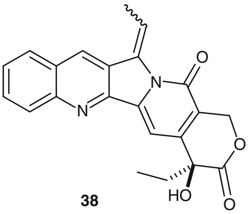
The α-hydroxylactone ring E was for a long time considered as an essential part responsible for the activity of CPT while being concurrently an instable part of the molecule easily, undergoing of hydrolytic lactone ring opening [125, 126]. α-Hydroxyacid **2** as a product of this equilibration occurring under physiological condition retains about 10% of CPT activity. For this reason most of the performed studies were undertaken to identify the factors increasing the stability of hydroxylactone ring or providing its stable and active equivalents. Replacing the lactone with more hydrolytically stable lactam, thiolactam, carbinol lactam or imide functionality has been reported by Hertzberg. Unfortunately none of these derivatives showed any activity as topoisomerase I inhibitor [142, 144].

Much more successful approach based on homologation of E ring by replacing α-hydroxylactone with seven-membered *β*-hydroxylactone was first reported by Lavergne [145, 146]. So-called homocamptothecin **14** displayed high activity and enhanced stability toward ring opening, served as a lead compound for designing of series of promising drug candidates [31–34]. Among them 10,11-difluorohomocamptothecin (**15**, diflomotecan) entered Phase I clinical trials [34]. Lipophilic 7-*tert*-butyldimethylsilylhomocamptothecin (analogue of DB-67) developed by Curran exhibited high stability both in serum and in a buffer [118, 147].

Derivatization of 20-hydroxy group increases stability of the lactone ring in CPTs by elimination of intramolecular hydrogen bonding between the hydroxy and the lactone carbonyl groups [148] as well as by increasing of sterical hindrance of the carbonyl group. A number of CPT esters have been prepared by using structurally diverse acids. They show increasing stability of the lactone ring as expected and in some cases higher than CPT activity [149–152]. SAR studies of alkyl esters of CPT have also been published [153].

The *S* absolute configuration at C-20 is another prerequisite for keeping the bioactivity of all camptothecins, *R*-enantiomer is inactive and racemic CPT exhibits much lower potency [127]. Replacement of the 20-hydroxy group, with hydrogen, amino group or halogen atom resulted in significantly weaker activity although some inhibition of topoisomerase I in vitro has been observed for such derivatives [142, 144, 154, 155].

It is worth to note that there are some examples of CPT analogues with non-lactone E ring possessing appreciable cytotoxicity. Hertzberg prepared two CPT analogues **41**–**42** which exhibited remarkable cytotoxicity but were not inhibitors of topoisomerase I, suggesting an alternative mode of biological action in this case [144]. Contrary, luotonin A **43** is a structurally related alkaloid isolated from the Chinese herbal medicinal plant *Peganum nigellastrum*, and despite absence of lactone ring E, either exhibits cytotoxicity against the murine leukemia P-388 cell line and stabilizes the human DNA topoisomerase I–DNA covalent binary complex by way similar to the CPT [156]. Active non-lactone analogues of CPT were also obtained by Lavielle et al. [157].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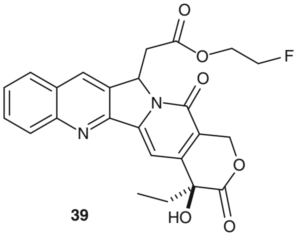
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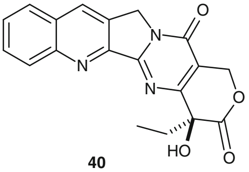
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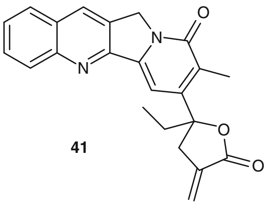
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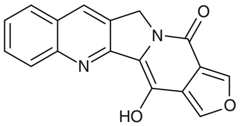
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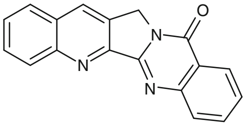

43 (luotonin A)

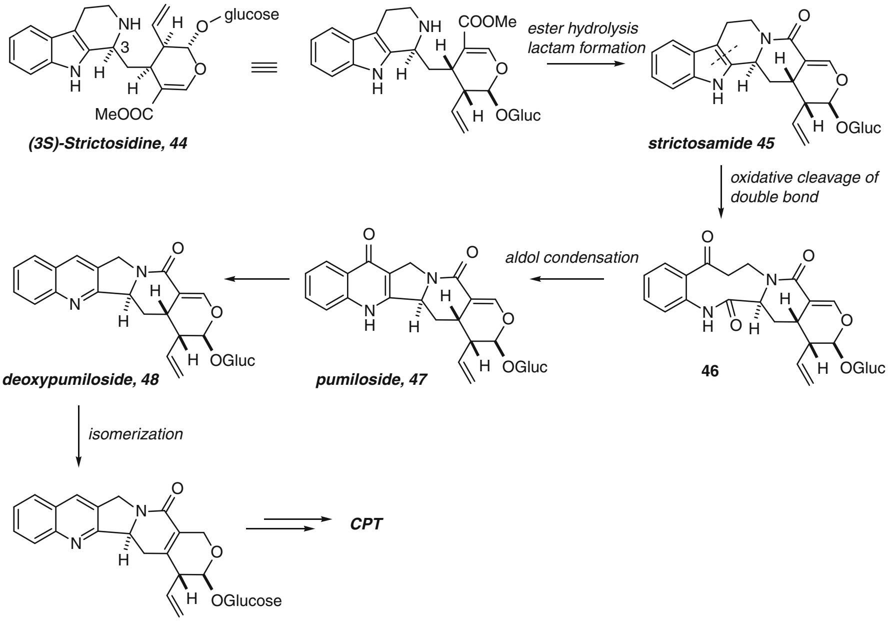
Detailed discussion of SAR of camptothecin is a subject of reviews [153, 158–160]. Some computational approaches to quantitative structure–antitumor activity relationship (QSAR) have also been reported [161].

An important area of medicinal chemistry of CPT is design of its prodrugs and conjugates for enhancing solubility, lowering toxicity, and improvement of pharmacokinetics and bioavailability. Specific examples of such drugs and their characteristics are given in biological activity section. The conjugation of CPT is typically completed by installation (typically esterification) of a dedicated linker undergoing enzymatic hydrolysis. Preferential site for the covalent attachment of CPT is 20-hydroxyl group (this also providing an additional stability of lactone ring of conjugate). Less common site of conjugation involves the use of polar substituents present in the quinoline ring of CPT and its derivatives.

4 Biosynthesis of Camptothecin

All monoterpenoid indole alkaloids are synthesized from strictosidine **44**, which is a general biosynthetic intermediate formed by enzymatic condensation of tryptamine with secologanin [162]. Recently, cDNA coding strictosidine synthase (E.C. 4.3.3.2) was isolated from *O. pumila* and expressed further in *E. coli*. The enzyme has been characterized regarding its substrate specificity toward a number of tryptamines and activity regulation [163]. In another work distribution of strictosidine activity in *O. pumila*, tissues were shown to correlate with the pattern of respective mRNA expression [164].

Strictosidine **44** is a precursor of strictosamide **45** which has been proposed as a key precursor in biosynthesis of CPT in *C. acuminata* on the basis of experiments with the ^{14}C and ^{3}H-labeled substrates carried out in the late 1970s [165] (for details of the labeling experiments, see also a review [52]). Strictosamide is a product of intramolecular cyclization of strictosidine. It is not clear whether this is an enzymatic process (no specific enzyme was identified so far) since strictosamide can be formed easily nonenzymatically under basic conditions. For example, complete conversion of strictosidine into strictosamide has been observed at pH 11 within 10 min at room temperature [164]. Unfortunately, further steps of CPT biosynthesis remained speculative because no specific enzymes and all intermediates were isolated. Two of the key postulated metabolites of the final stage of CPT biosynthesis were not isolated until 1989 when Haginiwa found pumiloside **47** and deoxypumiloside **48** in *Ophiorrhiza pumila* [166]. The presence of pumiloside **47** in *Camptotheca acuminata* was also confirmed by Westley one year later using both X-ray and NMR techniques [167]. Another problem was associated with absolute configuration of deoxypumiloside **48**; the expected (3*S*)-enantiomer (the same configuration at C-3 as its precursors strictosidine and strictosamide) has not been found; instead (3*R*)-epimer of **48** has been identified in regenerated *O. pumila* callus culture [168].


glucose
3
MeOOC
(3S)-Strictosidine, 44
COOMe
OGluc
ester hydrolysis
lactam formation
strictosamide 45
oxidative cleavage of
double bond
46
aldol condensation
pumiloside, 47
deoxypumiloside, 48
isomerization
OGlucose
CPT

Scheme 22.6 Biosynthesis of camptothecin

The plausible biosynthesis of camptothecin is outlined in Scheme 22.6. Strictosamide **45** may undergo oxidative cleavage of double bond giving diketo derivative **46** which provides pumiloside **47** after intramolecular aldol condensation (similar reaction sequence executed by chemical transformation was reported by Hutchinson [169]). Reduction of quinolone part in **47** afforded **48**, which forms CPT after double-bond rearrangement and hydrolytic cleavage of glucose.

This biosynthetic proposal became an indirect evidence by Brown et al. who demonstrated a biogenetically patterned synthesis of CPT and 20-deoxycamptothecin starting from strictosidine/vincoside. The complete route from **44** to racemic CPT has been completed by chemical transformation involving all of the postulated intermediates in the biosynthesis scheme [170]. Other biomimetic synthesis of CPT has also been reported [171].

Interesting studies on the early stages of CPT biosynthesis using in silico modeling (Atomic Reconstruction of Metabolism software) and in vivo tracer experiments with [1-^{13}C]glucose were conducted by Saito et al. The results have clearly shown that the monoterpene secologanin part is biosynthesized via 2C-methyl-D-erythritol-4-phosphate pathway but not via mevalonate route as postulated earlier. The quinoline part produced from tryptophan was suggested to be of shikimate origin [172].

The chemotaxonomical analysis of the distribution of CPT and their metabolites among plant genus suggests that the genes encoding the enzymes involved in CPT biosynthesis evolved early during evolution. The pool of the respective genes was probably not lost during evolution but might have not been expressed during a certain period of time and became active again at some later point. An interesting issue of self-resistance mechanism of CPT-producing plant was addressed by Saito. On the basis of observation that recombinant topoisomerase I from *O. pumila* expressed in yeast was resistant to CPT, he hypothesized that these plants may possess camptothecin-resistant topoisomerase I. In fact, aminoacid sequence analysis of *O. pumila* topoisomerase I revealed that a highly conserved residue adjacent to the catalytic part has been mutated [163, 173]. Similar mutations have been observed in all other CPT-producing plants as well as in CPT-resistant leukemia cancer in human [173]. Coevolution of CPT biosynthesis and self-resistance to CPT in plants were recently reviewed [174].

5 Biological Activity of Camptothecins

The most prominent activity of CPT and its derivatives is the inhibition of topoisomerase I which is a molecular basis of their anticancer properties. This specific mode of action of CPT has been proposed in the 1980s [5, 6, 175, 176], leading to reinvestigation of these alkaloids in medicinal and biological chemistry. DNA topoisomerases are essential enzymes present in all nucleated cells responsible for management of topology of DNA strands during replication, RNA transcription, recombination, repair, as well as chromatin assembly and remodeling [4, 177].

Topoisomerases were roughly classified as type I or type II depending of the type of DNA breakage mechanism. Type I breaks only one strand of duplex DNA giving 3′-phosphotyrosine intermediate **49** (so-called cleavable complex) in contrast to topoisomerases of type II which are able to break both strands of duplex DNA with the formation of a pair of 5′-phosphotyrosine covalent intermediates. In human topoisomerase I hydroxy group of Tyr^{723} residue is used for covalent modification of DNA. More specifically, the overall mechanism of enzymatic action involves four distinct steps: (1) DNA topoisomerase I binding; (2) breaking the single strand of DNA by reversible transesterification resulted in replacement of 5′-oxygen of phosphodiester bond for oxygen of enzyme Tyr^{723} (binary complex **49** formation); (3) DNA relaxation by single-strand passage around the unbroken strand; and (4) religation of cleaved DNA and concomitant release of the enzyme. The characteristics of human topoisomerase I and the details concerning its mechanism of action are given in a review [4, 177]. CPT non-covalently stabilizes the cleavable complex of DNA topoisomerase I, preventing the subsequent religation step (in this context the enzyme itself is a DNA-damaging agent). Stabilization of the cleavable complex **50** is not yet sufficient for the promotion of cell death mainly due to the complex reversibility. The lethal effect is a consequence of interaction between moving replication fork (or transcription process) and the CPT-stabilized cleavable complex **50**. This results in the formation of a double-strand break located at the fork and irreversible arrest of DNA replication in the S/G2 phase of cell cycle, leading to the apoptosis [178] (so-called fork-collision model). It is worth to note that cells in the S-phase division are up to 1,000-fold more sensitive to topoisomerase I inhibitors than in G1 or G2/M phases, so they are considered as S-phase-specific cytotoxic agents [179].

Interestingly, CPT does not bind to topoisomerase I and only weakly to B-DNA under physiological conditions [5], so both biomolecules and CPT are needed to form stable ternary complex **50**. Additional evidence supporting such mechanism comes from the observation of resistance of cell lines toward CPT which have specific mutations of enzyme [180–182] (for medical consequences of CPT resistance, see [4, 183]). Similarly, deletion of gene coding topoisomerase I from yeast (*Saccharomyces cerevisiae*) resulted in full resistance of viable cells to CPT, with restoration of sensitivity to CPT after expression of human or yeast topoisomerase I [184, 185]. The schematic mechanism of CPT action is given in Fig. 22.6.

Detailed mechanism of CPT stabilization of DNA–topoisomerase I complex is not perfectly understood and was a subject of three different proposals by Pommier [186, 187], Hol [188–190], and Kerrigan and Pilch [191]. Pommier suggested the intercalation of CPT in the binary complex in a manner capable of maximizing both stacking interaction and hydrogen bond formation to specific amino acids and DNA. Hol postulated stacking arrangement of CPT and +1 purine which flips out the DNA helix. Kerrigan and Pilch proposed a model of intercalation in which lactone E ring of CPT is located within minor groove interacting additionally via hydrogen bonding with binary complex. All of these proposals are summarized in a review [55–57].

Currently, the structure of the ternary complex received stronger experimental evidence by Staker et al. who reported X-ray crystal structure of human topoisomerase I covalently attached to duplex DNA and topotecan [192]. The drug intercalated into duplex DNA-enzyme complex and characteristic features are base-stacking interaction with both upstream and downstream base pairs and the use of only single Asp^{533} for hydrogen bonding with topotecan. Moreover, the quinoline part of the drug with C-7, C-9, and C-10 carbon atoms, located in the vicinity of the major groove of DNA, offers a space for accommodation of the substituents at these positions, which do not disrupt the activity of such modified CPT derivatives, in agreement with reported SAR studies. Interestingly, the report suggests also the possibility of intercalation of open carboxylate form of CPT in the pocket in the fashion similar to the lactone, although it was shown that carboxylate **2** does not bind to the binary complex **49** in solution [144].

Phase I clinical trial of CPT began in the 1970s, after demonstration of its high activity in preclinical studies. For this purpose, water-soluble sodium salt of CPT has been used intravenously. Although short-lasting remissions were observed in 5 out of 18 patients with primarily gastrointestinal cancers, further Phase I and Phase II trials in the USA did not confirm drug efficacy. In contrast, there was a single large trial in the People's Republic of China involving ca. 1,000 patients demonstrating a positive response (but caution is needed for its interpretation). Either unfavorable results and unpredictable and severe adverse effects such as significant hemorrhagic cystitis, life-threatening diarrhea, or myelosuppression

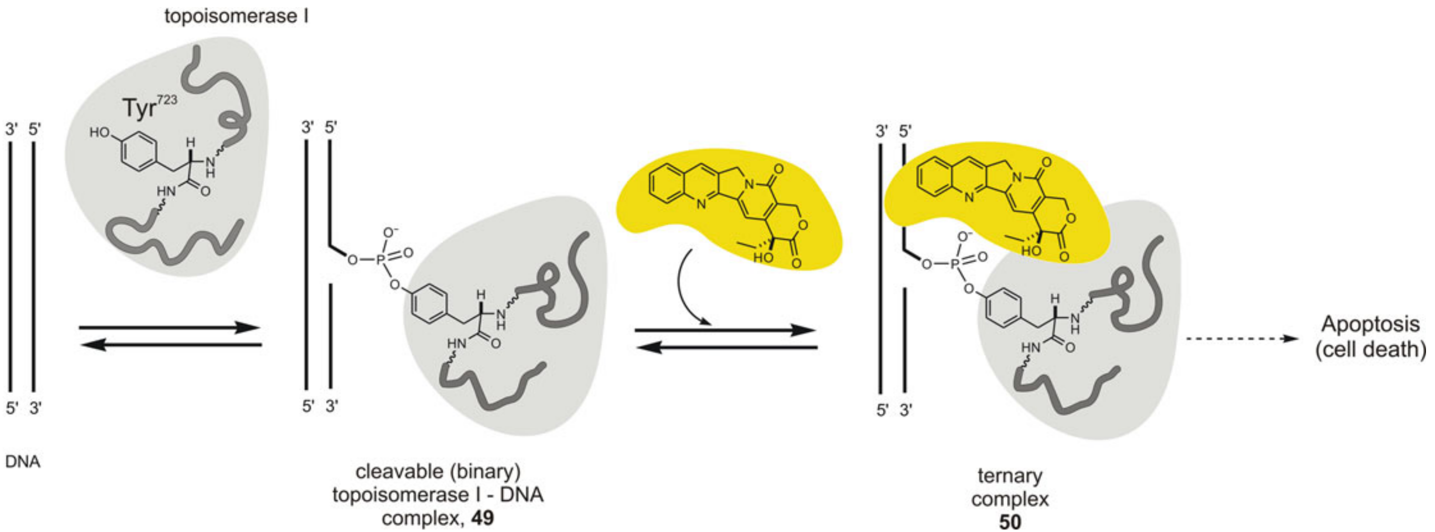

topoisomerase I
Tyr723
HO
H
N
H
HN
O
3' 5'
5' 3'
DNA
O⁻
O—P=O
O
N
O
HO
Apoptosis
(cell death)
cleavable (binary)
topoisomerase I - DNA
complex, 49
ternary
complex
50

Fig. 22.6 Mechanism of action of camptothecin (schematic representation)

(assumed to be the effect of poor solubility of drug) led to the discontinuation of trials [4, 193–195]. Almost 20 years later, water-soluble CPT derivatives topotecan and irinotecan were registered as anticancer drugs and approved for treatment of patients with cisplatin-refractory ovarian cancer and small-cell lung cancer (SCLC), after the failure of first-line chemotherapy (topotecan) and metastatic colorectal cancer (irinotecan) [4].

5.1 Irinotecan

Irinotecan (**5**) is a water-soluble derivative which is actually a prodrug of active 7-ethyl-10-hydroxycamptothecin (SN-38) formed by hydrolysis of dipiperidine carbamate residue at C-10. This active metabolite is ca. 100–1,000-fold more cytotoxic in vitro as compared with the irinotecan itself. Clinical use of irinotecan comprises typically the therapy of advanced or metastatic colorectal cancer [4, 7, 196–200] and gave in Phase II trial response rates of 10–35% independent of the applied schedule with median remission duration and median survival time 6–8 and 8–13 months, respectively. Further Phase III studies showed higher 1-year survival rate in patients refractory to previous treatment with 5-FU (36% vs. 14% of control group ($P < 0.01$)) [201] and similar survival advantage in patients with previously treated advanced colorectal cancer (1-year survival rates were 45% for irinotecan and 32% for 5-FU, respectively ($P < 0.05$)) [202]. Other Phase II trials with single-agent irinotecan encompassed small-cell lung, non-small-cell lung cancer (32–47% overall response rate), leukemia/lymphoma (17–38% response rate), as well as gastric, pancreatic, breast, and other cancer types (lowest efficacy) [203–206]. Apart from single use of irinotecan, there are a number of clinical studies showing beneficial effect of the use of irinotecan with combination of another anticancer drugs [207]. For example, first-line treatment of metastatic colorectal cancer by the combination of irinotecan, 5-fluorouracil (5-FU), and leucovorin (folinic acid) yielded a significantly higher remission rate, longer progression-free survival, and median survival [198, 208–210]. Similarly effective was a combination of irinotecan with bevacizumab which in the first-line regimen resulted in 67% of the overall response rate and median and overall survival of 12.3 and 23.7 months (95% CI), respectively [211].

Especially favorable irinotecan and platin-based drug combination often shows synergistic potency toward many tumors as it was demonstrated in many Phase II and III studies [212–220]. For example, in first-line chemotherapy of extensive SCLC, the improvement in the 1-year survival rate was 60% in comparison with conventional treatment with etoposide and cisplatin (40%) [221]. Other Phase III or II trials evaluated combination of irinotecan with etoposide [222–224], cetuximab [209, 225–229], docetaxel [230–233], mitomycin-C [234–238], and paclitaxel [239–241]. This topic has been reviewed in more detail in reviews [4, 7, 242, 243].

Pharmacology of irinotecan is very complex [4, 205, 244] depending on many enzymatic and protein systems, individual genetic factors, and being complicated by the fact that this drug exists in an equilibrium of the active lactone and an inactive carboxylate forms. SN-38 is detoxified in the liver by conjugation with glucuronide catalyzed by polymorphic enzyme uridine-diphosphate glucuronosyltransferase 1A1 (UGT1A1). Individual genetic profile of UGT1A1 was shown to be responsible for the disposition, efficacy, and toxicity of the drug [245–249].

5.2 Topotecan

Topotecan (9-[(dimethylamino)methyl]-10-hydroxy-CPT, **4**) is the second approved semisynthetic, water-soluble CPT derivative. Similarly to irinotecan, topotecan undergoes equilibration at physiological pH, giving only small fraction of active lactone form and most of the introduced dose as inactive carboxylate [250, 251] (tartaric acid is used in new parenteral formulation to prevent the lactone opening [252]).

Topotecan is currently approved in many countries for the second-line treatment of metastatic ovarian cancer after failure of initial or subsequent chemotherapy as well as for the treatment of patients with chemotherapy-sensitive SCLC after failure of first-line chemotherapy [4, 7, 253–258]. Other clinical use of topotecan as single drug in various schedules of administration has been evaluated in therapy of cervical, uterine, and brain cancers among others [259–261]. An advantage of topotecan is the possibility of its oral administration, which in Phase III trials was equally efficient in the treatment of SCLC in comparison to intravenous drug dosage [262]. Topotecan was also used in a combination with many other anticancer drugs such as cisplatin [263], carboplatin [264], taxol [265], amrubicin [266], or cyclophosphamide [267]. Despite a number of works evaluating such regimens, only few reported clear therapeutic benefits [265–268]; typically higher toxicity and lack of major improvement were observed. Topotecan pharmacology and toxicity are discussed in reviews [4, 205].

5.3 Other CPT-Related Anticancer Drugs

Belotecan ([7-(2-isopropylamino)ethyl]-CPT, Camtobell®, **6**) is a semisynthetic 7-substituted CPT approved in South Korea. Phase II clinical trials of single-agent belotecan against SCLC showed its remarkable efficacy (OR 24–42%) [8, 9]. Even more favorable results were reported for the treatment of SCLC and recurrent ovarian cancer by the combination of belotecan with cisplatin (OR 70%) and carboplatin, respectively (OR 57%) [10, 11].

The hexacyclic CPT analogue exatecan mesylate (DX-8951f, **12**) is a water-soluble, synthetic derivative with an additional six-membered ring with amino group and a methyl group and fluorine atom at C-10 and C-11, respectively. Exatecan showed superior and broader spectrum of antitumor activity in vitro and in vivo in comparison with other CPT analogues [269–271]. Disappointingly, clinical studies demonstrated only a modest activity against pancreatic, gastric, and metastatic breast cancers [272–275].

Gimatecan (ST1481, 7-*tert*-butoxyiminomethyl-CPT, **7**) is highly lipophilic rationally designed semisynthetic CPT derivative of potent antitumor activity (also against multidrug-resistant cancer), strong topoisomerase I inhibition, stable drug–target interactions, and favorable pharmacological profile [12–15, 276]. Very long biological half-life of 77 h and presence of only lactone form are additional advantages of this drug candidate. Gimatecan showed a high activity in large panel of human tumor xenografts model [12, 14] and entered currently to I and II Phase clinical trials against ovarian carcinoma and pediatric tumors [14, 15, 276].

Next two lipophilic drug candidates include silatecan (7-*tert*-butyldimethylsilyl-10-hydroxy-CPT, DB-67, **10**) [24, 25, 277] and karenitecin (7-[(2-trimethylsilyl)ethyl]-CPT, **8**) [16–19]. These newer generation camptothecin analogues contain trialkylsilyl group in position 7 (Fig. 22.3) and offer enhanced stability in blood and cell penetration, improved pharmacokinetics as well as increased lactone stability. Silatecan exhibited activity against the growth of in vivo-like histocultured human tumors and xenografted human tumors [25]. The results of clinical studies are not yet available. On the other hand, karenitecin was shown to be effective in Phase II trial for treatment of melanoma [16, 19], especially in combination with valproic acid [17] and in NSCLC [18].

Lurtotecan (GL147211C, 7-[(4-methylpiperazine)methyl]-10,11-(ethylenedioxy)-CPT, **13**) is another example of water-soluble, synthetic derivative of CPT bearing a dioxalane moiety between C-10 and C-11 [28–30, 278]. The antitumor activity of lurtotecan in Phase II trial was moderate in breast cancer patients and minimal in non-small-cell lung cancer patients, and no clear responses were obtained in colorectal patients [278]. Better results were observed with the use of liposomal lurtotecan formulation. Such prepared drug exhibited superior levels of activity and higher therapeutic index in preclinical models [279, 280]. Its clinical activity has also been confirmed in treatment of epithelial ovarian cancer [28].

Rubitecan (9-nitro-CPT, **9**) is a poorly water-soluble CPT analogue (formerly used for the preparation of 9-aminocamptothecin) which can be administrated orally, transdermally, or by inhalation with the use of liposomes as carriers [20–23]. It is not clear whether rubitecan acts itself as a drug or is a prodrug of 9-aminocamptothecin (which despite its considerable activity failed in clinical trials) [20]. Preclinical studies of rubitecan demonstrated its activity against broad spectrum of cancer types both in vitro and in vivo human tumor xenograft models. Unfortunately these favorable results were not usually observed in clinical trials [21].

Homocamptothecin (**14**) is an E-ring homologated camptothecin analogue (seven-membered E ring) that exhibited greater lactone stability in plasma compared with other topoisomerase I inhibitors and high activity in vitro studies [31]. Further SAR studies showed that even more potent are fluorinated derivatives of homocamptothecin [33], resulting a in development of diflomotecan

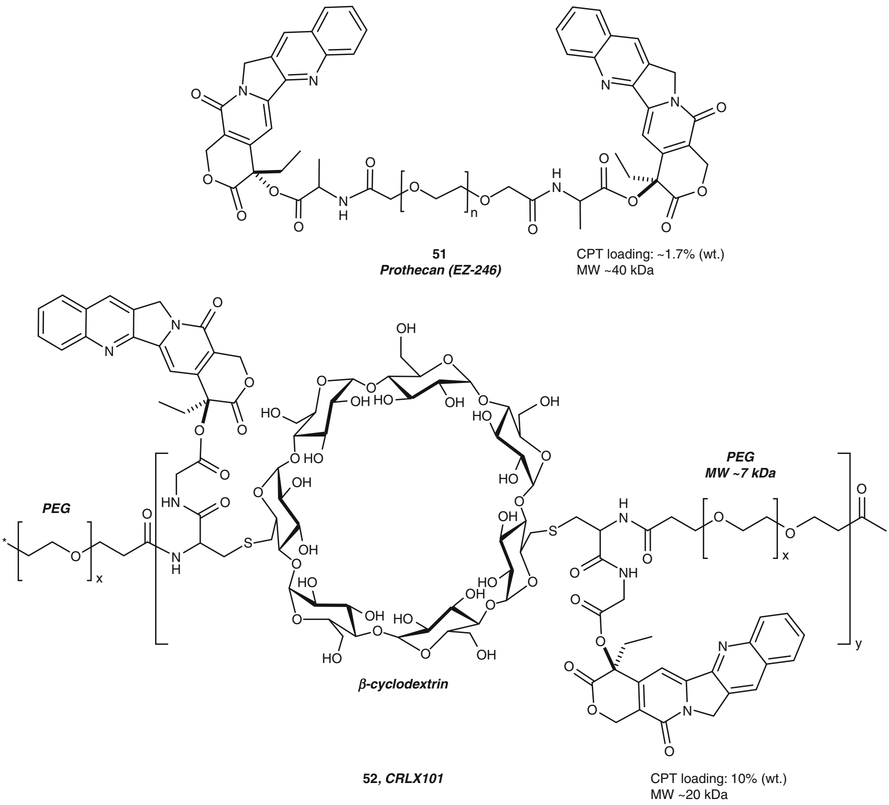
(10,11-difluoro-homocamptothecin, BN80915, **15**). **15** is a potent topoisomerase I inhibitor with enhanced plasma stability and superior preclinical antitumour activity as compared to irinotecan and topotecan [34, 281]. Diflomotecan is also the first homocamptothecin derivative which entered clinical studies. Phase I trial showed its better than other CPT-related drugs toxicity profile and highest oral bioavailability (ca. 70%) as compared with topotecan (30–44%) or lurtotecan (12–21%) [34, 281]. Reports from Phase II studies are not yet available. Several other homocamptothecin derivatives have also been prepared and screened as drug candidates [282–284].

Difficulties associated with CPTs administration, toxicity, and pharmacokinetics were a reason for the development of more favorable alternative forms of their delivery or prodrug systems [35–40]. Delivery of CPT and its derivatives was studied by using liposomes (see also lurtotecan). Examples of such lipid formulations of CPTs in clinical trials include CPX-1 (irinotecan-floxuridine liposome in Phase 1) from Celator Pharmaceuticals [285] and Brakiva® (Topotecan Optisome) liposomal formulation of topotecan from Hana Biosciences [286].

Micellar-drug formulation is another delivery system based on hydrophilic–hydrophobic block copolymers that self-assemble in aqueous solution into micellar particles with a hydrophobic core and a hydrophilic shell. NK012 is an example of micellar formulation of SN 38 (**11**) developed by Nippon Kayaku which is in Phase 1 clinical trial [287–289].

Newer delivery systems use the advantage of nanoscale and include CPT nanocrystals, nanoparticles, or nanobiohybrids. Most of these nano-sized formulations use CPT and were able to demonstrate their higher activity, cellular uptake, stability, and lower toxicity in vitro and in vivo tests as compared with CPT in solution [37–41]. For example, nanosuspension of CPT was compared with topotecan in vitro and in vivo cytostatic efficacy against MCF-7, HCT-8, and PC-3 cell lines using MTT assay and in antitumor activity in vivo against HCT-8 xenograft model. An about six times higher cytotoxicity against cell lines MCF-7 in vitro as compared with that of topotecan was reported, whereas similar level of antitumor potency was observed in vivo for both drugs. The lower toxicity of nano-sized CPT in comparison to that of topotecan was another advantage of this formulation [42]. Similarly, stable CPT nanocrystals were found to be more active in vitro toward MCF-7 cells as compared to CPT solution and exhibited significant suppression of tumor growth in MCF-7 xenografted BALB/c mice. Five times higher concentration of CPT at 24 h in the tumor was observed as compared with that obtained by using the drug salt solution [41].

Another important strategy used for the improvement of CPT delivery, distribution, stability, and lowering their toxicity is a construction of specific prodrugs which undergo a metabolitic activation, preferably at the place of requested action. Prodrugs of CPT are typically obtained by covalent modification of 20-hydroxy group, whereas semisynthetic CPTs are preferentially modified by using substituents located in AB (quinoline) moiety. Simplest types of CPT prodrugs were esters, amides, carbamates, and 20-*O*-carbonates of CPT [152], whereas newer attempts of their preparation involve rather the use of functionalized polymers as carriers,



51
Prothecan (EZ-246)

CPT loading: ~1.7% (wt.)
MW ~40 kDa

52*, CRLX101*

CPT loading: 10% (wt.)
MW ~20 kDa

Fig. 22.7 Selected prodrugs of camptothecin

typically poly(ethylene glycol) (PEG) or poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA) [148, 290, 291]. Simple PEG-CPT conjugates gave better level of tumor regression in HT-29 xenografts and decreased toxicity relative to the parent CPT [152]. Dimeric CPT conjugate linked by PEG (**51**, Prothecan, Pegamotecan, Fig. 22.7) entered in Phase II clinical trial [292]. Other examples of PEG conjugates of CPT are presented in reviews [55–57]. Semisynthetic CPTs have also been used for the construction of prodrugs. For example, PEG–irinotecan conjugate NKTR-102 is a prodrug developed by Nektar Therapeutics and evaluated currently in Phase II clinical trial against solid tumor malignancies, including colorectal, breast, ovarian, and cervical cancers [293]. Another example is PEGylated SN38 (EZN-2208) from Enzon Pharmaceuticals. This drug is in Phase II trial for patients with metastatic colon cancer [294].

Polymeric nanoparticles comprised of cyclodextrin-poly(ethylene glycol) copolymer with the covalently attached CPT at 10–12 wt% loading (CRLX101, **52**, Fig. 22.8) which self-assemble in solution into nanoparticles have been developed by Svenson et al. The unique composition of **52** resulted in an apparent solubility increase of >1,000-fold as compared to the parent CPT as well as gave

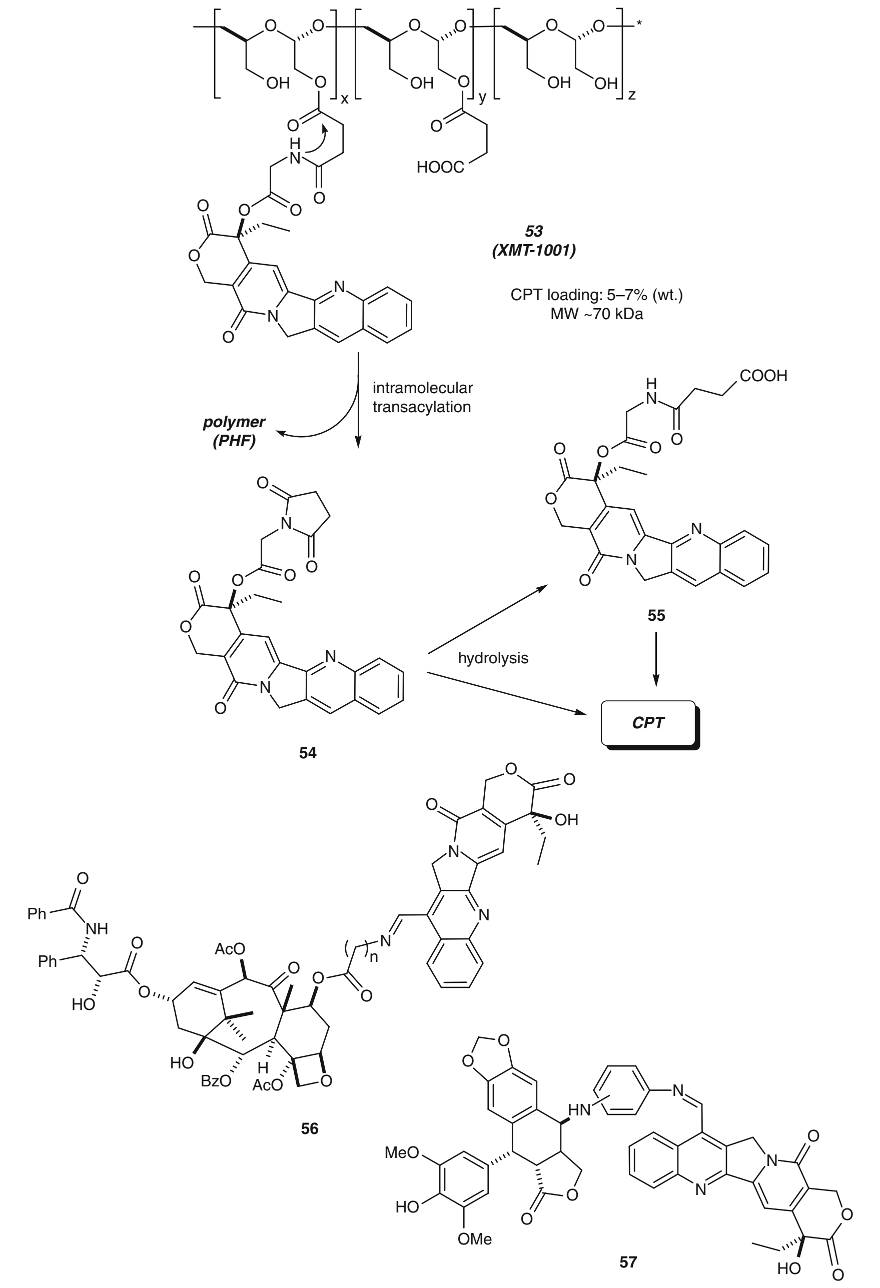

53
(XMT-1001)
CPT loading: 5–7% (wt.)
MW ~70 kDa
intramolecular
transacylation
polymer
(PHF)
54
55
hydrolysis
CPT
56
57

Fig. 22.8 Selected prodrugs and conjugates of camptothecin

superior preclinical pharmacokinetic results. CRLX101 was demonstrated to have significant antitumor activity superior to that of irinotecan across a broad range of xenograft models [45, 46] and is currently in Phase II clinical studies.

Another innovative product, XMT-1001 **53** from Mersana Therapeutics, is a CPT prodrug based on the carbohydrate-derived polyacetal matrix "Fleximer" (poly(1-hydroxymethylethylene hydroxymethylformal)). CPT is covalently linked to a hydrophilic and biodegradable polymer and releases CPT over an extended time period by dual-step activation comprising two major intermediates: CPT-20-*O*-(*N*-succinimidoglycinate) (**54**) and CPT-20-*O*-(*N*-succinamidoyl glycinate) (**55**). This mechanism was suggested to be responsible for broader therapeutic window of **53** compared with CPT and irinotecan in human tumor xenograft models and its lower toxicity. Currently, XMT-1001 is being evaluated in patients with advanced cancer in Phase 1 clinical trial [43, 44].

Conjugates of CPT with common anticancer drug paclitaxel were obtained by Lee. These compounds were found to be potent inhibitors of tumor cell replication with better activity relative to CPT. Specifically, selected conjugates such as **56** (Fig. 22.8) were more active against colon adenocarcinoma (HCT-8) cell replication as compared with simple mixture of paclitaxel and CPT. Interestingly, all of the conjugates were less potent than CPT as inhibitors of human topoisomerase I in vitro, suggesting their novel mechanism of action [295].

Chang et al. linked CPT and etoposide fragment (4b-amino-40-*O*-demethylepipodophyllotoxin). Rationale of this conjugation came from the observation that some CPT-resistant cell lines concurrently developed resistance toward etoposide which is topoisomerase II inhibitor. The bioconjugates **57** (Fig. 22.8) differing in a geometry of phenylenediamine linker were prepared, and their cytotoxic effects were reported to operate primarily through topoisomerase I inhibition. Interestingly, **57** was demonstrated to be more effective against human prostate cancer cells than either CPT or etoposide [296, 297]. Other examples of molecules conjugated with CPTs include 5-FU [298], somatostatin [299], bile acids [300], and glutathione [301].

Although the anticancer action of CPTs has received most attention both from academia and pharma community, wide biological screening of CPT and its derivatives resulted in finding several other potentially interesting activities. For example, antiviral efficacies of series CPT analogues on herpes simplex virus type 2 (HSV-2) in vitro were evaluated by Liu et al. [302]. It was found that several compounds exhibited high activity comparable or better than acyclovir (best compound $IC_{50} = 1.3\ \mu g\ mL^{-1}$). Preliminary biological tests suggested also that the intact E-lactone ring and 20-hydroxy group may not be a prerequisite for the antiviral activity [302]. Mappicine ketone **18** (Fig. 22.4) and its derivatives showed also antiviral action against herpes viruses (HSV) and human cytomegalovirus (HCMV) [303, 304].

In the 1990s inhibition of human immunodeficiency virus (HIV-1) replication in vitro by noncytotoxic doses of CPT has been demonstrated by Blair et al. [305]. On the other hand, Pardee et al. reported inhibition of Tat-mediated transactivation of HIV-1 by CPT [306]. These studies have not been continued until recently, when

Zheng et al. reported that 7-hydroxymethyl-CPT showed more potent anti-HIV activity compared with the parent CPT in vitro [307]. The anti-HIV activity has also been demonstrated for rubitecan [308].

CPTs exhibited also antitrypanosomal activity [309], and CPT itself was suggested to inhibit the biosynthesis of collagen [310].

Ecological role of CPT is not clear but indirect observations support the hypothesis that CPT may be a chemical defense system against insect pathogens [311, 312]. In fact, Liu observed that plantations of *C. acuminata* in the USA are relatively free of insect damages [311], and Cao et al. reported that leaves of the tree are toxic to goats [312]. Insecticidal activity of CPT and its derivatives received recently more attention [313–316]. Similarly, antifungal properties of CPT were studied in the context of the fungal diseases in *C. acuminata* that limit cultivation of the plants. Bioassays showed that pure CPT and flavonoids (trifolin and hyperoside) isolated from *Camptotheca* effectively control fungal pathogens of *Alternaria alternata*, *Epicoccum nigrum*, *Pestalotia guepinii*, *Drechslera* sp., and *Fusarium avenaceum* in vitro, although antifungal activity of these compounds in the plant is limited (full inhibition of mycelial growth was observed at 75–125 μg/mL CPT) [317].

6 Biotechnological Approaches

CPT bioproduction by plants has early been recognized as an attractive alternative to its isolation or total synthesis. In this context, since the 1970s CPT biosynthesis, its regulation and development of plant cell cultivation techniques were studied by several research groups. Unfortunately, most of initial efforts toward CPT bioproduction resulted in low alkaloid productivity, giving at best few milligrams for 100 g of dry weight of cultivated plant material or 1 L of liquid medium [318–320]. Further studies on optimization of the bioproduction of CPT involved the use of plant growth regulators and elicitors [321–324], metal ions [325], light, shading, and flooding [319, 326] as well as different cultivation techniques [327]. These efforts led eventually to the better control of the process and in many cases gave remarkable increase in the yield of CPT. For example, Roja et al. obtained by micropropagation *O. rugosa* plants which showed higher than natural plant CPT content, from 0.002% (roots) up to 0.09% (leaves) [322]. Similarly favorable results were reported by Madassery et al. who demonstrated that adventitious roots of *O. prostrata* exhibited stable level of CPT production in the range of 0.16–0.19% under optimized condition [323].

Saito et al. established a hairy root culture of *O. pumila* transformed by *Agrobacterium rhizogenes* strain 15834 characterizing by fast growth (16-fold during 5 weeks of cultivation) and high CPT productivity up to 0.1% per dry weight of the cells. Substantial amount of the alkaloid was excreted to the culture medium, especially in the presence of a polystyrene resin (Diaion HP-20) that absorbed CPT (with easy recovery of CPT from resin) [80]. The transformed hairy roots of *O. pumila* were later adapted to a larger-scale (3L bioreactor) culture affording

0.0085 % of CPT in the fresh wet tissue, and total amount of the alkaloid produced was 22 mg over 8 weeks culture [328].

10-Methoxy-CPT could be considered as a substrate in the semisynthesis of topotecan or irinotecan was produced in tissue culture of *O. liukiuensis* and *O. kuroiwai*, whereas hairy roots of these species gave predominantly CPT [324].

Endophytic fungi *Entrophospora infrequens* isolated form *N. foetida* were found to produce low amounts of CPT (maximal yield 0.5 mg/100 g of dry weight cell) when cultivated in bioreactor [329]. Similarly, low yield of CPT, 9-methoxy-CPT, and 10-hydroxy-CPT produced by some strains of *Fusarium solani* isolated from *Apodytes dimidiata* E. Mey. Ex Arn cultivated in broth medium was reported [88–90].

Genetic aspect of the biosynthesis of CPT and its regulation is a subject of a review [330].

7 Perspectives

The very successful story of camptothecin and related alkaloids confirms the importance of natural products chemistry as a major source of lead compounds for drug research. Although anticancer properties of CPTs focused most of the research attention, others, including antiviral, antiprotozoal, or insecticidal, are also considered in a research. Since CPT isolation in 1958 and first unsuccessful attempts in therapy, the dynamics of CPT research remains very high up to now, resulting in hundreds of published work each year. Although semisynthetic camptothecins such as topotecan and irinotecan hold their strong position in cancer chemotherapy, many other drug candidates were developed and are currently in various phases of the pre- or clinical testing. Recent works on CPTs involve also their new formulation (especially in a nanoscale) for the optimization of the drug delivery, stability and lowering their toxicity. Although substantial progress has also been observed in the total asymmetric syntheses of CPTs, further improvement is needed to provide cost-effective methods for their large-scale production. Biotechnological approaches to the CPT production are still inefficient and need more research to be competitive to the isolation of CPT from the plant material.

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Alkaloids Derived from Tryptophan: A Focus on Ergot Alkaloids

23

Peter Mai and Shu-Ming Li

Contents

1 Introduction 685
2 Occurrence and Structures of Ergot Alkaloids 687
2.1 Ascomycota as Producers of Diverse Ergot Alkaloids 687
2.2 Accumulation of Ergot Alkaloids in Fungi-Associated Plants 689
2.3 Symbiotic Communities 690
3 Cultivation, Extraction, Purification, and Analyses of Ergot Alkaloids 691
3.1 Cultivation of *Penicillium commune* as an Example for Ergot Alkaloid Production in Fungi 691
3.2 Extraction and Purification of Ergot Alkaloids 691
3.3 Analyses of Ergot Alkaloids in the Extracts 692
4 Versatile Biological Activities of Ergot Alkaloids 694
4.1 Therapeutically Significant Ergot Alkaloids and Those as Potential Pharmaceuticals 694
5 Biosynthesis of Ergot Alkaloids in Different Fungi 697
5.1 Biosynthesis of the Tetracyclic Ergoline as a General Structure of Ergot Alkaloids 697
5.2 Biosynthesis of Fumigaclavines 701
5.3 Formation of D-Lysergic Acid 702
5.4 Biosynthesis of Ergoamides and Ergopeptines 703
6 Industrial Production of Ergot Alkaloids 704
6.1 Life Cycle of *Claviceps purpurea* 704
6.2 Field Production of Ergot Alkaloids on Rye 704
6.3 Production of Ergot Alkaloids in Submerged Cultures 706
6.4 Semisynthetic Modifications of Natural Ergot Alkaloids Yield Significant Pharmaceuticals 707
7 Conclusion 707
References 707

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Abstract

Ergot alkaloids are secondary metabolites with significantly toxicological and pharmacological relevance and have been identified in different fungi of Ascomycota and several families of higher plants. Lysergic acid amides or peptides produced by the fungus *Claviceps purpurea* of the family Clavicipitaceae, e.g., ergometrine, ergotamine, or ergotoxine, and their semi-synthetic derivatives are widely used in modern medicine for treatment of diverse diseases. Large-scale productions of ergot alkaloids for pharmaceutical applications have been achieved by biotechnological processes including field cultivation of *Claviceps purpurea* on rye or in submerged cultures. Some members of the fungal family of Trichocomaceae such as *Aspergillus fumigatus* and *Penicillium commune* produce clavine-type ergot alkaloids. These substances consist merely of the ergoline ring system as a common structure of most ergot alkaloids and lack an amide or peptidyl moiety in comparison to ergoamides or ergopeptines. Diverse analytical methods were developed for detection and determination of ergot alkaloids as mycotoxins in foods, cereals, and livestock feeds. Significant progress has also been achieved on the molecular biological and biochemical investigations of ergot alkaloid biosynthesis in the last years. The reaction steps from prenylation of tryptophan to formation of the ergoline ring system have been studied in detail.

Keywords

Aspergillus fumigatus • biosynthesis • *Claviceps purpurea* • ergot alkaloids • mycotoxins • *Penicillium commune* • pharmaceuticals

Abbreviations

4-DMA-L-abrine	*N*-methyl-4-dimethylallyltryptophan
4-L-DMAT	4-dimethylallyltryptophan
5-HT	5-hydroxytryptamine
A. fumigatus	*Aspergillus fumigatus*
acetyl-CoA	Acetyl coenzyme A
C. purpurea	*Claviceps purpurea*
C. fusiformis	*Claviceps fusiformis*
DMAPP	Dimethylallyl diphosphate
DMATS	Dimethylallyl tryptophan synthase
E. coli	*Escherichia coli*
EA	Ergot alkaloid
ELISA	Enzyme-linked immunosorbent assay
GSH	Reduced glutathione
HPLC	High performance liquid chromatography
LSD	Lysergic acid diethylamide
N. lolii	*Neotyphodium lolii*
NADH	Nicotinamide adenine mononucleotide-phosphate
NFκB	Nuclear factor κB
NRPS	Nonribosomal peptide synthetase

P. commune	*Penicillium commune*
SAM	*S*-adenosyl methionine
TLC	Thin-layer chromatography
TLR 4	Toll-like receptor 4

1 Introduction

Ergot alkaloids (EAs) are a complex family of naturally occurring secondary metabolites derived from the amino acid L-tryptophan **1** and dimethylallyl diphosphate (DMAPP) [1–5]. The tetracyclic ergoline ring **2** is the common structural feature of all EAs (Fig. 23.1). Based on their structures, EAs can be classified into clavine-type alkaloids (clavines), ergoamides **3** and ergopeptines **4** with **2** as the common structure [5]. Clavines consist merely of **2**, while **3** and **4** are D-lysergic acid **5** derivatives linked with an alkyl amine or tripeptide at the carboxyl group [2, 5].

The fungus *Claviceps purpurea* (*C. purpurea)* of the family Clavicipitaceae represents the most important EA producer, which parasitizes mainly on rye and forms the so-called sclerotium as resting structure during its life cycle. EAs were also detected in *Aspergillus fumigatus* (*A. fumigatus*) and *Penicillium commune* (*P. commune*) of the fungal family of Trichocomaceae as well as in plants of the families Convolvulaceae,

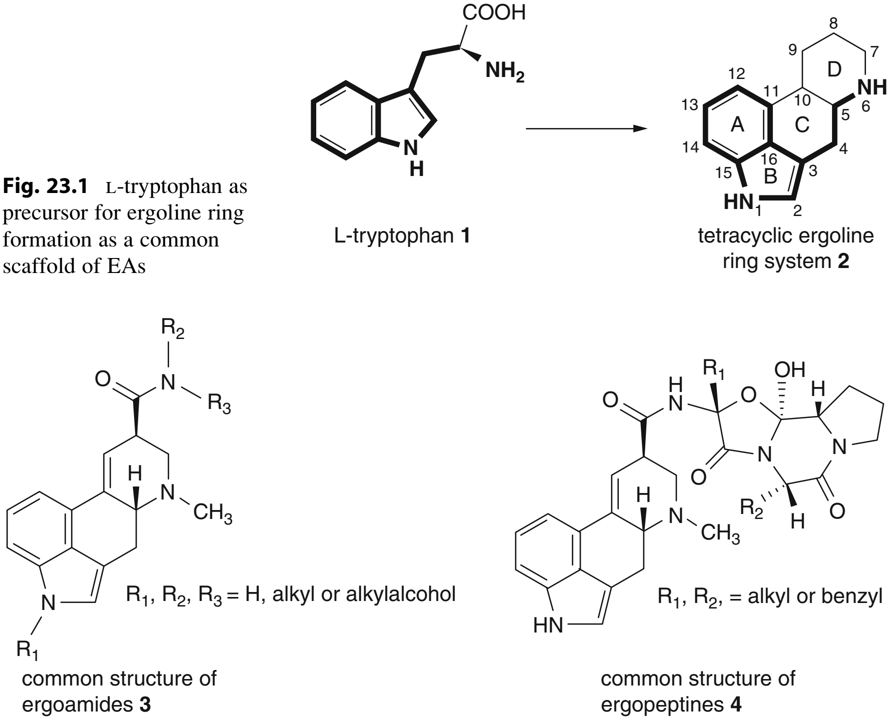


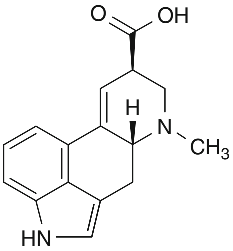
Fig. 23.1 L-tryptophan as precursor for ergoline ring formation as a common scaffold of EAs

L-tryptophan **1**

tetracyclic ergoline ring system **2**

common structure of ergoamides **3**

common structure of ergopeptines **4**

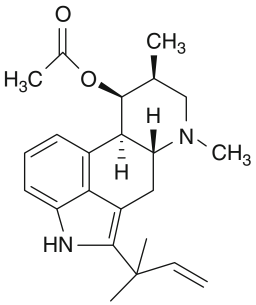


D-lysergic acid **5**

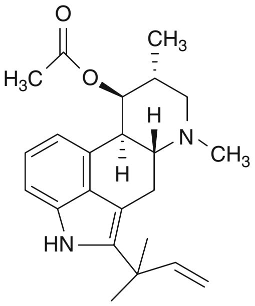
Poaceae, and Polygalaceae [1, 3, 5]. Recent investigation revealed however that the plant-associated fungi are responsible for EA production in plants [6, 7].

Due to their pharmacological and toxicological properties, EAs have attracted special attention as drugs in modern medicine, as mycotoxins in the food industry and ecological systems. In the middle ages, the consumption of rye bread contaminated with EAs led to severe epidemics, which have become known as ergotism or St. Anthony's fire. Ergotism can be distinguished in two forms dependent on observed symptoms. Ergotismus convulsivus is characterized by paranoia with hallucinations and ergotismus gangraenosus, which can lead to the loss of infected tissues [8]. The relationship between ergotism and infected rye was first recognized by Tulasne in 1853 [4]. Ergotism has been occasionally observed in livestock today [9]. Nowadays, EAs serve as important drugs in the modern medicine. They interact with serotonin, dopamine, and adrenergic receptors of the central nervous system and with adrenergic receptors of blood vessels. Natural and partial synthetic EAs are used in the treatment of postpartum hemorrhage, as antihypertensive drugs, in migraine therapy, in the treatment of Parkinson's disease, and in controlling the secretion of pituitary hormones such as prolactin [1, 4, 5, 10]. Infection of plants by endophytic fungi resulted in most cases in increased ecological fitness of the host. Infected plants show resistance to herbivores and extreme weather conditions such as drought. In addition, increased growth and mineral uptake is observed [11, 12].

First insights into the biosynthesis of EAs have been achieved by feeding experiments mainly carried out by the Gröger and Floss groups between the 1960s and 1990s [1, 3, 5, 13–15]. In 1999, the occurrence of EA biosynthetic gene cluster in *C. purpurea* has been proven by genome walking with the *dmaW* gene [16], which had been identified for the first pathway-specific enzyme dimethylallyl tryptophan synthase (DMATS) 4 years before [17]. The next important milestone in the research of EA biosynthesis was the identification of the biosynthetic gene cluster of fumigaclavine C **6** from the genome sequence of *A. fumigatus* Af293 by bioinformatic approaches [18, 19]. The identification of this cluster provided a convenient way to identify homologue genes in *C. purpurea* and *A. fumigatus* involved in the committed steps and different genes for specific steps in both pathways. Seven homologous genes were identified in both gene clusters and proposed for reactions from L-tryptophan to ergoline ring. In the last 7 years, the functions of most of these genes from both clusters were proven molecular biologically and biochemically.



(8S,9S) fumigaclavine C **6**



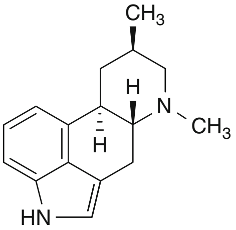
(8R,9S) fumigaclavine C **7**

2 Occurrence and Structures of Ergot Alkaloids

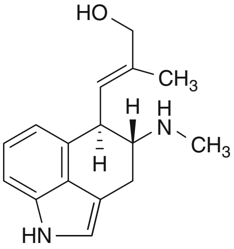
2.1 Ascomycota as Producers of Diverse Ergot Alkaloids

2.1.1 Fungi of the Family Trichocomaceae

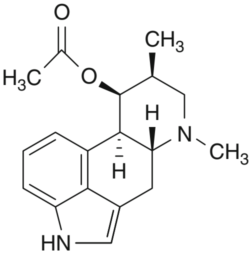
Some members of the family Trichocomaceae, e.g., *A. fumigatus* [20] and *P. commune* [21], produce clavines as the main EAs. Festuclavine **8**, chanoclavine-I **9**, fumigaclavines A **10** or **11**, B **12** or **13**, and C **6** or **7** have been isolated from members of the Trichocomaceae [20]. The accumulated EAs have been observed to be present in or on conidia of *A. fumigatus*. No EAs were detected in the conidiation-deficient strains of *A. fumigatus* by disruption of the gene *brlA*. Complementation of the deficient strain with the wild-type allele of *brlA* by homologous recombination restored conidiation and EA production [19]. In *A. fumigatus*, **6** is the end product of EA biosynthesis, whereas in *P. commune*, the biosynthetic pathway ends with the formation of **10** [22]. **12** or **13** as a precursor for **10** or **11** was found in both *Aspergillus* and *Penicillium* strains [21, 23–25]. The stereochemistry of fumigaclavines was not given in the literature in most cases. Conversion of chanoclavine-I aldehyde **14** leads to the formation of **8** in *A. fumigatus* as predominant product (see below) [26], while *Penicillium* strains produce **8** as main and its stereoisomer pyroclavine **15** and agroclavine **16** as minor products [21, 27–29].



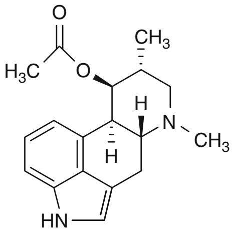
festuclavine **8**

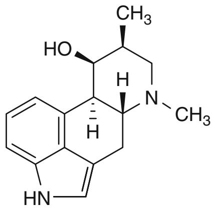


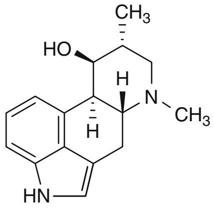
chanoclavine-I **9**

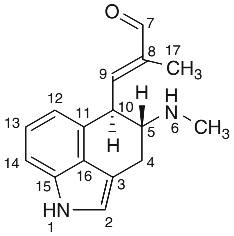


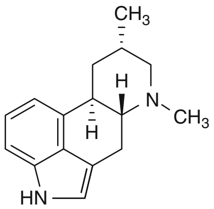
(8S,9S) fumigaclavine A **10**

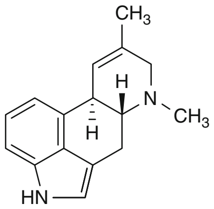

(8R,9S) fumigaclavine A **11**


(8S,9S) fumigaclavine B **12**


(8R,9S) fumigaclavine B **13**

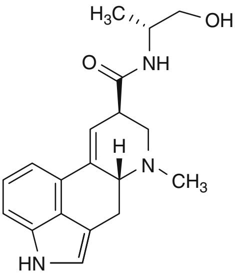

chanoclavine-I aldehyde **14**

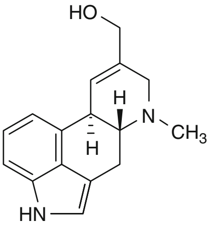

pyroclavine **15**

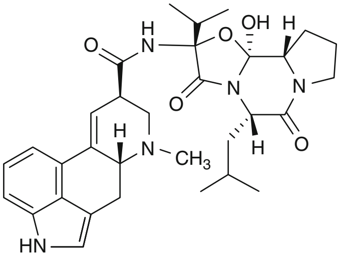

agroclavine **16**

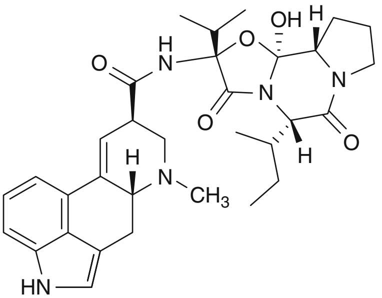
2.1.2 Fungi of the Family Clavicipitaceae

Fungi of the family Clavicipitaceae, e.g., *Claviceps fusiformis (C. fusiformis)*, *Claviceps paspali*, and *Claviceps hirtella*, were also reported to produce clavine-type EAs [2]. In comparison to *A. fumigatus* and *P. commune*, **14** is mainly converted to **16** in *Claviceps* species [30–34]. *Claviceps hirtella* produces clavines as major compounds and ergometrine **17** as minor component [35]. *C. fusiformis* is the sole exception of the family Clavicipitaceae, which produces clavines but no D-lysergic acid **5** derivatives such as **3** or **4** [31]. The most important **3** and **4** producing fungus of the family Clavicipitaceae is represented by *C. purpurea*. In this fungus, EAs are detected in the sclerotium [35]. In addition, *C. purpurea* strains differ from each other in the spectrum of EAs [35]. The strain P1 produces elymoclavine **18**, ergocryptine consisting of α-ergocryptine **19** and β-ergocryptine **20**, ergosine **21** and ergotamine **22** as major component [36]. In comparison, **22** and ergocristine **23** were found as major and **17** as minor EAs in the strain ECC93 [37, 38].

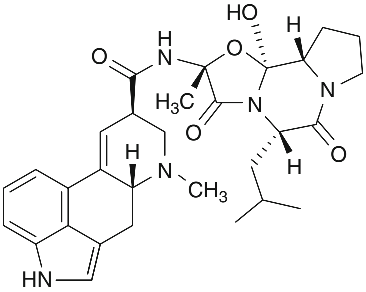

ergometrine **17**


elymoclavine **18**

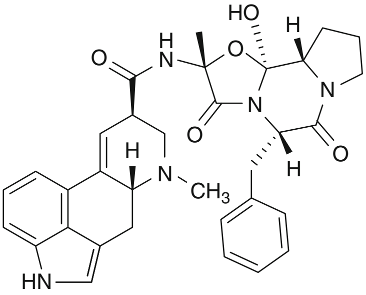

α-ergocryptine **19**



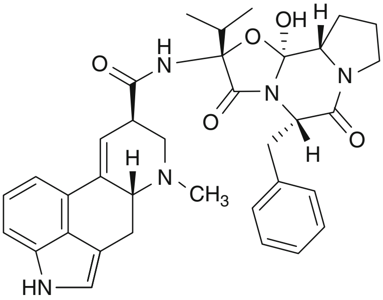
β-ergocryptine **20**



ergosine **21**



ergotamine **22**



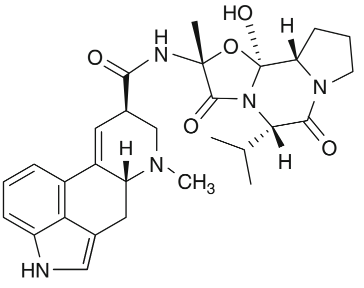
ergocristine **23**

2.2 Accumulation of Ergot Alkaloids in Fungi-Associated Plants

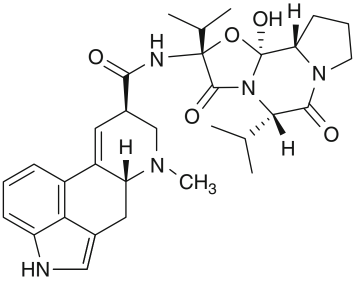
It was assumed that not only fungi but also plants of the families Convolvulaceae, Poaceae, and Polygalaceae can produce EAs. These plants should have obtained the ability through a horizontal gene transfer from fungi during evolution [3, 6]. Investigations of four different EA-containing plants of the family Convolvulaceae demonstrated however that EAs were only detectable in the presence of the plant-associated fungi. Treatment of *Ipomoea asarifolia* (Convolvulaceae) with fungicides resulted in complete abolishment of EA production [39]. In conclusion plant-associated and mostly seed-transmitted fungi, not the plants themselves produced EAs [40]. Comparison of the ribosomal DNA and *dmaW*, encoding the first enzyme in the EA biosynthetic pathway, revealed that these clavicipitalean fungi are closely related but not identical [41]. Recently, two new plant-associated EA-producing fungi were described and named *Periglandula ipomoeae* and *Periglandula turbineae* [42]. In Poaceen plants, sexual endophytic fungi of the genus *Epichloë* and their asexual counterparts *Neotyphodium* as well as the genus *Balansia* are responsible for EA production [7]. Ergovaline **24** and simple lysergic acid amides have been identified in *Epichloë festucae*, which is determined to be symbiotic with many grasses [7].

2.3 Symbiotic Communities

Clavicipitalean fungi and higher plants of the family Convolvulaceae as well as grasses of the family Poaceae exhibit often symbiotic communities. The endophytic fungi benefit from its host by receiving nutrients, protection, and dissemination. Plants have also advantages by associating with endophytic fungi, including defense against herbivores, insects, and nematodes. Other benefits include increased growth of the plant, resistance to drought, and nutrient status [43]. The beneficial implications of EAs in endophyte-infected tall fescue have been analyzed by application of root extracts including polyphenolic fraction, EAs, and loline alkaloids to *Pratylenchus scribneri*, a nematode of tall fescue grasses. It has been shown that *P. scribneri* affects endophyte-infected tall fescue in very low amount, compared with noninfected tall fescue. The toxicological tests of EAs revealed that **19** and **24** were toxic to nematodes at concentrations of 5 and 50 µg ml^{-1}. Also **17** and ergocornine **25** showed nematistatic effects at most tested concentrations [44]. Another study revealed a putative biosynthetic gene cluster of **24** in *Neotyphodium lolii* (*N. lolii*) by cloning and analysis of a nonribosomal peptide synthetase (NRPS) gene. A monomodular NRPS and other genes have been identified, which are orthologous to *C. purpurea* and *A. fumigatus* genes. Endophyte-associated grasses expressed genes, which encode enzymes catalyzing the biosynthesis of **24**, whereas no formation of **24** was found in *N. lolii* mycelia from axenic culture. It was proposed that plants associated with endophytes induce the gene expression by special signal molecules [45]. EA occurrence was also analyzed in the symbiotic community of a clavicipitalean fungus and *Ipomoea asarifolia* (Convolvulaceae). The *dmaW* gene encoding the first EA biosynthesis specific step was identified in clavicipitalean fungus, but not in *Ipomoea asarifolia* [40]. This demonstrates that only the fungus possesses the genetic information for EA biosynthesis. Nevertheless, the produced EAs were only detectable in plants, while no alkaloids were measured in the associated epibiotic fungus. A transport system is supposed to be responsible for transferring EAs from fungus to its host [40]. In contrast, no observed benefits have been achieved by symbiosis of native grass populations and endophytes in a 4-year experiment. Neither advantages in growth nor seed production or reproductive effort under full herbivory were detected in comparison to grasses, which are not infected or infected but not producing EAs [46].



ergovaline **24**



ergocornine **25**

3 Cultivation, Extraction, Purification, and Analyses of Ergot Alkaloids

3.1 Cultivation of *Penicillium commune* as an Example for Ergot Alkaloid Production in Fungi

The optimal cultivation conditions for EA production in fungi vary enormously. For example, different conditions for EA production were described for *C. purpurea* strains (see Sects. 6.2 and 6.3) [31–33, 47–49]. Here, we will just demonstrate the principle for EA production in *P. commune* used in our laboratory. Glucose peptone with soy flour medium (GPS) and wheat grains liquid media were inoculated with 1×10^5 spores of *P. commune* per 100 ml medium and cultivated at 25 °C for 14 days in the dark without shaking.

3.2 Extraction and Purification of Ergot Alkaloids

EAs are natural bases, due to the N-6 of **2**. The solubility of EAs can be therefore influenced by changing the pH value of the solution. At low pH value (e.g., $\text{pH} < 6$), the N-6 will be protonated and easier soluble in water. EAs exist, at high pH value (e.g., pH 9), in their free base and therefore are soluble in organic solvents. Furthermore, the residues at C-8 of **2** contribute to the different polarities of EAs. These properties are usually used in the extraction and purification procedures of EAs [22, 26, 50]. Initially, halogenated organic solvents such as chloroform or dichloromethane were applied for extraction of EAs. Ethyl acetate and acetonitrile have been demonstrated later to be alternatives to halogenated solvents [51]. Nevertheless, dichloromethane was better in certain extraction procedures. Ammonium hydroxide or sodium hydroxide was often found as component in the extraction agents for EAs as free bases [52–54]. As aforementioned, EAs can be also extracted as EA salts by using polar solvents with acid, e.g., methanol and 0.25% concentrated phosphoric acid (40/60) [55], acetonitrile with phosphate-buffer pH 6.0 (60/40) [56], or 2-propanol, H_2O, and lactic acid (50/49/1, V/V). These systems were able to extract EAs with different polarities [57].

Purification of the EA-containing extracts was performed either by liquid-liquid extraction [53] or on solid-phase extraction using silica gel as normal phase or C18 as reverse phase [54, 58]. EAs with a wide range of polarities can be separated by reverse phase HPLC (high performance liquid chromatography) using gradient elution system beginning often with an aqueous solvent, e.g., 5% acetonitrile in 50 mM ammonium acetate to a nonpolar solvent, e.g., 75% acetonitrile in 50 mM ammonium acetate [1]. Cation exchangers are often used for purification after extraction of EAs with acids. Protonation of EAs at the N-6 enables them to adsorb at the negatively charged material. Elution of EAs is performed by deprotonating with basic solvents [51].

3.2.1 Extraction of Ergot Alkaloids from Liquid Cultures of *Penicillium commune*

The mycelia of *P. commune* are to be separated from media by filtration and the pH value of the filtrate is adjusted to 8–9 with ammonium hydroxide. The filtrate is subsequently extracted twice with equal volume of dichloromethane. The organic phases are combined and evaporated to dryness in vacuum.

3.2.2 Extraction of Ergot Alkaloids from Cultures of *Penicillium commune* on Wheat Grains

The solid cultures with wheat grains are suspended in 300 ml ethyl acetate and shaked at 25 °C for 4 h. The organic phase is obtained by filtration, subsequently dried over sodium sulfate and evaporated to dryness in vacuum.

3.3 Analyses of Ergot Alkaloids in the Extracts

3.3.1 Colorimetric Assay

EAs can be identified by various analytical methods. The most important colorimetric analysis is the reaction of EAs with van Urk's reagent, a mixture of *p*-dimethylaminobenzaldehyde, $FeCl_3$, and H_2SO_4. This reagent is also used for quantification of EAs by measurement of the absorbance at 580 nm of the reaction products [59, 60].

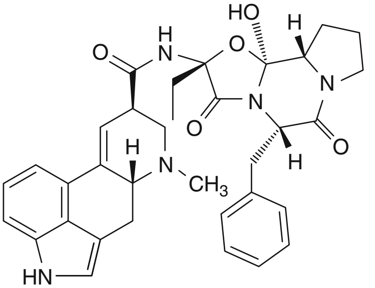
3.3.2 Thin Layer Chromatography (TLC)

TLC was performed on silica gel alone, impregnated with sodium hydroxide or formamide as well as aluminum oxide and used both for detection and separation of EAs [22, 49, 60–62].

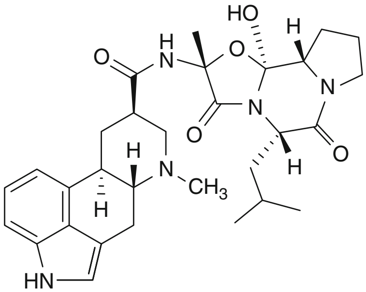
Detection of EAs on TLC was carried out by observation of their fluorescence or colors of reaction products with spray reagents. The presence of the double bond between C-9 and C-10 in EAs is prerequisite for a detection of their fluorescence under UV light. Clavine-type alkaloids show no auto-fluorescence due to the absence of the double bond in **2**. Various spray reagents are explored to detect clavine-type alkaloids or to distinguish between different EAs. Van Urk's reagent is also used as spray reagent to detect EAs [63]. To intensify the spots, TLC plates can be sprayed with sodium nitrite solution [64]. **22** was determined as a blue spot by using an acidic aqueous solution of 0.5% glyoxylic acid containing 0.05% ferric chloride. In case of ergoamides **3** additional heating of the sprayed plate at 110 °C is necessary for displaying spots [65]. Different colors of **22** can be achieved by spraying the TLC plates with π-acceptors such as 2, 3-dichloro-5, 6-dicyanobenzoquinone [66].

3.3.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Specificity in the determination of EAs in immunoassays depends on the applied antibodies [63]. Most ELISA techniques were only able to detect certain EAs [51]. A competitive inhibition ELISA was developed for specific reaction of polyclonal antibodies with phenylalanine residues of EAs in **22**, **23**, and ergostine **26** [67]. Development of a monoclonal antibody permitted the detection of various EAs, e.g., **24** and clavine alkaloids [68]. Another competitive ELISA was used for measuring dihydroergosine **27** in grain and animal feed. **27** conjugated to bovine serum albumin was recognized by specific mouse monoclonal antibodies and rabbit polyclonal antibodies [69]. Furthermore, the semiquantitative competitive ELISA is applied for the determination of EAs in endophyte-infected plants by specific reaction of the monoclonal antibody with the lysergic acid moiety [70].



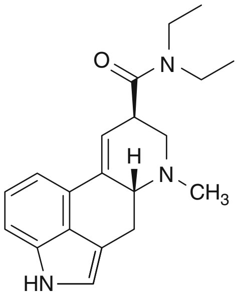
ergostine **26**



dihydroergosine **27**

3.3.4 High Performance Liquid Chromatography (HPLC)

HPLC was proven to be the most important instrumental analytical method for determination of EAs. Separation on HPLC and downstream detection by fluorescence detector or tandem mass spectrometer was performed. In the early 1970s, when the semisynthetic lysergic acid diethylamide **28** (LSD) became available in the drug scene, there was the need of a suitable analytical method for the detection of LSD. Since then, many HPLC analyses have been developed for the detection of EAs. Initially, normal phase HPLC was used for the determination of **4** with subsequent fluorescence detection. Today, reversed phase with C18 column materials is used more frequently for analysis of EAs [63, 71–73]. As mentioned before, EAs differ often in the position of the double bond in ring D of the **2**. Clavine-type EAs contain sometimes a double bond at C-8 and C-9, whereas **3** and **4** carry a double bond at C-9 and C-10 instead, which influences the chromophoric features and is therefore a key parameter for the choice of excitation and emission wavelength of fluorescence detection. Extract mixtures containing EAs with a double bond at different positions (C-8 and C-9 or C-9 and C-10) should be analyzed in two runs to ensure the detection and quantification of the complete EAs. Another possibility is to use two fluorescence detectors subsequently [74].



lysergic acid
diethylamide **28**

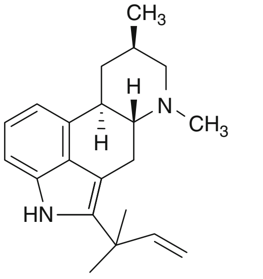
4 Versatile Biological Activities of Ergot Alkaloids

4.1 Therapeutically Significant Ergot Alkaloids and Those as Potential Pharmaceuticals

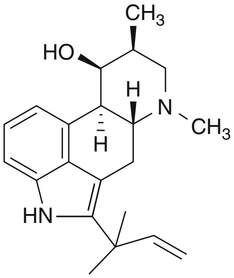
Comparison of the general ergoline structure of EAs with the neurotransmitters noradrenaline, dopamine, and serotonin display structural similarities [75] and accordingly explain their interaction with α-adrenergic receptors and serotonin receptors as agonists, partial agonists, and antagonists [76]. In addition, they act as agonists or partial agonists at dopamine receptors of the central nervous system. In the modern medicine, natural EAs and their semisynthetic derivatives are important therapeutic drugs. Due to the broad interaction with receptors and some adverse effects, pharmacologically used compounds are characterized as “dirty drugs.” To obtain desired affinity of EAs toward the various receptors, chemical modifications on the substituents linked to the carboxyl group of **5** were carried out [77].

4.1.1 Clavine-Type Alkaloids

An important compound with biological activity of clavines is represented by **6** as end product of EA biosynthesis in *A. fumigatus*. In animal experiments using rats, **6** exhibited vasorelaxant effects on isolated thoracic aortic rings independent of endothelial mediators. Several mechanisms are proposed for the vasorelaxant effect of **6**. These results indicate that **6** has potential capacity in vascular protection and may be used as therapeutic drug against cardiovascular diseases [78]. Treatment of mice with induced liver damage and colitis with **6** showed clear beneficial effects on these diseases [79, 80]. In addition, it has also been demonstrated that **6** exhibits potential relevance as an antiatherosclerotic agent. Inflammation processes like activation of toll-like receptor 4 (TLR 4) and nuclear factor κB (NFκB) have been shown to be responsible in the development of atherosclerosis. **6** interferes with the TLR 4/NFκB signaling pathway leading to a decrease of inflammatory factors [81]. Studies with other biologically active clavine-type alkaloids, e.g., **8**, **15**, **16**, and **18**, revealed that these compounds act as pure antagonists or partial agonists at $5HT_{2A}$ (5-hydroxytyptamine) receptors of rat tail artery and as antagonists at α_1-adrenoceptors of rat aorta [82]. It has been furthermore reported that clavines, e.g., 9-deacetoxyfumigaclavine C **29** and 9-deacetylfumigaclavine C **30** exhibit selective and potent cytotoxicity against human leukemia cells [83].



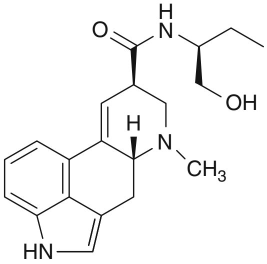
9-deacetoxyfumigaclavine C **29**



9-deacetylfumigaclavine C **30**

4.1.2 Ergoamides

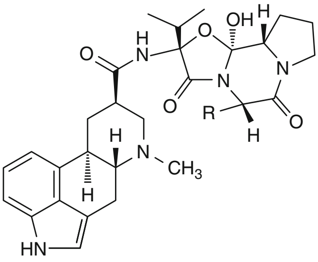
17 and its semisynthetic derivative methylergometrine **31** are the main ergoamides used in modern medicine. **17** and **31** are assembled from **5** and amidated with 2-aminopropanol and 2-aminobutanol, respectively. Their pharmacological effects are based on the selective antagonistic interaction with 5-HT receptors on the smooth muscles and as partial agonists or antagonists to 5-HT receptors of the central nervous system. Additionally, they act on blood vessels as antagonists of dopaminergic receptors and partial agonists of α-adrenergic receptors. **17** and **31** are used due to the direct stimulatory effect on uterine contraction in obstetrics and for both prevention and treatment of postpartum hemorrhage [84]. **28** is also a semisynthetic ergoamide and it should be mentioned here. Due to the strong hallucinogenic side effects and abuse potential, **28** is no longer used for treatment of psychiatric disorders [85, 86].



methylergometrine **31**

4.1.3 Ergopeptines

Peptide alkaloids or ergopeptines **4** are composed of **5** with proline-containing tripeptidyl residues attached to the carboxyl group resulting in the formation of a tricyclic system. **22** is one of the therapeutically important peptide alkaloids. **22** interacts as partial agonist and antagonist with 5-HT- and α-adrenergic receptors in blood vessels and smooth muscles. The most significant effect of **22** is based on the agonistic effect at 5-$HT_{1B/1D}$ receptors at the blood


O
H
N
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OH
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R
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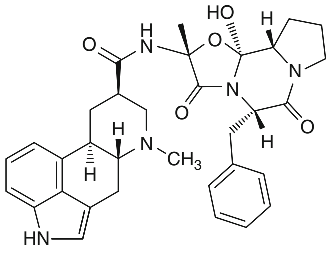
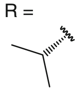
N
CH3
H
HN

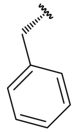
dihydroergotoxine **33–36**


O
H
N
HO
O
H
N
N
O
H
O
H
N
CH3
H
HN

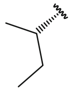
dihydroergotamine **32**



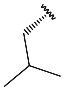
dihydroergocornine **33**



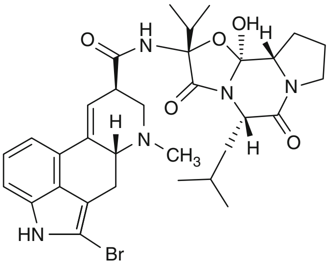
dihydroergocristine **34**



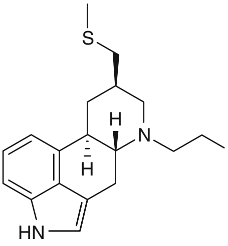
α-dihydroergocryptine **35**



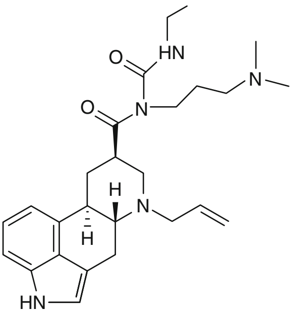
β-dihydroergocryptine **36**


O
H
N
OH
H
O
N
N
O
H
N
CH3
H
O
HN
Br

bromocryptine **37**



pergolide **38**


O
HN
N
O
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H
N
H
HN

cabergoline **39**

vessels in the central nervous system, where the constriction of intracranial blood vessels leads to reduction of neurogenic inflammation in the trigemino-vascular system. **22** is used for treatment of mild to severe migraine attacks and cluster headache [87, 88]. The hydration of the double bond at C-9 and C-10 of **22** results in the semisynthetic derivative dihydroergotamine **32**, which is also used in the therapy of migraine headache [89, 90] and furthermore in the therapy of orthostatic hypotension [91]. The ergopeptine alkaloids **19**, **20, 23**, and **25** are components of ergotoxine, which were initially thought to be a single compound. Dihydroergotoxine consisting of the semisynthetic derivatives dihydroergocornine **33**, dihydroergocristine **34**, α-dihydroergocryptine **35**, and β-dihydroergocryptine **36**, also named ergoloid, is used for treatment of age-related cerebral dysfunction like dementia [84, 92]. Furthermore **35** and bromocryptine **37**, a semisynthetic derivative of **19**, as well as pergolide **38** and cabergoline **39**, are used therapeutically for treatment of early-onset Parkinson's disease [93–95]. Their effects are based on their potent agonism on dopamine (D_2) receptors. Dopamine is a prolactin-inhibiting factor. The agonistic effect of **37** and **39** to dopaminergic receptors results also in inhibition on the release of the peptide hormone prolactin [96]. Therefore, they are also used in treatment of prolactin-related disorders such as galactorrhea, prolactin-dependent mammary carcinoma, amenorrhea, and acromegaly [97–99].

5 Biosynthesis of Ergot Alkaloids in Different Fungi

5.1 Biosynthesis of the Tetracyclic Ergoline as a General Structure of Ergot Alkaloids

Different approaches have been carried out to investigate the biosynthesis of EAs. Early feeding experiments with isotope-labeled precursors, e.g., [^{14}C]-L- tryptophan **1**, to rye ears infected with *C. purpurea* or to fermentation cultures of different *Claviceps* species proved its incorporation into **2** [100–102]. Further biochemical experiments with crude and purified enzyme extracts from producers revealed the determinant step in the biosynthesis of EAs by the key enzyme 4-dimethylallyltransferase (4-DMATS) [103, 104], i.e., the prenylation of **1** at C-4 position [17, 104, 105].

The gene *dmaW* encoding for the first pathway-specific DMATS was cloned firstly from *C. fusiformis* [17]. This was done by PCR amplification using degenerate primers deduced from partial amino acid sequences obtained from purified DMATS of *C. fusiformis* and cDNA (copy DNA) library from the same strain [17]. The identified nucleotide sequence of *dmaW* led later to detection of an EA gene cluster in *C. purpurea* containing 14 genes by chromosome walking (Fig. 23.2) [16]. This cluster was proven to be responsible for the biosynthesis of ergoamides,

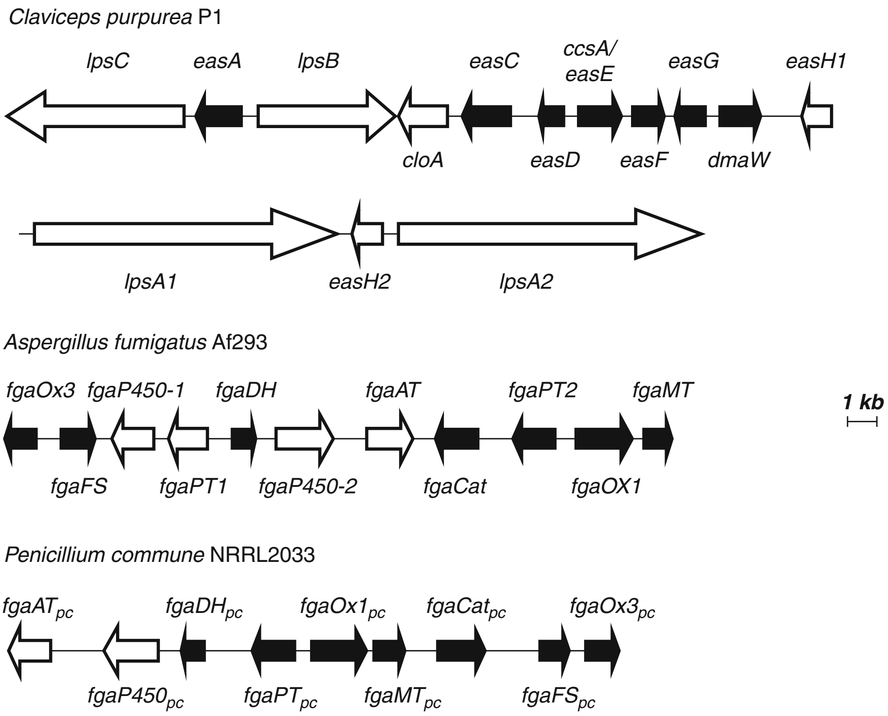

Claviceps purpurea P1
lpsC
easA
lpsB
easC
ccsA/ easE
easG
easH1
cloA
easD
easF
dmaW
lpsA1
easH2
lpsA2
Aspergillus fumigatus Af293
fgaOx3
fgaP450-1
fgaDH
fgaAT
fgaPT2
fgaMT
1 kb
fgaFS
fgaPT1
fgaP450-2
fgaCat
fgaOX1
Penicillium commune NRRL2033
fgaATpc
fgaDHpc
fgaOx1pc
fgaCatpc
fgaOx3pc
fgaP450pc
fgaPTpc
fgaMTpc
fgaFSpc

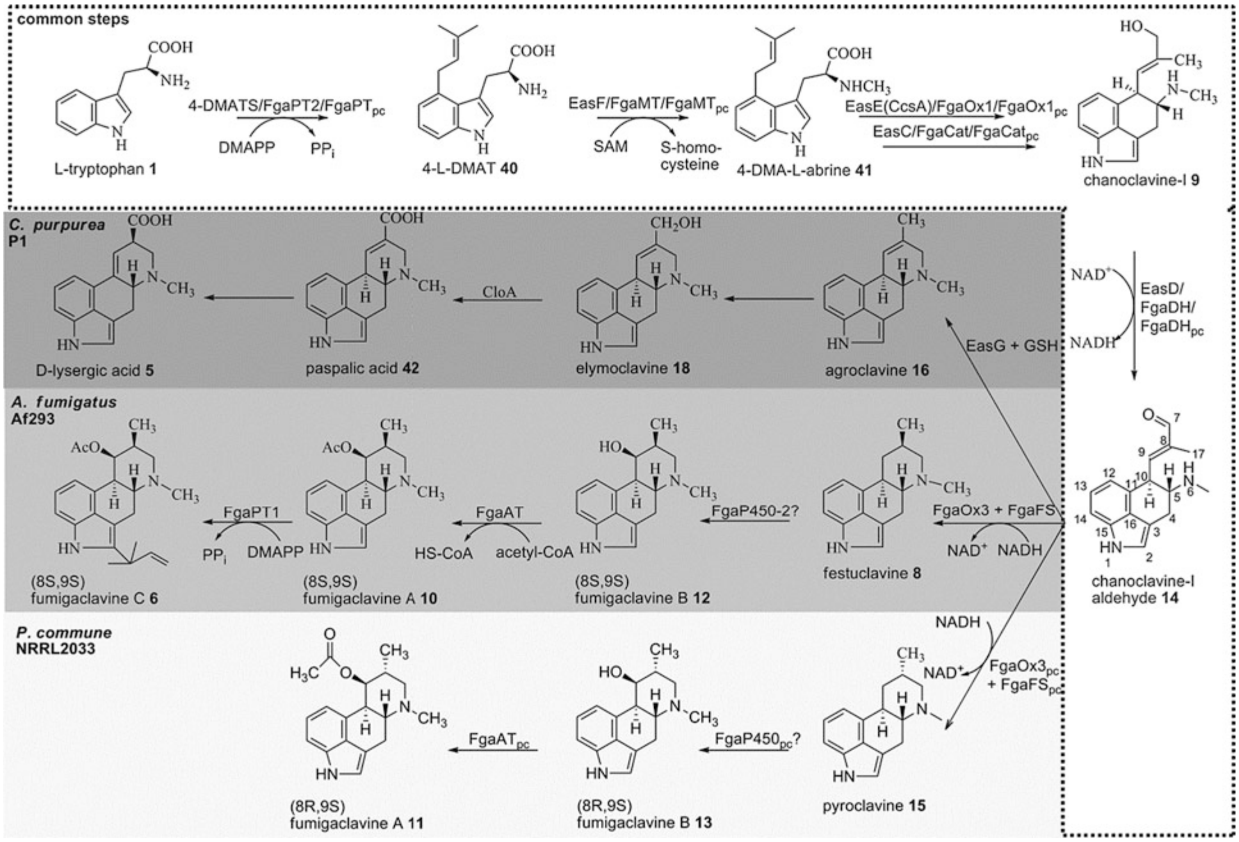
Fig. 23.2 EA gene clusters of *C. purpurea*, *A. fumigatus*, and *P. commune*. The *black arrows* indicate the homologous genes of all clusters (Modified after [22, 35, 109])

e.g., **17** and ergopeptines, e.g., **22** [35, 38, 106]. Blasting the genome sequence of the first *Aspergillus* species, i.e., *A. fumigatus* Af293 [107] with *dmaW* sequence from *C. purpurea* revealed the presence of an EA gene cluster in *A. fumigatus* containing 11 genes on chromosome 2 [18] (Fig. 23.2). This cluster contains genes necessary for the biosynthesis of the clavine alkaloid **6** lacking an amide or a peptidyl moiety [5, 22]. To identify the biosynthetic gene cluster of **11**, a cosmid library with genomic DNA from *P. commune* NRRL2033 was constructed and screened with *fgaPT*, a homologous gene of *dmaW* from *P. commune* NRRL2033. Sequencing and analysis of three positive cosmids revealed a putative cluster consisting of nine genes for the biosynthesis of **11** (Fig. 23.2) [108].

Identification of the biosynthetic gene clusters for **6** and **11** as well as comparison of the EA biosynthetic genes in *C. purpurea*, *A. fumigatus*, and *P. commune* provided a convenient way to identify candidate genes for the formation of the common structure **2** and for the specific steps in the three fungi [5, 18, 22]. Comparison of the gene clusters from *C. purpurea, A. fumigatus*, and *P. commune* revealed the presence of seven orthologous/homologous genes (black arrows) in the three clusters (Fig. 23.2), which were speculated to be responsible for the formation of the ergoline scaffold **2** [5, 18, 22]. The NRPS genes *lpsA1, lpsA2, lpsB*, and *lpsC* from the cluster of *C. purpurea* P1 will be needed for the biosynthesis and attachment of the alcohol amine and peptidyl moiety to the carboxyl group of **5**. The presence of the putative acetyltransferase gene *fgaAT* in the cluster of **6** in *A. fumigatus* or $fgaAT_{Pc}$ in the cluster of **11** in *P. commune* would be used for the transfer of an acetyl group from acetyl-CoA to a hydroxyl group of **12** or **13**. The second prenyltransferase gene *fgaPT1* in the cluster of *A. fumigatus* could be assigned for the conversion of **10** to **6** [110]. This gene is absent in the cluster of *P. commune*, indicating that **11** instead **7** is the end product of the gene cluster. This was proven by isolation and identification of **11** as the main EA in *P. commune* NRRL 2033 [22, 108, 110].

Functional proof of genes was carried out by knock-out experiments and identification of the accumulated intermediates [111–115] or by biochemical approaches using purified recombinant enzymes [18, 26, 50, 110, 116–119].

The biosynthesis of **2** begins with the prenylation of **1** by the enzyme 4-DMATS (DmaW), e.g., FgaPT2 from *A. fumigatus* (Scheme 23.1). The essential role of *fgaPT2* for the biosynthesis of **6** in *A. fumigatus* has been proven by knock-out experiments [113]. Incubation of **1** with recombinant FgaPT2 from *Saccharomyces cerevisiae* in the presence of DMAPP and subsequent isolation and structure elucidation of the enzyme product proved unequivocally its conversion to 4-dimethylallyltryptophan (4-L-DMAT) **40** [18]. **40** was then converted to *N*-methyl-4-dimethylallyltryptophan (4-DMA-L-abrine) **41** by a methyltransferase, e.g., FgaMT from *A. fumigatus*, in the presence of *S*-adenosyl methionine, which was verified by using the purified recombinant FgaMT [117]. Conversion of **41** to the next detected intermediate **9** requires one decarboxylation and two oxidation steps. The first proposed oxidation starts with the formation of a diene by hydroxylation at C-9 resulting in formation of an unstable intermediate and subsequent spontaneous dehydration [3]. The gene *ccsA* (also termed *easE*) encodes a flavin adenine dinucleotide containing oxidoreductase, which plays an important role in the biosynthesis of **9**. Experiments with knock-out mutants of *C. purpurea* strain P1 lacking *ccsA* led to accumulation of **41** and traces of **40** [114]. In addition, the gene *easC* has been proven to encode a catalase and plays also an essential role in the biosynthesis of **9**. Disruption of *easC* in *A. fumigatus* by homologous recombination resulted in accumulation of **41** and abolishment of downstream EAs, similar to the results obtained from disruption mutants of *ccsA* mentioned above [111]. Feeding experiments with **9** to *easC* and *easE* mutants of *A. fumigatus* led to restoration of EA biosynthesis, which proved that the conversion of **41** to **9** required both EasC and EasE. Recombinant EasC from *Escherichia coli (E. coli)* showed catalase activity in the presence of H_2O_2. It was therefore speculated that two different classes of enzymes, a catalase and a flavin adenine dinucleotide-dependent oxidoreductase are together responsible for the formation of **9** (Scheme 23.1). Biochemical evidence for this hypothesis has to be provided in the future. The common biosynthetic pathway of EA-producing fungi, e.g., *Aspergillus*,


common steps
L-tryptophan 1
4-DMATS/FgaPT2/FgaPT$_{pc}$
DMAPP
PP$_i$
4-L-DMAT 40
EasF/FgaMT/FgaMT$_{pc}$
SAM
S-homo-cysteine
4-DMA-L-abrine 41
EasE(CcsA)/FgaOx1/FgaOx1$_{pc}$
EasC/FgaCat/FgaCat$_{pc}$
chanoclavine-I 9
NAD$^+$
NADH
EasD/ FgaDH/ FgaDH$_{pc}$
chanoclavine-I aldehyde 14
C. purpurea P1
D-lysergic acid 5
paspalic acid 42
CloA
elymoclavine 18
agroclavine 16
EasG + GSH
A. fumigatus Af293
(8S,9S) fumigaclavine C 6
FgaPT1
PP$_i$
DMAPP
(8S,9S) fumigaclavine A 10
FgaAT
HS-CoA
acetyl-CoA
(8S,9S) fumigaclavine B 12
FgaP450-2?
festuclavine 8
FgaOx3 + FgaFS
NAD$^+$
NADH
P. commune NRRL2033
(8R,9S) fumigaclavine A 11
FgaAT$_{pc}$
(8R,9S) fumigaclavine B 13
FgaP450$_{pc}$?
pyroclavine 15
NADH
NAD$^+$
FgaOx3$_{pc}$ + FgaFS$_{pc}$

Scheme 23.1 EA biosynthesis in *A. fumigatus*, *P. commune*, and *C. purpurea* modified after [22, 26, 115, 124]

Penicillium, and *Claviceps*, ends with enzymatic formation of **14** (Scheme 23.1). Earlier experiments in the 1970s and 1980s by feeding *Claviceps* cultures with isotope-labeled precursors identified **14** as further intermediate in the conversion of **41** to **16** [13, 120]. Biochemical investigation showed that a short-chain dehydrogenase FgaDH from *A. fumigatus* catalyzed the conversion of **9** to **14** in the presence of nicotinamide adenine nucleotide (NAD^+) [50]. The orthologous EasD from *C. purpurea* and $FgaDH_{Pc}$ from *P. commune* are expected to catalyze the same reaction (Scheme 23.1).

14 was found to be the branch point in the biosynthesis of different EAs [112]. A lot of efforts have been made to investigate the conversion of **14** to downstream intermediates. *EasA* from *C. purpurea* and its homologous *fgaOx3* from *A. fumigatus* and *fgaOx3*$_{Pc}$ from *P. commune* belong to the old yellow enzyme family and encode for flavin adenine dinucleotide-dependent oxidoreductases. Investigations with purified recombinant proteins showed that in *A. fumigatus*, FgaOx3 catalyzed together with the festucalvine synthase the formation of **8** from **14** (Scheme 23.1) [26]. In the absence of FgaFS, FgaOx3 catalyzed the conversion of **14** to two shunt product isomers [121]. It has been proposed that conversion of **14** to **8** consist of two reduction steps. The double bond between C-8 and C-9 would be firstly reduced by FgaOx3 and the resulted dihydrochanoclavine-I aldehyde would undergo spontaneous cyclization to an iminium intermediate, which is subsequently reduced by FgaFS [26]. Similar to their orthologous FgaOx3 and FgaFS in *A. fumigatus*, $FgaOx3_{Pc}$ and $FgaFS_{Pc}$ from *P. commune* also catalyzed the conversion of **14** to **8** [22]. Interestingly, **15** was found as the second enzyme product in the incubation mixture of **14** with $FgaOx3_{Pc}$ and $FgaFS_{Pc}$. Incubation with different enzyme combinations proved that the second reduction catalyzed by FgaFS or $FgaFS_{Pc}$ controlled the stereochemistry of the product, i.e., those of **8** and **15** [22].

In contrast, EasA from *C. purpurea* was proven to be inactive and will be not involved in the conversion of **14** to **16** [22, 116]. The agroclavine synthase EasG in *C. purpurea* alone was responsible for conversion of **14** to **16**, via a nonenzymatic adduct with reduced glutathione (GSH) (Scheme 23.1). GSH was necessary for the isomerization of **14** to form isochanoclavine-I aldehyde, which would undergo spontaneous cyclization to an iminium intermediate. This imine is subsequently reduced by EasG, as in the case of FgaFS [116]. **8**, **15**, and **16** serve as precursors for **6** in *A. fumigatus*, **11** in *P. commune*, and lysergic acid **5** in *C. purpurea*, respectively.

5.2 Biosynthesis of Fumigaclavines

The first main tetracyclic intermediate with an ergoline **2** scaffold in the biosynthesis of fumigaclavines was detected as **8** in *A. fumigatus*, whereas another intermediate with tetracyclic scaffold was determined as **15** in *P. commune*. Both structures contain a saturated D-ring, which are different in their stereochemistry at C-8. In comparison, **8** and downstream enzymatic products of *A. fumigatus*, e.g., **6**, **10**, and **12** have *S*-configuration at C-8, while **15** and the subsequent enzymatic products, e.g., **11** and **13** have R-configuration at C-8 position. In addition, the biosynthetic pathway in *A. fumigatus* ends with the formation of **7**, whereas **11** is the last detectable product of *P. commune*. The difference of structural features of **6** and **11** depends on the additional gene *fgaPT1* encoding for a second prenyltransferase in *A. fumigatus* biosynthetic gene cluster, while the gene cluster of *P. commune* contains only one gene *fgaPT* encoding for a dimethylallyl tryptophan synthase [22]. The comparison of the structures **6** and **8**, which differ in an acetoxy group at position C-9 and a reverse prenyl moiety at position C-2 of **6**, indicates that three enzymatic reactions must be involved in the formation of **6** from **8**. These reactions must be hydroxylation, acetylation, and prenylation. Three genes in the cluster of *A. fumigatus*, *fgaP450-2*, *fgaAT*, and *fgaPT1*, encode a putative hydroxylase, acetyltransferase, and prenytransferase and are proposed to be responsible for the enzymatic synthesis of **6** from **8**. Two reactions, i.e., hydroxylation and acetylation, are needed in the biosynthesis of **11** from **15** in *P. commune*. It can be expected that the orthologous enzymes catalyze same reactions with different stereochemistry at C-9. Indeed, cloning of the putative genes *fgaAT* and *fgaPT1* from *A. fumigatus*, overexpression in *E. coli* and biochemical investigations revealed that FgaAT catalyzes the acetylation of **13** isolated from *P. commune* in the presence of acetyl-CoA, resulting in the formation of **11** [122]. FgaPT1 catalyzes the attachment of a prenyl moiety to C-2 of **11** isolated from *P. commune* in the presence of DMAPP [110]. Until now, the enzymatic conversion of **8** to **12** or **15** to **13** is not yet investigated. It is speculated that this reaction is catalyzed by a cytochrome P450 monooxygenase (Scheme 23.1).

5.3 Formation of D-Lysergic Acid

The conversion of **16** to **5**, as the acyl component of **3** and **4** in *C. purpurea*, requires two oxidation steps to form paspalic acid **42** via **18** and subsequent isomerization of **42** to **5**. Disruption of *cloA*, encoding a cytochrome P450 monooxygenase in the EA gene cluster of *C. purpurea*, resulted in the abolishment of **4** production, whereas the immediate precursor of **16**, **18**, and **42** accumulated in the *cloA* deficient mutant. Restoration of ergopeptine **4** biosynthesis was observed, on the one hand, by feeding the *cloA* deficient mutant with **5** and, on the other hand, by complementation of the mutant with intact *cloA* gene [123]. In addition, cross-complementation analyses of *cloA* between *C. pupurea* and *C. fusiformis* confirmed the function of CloA as elymoclavine oxidase. The EA biosynthesis in *C. purpurea* proceeds via **16**, **18**, and **42** to **5** and the ergopeptines **4**, while *C. fusiformis* is only able to produce **16** and **18**. Due to mutations and rearrangements of *cloA* in *C. fusiformis*, the biosynthetic pathway ends on the level of **18**. Integration of intact *cloA* from *C. purpurea* into *C. fusiformis* led to the production of **4**. In summary, these experiments showed the catalytic role of CloA bridging the biosynthesis of clavine alkaloids and D-lysergic acid-derived EAs in *C. purpurea* [115]. Until now, the conversion of **16** to **18** as well as **42** to **5** is not investigated biochemically. No information on the structural genes is available (Scheme 23.1).

5.4 Biosynthesis of Ergoamides and Ergopeptines

C. purpurea produces simple D-lysergic acid alkyl amides like **17** and ergopeptines **4**. The tripeptidyl moiety of **4** in *C. purpurea* contains in the first and second position variable unpolar amino acids and L-proline in the third position. Biochemical investigations revealed that biosynthesis of **4** are catalyzed by two interacting NRPSs. The biosynthetic gene cluster of EAs in *C. purpurea* includes four NRPS genes, *lpsA1*, *lpsA2*, *lpsB*, and *lpsC*. The lysergyl peptidyl synthetases LPSA1 and LPSA2, encoded by *lpsA1* and *lpsA2*, contain both trimodular domains ($A_1TC_1A_2TC_2A_3TC_3$). In contrast, LPSB and LPSC encoded by *lpsB* and *lpsC* contain only monomodular domains (ATC). In addition, LPSC includes a further domain termed as R-domain (reduction). The function of LPSA1 was investigated by deletion experiments in *C. purpurea* strain P1, which produces **19**, **20**, and **22**. **22**, with a tripeptide moiety of phenylalanine, alanine, and proline, was absent in the resulted deficient mutant, while **19** and **20**, with a tripeptide moiety of valine, proline, and leucine or isoleucine, were still produced by the mutant. These results prove that LPSA1 is involved in the formation of the tripeptide moiety of **22**, but not in the formation of the tripeptide moiety of **19** and **20**. In addition, these findings indicate that the formation of **4** with altered peptidyl residues is determined by NRPS specificity [37]. The second NRPS gene *lpsB* was investigated by several approaches. Analysis of the *lpsB* disruption mutant in *C. purpurea* P1 showed inability to produce any ergopeptines, whereas accumulation of **5** was observed. After heterologous expression of *lpsB* in *E. coli*, the purified enzyme LPSB was assayed with **5** and amino acids presented in the peptidyl moieties of **4**. LPSB was shown to be responsible only for **5** activation and incorporation into the ergopeptine skeleton. The connection of **5** with the tripeptidyl residue containing alanine, phenylalanine, and proline catalyzed by NRPSs is proposed to start with activation of **5** by the A-domain of the monomodular LPSB and subsequent transfer to the T-domain, which acts as peptidyl carrier protein binding D-lysergic acid as thioester. In comparison, the trimodular LPSA1 catalyzes the activation of alanine, phenylalanine, and proline by the A-domains and transfers them to the 4′-phosphopantetheinyl residues of the three T-domains. **5** is transferred to the trimodular LPSA1 and is then incorporated to the D-lysergyl mono-, di-, and tripeptides. It is presumed that the formation of the peptide bond between **5** and alanine is catalyzed by the C-domain of LPSB in a condensation reaction. The elongation to a tripeptide attaching phenylalanine and proline is then catalyzed by the C_1- and C_2-domain of LPSA1. The C_3-domain of LPSA1 releases the D-lysergyl tripeptide from the enzyme complex as lysergyl peptide lactam, after the cyclization between phenylalanine and proline. To obtain ergopeptine **4** as the end product one further heterocyclization is needed, which is catalyzed by a putative cytochrome P450 monooxygenase (Scheme 23.2).

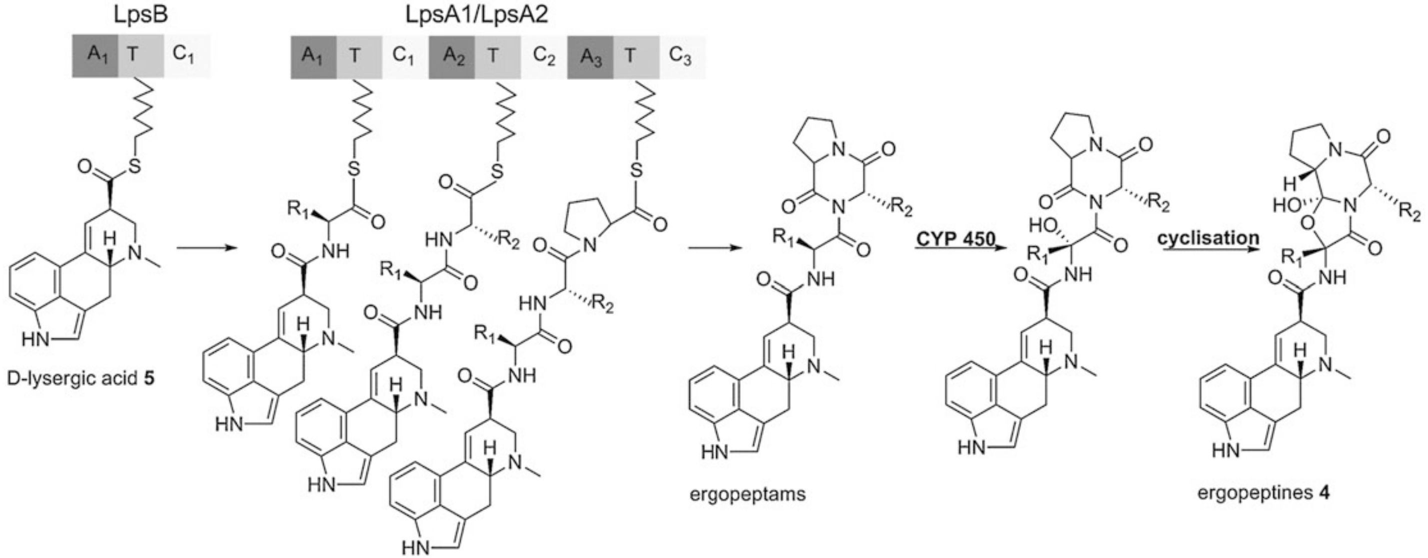
6 Industrial Production of Ergot Alkaloids

6.1 Life Cycle of *Claviceps purpurea*

As aforementioned, *C. purpurea* is the most important fungus in the *Claviceps* genus. Approximately 400 species of grasses serve as hosts for the infection by *C. purpurea* [125, 126]. At the beginning of the life cycle, ascospores of *C. purpurea* infect unfertilized rye ovaries. Under unfavorable conditions, a sclerotium is formed as resting structure of the fungus. At springtime, fruiting bodies composing of perithecia with haploid ascospores are developed from the sclerotium. After releasing the ascospores from the tubular asci, they are transported by wind to flourishing rye ears and initiate the primary infection of the host. The spores attach to the stigmatic hairs of the ovary style and begin to germinate. Penetration of the hyphae into the host is probably enforced by the release of cell wall-dissolving enzymes [4]. The hyphae grow toward the ovary base and after reaching the base a massive colonization of the entire ovary occurs by extensive branching of the hyphae. Tissues below the base (rachilla) are not affected by fungal hyphae. Constriction of the hyphae leads to the formation of conidiospores which serve for vegetative propagation of the fungus before the sclerotium is formed again. Approximately one week after infection, a syrupy fluid is discarded, which is referred to as honeydew. The conidiospores are exuded into the honeydew. The sugary honeydew attracts insects to spread the spores to other unfertilized ovaries in a secondary infection [127–129]. After about 2 weeks, the production of honeydew stops and leads to the maturation of the horny black sclerotium containing hardened fungal mycelium [128, 130]. After 5 weeks, the maturation of sclerotium is complete. The sclerotium ensures the survival of the fungus at low temperatures in winter. Reaching of temperature to 20 °C in springtime, germination can occur beneath the soil surface [131].

6.2 Field Production of Ergot Alkaloids on Rye

Initially, EAs were produced on a large scale by field cultivation on infected rye or triticale. *C. purpurea, C. fusiformis*, and *Claviceps paspali* are generally applied as parasitic fungi to produce ergopeptines **4**, clavine alkaloids, and single lysergic acid derivatives as precursors for partial synthetic drugs, e.g., **22** [2, 77]. Chemical synthesis is time-consuming and expensive. Inoculation of hosts with parasitic cultures can be performed either with specific pricking machinery or by spraying conidial suspension initiating the primary infection cycle. Improvement of field


LpsB
A_1 T C_1
LpsA1/LpsA2
A_1 T C_1 A_2 T C_2 A_3 T C_3
D-lysergic acid 5
ergopeptams
CYP 450
cyclisation
ergopeptines 4

Scheme 23.2 Formation of Ergopeptines by LpsB, LpsA1, and LpsA2, respectively (Modified after [4]) cultivation technology results in higher yield of generated EAs [132]. Several cell lines of rye were interbred with each other to obtain a hybrid cell line with induced male-sterility. Due to prevented self-pollination the florets stay open longer increasing the probability of contact of the spore suspension with the stigmatic hairs of the ovary style [133]. The EAs are synthesized during the sexual life cycle of the parasitic fungi on their hosts. Mutants of *Claviceps* strains lead to increase of generated sclerotia from 400 to 1,000 kg per hectare [134]. Field cultivation on rye exhibits both advantages and disadvantages. The genetic variability of *Claviceps* strains leads to the production of a wide range of EAs. In addition, *Claviceps* strains used for cultivation on rye show genetic stability in comparison to submerged cultures. Furthermore, hybrid ryes with prolonged flowering time were used for field cultivation to ensure efficient infection by conidial suspension. However, the disadvantages of parasitic EA production are the dependency on environmental conditions such as weather, alternating quality of hybrid rye, and expensive purification processes [77].

6.3 Production of Ergot Alkaloids in Submerged Cultures

The submerged cultivation is also used for the production of EAs, e.g., **3**,**4**, and **5** [135]. The submerged cultivation requires a special media and is accomplished independently of the host organisms. The prerequisite for successful cultivation are suitable strains, which are capable to produce EAs in submerged cultures. To obtain appropriate fungi cultures, various *Claviceps* strains were isolated from its sclerotia modified by mutagenesis and selected according to desired properties. **5** serves as a starting compound for the production of partially synthetic drugs such as **31** or **39**. Only low concentrations of pure **5** can be obtained in cultivation processes [77]. Therefore, isomerization of **42** or hydrolysis of **3** or **4** to **5** was performed [136]. To assure a high quality of selected strains for EA production, storage in liquid nitrogen or freeze drying in a preservative solution consisting of sucrose and skimmed milk should be performed [137]. In nature, EA production is dependent on the formation of sclerotium during the life cycle of *Claviceps*. Mycelium at sphacelia stage is unable to synthesize EAs. The strains used in submerged cultures show different cell morphology [138]. The sclerotia-like cells possess the ability to produce EAs. Suitable medium for *Claviceps* cultivation in submerged culture contains characteristic components [139, 140]. Sucrose is used in a concentration of 300 g $L^{-\ 1}$ and glucose in concentration of 100 g L^{-1} [48]. An osmotic pressure of 10–20 bars is required to induce the formation of sclerotia-like cells, while the conidiation is inhibited at these conditions [141]. Furthermore, a noninhibitory, slowly metabolized carbon source such as mannitol, sorbitol, or sucrose is needed, which acts as nutrient for the fungus [142, 143]. Organic acids are used as carbon source for saturation of tricarboxylic acid cycle [48, 144] leading to increased synthesis of acetyl-CoA at glycolysis and citric acid cycle [145, 146]. Acetyl-CoA is the key substrate in the alkaloid and lipid synthesis [77]. Glyoxylate and pentose phosphate pathway supply the reducing agent which is provided for the mevalonate pathway and lipid biosynthesis [147]. Limitation of phosphate increases oxidative metabolism in cells of *Claviceps* strains and promotes the production of secondary metabolites such as EAs [147, 148].

6.4 Semisynthetic Modifications of Natural Ergot Alkaloids Yield Significant Pharmaceuticals

Dihydroergotamine **32** is produced by partial synthesis using the naturally occurring ergotamine **22**. Hydrogenation of the double bond at C-9 and C-10 of the **22** or amidation of dihydrolysergic acid with synthesized tripeptide results in the formation of **32** [149]. Dihydroergotoxine is a mixture consisting of **33**, **34**, **35**, and **36**. The peptidyl moieties of these compounds contain two identical amino acids, valine and proline, whereas the third amino acid differs from each other. Dihydroergotoxine is also obtained by hydrogenation of double bond between C-9 and C-10 of the natural EAs [76, 84]. Bromocryptine **37** is also an unnatural ergot alkaloid. It is maintained by brominating the C-2 of the α-ergocryptine indole ring [149].

7 Conclusion

The relevance of EAs for modern medicine, pharmaceutical and food industry, as well as for agriculture, leads to significant progresses in EA research. Identification of EA gene clusters in different Ascomycota facilitated the molecular biological and biochemical investigations of genes/enzymes in the EA biosynthesis. Based on the obtained genetic information, new EA-producing strains could be reconstructed by recombination of the biosynthetic genes and serve as sources for production of EAs with new structural features. The total yield could be improved by genetic manipulation. This field will be attractive for research on EAs in the near future.

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Alkaloids Derived from Anthranilic Acid: Quinoline, Acridone, and Quinazoline

24

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Contents

1 Introduction 717
2 Biosynthesis and Biogenesis 731
2.1 Quinoline and Acridone Alkaloids 731
2.2 Alkyl, Aryl, Alkylarylquinoline Alkaloids 824
2.3 Quinazoline Alkaloids 838
3 Biological Activity 842
3.1 Antiprotozoal Activity 844
3.2 Molluscicidal Activity 846
3.3 Antimicrobial Activity 847
3.4 Antitumor Activity 847
3.5 Enzyme Inhibitors 848
3.6 Antiplatelet Activity 848
3.7 Insecticide Activity 849
3.8 Miscellaneous Biological Activities 849
4 Conclusions 850
References 852

Abstract

In this chapter, some of the latest developments in the groups of natural products collectively known as the quinolin/one, quinazolin/one, and acridone alkaloids are presented. Emphasis is placed on their biogenesis, biological activities, and

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natural distribution. Quinolin/ones and acridones undoubtedly occur in greatest abundance in plants from the Rutaceae family. The 2-alkylquinolin/4(1*H*)-ones are typical constituents of the Rutaceae but, surprisingly, they have also been isolated from some bacteria, mainly from *Pseudomanas*. Fungus *Penicillium* has yielded a novel class of quinolin/ones, which is based on the combination of amino acids L-valine and L-isoleucine, anthranilic acid, and acetic acid, or these amino acids and tryptamine. They constitute two small groups quinolactacins and quinocitrinines, which are alkaloid types at present unknown from any other source. Quinazoline derivatives are less common in Rutaceae, and a variety of them are produced by bacteria, fungi, and marine animals. In recent years, a number of these alkaloids have been found to possess real pharmacological activity, which is discussed.

Keywords
Acridone • Biological activity • Biosynthesis • *Penicillium* • *Pseudomonas* • Rutaceae • Quinoline • Quinazoline

Abbreviations

ACP	Acyl carrier protein
AMP	Adenosine monophosphate
Ant	Anthranilic acid
ATP	Adenosine triphosphate
AuaAE	Genes required for quinoline alkaloid aurachin synthesis
CF	Cystic fibrosis
CML	Chronic myelogenous leukemia
CoA	Coenzyme A as part of a thioester, e.g., acetyl-CoA $(CH_3COSCoA)$
CoASH	Coenzyme A
DMAPP	Dimethylallyl diphosphate
FPP	Farnesyl diphosphate
GC-MS	Gas chromatograph-mass spectrometry
Ger	Geranyl
Gln	Glutamine
INCA	Instituto Nacional do Cancer (Brazil – www.inca.gov.br)
L-Ala	L-alanine
L-Asp	Aspartic acid
L-Trp	L-tryptophan
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NMR	Nuclear magnetic resonance spectroscopy

NRPS	Nonribosomal peptide synthetase
Phe	Phenylalanine
PKSs	Polyketide synthases
PPi	Inorganic diphosphate
PQS	Pseudomonas quinolone signal
pqsABCD	Genes required for PQS synthesis
pqsE	The function of the *pqsE* gene is not known but it is required for PQS synthesis
Pro	Proline
TNF	Tumor necrosis factor
Val	Valine

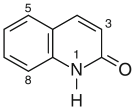
1 Introduction

The distribution, biogenesis, and biological activity of quinoline, quinazoline, and acridone alkaloids have been reviewed since 1953. Annual coverage has been provided by Openshaw (1953–1967), Snieckus (1972–195), Grundon (1976–1990), Michael (1991–2008), in Specialist Periodical Reports "The Alkaloids" (R. H. F. Manske and H. L. Holmes, ed; and The Chemical Society, Burlington House, London) and subsequently in "Natural Product Reports" [1–40]. In this chapter, some of the latest developments in the groups of natural products collectively known as the quinolin/one, quinazolin/one, and acridone alkaloids are presented. Emphasis is placed on their biogenesis, biological activities, and natural distribution.

In the context of this chapter, the term quinolin/one covers direct derivatives of anthranilic acid. The primary role of anthranilic acid in the formation of the quinoline nucleus has been substantiated by tracer experiments confirming the retention of the carbon atom of the carboxylic acid group and thereby eliminating the possibility of derivation via either the catabolism of tryptophan or some rearrangement analogous to that occurring in the biogenesis of quinine [41]. The term "pseudoquinoline" has been used to distinguish this group of alkaloids, and they were not included in this chapter.

Tables 24.1–24.31 list the different types of alkaloids indicated with their sources [1–162], and include research on quinolin/ones, quinazolin/one, and acridone alkaloids also listed in other reviews [1–42]. In order to bring up-to-data the distribution of these alkaloids the search was carried out in *Chemical Abstracts* (SciFinder) and Web of Science (to June, 2012). The biosynthesis of the alkaloids is discussed. In recent years, a number of these alkaloids have been found to possess real pharmacological activity, which will be discussed in the following sections.

Table 24.1 Occurrence of quinolin-2(1*H*)-one alkaloids in Rutaceae species



Alkaloids	Substituents	Occurrence
–	1-Me	*Galipea officinalis* [42]
–	4-OH	*Haplophyllum bucharicum* [34]
–	4-OMe	*Haplophyllum bucharicum* [34], *H. bungei* [23], *Hortia longifolia* [44], *Pilocarpus grandiflorus* [40]
–	1-Me, 4-OMe	*Aegle marmelos* [31], *Afraegle paniculata* [18], *Andreadoxa flava* [38], *Casimiroa edulis* [33], *Clausena lansium* [33], *Drummondita calida* [26], *Esenbeckia grandiflora* [30], *E. pentaphylla* [40], *Euodia lunu-ankenda* [19], *E. pilulifera* [24], *Geijera balansae* [21], *Glycosmis mauritiana* [43], *Haplophyllum dauricum* [18], *Hesperethusa crenulata* [42], *Hortia longifolia* [44], *Myrtopsis sellingii* [44], *Ruta chalepensis* [35], *R. montana* [34], *Sarcomelicope dogniensis* [24], *S. glauca* [21], *Tetradium glabrifolium* (= *Euodia meliaefolia*) [30], *Ticorea longiflora* [31], *Toddalia asiatica* [26], *Zanthoxylum beniensis* (*Fagara beniensis*) [42], *Z. ailanthoides* [17], *Z. cuspidatum* [44], *Z. decaryi* [44], *Z. dissitum* [30], *Z. integrifoliolum* [16], *Z. nitidum* [30], *Z. schinifolium* [32], *Z. simulans* [30]
Katimborine	1-Me, 4-O-β-neohesperidosyl	*Citropsis articulata* [45]

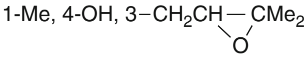
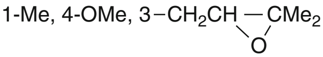
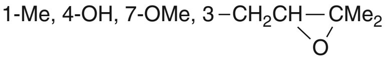
(*continued*)

Table 24.1 (continued)

Alkaloids	Substituents	Occurrence
Haplafine	4-$OCH_2CH = CMe_2$	*Haplophyllum myrtifolium* [25], *H. perforatum* [21]
Haplotusine	1,4-OMe	*Haplophyllum obtusifolium* [19]
Swietenidine B	3,4-diOMe	*Chloroxylon swietenia* [44]
–	3-OMe, 4-OH, 1-Me	*Micromelum falcatum* [46]
N-Methylswietenidine B	3,4-diOMe, 1-Me	*Clausena anisata* [23], *Micromelum falcatum* [46], *Severinia buxifolia* [36]
Edulitine	4,8-diOMe	*Casimiroa edulis* [44], *Haplophyllum dauricum* [18], *H. bungei*, *H. foliosum*, *H. robustum* [42], *Murraya paniculata* [23], *Zanthoxylum ailanthoides* [38], *Z. nitidum* [30], *Z. simulans* [28]
Folifidine	1-Me, 4-OMe, 8-OH	*Haplophyllum bucharicum*, *H. dubium*, *H. foliosum* [42]
Glycolone	4,8-diOMe	*Glycosmis pentaphylla* [19]
Folimine	1-Me, 4,8-diOMe	*Almeidea rubra* [32], *Boronia pinnata* [35], *Haplophyllum bungei* [23], *H. cappadocicum* [22], *H. dauricum* [18], *H. foliosum*, [42], *H. obtusifolium* [19], *H. perforatum* [44]
Integriquinolone	1-Me, 4-OMe, 6-OH	*Aegle marmelos* [31], *Limonia acidissima* [27], *L. crenulata* [36], *Toddalia asiatica* [26], *Zanthoxylum ailanthoides* [38], *Z. integrifoliolum* [16]
–	1-Me, 4,6-diOMe	*Agathosma barosmae folia* [31], *A.* spec. nov. [24]
Folidine	1-Me, 4-OMe, 8-$OCH_2COCHMe_2$	*Haplophyllum foliosum* [21]

(*continued*)

Table 24.1 (continued)

Alkaloids	Substituents	Occurrence
Casimiroine	1-Me, 4-OMe, 7-OCH_2O-8	*Casimiroa edulis* [42], *C. pubescena* [39], *Dictyoloma vandellianum* [23]
Halfordamine	4,6,8-triOMe	*Agathosma bisulca*, *A. capensis*, *A.* sp. nov. [21], *Halfordia scleroxyla* [44]
Swietenidine A	1-Me, 3,8-diOMe, 4-OH	*Chloroxylon swietenia* [44]
Ravenine	1-Me, 4-$OCH_2CH = CMe_2$	*Ravenia spectabilis* [42]
Atanine	3-$CH_2CH = CMe_2$, 4-OMe	*Afraegle paniculata* [44], *Euodia rutaecarpa* [29], *Haplophyllum canaliculatum* [47], *Ravenia spectabilis* [42], *Zanthoxylum xanthoxyloides* (*Fagara xanthoxyloides*) [42], *Z. integrifolium* [33]
–	1-Me, 3-$CH_2CH = CMe_2$, 4-OH	*Almeidea guyanensis* [44]
Ravenoline	1-Me, 3-CH(Me)C(=CH_2)Me, 4-OH	*Ravenia spectabilis* [42]
Paraensine	1-Me, 3-CH(Me)C(OH)Me_2, 4-OH	*Euxylophora paraensis* [17], *Ravenia spectabilis* [25]
Semecapifoline	3-CH_2OMe, 4,7-diOMe	*Melicope semecarpifolia* (= *M. confusa*, *Euodia merrillii*) [36]
Melisemine	3-CHO, 4,6,7-triOMe	*Melicope semecarpifolia* [37]
–	1-H, 3,4,8-triOMe	*Eriostemon gardineri* [29]
Haplobungine	1-H, 4,7,8-triOMe	*Haplophyllum bungei* [23]
–	1-Me, 4,7,8-triOMe	*Dictyoloma vandellianum* [23], *Spathelia sorbifolia* [44]
–		*Galipea officinalis* [35]
–		*Drummondita cálida* [48]
–		*Toddalia aculeata* (= *T asiatica*) [40]
–	1-Me, 4,7-diOMe, 3-$CH_2CH(OH)C(OH)Me_2$	*Toddalia aculeata* (= *T asiatica*) [40]

(*continued*)

Table 24.1 (continued)

Alkaloids	Substituents	Occurrence
N-Methylatanine	1-Me, 3-$CH_2CH = CMe_2$, 4-OMe	*Almeidea guyanensis* [44], *Citrus grandis* [17], *Drummondita calida* [48], *Esenbeckia alata* [49], *Glycosmis trichanthera* (*G. calcicola*) [33], *Melicope indica* [44], *Thamnosma africana* [33], *Zanthoxylum beecheyanum* (*Z. arnottianum*) [39]
Schinifoline	3-$CH_2CH = C(=CH_2)Me$, 4-OMe	*Glycosmis cyanocarpa* [28], *Zanthoxylum schinifolium* [27], *Z. simulans* [27]
N-Methylschinifoline	1-Me, 3-$CH_2CH = C(=CH_2)Me$, 4-OMe	*Zanthoxylum schinifolium* [27], *Z. simulans* [27]
Haplosamine	1-Me, 3-$CH_2CH(OH)C(OH)(CH_2OH)Me$, 4 OMe	*Hapophyllum perforatum* [30]
Glycocitlone-A	1-Me, 3-$CH = CHC(OH)Me_2$, 4-OMe	*Glycosmis citrifolia* [34]
Glycosolone	1-Me, 3-$CH_2CH = CMe_2$, 4-OMe, 8-OH	*Glycosmis citrifolia* [34], *G. pentaphylla* [44]
Glycocitlone-B	1-Me, 3-$CH = CHC(OH)Me_2$, 4-OMe, 8-OH	*Glycosmis citrifolia* [34]
Glycocitlone-C	1-Me, 3-$CH = CHC(OH)Me_2$, 4-OMe, 8-OMe	*Glycosmis arborea* [39], *G. citrifolia* [34]
Homo-glycosolone	1-Me, 3-$CH_2CH = CMe_2$, 4-OCH_2CH_3, 8-OH	*Glycosmis pentaphylla* [21]
Glycolone	1-Me, 3-$CH_2CH = CHMe_2$, 4-OCH_2CH_3, 8-OH	*Glycosmis pentaphylla* [23], *Zanthoxylum scandens* [27]
–	1-Me, 4-$OCH_2CH(OH)C(OH)Me_2$	*Euxylophora paraensis* [44]
O-Methylglycosolone	1-Me, 3-$CH_2CH = CMe_2$, 4,8-diOMe	*Glycosmis arborea* [33], *G. citrifolia* [18], *G. mauritiana* [44], *Zanthoxylum scandens* [27]
Zascanol epoxide	1-Me, 4,8-diOMe, 3–$CH_2\underbrace{CH-C}_{O}(CH_2OH)Me$	*Zanthoxylum scandens* [27]
Acutifolidin	3-$CH(OH)CH_2C(=CH_2)Me$, 4,8-diOMe	*Zanthoxylum acutifolium* [27]
Acutifolin	1-Me, 3-$CH(OH)CH_2C(=CH_2)Me$, 4,8-diOMe	*Glycosmis arborea* [39], *Zanthoxylum acutifolium* [27]

(*continued*)

Table 24.1 (continued)

Alkaloids	Substituents	Occurrence
(−)-*O*-Methylacutifolin	1-Me, 3-CH(OMe)$CH_2C(=CH_2)$Me, 4,8-diOMe	*Zanthoxylum acutifolium* [27]
Acutifolin palmitate	1-Me, 3-CH[OCO$(CH_2)_{14}$Me]$CH_2C(=CH_2)$ Me, 4,8-diOMe	*Zanthoxylum acutifolium* [27]
Glycophylone	1-Me, 3-$CH_2CH = CMe_2$, 4-OH, 8-OMe	*Glycosmis citrifolia* [34], *G. pentaphylla* [18]
–	3-$CH_2CH = CMe_2$, 4-OMe, 7-OCH_2O-8	*Ptelea trifoliata* [42]
Edulinine	1-Me, 3-$CH_2CH(OH)C(OH)Me_2$, 4-OMe	*Amyris diatrypa* [19], *Boronella pancheri* [28], *Casimiroa edulis* [44], *Citrus macroptera* [42], *Dutaillyea drupacea* [44], *Euodia gracilis* [23], *Haplophyllum foliosum* [15], *H. patavinum* [36], *Melicope semecarpifolia* [37], *Orixa japonica* [39], *Ruta graveolens* [42], *Teclea nobilis* [22], *T. simplicifolia* [22], *Zanthoxylum mayu* (*Fagara mayu*) [44], *Z. simulans* [16], *Z. usambarense* [30], *Z. williamsii* [44]
(−)-Acetoxyedulinine	1-Me, 3-$CH_2CH(OAc)C(OH)Me_2$, 4-OMe	*Skimmia laureola* [32]
(−)-Ptelefoliarine	1-Me, 3-$CH_2CH(OH)C(=CH_2)$Me, 4-OMe	*Skimmia laureola* [32]
(−)-Acetoxyptelefoliarine	1-Me, 3-$CH_2CH(OAc)C(=CH_2)$Me, 4-OMe	*Skimmia laureola* [32]
Orixiarine	1-Me, 3-$CH_2COCHMe_2$, 4-OMe	*Orixa japonica* [35], *Skimmia laureola* [32]
–	3-$CH_2CH = CMe_2$, 4-$OCH_2CH = CMe_2$	*Euodia roxbughiana* [30], *Haplophyllum bucharicum* [17], *H. tuberculatum* [42]
Pteleprenine	1-Me, 3-$CH_2CH = CMe_2$, 4-OMe, 7-OCH_2O-8	*Orixa japonica* [28], *Ptelea trifoliata* [44]
N-demethyllunidonine	3-$CH_2COCHMe_2$, 4-OMe, 7-OCH_2O-8	*Orixa japonica* [28], *Ptelea trifoliata* ssp. *pallida* var. *confinis* [44]

(*continued*)

Table 24.1 (continued)

Alkaloids	Substituents	Occurrence
Preskimmianine	3-$CH_2CH = CMe_2$, 4,7,8-triOMe	*Acronychia oligophylebia* [19], *Boronia bowmanii* [28], *B. pinnata* [35], *Citrus grandis* [17], *Dictaminus albus*, *D. angustifolius* [42], [44], *D. dasycarpus* [33], *Orixa japonica* [39]
Lunacridine	1-Me, 3-$CH_2CH(OH)CHMe_2$, 4,8-diOMe	*Lunasia amara*, *L. amara* var. *repanda*, *L. costulata* [42], *Zanthoxylum budrunga* [37]
Orixalone A	1-Me, 3-$CH_2COCHMe_2$, 4,8-diOMe	*Lunasia amara* [50], *Orixa japonica* [39]
Orixalone B	1-Me, 3-$CH_2COC(=CH_2)$ Me, 4,8-diOMe	*Orixa japonica* [39]
Orixalone C	3-$CH_2COC(=CH_2)$ Me, 4,8-diOMe	*Orixa japonica* [39]
Foliosidine	1-Me, 4-OMe, 8-$OCH_2CH(OH)C(OH)Me_2$	*Haplophyllum dubium*, *H. foliosum* [42], *H. perforatum* [44]
Foliphorin	1-Me, 4-OMe, 8-$OCH_2CH(OAc)C(OH)Me_2$	*Haplophyllum foliosum* [34]
Daurine	1-Me, 4-OMe, 8-$OCH_2CH = CMe_2$	*Haplophyllum dauricum* [17]
–	1-Me, 3,3-di$CH_2CH = CMe_2$ 1,2,3,4-tetrahydro, 4 = O	*Esenbeckia almawillia* [39], *E. flava* [44]
Buchapsine (Buchapine)	3-$CH_2CH = CMe_2$, 3-$C(Me)_2CH = CH_2$, 1,2,3,4-tetrahydro, 4 = O	*Euodia roxbughiana* [30], *Haplophyllum bucharicum* [17], *H. tuberculatum* [22]
rac-Haplotubinone	3-CH(1′,3-epoxide)CH [$C(OH)Me_2$] $CH_2CH = CMe_2$, 3,4-dihydro, 4 = O	*Haplophyllum tuberculatum* [35]
(−)-Pinolinone	1-Me, 3-$CH_2CH = CMe_2$, 3-α-OH, 4-β-OH, 3,4-dihydro	*Boronia pinnata* [35]
Sveribuxine	3,3-di$CH_2CH = C(Me)CH_2CH_2CH = CMe_2$, 6-OH	*Severinia* (= *Atalantia*) *buxifolia* [33]
–	1-Me, 3-$CH_2CH = CMe_2$, 4,6,8-triOMe, 3,4-dihydro	*Ptelea trifoliata* [44]
Ptelefolidine	1-Me, 3-$CH_2CH(OH)C(=CH_2)Me$, 4-OMe, 7-OCH_2O-8	*Ptelea trifoliata* [42], *P. trifoliata* ssp. *pallida* var. *confinis* [44]
Lunidonine	1-Me, 3-$CH_2COCHMe_2$, 4-OMe, 7-OCH_2O-8	*Lunasia amara* var. *repanda* [42], *Orixa japonica* [39], *Ptelea trifoliata*, *P. trifoliata* ssp. *pallida* var. [44]

(*continued*)

Table 24.1 (continued)

Alkaloids	Substituents	Occurrence
2′-Hydroxylunidonine	1-Me, 3-$CH_2CH(OH)CHMe_2$, 4-OMe, 7-OCH_2O-8	*Lunasia amara* [50]
N-Methylpreskimmianine	1-Me, 3-$CH_2CH = CMe_2$, 4,7,8-triOMe	*Aralipsis tabouensis* [23], *Vepris louisii* [44]
Lunidine	1-Me, 3-$CH_2CH(OH)CHMe_2$, 4-OMe, 7-OCH_2O-8	*Lunasia amara* var. *repanda* [42], *Ptelea trifoliata* [42]
(−)-Balfourolone	1-Me, 3-$CH_2CH(OH)C(OH)Me_2$, 4,8-diOMe	*Balfourodendron riedelianum* [42]
(+)-Hydroxylunacridine	1-Me, 3-$CH_2CH(OH)C(OH)Me_2$, 4,8-diOMe	*Lunasia amara* [42], *L. amara* var. *repanda* [42], *Orixa japonica* [42]
Nororixine	3-$CH_2CH(OH)C(OH)Me_2$, 4-OMe, 7-OCH_2O-8	*Orixa japonica* [42]
Ptelefolidine methyl ether	1-Me, 3-$CH_2CH(OMe)C(=CH_2)Me$, 4-OMe, 7-OCH_2O-8	*Ptelea trifoliata* [42]
Ptelecortine	1-Me, 3-$CH_2CH = CMe_2$, 4,8-diOMe, 6-OCH_2O-7	*Ptelea trifoliata* [44]
Ptelefoline	1-Me, 3-$CH_2CH(OH)C(=CH_2)Me$, 4,6,8-triOMe	*Ptelea trifoliata* [42]
Hydroxylunidonine	1-Me, 3-$CH_2COC(OH)Me_2$, 4-OMe, 7-OCH_2O-8	*Ptelea trifoliata* [44]
Isoptelefoline	1-Me, 3-$CH_2CH(OH)C(=CH_2)Me$, 4,7,8-triOMe	*Ptelea trifoliata* [42]
Hydroxylunidine	1-Me, 3-$CH_2CH(OH)C(OH)Me_2$, 4-OMe, 7-OCH_2O-8	*Lunasia amara* [42], *L. amara* var. *repanda* [42], *Ptelea trifoliata* [42]
Ptelefoline methyl ether	1-Me, 3-$CH_2CH(OMe)C(=CH_2)Me$, 4,6,8-triOMe	*Ptelea trifoliata* [44]
Pteleoline	1-Me, 3-$CH_2CH_2CH(Me)COOMe$, 4-OMe, 7-OCH_2O-8	*Ptelea trifoliata* [42]
Ptelefructine	1-Me, 3-$CH_2CH(OH)C(=CH_2)Me$, 4,8-diOMe, 6-OCH_2O-7	*Ptelea trifoliata* [42]
–	1-Me, 3-$CH_2COCHMe_2$, 4,6-diOMe, 7-OCH_2O-8	*Ptelea trifoliata* [44]
–	1-Me, 3-$CH_2CH(OH)CHMe_2$, 4,6-diOMe, 7-OCH_2O-8	*Ptelea trifoliata* [44]
Veprisilone	1-Me, 3-$CH_2COC(OH)Me_2$, 4,7,8-triOMe	*Vepris louisii* [44]
Evomeliaefolin	3-$CH(OH)CH_2COMe$, 4,7,8-triOMe	*Glycosmis citrifolia* [30], *Tetradium glabrifolium* (= *Euodia meliaefolia*) [30]
Dasycarine	3-$CH_2CH = CMe_2$, 4,5,8-triOMe	*Dictamnus dasycarpus* [35]

(*continued*)

Table 24.1 (continued)

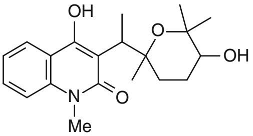
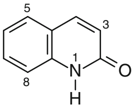
Alkaloids	Substituents	Occurrence
Glycocitridine	3-CHO, 4,7,8-triOMe	*Glycosmis citrifolia* [30], *Melicope semecarpifolia* [37]
–	1-Me, 3-$CH_2CH(OH)C(OH)Me_2$, 4,6-diOMe, 7-OCH_2O-8	*Ptelea trifoliata* [44]
–	1-Me, 2,4- di = O, 3-OH, 3-CH_2COOMe	*Micromelum falcatum* [46]
–	1-Me, 2,4- di = O, 3-OH, 3-CH_2COMe	*Micromelum falcatum* [46]
Megistoquinone II	3-CH = CH-OMe, 4,7-diOMe, 4,8-di = O	*Sarcomelicope megistophylla* [36]
Bucharaine	4-OCH_2CH = $C(Me)CH_2CH_2CH(OH)C(OH)Me_2$	*Haplophyllum bucharicum* [42]
Bucharidine		*Haplophyllum bucharicum* [42]

Table 24.2 Occurrence of quinolin-2(1*H*)-one alkaloids in the non-rutaceous species



Alkaloids	Substituents	Occurrence
–		*Aconitum ferox* (after Ayurvedic processing) (Ranunculaceae) [28]
–	3,4-dihydro, 6-OH	*Aconitum ferox* (after Ayurvedic processing) [28]
–	4-*p*-OMePh, 5,7-diOMe	*Chiococca alba* (Rubiaceae) [24]
–	4-*p*-OMePh, 5,7-diOMe, 8-OH	*Chiococca alba* [24]
–	4-$COOCH_2CH_3$	*Brucea javanica* (Simaroubaceae) [24]
(+)-Sterculinine I	4-CONHCH(COOMe)CH_2COO-*n*-Bu	*Sterculia lychnophora* (Sterculiaceae) [37]
(+)-Sterculinine II	4-CONHCH(COOMe)CH_2COOMe	*Sterculia lychnophora* (Sterculiaceae) [37]
–	4-COOMe, 6-OH	*Oryza sativa* cv. *Heugjinmi* (Poaceae) [36]
–	4-COOMe, 6-OMe	*Oryza sativa* cv. *Heugjinmi* [36]
–	4-OMe	*Strychnos cathayensis* (Loganiaceae) [35]
–	4-OMe, 8-Oglucose	*Echinops gmelinii* (Asteraceae) [39]

(*continued*)

Table 24.2 (continued)

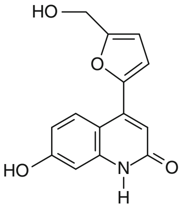
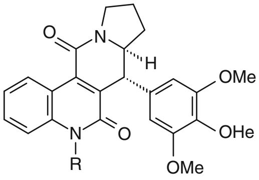
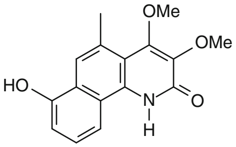
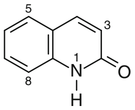
Alkaloids	Substituents	Occurrence
–	1-Me, 4-OMe, 8-Oglucose	*Echinops gmelinii* (Asteraceae) [39]
–	3,4,8-triOMe	*Sebastiania corniculata* (Euphorbiaceae) [39]
–		*Aquilegia ecalcarata* (Ranunculaceae) [36]
(+)-Isaindigotidione		*Isatis indigotica* (Brassicaceae) [32]
Asimicilone		*Asmina parviflora* (Annonaceae) [27]

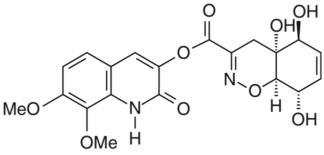
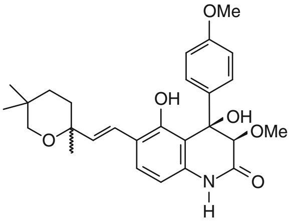
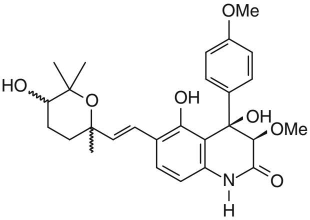
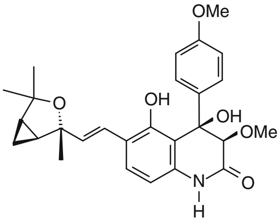
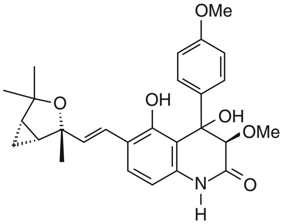
Table 24.3 Occurrence of quinolin-2(1*H*)-one alkaloids in fungi, bacteria, and marine organisms



Alkaloids	Substituents	Occurrence
Viridicatin	3-OH, 4-Ph	*Penicillium citrinum* [24], *P.crustosum* [39]
Viridicatol	3-OH, 4-*o*-OH-Ph	*Penicillium crustosum* [39]
–	4α-*p*-OMePh, 4β-OH, 3β-OMe, 3,4-dihydro	*Penicillium* sp. NTC-47 [31], *P*. cf. *simplicissimum* [35]
–	4α-*p*-OMePh, 4β-OH, 3β-OMe, 5-OH, 3,4-dihydro	*Penicillium* sp. NTC-47 [31], *P*. cf. *simplicissimum* [35]
(−)-Peniprequinolone	4α-*p*-OMePh, 4β-OH, 3β-OMe, 5-OH, 6-$CH_2CH = CMe_2$, 3,4-dihydro	*Penicillium*. cf. *simplicissimum* [35], *P. janczewskii* [39]

(*continued*)

Table 24.3 (continued)

Alkaloids	Substituents	Occurrence
–	4-OH, 6-Br	*Hyrtios erecta* (sponge) [36]
–	4-OH, 6,7-diBr	*Hyrtios erecta* (sponge) [36]
–	1-SH, 4-COH	*Pseudomonas fluorescens* G308 [36]
Penicillazine	 O OH OH O N O H OH MeO N O OMe H	*Penicillium* sp. no. 386 [35]
(+)-Penigequinolones A and B (diastereoisomers)	 OMe OH OH OMe O N O H	*Penicillium* sp. no. 410 [31], *Penicillium* sp. NTC-47 [31], *P.* cf. *simplicissimum* [35]
Aspoquinolone C and D (diastereomers)	 OMe HO O OH OH OMe N O H	*Aspergillus nidulans* [51]
Aspoquinolone A	 OMe O OH OH OMe N O H	*Aspergillus nidulans* [51]
Aspoquinolone B	 OMe O OH OH OMe N O H	*Aspergillus nidulans* [51]

(*continued*)

Table 24.3 (continued)

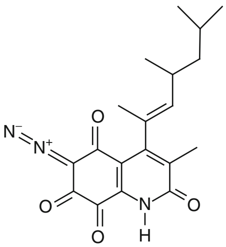
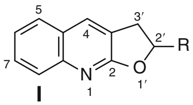
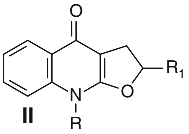
Alkaloids	Substituents	Occurrence
Lagunamycin		*Streptomyces* strain AA0 [44]

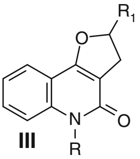
Table 24.4 Occurrence of dihydrofuroquinoline/one alkaloids in Rutaceae species



I



II



III

Alkaloids	Substituents	Occurrence
Platydesmine	**I**: 2′-C(OH)Me_2, 4-OMe	*Amyris diatrypa* [19], *Choisya ternata* [37], *Comptonella sessilifoliola* [25], *Dictamnus dasycarpus* [29], *Euodia macrocarpa* [24], *Flindersia fournieri* [15], *Geijera balansae* [21], *G. salicifolia* [42], *Haplophyllum perforatum* [44], *Melicope perspicuinervia* [44], *M. semecarpifolia* (*M. confusa*, = *Euodia merrillii*) [30], *Pilocarpus grandiflorus* [40], *Platydesma campanulata* [42], *Zanthoxylum acanthopodium* [24], *Z. ailanthoides* [38], *Z. avicennae* [44], *Z. integrifoliolum* [16], *Z. parviflorum* [42], *Z. schinifolium* [35], *Z. simulans* [37]
(+)-(R)-Platydesminium	**I**: 2′-C(OH)Me_2, 4-OMe, 1-Me	*Araliopsis tabouensis* [44], *Flindersia fournieri* [44], *Ruta graveolens* [42, 44], *Skimmia japonica* [42], *Zanthoxylum chalybeum* [30], *Z. usambarense* [29]
(−)-(S)-Platydesminium	**I**: 2′-C(OH)Me_2, 4-OMe, 1-Me	*Choisya ternata* [44]
Dubinidine	**I**: 2′-C(OH)(CH_2OH)Me, 4-OMe	*Dictamnus angustifolius* [42], *Haplophyllum dubium* [42], *H. foliosum* [42, 44], *H. perforatum* [44]

(*continued*)

Table 24.4 (continued)

Alkaloids	Substituents	Occurrence
(+)-Riedelianine	**I**: 2′-C(OH)Me_2, 6-OH	*Balfourodendrom riedelianum* [18]
Myrtopsine	**I**: 2′-C(OH)Me_2, 3′-OH, 4-OMe	*Haplophyllum foliosum* [16], *Myrtopsis sellingii* [44], *Zanthoxylum integrifoliolum* [16]
7,8-Dimethoxymyrtopsine	**I**: 2′-C(OH)Me_2, 3′-OH, 4,7,8-triOMe	*Boronella pancheri* [28], *Dictamnus dasycarpus* [29], *Dutaillyea baudouinii* [26], *Melicope semecarpifolia* (= *M. confusa*, *Euodia merrillii*) [36]
Lunasine	**I**: 2′-CHMe_2, 4,8-diOMe, 1-Me	*Lunasia costulata*, *L. quercifolia* [42]
Ribalinium	**I**: 2′-C(OH)Me_2, 4-OMe, 6-OH, 1-Me	*Balfourodendron riedelianum* [42], *Ruta graveolens* [42], *R. graveolens* spp. *hortensis* [44]
Pteleatinium	**I**: 2′-C(OH)Me_2, 4-OMe, 8-OH, 1-Me	*Ptelea trifoliata* [42]
Platidesmine acetate	**I**: 2′-C(OAc)Me_2, 4-OMe	*Geijera salicifolia* [42]
8-Methoxyplatydesmine	**I**: 2′-C(OH)Me_2, 4,8-diOMe	*Melicope semecarpifolia* [37]
(+)-3′-*O*-Acetylisopteleflorine	**I**: 2′-C(OAc)Me_2, 4-OMe, 7-OCH_2O-8	*Orixa japonica* [30]
(−)-Luninium	**I**: 2′-CHMe_2, 4-OMe, 7-OCH_2O-8, 1-Me	*Ptelea trifoliata* [42]
Ptelefolidonium	**I**: 2′-C(=CH_2)Me, 4-OMe, 7-OCH_2O-8, 1-Me	*Ptelea trifoliata* [23]
(+)-(R)-Balfourodinium	**I**: 2′-C(OH)Me_2, 4,8-diOMe, 1-Me	*Balfourodendron riedelianum* [42], *Orixa japonica* [42]
(−)-(S)-Balfourodinium	**I**: 2′-C(OH)Me_2, 4,8-diOMe, 1-Me	*Choisya ternata* [42], *Lunasia amara* [50]
(+)-Ptelefolonium	**I**: 2′-C(=CH_2)Me, 4,6,8-triOMe, 1-Me	*Ptelea trifoliata* [44], *P. trifoliata* ssp. *trifoliata* var. *trifoliata* [44]
–	**I**: 2′-C(=CH_2)Me, 4,7,8-triOMe, 1-H	*Dictamnus dasycarpus* [52]
(+)-Isoptelefolonium	**I**: 2′-C(=CH_2)Me, 4,7,8-triOMe, 1-Me	*Ptelea trifoliata* [13], *P. trifoliata* ssp. *trifoliata* var. *trifoliata* [44]
Dubinine	**I**: 2′-C(OH)(CH_2OAc)Me, 4-OMe	*Haplophyllum dauricum* [42]
(−)-Isopteleflorine	**I**: 2′-C(OH)Me_2, 4-OMe, 7-OCH_2O-8	*Orixa japonica* [35]
(−)-(S)-Hydroxyluninium	**I**: 2′-C(OH)Me_2, 4-OMe, 7-OCH_2O-8, 1-Me	*Choisya ternata* [13], *Ptelea trifoliata* [42], *P. trifoliata* ssp. *trifoliata* var. *trifoliata* [44]
7,8-Dimethoxyplatydesmine	**I**: 2′-C(OH)Me_2, 4,7,8tri-OMe	*Dictamnus dasycarpus* [29], *Dutaillyea baudouinii* [26], *Melicope semecarpifolia* (= *M. confusa*, *Euodia merrillii*) [36]

(*continued*)

Table 24.4 (continued)

Alkaloids	Substituents	Occurrence
Veprisinium	**I**: 2′-C(OH)Me_2, 4,7,8tri-OMe, 1-Me	*Vepris louisii* [44]
Ptelecultinium	**I**: 2′-C(=CH_2)Me, 4,8-diOMe, 1′,2′-dehydro, 1-Me (ClO_4^-)	*Ptelea trifoliata* [22]
2-Acetylevolitrine	**I**: 2′-COMe, 4,7-diOMe, 1′,2′-dehydro	*Melicope semecarpifolia* (= *M. confusa*, *Euodia merrillii*) [36]
2-Acetylpteleine	**I**: 2′-COMe, 4,6-diOMe, 1′,2′-dehydro	*Melicope semecarpifolia* (= *M. confusa*, *Euodia merrillii*) [36]
Isoplatydesmine	**II**: 2′-C(OH)Me_2, 1-Me	*Araliopsis soyauxii* [44], *A. tabouensis* [44], *Boronella pancheri* [28], *Citrus macroptera* [29], *Euodia gracilis* [23], *Haplophyllum patavinum* [36], *Melicope semecarpifolia* [37], *Orixa japonica* [39], *Pelea barbigera* [44], *Teclea nobilis* [22], *T. simplicifolia* [22], *Zanthoxylum nitidum* [30]
Methylisoplatidesmine	**II**: 2′-C(OMe)Me_2, 1-Me	*Skimmia laureola* [33]
Lunacrine	**II**: 2′-CHMe_2, 8-OMe, 1-Me	*Lunasia* amara, *L. amara* var. *repanda*, *L. costulata*, *L. quercifolia* [42]
(+)-Neoacutifolin	**II**: 2′-C(=CH_2)Me, 8-OMe, 1-Me	*Zanthoxylum acutifolium* [27]
Ribaline	**II**: 2′-C(OH)Me_2, 6-OH, 1-Me	*Balfourodendron riedelianum* [42], *Ruta graveolens* [42], *R. graveolens* ssp. *hortensis* [44]
Folisine	**II**: 2′-C(OH)(CH_2OH) Me, 1-Me	*Haplophyllum foliosum* [42]
Ptelefolidone	**II**: 2′-C(=CH_2)CH_2OH, 7-OCH_2O-8, 1-Me	*Ptelea trifoliata* [44]
Lunine	**II**: 2′-CHMe_2, 7-OCH_2O-8, 1-Me	*Lunasia amara*, *L. quercifolia* [42], *Ptelea trifoliata* [42]
(+)-Balfourodine	**II**: 2′-C(OH)Me_2, 8-OMe, 1-Me	*Balfourodendron riedelianum* [42], *Lunasia amara* [50], *Ptelea trifoliata* ssp. *pallida* var. *confinis* [44]
(−)-Hydroxylunacrine	**II**: 2′-C(OH)Me_2, 8-OMe, 1-Me	*Lunasia amara* [42]
Ptelefolone	**II**: 2′-C(=CH_2)Me, 6,8-diOMe, 1-Me	*Ptelea trifoliata* [42]
Hydroxylunine	**II**: 2′-C(OH)Me_2, 7-OCH_2O-8, 1-Me	*Lunasia amara* [42], *Orixa japonica* [42], *Ptelea trifoliata* [42], *P. trifoliata* ssp. *pallida* var. *confinis* [44]
Ifflaiamine	**II**: 1,2′,3′,3′-tetraMe	*Flindersia ifflaiana* [42] *Lunasia amara* [50]
8-Methoxyifflaiamine	**II**: 1,2′,3′,3′-tetraMe, 8-OMe	*Lunasia amara* [50, 54]
Lemobiline	**II**: 1,2′,2′,3′-tetraMe	*Euxylophora paraensis* [44], *Flindersia ifflaiana* [42], *Ravenia spectabilis* [42]

(*continued*)

Table 24.4 (continued)

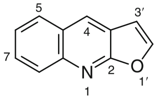
Alkaloids	Substituents	Occurrence
Bucharaminol	**II**: 2′,3′-diMe, 3′-$CH_2CH_2CH(OH)C(OH)Me_2$	*Haplophyllum acutifolium* [44]
Almeine	**III**: 2′-C(=CH_2)Me, 1-Me	*Almeidea guyanensis* [17, 44], *Andreadoxa flava* [38]
Araliopsine	**III**: 2′-C(OH)Me_2, 1-Me	*Araliopsis soyauxii* [44], *Zanthoxylum simulans* [16]
–	**III**: 2′,3′,3′-triMe, 1-H	*Euxylophora paraensis* [17]
Oligophyline	**III**: 2′,3′,3′-triMe, 1-Me	*Acrocychia oligophylebia* [19], *Andreadoxa flava* [38], *Euxylophora paraensis* [17]
Oligophylicine	**III**: 2′,3′,3′-triMe, 1-H, 8-OMe	*Acrocychia oligophylebia* [19]
Oligophylicidine	**III**: 2′,3′,3′-triMe, 1-H, 7,8-diOMe	*Acrocychia oligophylebia* [19]

2 Biosynthesis and Biogenesis

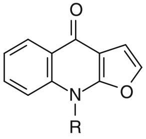
2.1 Quinoline and Acridone Alkaloids

Alkaloids derived from anthranilic acid undoubtedly occur in greatest abundance in plants from the Rutaceae family. Particularly well represented are alkaloids based on quinoline and acridone skeletons. A more direct route to the quinoline ring system is by the combination of anthranilic acid and acetate/malonate. Anthranilic acid-specific enzymes involved in the biosynthesis of dihydrofuro- and dihydropyranoquinolin/one alkaloids have been detected in tissue cultures of *Choisya ternata* and *Ruta graveolens* [101]. The biosynthesis pathway illustrated in Scheme 24.1 is supported by tracer feeding experiments with *Skimmia japonica*, *Choisya ternata*, and *Ptelea trifoliata*, in which the alkaloids were formed by the initial condensation of anthraniloyl-CoA and a 2-carbon unit (malonyl-CoA) to give rise to the quinoline ring system, normally in the form of a quinolin-2-one (**1**). This may then be followed by addition to **1** of a 3,3-dimethylallyl unit at the highly activated C-3 position to yield the 3-(3,3-dimethylallyl)-4-hydroxyquinolin-2-one (**2**). Methylation of the 4-hydroxyl group then occurs preferentially and ensures that subsequent oxidative cyclization gives compounds with linear annelation. The cyclization of **2** between C-2-*O* and C-2′ [probably via initial epoxidation of the side-chain, (**3**)] to 2′-isopropyl-2′,3′-dihydrofuroquinolines (**4**) and then, by loss of the isopropyl moiety, to furoquinolines (**7**) is now well established [102–107]. The mechanism by which loss of the isopropyl substituent occurs has not, as yet, been satisfactorily established, however, the structures of some alkaloids isolated have provided substantial indirect evidence that oxidation at C-3′ may be involved, e.g., 2′-isopropyl-3′-hydroxy-2′,3′-dihydrofuroquinoline myrtopsine (**6**, R = Me) isolated from *Myrtopsis sellingii* [108].

Table 24.5 Occurrence of furoquinolin/one alkaloids in Rutaceae species



I



II

Alkaloids	Substituents	Occurrence
Dictamnine	**I**: 4-OMe	*Acronychia pedunculata* [23], *A. pubescens* [31], *Aegle marmelos* [42], *Afraegle paniculata* [44], *Almeidea coerulea* [40], *Amyris pinnata* [44], *Andreadoxa flava* [38], *Balfourodendron riedelianum* [42], *Boenninghausenia albiflora* var. *japonica* [42], *Boronella* aff. *verticillata* [44], *Boronia bowmanii* [28], *B. coerulescens* spp. *spinescens* [29], *B. inornata* [29], *B. pinnata* [35], *Bouchardatia neurococca* [38], *Casimiroa edulis* [44], *Chorilaena quercifolia* [42], *Comptonella sessilifoliola* [25], *Decatropis bicolor* [42], *Dictamnus albus* [42], *D. angustifolius* [42, 44], *D. caucasicus* [42], *D. dasycarpus* [26], *Dutaillyea drupacea* [44], *D. oreophila* [44], *Drummondita calida* [26], *Esenbeckia alata* [49], *E. flava* [44], *E. leiocarpa* [24], *E. litoralis* [44], *E. pentaphylla* [40], *Euodia belahe* [42], *E. fargesii* [40], *E. lepta* [39], *E. litoralis* [42], *E. lunu-ankenda* [19], *E. macrocarpa* [24], *E. pachyphylla* [24], *E. roxburghiana* [24], *Feronia limonia* [19], *Flindersia acuminate* [42], *F. fournieri* [44], *F. maculosa* [42], *F. pimenteliana* [44], *F. pubescens* [42], *Geijera balansae* [21], *Glycosmis biloculares* [44], *G. mauritiana* [44], *G. pentaphylla* [42], *G. trichanthera* (*G. calcicola*) [33], *Halfordia kendack* [44], *Haplophyllum bucharicum* [42], *H. bungei* [42], *H. buxbaumii* [22], *H.coppadocicum* [22], *H. dauricum* [18], *H. obtusifolium* [21], *H. myrtifolium* [38], *H. robustum* [44], *H. suaveolens* [42], *H. vucanicum* [30], *Helietta longifoliata* [42], *Hortia arborea* [42], *H. longifolia* [44], *Medicosma cunninghamii* [42], *Melicope indica* [44], *M. pteleifolia* [55], *M. semecarpifolia* (= *M. confusa*, *Euodia merrillii*) [36], *M. triphylla* [24], *Monnieria trifolia* [44], *Myrtopsis macrocarpa* [44], *M. myrtoidea* [44],

		M. novae-caledoniae [44], *M. sellingii* [44], *Neoraputia paraensis* [38], *Phebalium nudum* [42], *Phelodendron amurense* (callus tissue) [32], *Pilocarpus grandiflorus* [40], *Pitavia punctata* [42], *Ptelea trifoliata* [42], *Raputia heptaphylla* [56], *Ruta angustifolia* [42], *R. chalepensis* [42, 44], *R. chalepensis* var. *latifolia* [23], *R. corsica* [38], *R. graveolens* [42], *R. montana* [42], *Sarcomelicope argyrophylla* [21], *S. dogniensis* [24], *S. glauca* [21], *S. megistophylla* [34], *Skimmia foremanii* [42], *S. japonica* [42], *S. laureola* [57], *S. repens* [42], *Spiranthera odoratissima* [40], *Teclea amanuensis* [64], *T. natalensis* [39], *T. trichocarpa* [37], *Tetradium trichotomum* [25], *Ticorea longiflora* [31], *Toddalia aculeata* [44], *T. aculeate* var. *gracilus* [44], *T. asiatica* var. *gracili* [16], *T. asiatica* [33], *Zanthoxylum mayu* (*Fagara mayu*) [44], *Z. ailanthoides* [42], *Z. acanthopodium* [24], *Z. alatum* [42], *Z. arnottianum* [44], *Z. austrosinense* [23], *Z. avicennae* [26], *Z. belizense* [44], *Z. budrunga* [16], *Z. cuspidatum* [44], *Z. decaryi* [44], *Z. dissitum* [30], *Z. ekmanii* [39], *Z. inerme* [44], *Z. integrifoliolum* [16], *Z. nitidum* [60], *Z. parviflorum* [42], *Z. pistaciiflorum* [38], *Z. regnellianum* [30], *Z. schinifolium* [27], *Z. simulans* [44], *Z. stelligerum* [37], *Z. wutaiense* [59]
O-Ethylnordictamnine	**I**: 4-OCH_2CH_3	*Dictamnus dasycarpus* [21]
Confusameline	**I**: 4-OMe, 7-OH	*Euodia elleryana* [42], *Melicope confusa* [42], *M. lasioneura* [44], *Myrtopsis sellingii* [44], *Zanthoxylum ailanthoides* [38]
Robustine	**I**: 4-OMe, 8-OH	*Dictamnus albus* [39], *D. caucasicus* [42], *Euodia fargesii* [40], *Haplophyllum bucharicum* [42], *H. cappadocicum* [27], *H. dauricum* [18], *H. dubium* [42], *H. myrtifolium* [38], *H. pedicellatum* [42], *H. obtusifolium* [21], *H. ramosissimum* [22], *H. robustum* [42], *H. vulcanicum* [19], *Philotheca deserti* var. *deserti* [40], *Tetradium glabrifolium* (*Euodia meliaefolia*) [30], *Thamnosma montana* [44], *Toddalia aculeata* [44], *Zanthoxylum ailanthoides* [38], *Z. alatum* [44], *Z. arnottianum* [44], *Z. cuspidatum* [44], *Z. integrifoliolum* [16], *Z. schinifolium* [35], *Z. simulans* [28], *Z. stelligerum* [37]

(*continued*)

Table 24.5 (continued)

Alkaloids	Substituents	Occurrence
5-Methoxydictamnine	**I**: 4,5-diOMe	*Ruta chalepensis* var. *latifolia* [23], *Zanthoxylum nitidum* [60]
Pteleine	**I**: 4,6-diOMe	*Acronychia baeurlenii* [31], *Comptonella sessilifoliola* [25], *Drummondita calida* [26], *Dutaillyea drupacea*, *D. oreophila* [44], *Euodia macrocarpa* [24], *E. merrilli* [26], *Haplophyllum thesioides* [28], *Helietta longifoliata* [42], *Medicosma cunninghamii* [42], *Melicope triphylla* [24], *Philotheca deserti* var. *deserti* [40], *Platydesma campanulata* [42], *Ptelea trifoliata* [42], *Ruta graveolens* [42], *R. chalepensis* [35], *Teclea nobilis* [38], *Zanthoxylum ailanthoides* [40]
Evolitrine	**I**: 4,7-diOMe	*Acronychia laurifolia* (*A. pedunculata*) [34], *A. oligophylebia* [19], *A. pubescens* [31], *A. pedunculata* [44], *Almeidea guyanensis* [44], *A. rubra* [32], *Boronella koniambiensis* [40], *B. pancheri* [28], *Boronia bowmanii* [28], *B. inornata* [29], *B. pinnata* [35], *Comptonella sessilifoliola* [25], *Cusparia macrocarpa* [42], *Drummondita calida* [26], *Dutaillyea drupacea*, *D. oreophila* [44], *Esenbeckia flava* [44], *E. litoralis* [44], *Euodia belahe* [42], *E. fatraina* [29], *E. lepta* [39], *E. litoralis* [42], *E. lunu-ankenda* [19], *E. macrocarpa* [24], *E. pachyphylla* [24], *E. pilulifera* [24], *E. roxburghiana* [24], *Galipea longiflora* [24], *Glycosmis cyanocarpa* [44], *Medicosma subsessilis* [38], *Melicope confusa* [34], *M. indica* [44], *M. lasioneura* [44], *M. pteleifolia* [55], *M. semecarpifolia* (= *M. confusa*, *Euodia merrillii*) [36], *M. triphylla* [24], *Nematolepis phebalioides* [28], *Orixa japonica* [42], *Phebalium nudum* [42], *Philotheca deserti* var. *deserti* [40], *Platydesma campanulata* [42], *Ruta montana* [34], *Sarcomelicope argyrophylla* [21], *S. dogniensis* [24], *S. glauca* [21], *Ticorea longiflora* [31], *Zanthoxylum ailanthoides* [40], *Zieridium ignambiensis* [26]

γ-Fagarine	**I**: 4,8-diOMe	*Acronychia laurifolia* (*A. pedunculata*) [34], *Aegle marmelos* [42, 44], *Amyris texana* [26], *Asterolasia drummondii* [28], *Boronia bowmanii* [28], *Casimiroa edulis* [44], *Chloroxylon swietenia* [42], *Dictamnus albus* [42], *D. angustifolius* [44], *D. caucasicus* [42], *D. dasycarpus* [29], *Drummondita calida* [26], *D. hasselli* [26], *Eriostemon coccineus* [26], *Ertela* (*Monnieria*) *trifolia* [61], *Erythrochiton brasiliensis* [44], *Esenbeckia alata* [49], *E. febrifuga* [62], *E. grandiflora* ssp. *brevipetiolata* [38], *E. hieronimi* [30], *E. pentaphylla* [40], *Euodia lunu-ankenda* [24], *Flindersia fournieri* [44], *Geijera balansae* [21], *G. salicifolia* [42], *Glycosmis arborea* [42], *G. citrifolia* [30], *G. pentaphylla* [42], *G. trichanthera* (*G. calcicola*) [33], *Haplophyllum bucharicum* [42], *H. buxbaumii* [19], *H. dahuricum* [16], *H. dubium* [14], *H. duricum* [44], H. *glabrinum* [21], *H. kowalenskyi* [44], *H. leptomerum* [21], *H. myrtifolium* [25], *H. obtusifolium* [21], *H. pedicellatum* [42], *H. robustum* [42], *H. schelkovnikovii* [44], *H. suaveolens* [18], *H. tenue* [44], *H. tuberculatum* [44], *H. villosum* [44], H. *vulcanicum* [19], *Helietta logifoliata* [37], *Hortia arborea* [42], *H. colombiana* [32], *Monnieria trifolia* [18], *Metrodorea flavida* [33], *Myrtopsis myrtoidea* [44], *M. novae-caledoniae* [44], *M. sellingii* [44], *Orixa japonica* [39], *Phebalium nudum* [42], *Philotheca deserti* var. *deserti* [40], *Phelodendron amurense* (callus tissue) [32], *Pitavia punctata* [42], *Ravenia spectabilis* [42], *Ruta chalepensis* [42], *R. corsica* [38], *R. graveolens* [42], *Ruta* callus cultures (*R. bracteosa*, *R. chalepensis*, *R. macrophylla*) [26], *Spiranthera odoratissima* [38], *Tetradium glabrifolium* [22], *Thamnosma montana* [42], *Ticorea longiflora* [31], *Toddalia aculeata* [44], *T. aculeate* var. *gracilus* [44], *T. asiatica* [14], *T. asiatica* var. *gracilis* [16], *T. asiatica* [33], *Vepris stolzii* [44], *Zanthoxylum coco* (*Fagara coco*) [42], *Z. mayu* (*F. mayu*) [44], *Z. zanthoxyloides* (*F. zanthoxyloides*, cell cutures) [29], *Z. ailanthoides* [38], *Z. alatum* [42], *Z. cuspidatum* [44], *Z. budrunga* (*Z. rhetsa*) [39], *Z. dimorphophyllum* [36], *Z. dissitum* [30], *Z. integrifoliolum* [39], *Z. monophyllum* [33], *Z. nitidum* [30], *Z. oxyphyllum* [44], *Z. pistaciiflorum* [38], *Z. rubescens* [19], *Z. schinifolium* [35], *Z. simulans* [44], *Z. tsihanimposa* [44], *Z. wutaiense* [59]

(*continued*)

Table 24.5 (continued)

Alkaloids	Substituents	Occurrence
O-Ethylnor-γ-fagarine	**I**: 4-OCH_2CH_3, 8-OMe	*Dictamnus dasycarpus* [21]
–	**I**: 4-OMe, 7-OH, 8-$CH_2CH = CMe_2$	*Haplophyllum tuberculatum* [27], *Sarcomelicope glauca* [21]
–	**I**: 4-OMe, 7-OH, 8-$C(Me_2)CH = CH_2$	*Haplophyllum glabrinum* [22]
–	**I**: 4,7-diOMe, 8-$C(Me_2)CH = CH_2$	*Almeidea coerulia* [32]
Evomerrine	**I**: 4-OMe, 7-OH, 8-CHO	*Melicope semecarpifolia* (*M. confusa*, = *Euodia merrillii*) [30]
(+)-Roxiamine A	**I**: 4-OMe, 7-$OCH_2CH_2CH(Me)$ COOMe	*Euodia roxburghiana* [30]
Roxiamine B	**I**: 4-OMe, 7-$OCH_2CH = C(Me)$ COOMe	*Euodia roxburghiana* [30]
(+)-Roxiamine C	**I**: 4-OMe, 7-$OCH_2CH_2CH(OH)Me$	*Euodia roxburghiana* [30]
(−)-Confusadine	**I**: 4-OMe, 7-$OCH_2CH(OH)C(=CH_2)$ Me	*Melicope semecarpifolia* [37]
Maculine	**I**: 4-OMe, 6-OCH_2O-7	*Araliopsis soyauxii* [44], *Esenbeckia febrifuga* [42], *E. grandiflora* [30], *E. hieronimi* [30], *E. leiocarpa* [24], *E. litoralis* [44], *E. pilocarpoides* [19], *Flindersia acuminata*, *F. bennettiana* [42], *F. dissosperma*, *F. maculosa*, *F. schottiana*, *F. xanthoxyla* [42], *Helietta longifoliata* [42], *H. parvifolia* [21], *Metrodorea flavida* [33], *Oricia suaveolens* [63], *Raulinoa echinata* [36], *Ptelea aptera* [42], *Sargentia gregii* [44], *Teclea nobilis* [22], *T. simplicifolia* [44], *T. sudanica* [44], *Vepris biloculares* [42], *V. punctata* [37]
Kokusagine	**I**: 4-OMe, 7-OCH_2O-8	*Esenbeckia pilocarpoides* [19], *Euodia lunu-ankenda* [19], *Lunasia amara* [42], *Orixa japonica* [42, 44]
Delbine	**I**: 4,7-diOMe, 6-OH	*Esenbeckia grandiflora* ssp. *brevipetiolata* [38], *Haplopyllum vulcanicum* [19], *Monnieria trifolia* [44]
Isohaplopine	**I**: 4,7-diOMe, 8-OH	*Teclea simplicifolia* [22], *Zanthoxulum arborescens* [16]
Heliparvifoline	**I**: 4,6-diOMe, 7-OH	*Haplophyllum thesioides* [28], *Helietta parvifolia* [44], *Melicope confusa* [21]

Haplopine	**I**: 4,8-diOMe, 7-OH	*Aegle marmelos* [44], *Afraegle paniculata* [44], *Dictamnus albus* [39], *D. dasycarpus* [32], *Dutaillyea baudouinii* [26], *Euodia fargesii* [40], *Geijera balansae* [21], *Haplophyllum acutifolium* [22], *H. bucharicum* [42], *H. cappadocicum* [27], *H. dauricum* [18], *H. dubium*, *H. ferganicum* [17], *H. foliosum* [42], *H. latifolium* [44], *H. obtusifolium* [18], *H. patavinum* [36], *H. pedicellatum* [42], *H. perforatum* [42, 44], *H. ramosissimum* [23], *H. robustum* [42], *H. vucanicum* [30], *Melicope lasioneura* [44], *M. semecarpifolia* (*M. confusa*, = *Euodia merrilli*) [30], *Monnieria trifolia* [44], *Oricia suaveolens* [63], *Sarcomelicope glauca* [21], *Skimmia laureola* [32], *S. reevesiana* [21], *Toddalia asiatica* [33], *Zanthoxylum ailanthoides* [38], *Z. arnottianum* [44], *Z. bungeanum* [19], *Z. cuspidatum* [44], *Z. dissitum* [30], *Z. integrifoliolum* [16], *Z. microcarpum* [44], *Z. nitidum* [39], *Z. rhoifolium* [26], *Z. simulans* [28]
(−)-Haplotubine	**I**: 4,8-diOMe, 7-$OCH_2CH=C(Me)CH_2CH_2CH(OH)C(OH)Me_2$	*Haplophyllum tuberculatum* [35]
–	**I**: 4,5,6-triOMe	*Haplophyllum buxbaumii* [19]
–	**I**: 4,5,7-triOMe	*Haplophyllum buxbaumii* [19], *H. myrtifolium* [25]
Kokusaginine	**I**: 4,6,7-triOMe	*Acronychia baueri* [42], *A. baeurlenii* [31], *A. laurifolia* (*A. pedunculata*) [34], *A. oligophylebia* [19], *A. pedunculata* [44], *A. pubescens* [31], *Almeidea rubra* [32], *Araliopsis soyauxii* [44], *Balfourodendron riedelianum* [42], *Bauerella simplicifolia* ssp. *neo-scotica* [44], *Brombya* sp. nov [40], *Choisya arizonica*, *C. mollis*, *C. ternata* [42], *Clausena dunniana* [38], *Comptonella sessilifoliola* [25], *Dutaillyea drupacea*, *D. oreophila* [44], *Esenbeckia alata* [49], *E. belizencis* [27], *E. febrifuga* [62], *E. grandiflora* [30], *E. grandiflora* ssp. *brevipetiolata* [38], *E. hieronimi* [30], *E. leiocarpa* [24], *E. litoralis* [44], *E. pentaphylla* [40], *E. pilocarpoides* [19], *Euodia alata*, *E. belahe*, *E. euneura* [24], *E. lepta* [23], *E. litoralis* [24], *E. macrocarpa* [24], *E. pachyphylla* [24], *E. pilulifera* [24], *E. roxburghiana* [24], *E. xanthoxyloides* [42], *Flindersia collina*, *F. maculosa*, *F. pubescens*, *F. schottiana* [42], *Glycosmis bilocularis* [30, 44], *G. cyanocarpa* [26], *G. mauritiana* [28], *G. pentaphylla* [42], *Halfordia kendack* [44],

(*continued*)

Table 24.5 (continued)

Alkaloids	Substituents	Occurrence
		Haplophyllum buxbaumii [19], *H. myrtifolium* [25], *H. suaveolens* [42], *H. thesioides* [28], *H. vulcanicum* [18], *Helietta longifoliata*, *H. parvifolia* [42], *Melicope bonwickii* [40], *M. confusa* [42], *M. erromangensis* [31], *M. lasioneura* [44], *M. leptococca* [18], *M. perspicuinervia* [44], *M. triphylla* [23], *Metrodorea flavida* [33], *Monnieria trifolia* [18], *Oricia renieri* [44], *O. suaveolens* [44], *Orixa japonica* [42], *Pelea barbigera* [44], *Phebalium nudum* [42], *Platydesma campnulata* [42], *Ptelea aptera*, *P. trifoliata* [42], *Raputia heptaphylla* [56], *Raulinoa echinata* [36], *Ruta chalepensis* [42,44], *R. chalepensis* var. *latifolia* [23], *R. graveolens*, *R. montana* [42], *Sarcomelicope glauca* [21], *S. dogniensis* [24], *Sargentia gregii* [44], *Skimmia laureola* [32], *Teclea amanuensis* [64], *T. grandifolia* [23], *T. nobilis* [22], *T. ouabanguiensis* [44], *T. unifoliata* [44], *T. verdoorniana* [44], *Vepris ampody*, *V. biloculares* [42], *V. dainelli* [23], *V. glomerata* [23], *V. heterophylla* [29], *V. pilosa* [44], *V. punctata* [37], *Zanthoxylum bungeanum* [19], *Z. pluviatile* [42], *Z. setulosum* [38]
Maculosidine	**I**: 4,6,8-triOMe	*Acronychia laurifolia* (*A. pedunculata*) [34], *A. oligophylebia* [19], *Asterolasia drummondii* [28], *Balfourodendron riedelianum* [42], *Drummondita calida* [26], *Eriostemon brucei*, *E. coccineus*, *E. difformis*, *E. thryptomenoides*, *E. tomentellus* [42], *Esenbeckia almawillia* [30], *E. conspecta* [36], *E. flava* [44], *E. hartmanii* [42], *Flindersia maculosa*, *F. pubescens* [42], *Galipea officinalis* [36], *Helietta logifoliata* [37], *Oricia renieri* [44], *Philotheca deserti* var. *deserti* [40], *Ptelea trifoliata* [42],*Teclea grandifolia* [23], *Ruta chalepensis* [35]

Skimmianine	**I**: 4,7,8-triOMe	*Acronychia baueri* [42], *A. laurifolia* (*A. pedunculata*) [34], *A. oligophylebia* [19], *A. pedunculata* [23], *Aegle marmelos* [42], *Agathosma barosmae folia* [31], *A. bisulca* [21], *A. capensis* [21], *A. pegleare* [21], *A.* sp. nov., [21], *A. thymifolia* [21], *Almeidea coerulea* [40], *A. guyanensis* [44], *A. rubra* [32], *Amyris pinnata* [44], *A. texana* [26], *Araliopsis soyauxii*, *A. tabouensis* [44], *Balfourodendron riedelianum* [42], *Boronia ternata* [42], *Casimiroa edulis* [44], *C. pubescens* [38], *Chloroxylon swietenia* [42], *Choisya arizonica*, *C. mollis*, *C. ternata* [42], *Chorilaena quercifolia* [31], *Clausena dunniana* [38], *Decatropis bicolor* [42], *Dictamnus albus* [42], *D. angustifolius* [42, 44], *D. caucasicus* [42], *D. dasycarpus* [33], *D. hispanicus* [44], *Diphasia klaineana* [44], *Drummondita calida* [48], *Eriostemon coccineus*, *E. difformis*, *E. thryptomenoides*, *E. tomentellus* [42], *Esenbeckia febrifuga* [42], *E. flava* [44], *E. grandiflora* ssp. *brevipetiolata* [38], *E. hatmanii* [42], *E. hieronimi* [30], *E. litoralis* [14], *Ertela* (*Monnieria*) *trifolia* [61], *Euxylophora paraensis* [44], *Euodia alata* [42], *E. elleryana* [42], *E. lepta* [23], *E. lunu-ankenda* [24], *E. meliaefolia* [28], *E. merrilli* [26], *E. pachyphylla* [24], *Flindersia bennettiana*, *F. bourjotiana*, *F. dissosperma* [42], *F. fournieri* [44], *F. laevicarpa*, *F. maculosa* [42], *F. pimenteliana* [44], *F. pubescens* [42], *Galipea longiflora* [28], *Geijera balansae* [44], *G. salicifolia* [42], *Geleznowia verrucosa* [42], *Glycosmis arborea* [42], *G. biloculares* [44], *G. citrifolia* [30], *G. cyanocarpa* [26], *G. mauritiana* [44], *G. pentaphylla* [42], *G. trichanthera* (*G. calcicola*) [33], *Haplophyllum acutifolium*, *H. alberti* [16], *H. bucharicum*, *H. bungei* [42], *H. buxbaumii* [19], *H. canaliculatum* [47], *H. cappadocicum* [27], *H. dauricum* [44], *H. dubium*, *H. foliosum* [42], *H. glabrinum* [21], *H. kowalenskyi* [44], *H. latifolium* [44], *H. leptomerum* [21], *H. myrtifolium* [25], *H. obtusifolium*, *H. patavinum* [36], *H. pedicellatum* [42], *H. perforatum* [42, 44], *H. popovii*, *H. ramossisimum* [42], *H. robustum* [42], *H. schelkovnikovii* [44], *H. suaveolens* [42], *H. tenue* [44], *H. thesioides* [28], *H. tuberculatum* [44], *H. villosum* [44], *H. vulcanicum* [19], *Helietta longifoliata*, *H. parvifolia* [42], *Hortia arborea* [42], *H. colombiana* [32], *H. longifolia* [44], *H. regia*

(*continued*)

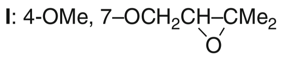
Table 24.5 (continued)

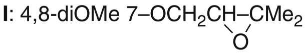
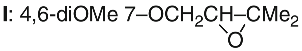
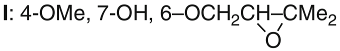
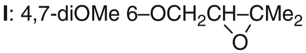
Alkaloids	Substituents	Occurrence
		[22], *Lunasia amara* [42], *Melicope confusa*, *M. erromangensis* [31], *M. fareana* [42], *M. lasioneura* [44], *M. leratii* [44], *M. perspicuinervia* [44], *M. pteleifolia* [55], *M. triphylla* [23], *Monnieria trifolia* [42], *Murraya omphalocarpa* [42], *M. paniculata* [44], *Myrtopsis macrocarpa* [44], *M. myrtoidea* [44], *M. novae-caledoniae* [44], *M. sellingii* [44], *Nematolepis phebalioides* [28], *Neoraputia paraensis* [38], *Oricia gabonensis* [44], *O. renieri* [16], *O. suaveolens* [63], *Orixa japonica* [42], *Phebalium nudum* [42], *Phelodendron amurense* (callus tissue) [32], *Ptelea aptera*, *P. crenulata*, *P. trifoliata* [42], *Raputia heptaphylla* [56], *Raulinoa echinata* [36], *Ruta chalepensis* [42, 44], *R. corsica* [38], *R. graveolens*, *R. montana* [42], *R.* callus cultures (*R. bracteosa*, *R. chalepensis*, *R. macrophylla*) [26], *Sarcomelicope glauca* [21], *S. follicularis* [38], *Skimmia arisanensis*, *S. japonica*, *S. laureola* [42], *S. reevesiana* [21], *Spiranthera odoratissima* [38], *Stauranthus perforatus* [35], *Teclea gerrardii* [65], *T. nobilis* [22], *T. sudanica* [44], *T. simplicifolia* [22], *T. trichocarpa* [37], *T. unifoliata* [44], *T. verdoorniana* [44], *Thamnosma montana* [42], [44], *Ticorea longiflora* [31], *Toddalia asiatica* [14], *T. asiatiaca* var. *gracilis* [16], *T. aculeata* [42], *T. aculeata* var. *gracilus* [44], *Vepris bilocularis* [42], *V. dainelli* [23], *V. glomerata* [23], *V. punctata* [37], *V. stolzii* [44], *Zanthoxylum angolensis* (*Fagara angolensis*), *Z. capensis* (*F. capensis*), *Z. chalybea* (*F. chalybea*), *Z. coco* (*F. coco*), *Z. macrophylla* (*F. macrophylla*), *Z. mantsurica* (*F. mantsurica*) [42], *Z. mayu* (*F. mayu*) [44], *Z. okinawensis* (*F. okinawensis*), *Z. rubescens* (*F. rubescens*), *Z. viridis* (*F. viridis*), *Z. xanthoxyloides* (*F. xanthoxyloides*) [42], *Z. zanthoxyloides* (*F. zanthoxyloides*, cell cutures) [29], *Z. acanthopodium* [21], *Z. ailanthoides*, *Z. alatum* [42], *Z. arnottianum* [44], *Z. avicennae* [26], *Z. belizense* [44], *Z. bouetense* [44], *Z. bungeanum* [44], *Z. caribaeum* [44], *Z. coreanum* [23], *Z.* culantrillo [44], *Z. cuspidatum* [44], *Z. davyi* [35], *Z. decaryi*, *Z. dinklagei* [44], *Z. dissitum* [30], *Z. ekmanii* [39], *Z. fagara* [44], *Z. heitzii* [40],

		Z. integrifoliolum [16], *Z. lemairie* [32], *Z. leprieurii* [44], *Z. limoncillo* [44], *Z. monophyllum* [33], *Z. nitidum* [44], *Z. ovalifolium* [44], *Z. parviflorum*, *Z. pistacifolium* [22], *Z. pistaciiflorum* [38], *Z. pluviatile*, *Z. rhetsa*, *Z. rhoifolium* [26], *Z. rugosum* (*Z. chiloperone*, *Fagara chiloperone*) [34], *Z. sarasinii* [23], *Z. schinifolium* [42], *Z. simulans* [44], *Z. stelligerum* [37], *Z.* sp. Sévenet –Pusset 1345 [41], *Z. syncarpum* [33], *Z. tessmannii* [21], *Z. tsihanimposa* [44], *Z. utile* [26], *Z. williamsii* [44]
O-Ethylnorskimmianine	**I**: 4-OCH_2CH_3, 7,8-diOMe	*Dictamnus dasycarpus* [21]
Tecleine	**I**: 4-OMe, 6-OCH_2O-7, 8-OH	*Teclea grandifolia* [23], *T. ouabanguiensis* [17], *T. verdoorniana* [16]
Flindersiamine	**I**: 4,8-diOMe, 6-OCH_2O-7	*Araliopsis soyauxii* [44], *A. tabouensis* [40], *Balfourodendron riedelianum* [42], *Esenbeckia alata* [49], *E. almawillia* [30], *E. belizencis* [27], *E. conspecta* [36], *E. febrifuga* [42], *E. flava* [44], *E. grandiflora* [30], *E. grandiflora* ssp. *brevipetiolata* [38], *E. hieronimi* [30], *E. leiocarpa* [24], *E. litoralis* [37], *E. pentaphylla* [40], *E. pilocarpoides* [19], *E. yaaxhokob* [38], *Flindersi bennettiana*, *F. bourjotiana*, *F. collina*, *F. dissosperma* [42], *F. maculosa*, *F. pubescens*, *F. xanthoxyla* [42], *Helietta longifoliata* [42], *H. parvifolia* [44], *Melicope erromangensis* [31], *Metrodorea flavida* [33], *Nematolepis phebalioides* [28], *Oricia suaveolens* [63], *Oriciopsis glaberrima* [44], *Raputia heptaphylla* [56], *Raulinoa echinata* [36], *Teclea grandifolia* [23], *T. natalensis* [39], *T. nobilis* [22], *T. ouabanguiensis* [44], *T. sudanica* [42, 44], *T. verdoorniana* [44], *Vepris biloculares* [42], *V. punctata* [37]
5-Methoxymaculine	**I**: 4,5-diOMe, 6-OCH_2O-7	*Oricia suaveolens* [63], *Vepris punctata* [37]
Acronycidine	**I**: 4,5,7,8-tetraOMe	*Acronychia baueri* [42, 44], *Bauerella simplicifolia* ssp. *neo-scotica* [44], *Medicosma subsessilis* [38], *Melicope foreana* [42], *Sarcomelicope leiocarpa* [19], *S. megistophylla* [29]
Halfordinine	**I**: 4,6,7,8-tetraOMe	*Araliopsis tabouensis* [44], *Diphasia angolensis* [44], *Halfordia scleroxyla* [44], *Melicope leptococca* [18], *M. perspicuinervia* [44], *Nematolepis phebalioides* [28], *Oricia suaveolens* [44], *Sarcomelicope argyrophylla* [21], *Teclea verdoorniana* [44]
–	**I**: 4,5,6,7-tetraOMe	*Brombya* sp. nov [40]
–	**I**: 4,5,6,7,8-pentaOMe	*Vepris punctata* [37]

(*continued*)

Table 24.5 (continued)

Alkaloids	Substituents	Occurrence
5,8-Dimethoxymaculine	**I**: 4,5,8-triOMe, 6-OCH_2O-7	*Vepris punctata* [37]
Melineurine	**I**: 4-OMe, 7-$OCH_2CH = CMe_2$	*Melicope lasioneura* [44]
Haplophydine	**I**: 4-OMe, 8-$OCH_2CH = CMe_2$	*Haplophyllum glabrinum* [21], *H. perforatum* [44]
–	**I**: 4-OMe, 7-$OCH_2CH(OH)C(=CH_2)$ Me	*Euodia xanthoxyloides* [42]
Evellerine	**I**: 4-OMe, 7-$OCH_2CH(OH)C(OH)Me_2$	*Euodia elleryana* [42], *E. euneura* [24], *Melicope bonwickii* [40]
–	**I**: 4-OMe, 7-$OCH_2CH(OH)C(Cl)Me_2$	*Melicope bonwickii* [40]
–		*Melicope bonwickii* [40]
Folifinine	**I**: 4-OMe, 7-$OCH_2CH_2C(OH)Me_2$, 8-OH	*Haplophyllum foliosum* [44]
7-Prenyloxy-γ-fagarine	**I**: 4,8-diOMe, 7-$OCH_2CH = CMe_2$	*Ertela* (*Monnieria*) *trifolia* [61], *Haplophyllum canaliculatum* [47], *H. ferganicum* [17], *H. glabrinum* [21], *H. latifolium* [44], *H. perforatum* [44], *H. tuberculatum* [34], *Melicope lasioneura* [17], *Ptelea aptera* [42], *Teclea gerrardii* [66], *Skimmia reevesiana* [21], *Teclea nobilis* [38]
Haplobine	**I**: 4,8-diOMe, 7-$OCH_2CH = C(CH_2Cl)$ Me	*Haplophyllum obtusifolium* [21]
Haplatine (Myrtifoline)	**I**: 4,8-diOMe, 7-$OCH_2CH = C$ $(CH_2OH)Me$	*Haplophyllum latifolium* [44], *H. myrtifolium* [25]
Haplatine acetate	**I**: 4,8-diOMe, 7-$OCH_2CH = C$ $(CH_2OAc)Me$	*Haplophyllum obtusifolium* [18]

Anhydroevoxine		*Euodia xanthoxyloides* [42], *Haplophyllum ferganicum* [17], *H. perforatum* [19], *H. sieversii* [40], *Teclea nobilis* [38]
Evodine	**I**: 4,8-diOMe, 7-OCH$_2$CH(OH)C(=CH$_2$)Me	*Euodia xanthoxyloides* [42], *Haplophyllum ferganicum* [17], *H. glabrinum* [21], *H. obtusifolium* [18], *H. perforatum* [44], *H. ramosissimum* [22], *Skimmia reevesiana* [21]
Evoxoidine	**I**: 4,8-diOMe, 7-OCH$_2$COCHMe$_2$	*Euodia xanthoxyloides* [42], *Haplophyllum perforatum* [44]
Nobiline	**I**: 4,6-diOMe, 7- OCH$_2$CH = CMe$_2$	*Teclea nobilis* [22]
Isotecleoxine		*Teclea nobilis* [38]
Evolatine	**I**: 4,6-diOMe, 7-OCH$_2$CH(OH)C(OH)Me$_2$	*Euodia alata* [42], *Vepris heterophylla* [29]
Teclealbine	**I**: 4,6-diOMe, 8-OCH$_2$CH = CMe$_2$	*Teclea nobilis* [38]
Tecleanatalensine B	**I**: 4,7-diOMe, 6-OCH$_2$CH = CMe$_2$	*Ertela* (*Monnieria*) *trifolia* [61], *Teclea natalensis* [39]
Quinosuaveoline A		*Oricia suaveolens* [63]
(+)-Tecleoxine (Tecleanatalensine A)		*Teclea natalensis* [39], *T. nobilis* [38]
Montrifoline [(+)-(S)-Nkolbisine]	**I**: 4,7-diOMe, 6-OCH$_2$CH(OH)C(OH)Me$_2$	*Haplophyllum myrtifolium* [25], *H. thesioides* [28], *H. vulcanicum* [19], *Monnieria trifolia* [44], *Oricia suaveolens* [63], *Teclea grandifolia* [23], *T. nobilis* [22], *T. ouabanguiensis* [44], *T. simplicifolia* [22], *T. verdoorniana* [44]
(+)-Chlorodesnkolbisine	**I**: 4,7-diOMe, 6-OCH$_2$CH(OH)C(Cl)Me$_2$	*Ertela* (*Monnieria*) *trifolia* [61], *Teclea nobilis* [38]

(*continued*)

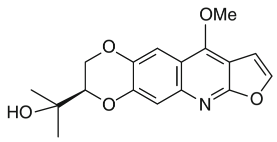
Table 24.5 (continued)

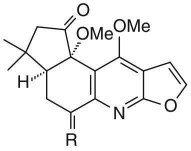
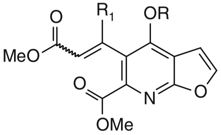
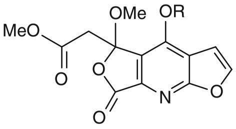
Alkaloids	Substituents	Occurrence
(−)-Methylnkolbisine	**I**: 4,7-diOMe, 6-$OCH_2CH(OH)C(OMe)Me_2$	*Ertela* (*Monnieria*) *trifolia* [61], *Teclea nobilis* [38]
–	**I**: 4,7-diOMe, 8-$OCH_2CH = CMe_2$	*Teclea natalensis* [39], *T. simplicifolia* [22], *Zanthoxylum arborescens* [16]
–	**I**: 4,7-diOMe, 5-OH, $6\text{–}CH_2\underset{\diagdown O \diagup}{CH\text{–}CMe_2}$	*Teclea amanuensis* [64]
Evoxine	**I**: 4,8-diOMe, 7-$OCH_2CH(OH)C(OH)Me_2$	*Choisya ternate* [42], *Dictamnus angustifolius* [42], *Dutaillyea baudouinii* [26], *Ertela* (*Monnieria*) *trifolia* [61], *Euodia alata*, *E. xanthoxyloides* [42], *Haplophyllum acutifolium* [22], *H. alberti* [16], *H. dubium*, *H. ferganicum* [17], *glabrinum* [21], *H. hispanicum* [42], *H. latifolium* [44], *H. obtusifolium*, *H. perforatum*, *H. popovii*, *H. ramossisimum*, *H. suaveolens* [42], *H. tuberculatum* [44], *Monnieria trifólia* [44], *M. trifoliata* [16], *Orixa japonica* [44], *Teclea gerrardii* [66], *Skimmia caureola* ssp. *multinervia* [30], *S. laureola* [32], *S. reevesiana* [21], *Teclea boiviniana* [44]
Methylevoxine	**I**: 4,8-diOMe, 7-$OCH_2CH(OH)C(OMe)Me_2$	*Ertela* (*Monnieria*) *trifolia* [61], *Haplophyllum glabrinum* [21], *H. obtusifolium* [18], *H. perforatum* [44], *H. ramosissimum* [22]
Evoxine acetate	**I**: 4,8-diOMe, 7-$OCH_2CH(OAc)C(OH)Me_2$	*Haplophyllum ferganicum* [17], *H. hispanicum* [42], *H. ramosissimum* [24]
–	**I**: 4,8-diOMe, 7-$OCH_2CH(OH)C(Cl)Me_2$	*Ertela* (*Monnieria*) *trifolia* [61]
Maculosine	**I**: 4-$OCH_2CH(OH)C(OH)Me_2$, 6-OCH_2O-7	*Flindersia maculosa* [42]
Nigdenine	**I**: 4-$OCH_2CH(OH)C(OH)Me_2$, 7,8-diOMe	*Haplophyllum vulcanicum* [19]
–	**I**: 4-$OCH_2CH(OH)C(OH)Me_2$, 6,7-diOMe	*Clausena dunniana* [38]

–	**I**: 4,6,7-triOMe, 5-$CH_2CH = CMe_2$	*Teclea amanuensis* [64]
Tecleamine	**I**: 4-OMe, 6-OCH_2O-7, 8-$OCH_2CH = CMe_2$	*Teclea grandifolia* [23], *T. ouabanguiensis* [44]
Tecleaverdoornine	**I**: 4-OMe, 6-OCH_2O-7, 8-OH, 5-$CH_2CH = CMe_2$	*Oricia suaveolens* [63], *Teclea grandifolia* [23], *T. ouabanguiensis* [44], *T. verdoorniana* [44], *Vepris heterophylla* [29]
Tecleaverdine	**I**: 4-OMe, 6-OCH_2O-7, 8-OH, 5-$CH_2CH(OH)C(OH)Me_2$	*Teclea verdoorniana* [16]
Glycoperine	**I**: 4,8-diOMe, 7-O-rhamnose	*Haplophyllum acutifolium* [22], *H. ferganicum* [17], *H.latifolium* [44], *H. perforatum* [44]
Glycohaplopine	**I**: 4,8-diOMe, 7-O-glucose	*Haplophyllum perforatum* [44]
Monoacetylglycoperine	**I**: 4,8-diOMe, 7-O-rhamnose monoacetate	*Haplophyllum perforatum* [44]
Diacetylglycoperine	**I**: 4,8-diOMe, 7-O-rhamnose dicetate	*Haplophyllum perforatum* [44]
Triacetylglycoperine	**I**: 4,8-diOMe, 7-O-rhamnose triacetate	*Haplophyllum perforatum* [44]
Haplosinine	**I**: 4,8-diOMe, 7-O-[β-D-glucopyranosyl(1 → 3)]-α-L-rhamnopyranoside	*Haplophyllum perforatum* [23]
Haplosidine	**I**: 4,8-diOMe, 7-O-[β-D-glucopyranosyl(1 → 3)]-α-L-(2′-*O*-acetyl)-rhamnopyranoside	*Haplophyllum perforatum* [23]
Perfaminole	**I**: 4,8-diOMe, 8-$CH_2CH = CMe_2$, 7-α-OH, 7,8-dihydro	*Haplophyllum glabrinum* [21]
Perfamine	**I**: 4,8-diOMe, 8-$CH_2CH = CMe_2$, 7 = O, 7,8-dihydro	*Haplophyllum canaliculatum* [47], *H. glabrinum* [21], *H. perforatum* [44]

(*continued*)

Table 24.5 (continued)

Alkaloids	Substituents	Occurrence
(−)-Sarcomegistine	**I**: 4,8-diOMe, 5-OH, 8-$CH_2CH = CMe_2$, 7 = O, 7,8-dihydro	*Sarcomelicope megistophylla* [29]
Dihydoperfamine	**I**: 4,8-diOMe, 8-$CH_2CH = CMe_2$, 7 = O, 5,6,7,8-tetrahydro	*Haplophyllum glabrinum* [21], *H. tuberculatum* [23]
Perforine	**I**: 4,8-diOMe, 7-OH, 8-$CH_2CH_2C(OH)Me_2$, 5,6,7,8-tetrahydro	*Haplophyllum perforatum* [42]
Haplophyllidine	**I**: 4,8-diOMe, 7-β-OH, 8-$CH_2CH = CMe_2$, 5,6,7,8-tetrahydro	*Haplophyllum perforatum* [42]
7-(*O*-Acetyl) haplophyllidine	**I**: 4,8-diOMe, 7-β-OAc, 8-$CH_2CH = CMe_2$, 5,6,7,8-tetrahydro	*Almeidea coerulia* [32], *Haplophyllum perforatum* [34]
Dihydroperfaminole	**I**: 4,8-diOMe, 7-α-OH, 8-$CH_2CH = CMe_2$, 5,6,7,8-tetrahydro	*Haplophyllum glabrinum* [22]
–	**I**: 4,8-diOMe, 7-α-OAc, 8-CH = CHC(OH)Me_2, 5,6,7,8-tetrahydro	*Almeidea rubra* [39]
Megistoquinone I	**I**: 4,7-diOMe, 5,8- di = O,	*Sarcomelicope megistophylla* [36]
Melicarpine	**I**: 4,8,8-triOMe, 5- = O, 7-OMe	*Melicope semecarpifolia* [37]
Melicarpinone	**I**: 4-OMe, 7- = O	*Melicope semecarpifolia* [37]
Quinosuaveoline B		*Oricia suaveolens* [63]

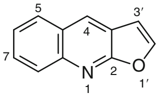
(+)-Megistosarcimine R = NH (+)-Megistosarconine R = O		*Sarcomelicope megistophylla* [34]
		
(*E* and *Z*)-Rhoifolinic acid dimethyl ester (R = Me, R$_1$ = H)		*Glycosmis citrifolia* [30], *Melicope semecarpifolia* [37], *Metrodorea nigra* [30], *Zanthoxylum rhoifolium* [26]
Furomegistine I (R = Me, R$_1$ = OMe)		*Sarcomelicope megistophylla* [35]
Furomegistine II		*Sarcomelicope megistophylla* [35]

(*continued*)

Table 24.5 (continued)

Alkaloids	Substituents	Occurrence
Isodictamnine	**II**: 1-Me	*Boronia inornata* [29], *Dictamnus albus* [42], *D. angustifolius* [44], *D. caucasicus* [42], *D. dasycarpus* [33], *Glycosmis arborea* [39], *Helietta longifoliata* [42]
Dictangustine-A	**II**: 6-OH, 1-Me	*Dictamnus angustifolium* [67], *D. dasycarpus* [33], *Glycosmis mauritiana* [43]
Isopteleine	**II**: 6-OMe, 1-Me	*Dictamnus angustifolius* [44], *D. caucasicus* [42]
Taifine	**II**: 7-OMe, 1-Ethyl	*Ruta chalepensis* [44]
Iso-γ-fagarine	**II**: 8-OMe, 1-Me	*Dictamnus angustifolium* [67], *D. dasycarpus* [33], *Glycosmis arborea* [39], *G. citrifolia* [34]
Isotaifine	**II**: 8-OMe, 1-Ethyl	*Ruta chalepensis* [16]
8-Methoxytaifine	**II**: 7,8-diOMe, 1-Ethyl	*Ruta chalepensis* [16]
Isokokusagine	**II**: 7-OCH$_2$O-8, 1-Me	*Almeidea rubra* [39]
Isoskimmianine	**II**: 7,8-diOMe, 1-Me	*Almeidea rubra* [39]
Glycarpine	**II**: 5,7-diOMe, 1-Me	*Glycosmis cyanocarpa* [44]
Isomaculosidine	**II**: 6,8-diOMe, 1-Me	*Dictamnus albus*, *D. angustifolium* [67], *D. caucasicus* [42], *D. dasycarpus* [21], *Ptelea trifoliate* [44]
Isomaculine	**II**: 6-OCH$_2$O-7, 1-Me	*Esenbeckia pilocarpoides* [19]
Isoflindersiamine	**II**: 6-OCH$_2$O-7, 8-OMe, 1-Me	*Helietta parvifolia* [44]
Acrophylline	**II**: 7-OMe, 1-CH$_2$CH = CMe$_2$	*Acronychia haplophylla* [42]
Acrophyllidine	**II**: 7-OMe, 1-CH$_2$CH$_2$C(OH)Me$_2$	*Acronychia haplophylla* [42]

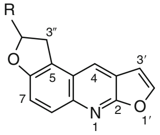
Table 24.6 Occurrence of furoquinolin alkaloids in non-rutaceous species



I

Alkaloids	Substituents	Occurrence
Dictaminine	**I**: 4-OMe	*Aralia bipinnata* (Araliaceae) [29], *Oxytropis glabra* (Fabaceae) [25]
–	**I**: 4,8-diOMe, 5-OH	*Alstonia mairei* (Apocinaceae) [24]
Kokusaginine	**I**: 4,6,7-triOMe	*Tinospora malabarica* (Menispermaceae) [21]
Skimmianine	**I**: 4,7,8-triOMe	*Aralia bipinnata* (Araliaceae) [29], *Alstonia mairei* (Apocynaceae) [24], *Toona ciliata* (Meliaceae) [38]
Dictamnine-7-β-D-mannopyranoside	**I**: 4-OMe, 7-Ogl	*Solidago canadenses* (Asteraceae) [68]
8-Methoxydictamnine-7-β-D-mannopyranoside	**I**: 4,8-diOMe, 7-Ogl	*Solidago canadenses* (Asteraceae) [68]

Table 24.7 Occurrence of dihydro-, difuroquinoline, dihydro- and pyranofuroquinoline alkaloids in Rutaceae species



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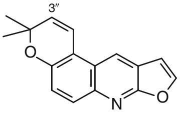

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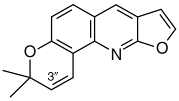

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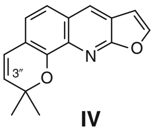

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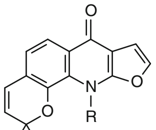
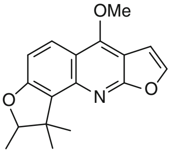
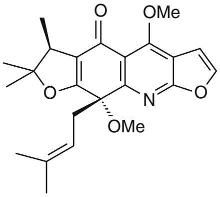
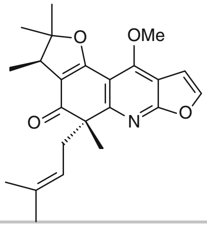


V

Alkaloids	Substituents	Occurrence
(−)-Desmethoxychoisyne	**I**: 2″-C(OH)Me$_2$, 4-OMe	*Choisya ternata* [37]
Choisyne	**I**: 2″-CHMe$_2$, 4,7-diOMe	*Choisya arizonica*, *C. mollis*, *C. ternata* [42]
Medicosmine	**II**: 4-OMe	*Boronella koniambiensis* [40], *Medicosma cunninghamii* [42]
Acronydine	**II**: 4,7-diOMe	*Acronychia baueri* [42], *Melicope leptococca* [18], *Sarcomelicope dogniensis* [24], *S. leiocarpa* [19], *S. megistophylla* [29]
–	**II**: 4-OMe, 3″-α-OH, 4″-α-OH, 3″,4″-dihydro	*Boronella koniambiensis* [40]

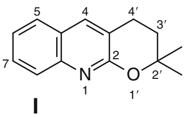
(*continued*)

Table 24.7 (continued)

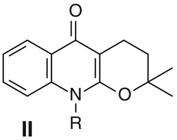
Alkaloids	Substituents	Occurrence
-(*Cis*)-	**II**: 4,7-diOMe, 3″-α-OH, 4″-α-OH, 3″,4″-dihydro	*Sarcomelicope dogniensis* [24]
-(*Trans*)-	**II**: 4,7-diOMe, 3″-α-OH, 4″-β-OH, 3″,4″-dihydro	*Sarcomelicope dogniensis* [24]
Dutadrupine	**III**: 4-OMe	*Almeidea coerulia* [32], *Comptonella sessilifoliola* [25], *Dutaillyea drupacea* [44], *Melicope erromangensis* [31], *M. semecarpifolia* [37]
Anhydroperforine	**III**: 4,8-diOMe, 5,6,7,8-tetrahydro, 3″,4″-dihydro	*Haplophyllum perforatum* [44]
Foliminine	**IV**: 3″,4″-dihydro, 4-OMe	*Haplophyllum foliosum* [44]
Isodutadrupine	**V**: 1-Me	*Almeidea coerulia* [32], *A. rubra* [39]
(+)-Semecarpine		*Melicope semecarpifolia* [37]
(−)-Sarcodifurine A		*Sarcomelicope folliculares* [39]
(+)-Sarcodifurine B		*Sarcomelicope folliculares* [39]

Dictamnine (**7**, R = Me) was a specific precursor of skimmianine (**7**, R = Me, 7,8-diOMe) and other furoquinoline alkaloids oxygenate in the aromatic ring in *Skimmia japonica* and *Choisya ternate* [41]; similar results were obtained with cell cultures of *Ruta graveolens* [104]. Dihydro- (**5**) and furoquinolin-4-ones (**8**) are also known.

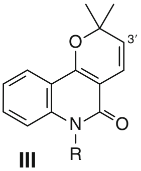
Table 24.8 Occurrence of linear dihydro-, pyranoquinoline/4(1*H*)-one and angular dihydro-, pyranoquinolin-2(1*H*)-one alkaloids in Rutaceae species



I



II



III

Alkaloids	Substituents	Occurrence
Geibalansine	**I**: 3′-OH, 4-OMe	*Citrus grandis* f. *buntan* [28], *Geijera balansae* [44], *Melicope semecarpifolia* [40]
(−)-Tabouensinium chloride	**I**: 3′-OH, 4-OMe, 1-Me Cl^-	*Araliopsis tabouensis* [40]
Rutalinium	**I**: 3′,6-diOH, 4-OMe, 1-Me	*Ruta graveolens* [42], *R. graveolens* ssp. *hortensis* [44]
O-Acetylgeibalansine	**I**: 3′-OAc, 4-OMe	*Geijera balansae* [44]
Peteleflorine	**I**: 3′-OH, 4-OMe, 7-OCH_2O-8	*Ptelea trifoliata* [44]
7,8-Dimethoxygeibalansine	**I**: 4,7,8-OMe, 3′-OH	*Dictamnus dasycarpus* [52], *Dutaillyea baudouinii* [26], *Melicope semecarpifolia* (= *M. confusa*, *Euodia merrillii*) [36]
3′-Chloro-7,8-dimethoxygeibalansine	**I**: 4,7,8-OMe, 3′-Cl	*Dictamnus dasycarpus* [52]
–	**I**: 4-OMe	*Euodia pilulifera* [24]
–	**I**: 4,7,8-triOMe	*Boronella pancheri* [28], *Euodia pilulifera* [24]
–	**I**: 4-OMe, 3′,4′-dehydro	*Euodia pilulifera* [24]
–	**I**: 4,8-diOMe, 3′,4′-dehydro	*Dictamnus dasycarpus* [52]
–	**I**: 4,7,8-triOMe, 3′,4′-dehydro	*Euodia pilulifera* [24]
Khaplofoline	**II**	*Haplophyllum foliosum*, *H. suaveolens* [42]
N-Methylkhaplofoline	**II**: 1-Me	*Almeidea guyanensis* [17, 44]
(+)-Folifine	**II**: 3′-OH, 1-Me	*Araliopsis tabouensis* [44], *Haplophyllum bucharicum*, *H. foliosum* [42]
(−)-Ribalinine	**II**: 3′-OH, 1-Me	*Araliopsis soyauxii* [44], *Euodia gracilis* [23], *Haplophyllum patavinum* [36], *Skimmia laureola* [69], *Teclea nobilis* [22], *Zanthoxylum mayu* (*Fagara mayu*) [44]
(−)-Acetylribalinine	**II**: 3′-OAc, 1-Me	*Skimmia laureola* [39]
(±)-Ribalinine	**II**: 3′-OH, 1-Me	*Balfourodendrom riedelianum* [42], *Citrus macroptera* [29], *Teclea simplicifolia* [22], *Zanthoxylum nitidum* [30], *Z. simulans* [16]

(*continued*)

Table 24.8 (continued)

Alkaloids	Substituents	Occurrence
(−)-(S)-Ribalinidine	**II**: 3′,6-OH, 1-Me	*Balfourodendrom riedelianum* [42], *Ruta chalepensis* [44], *R. graveolens* [42], *R. graveolens* ssp. *hortensis* [44]
(+)-Ribalinidine	**II**: 3′,6-OH, 1-Me	*Balfourodendrom riedelianum* [18]
(+)-Isobalfourodine	**II**: 3′,8-OH, 1-Me	*Balfourodendrom riedelianum* [42], *Orixa japonica* [35]
(−)-Lunacrinol (lunasia II)	**II**: 3′-OH, 1-Me, 8-OMe	*Esenbeckia hieronimi* [30], *Lunasia amara* [42]
(+)-Ribaliprenylene	**II**: 3′-β-OH, 1-Me, 8-$CH_2CH = CMe_2$	*Skimmia laureola* [39]
Haplobucharine	**II**: 1-$CH_2CH = CMe_2$	*Haplophyllum bucharicum* [44]
Neohydroxylunine	**II**: 3′-OH, 7-OCH_2O-8, 1-Me	*Ptelea trifoliata* [44]
Flindersine	**III**	*Atalantia roxburghiana* [44], *Flindersia australis* [42], *Geijera balansae* [21], *G. parviflora* [42], *Glycosmis parviflora* (*G. citrifolia*) [36], *Haplophyllum acutifolium* [36], *H. canaliculatum* [47], *H. glabrinum* [21], *H. mytifolium* [25], *H. perforatum* [44], *H. sieversii* [40], *H. suaveolens* [18], *H. tuberculatum* [42], *H. thesioides* [28], *Hortia colombiana* [32], *Micromelum minutum* [44], *Neoraputia paraensis* [38], *Zanthoxylum beecheyanum* (*Z. arnottianum*) [39], *Z. coco* [16], *Z. simulans* [27]
N-Methylflindersine	**III**: 1-Me	*Almeidea guyanensis* [44], *Andreadoxa flava* [38], *Dictamnus angustifolium* [67], *D. dasycarpus* [33], *Esenbeckia alata* [49], *E. pentaphylla* [40], *Euxylophora paraensis* [44], *Glycosmis parviflora* (*G. citrifolia*) [36], *Hortia colombiana* [32], *Lunasia amara* [50], *Melicope ptelefolia* (*Euodia lepta*) [33], *Myrtopsis novae-caledoniae* [44], *Orixa japonica* [28], *Phellodendron chinense*[25], *Ptelea trifoliata* [44], *Spathelia sorbifolia* [44],

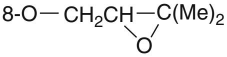
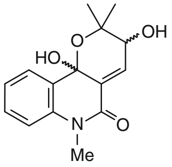
(*continued*)

Table 24.8 (continued)

Alkaloids	Substituents	Occurrence
		Toddalia asiatica [26], *Zanthoxylum chalybea* (*Fagara chalybea*) [44], *Z. heitzii* (*F. heitzii*) [18], *Z. holstii* (*F. hostii*) [13], *Z. beecheyanum* (*Z. arnottianum*) [39], *Z. budrunga* (*Z. rhetsa*) [39], *Z. schinifolium* [27], *Z. simulans* [27]
–	**III**: 1-CH_2OH	*Haplophyllum buxbaumii* [19]
N-Acetoxymethylflindersine	**III**: 1-CH_2OAc	*Haplophyllum suaveolens* [34], *Zanthoxylum simulans* [27]
Haplophylline	**III**: 1-$CH_2OCOCH = CMe_2$	*Haplophyllum suavelolens* [18]
Haplamine	**III**: 6-OMe	*Haplophyllum acutifolium* [21], *H. perforatum* [44], *H. sieversii* [40], *H. suaveolens* [34], *Vepris bilocularis* [30]
Dihydrohaplamine	**III**: 6-OMe, 3′,4′-dihydro	*Haplophyllum perforatum* [34]
N-Methylhaplamine	**III**: 1-Me, 6-OMe	*Agathosma barosmae folia* [31], *A.* spec. Nov. [24]
–	**III**: 8-OMe	*Andreadoxa flava* [38], *Dictyoloma vandellianum* [37], *Myrtopsis macrocarpa* [44], *Neoraputia paraensis* [38], *Teclea nobilis* [22], *Zanthoxylum simulans* [27]
Haplophytin-A	**III**: 5-OMe	*Haplophyllum acutifolium* [36]
–	**III**: 3′-OH, 3′,4′-dihydro, 1-Me	*Skimmia laureola* [69]
–	**III**: 4′-OH, 3′,4′-dihydro, 1-Me	*Euxylophora paraensis* [44]
–	**III**: 4′-OH, 3′,4′-dihydro, 1-H	*Geijera balansae* [21]
- (−)	**III**: 3′,4′-diOH (*cis*), 3′,4′-dihydro, 1-H	*Geijera balansae* [21]
(+)-Orixalone D	**III**: 3′,4′-diOH (*trans*), 3′,4′-dihydro, 1-Me	*Orixa japonica* [39]
–	**III**: 7-OMe, 1-Me	*Oricia renieri* [44]
–	**III**: 7-OMe	*Vepris bilocularis* [30]
Zanthobungeanine	**III**: 8-OMe, 1-Me	*Andreadoxa flava* [38], *Esenbeckia conspecta* [36], *Geijera balansae* [21], *Orixa japonica* [28], *Raputia heptaphylla* [56], *Zanthoxylum rhetza* (*Fagara rhetza*) [27], *Z. beecheyanum* (*Z. arnottianum*) [39], *Z. budrunga* [25], *Z. bungeanum* [41], *Z. planispinum* [27], *Z. simulans* [44]

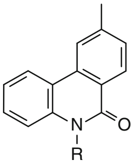
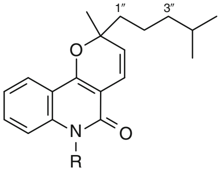
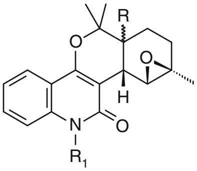
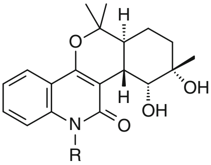
(*continued*)

Table 24.8 (continued)

Alkaloids	Substituents	Occurrence
Oricine	**III**: 6,7-diOMe, 1-Me	*Oricia renieri* [44], *O. suaveolens* [42]
–	**III**: 6-OMe, 8-OH, 1-H	*Haplophyllum telephioides* [28]
Veprisine	**III**: 7,8-diOMe, 1-Me	*Araliopsis tabouensis* (*A. soyauxii*) [23], *Glycosmis mauritiana* [43], *Oricia renieri* [44], *Stauranthus perforatus* [35], *Vepris louisii* [44], *V. stolzii* [44]
Stauranthine	**III**: 7-OCH_2O-8, 1-Me	*Stauranthus perforatus* [37]
–	**III**: 8-OH, 1-CH_2OAc	*Zanthoxylum monophyllum* [44]
–	**III**: 8-$OCH_2CH = CMe_2$, 1-Me	*Vepris stolzii* [44]
Revesilone	**III**: 3′,4′-dihydro, 8-OH, 1-Me	*Ravenia spectabilis* [18]
(−)-Zanthodioline	**III**: 3′,4′-diOH (*trans*), 3′,4′-dihydro, 8-OMe, 1-Me	*Zanthoxylum beecheyanum* (*Z. arnottianum*) [39], *Z. simulans* [32, 44]
Araliopsinine	**III**: 3′,4′-diOH (*trans*), 3′,4′-dihydro, 7,8-diOMe, 1-Me	*Araliopsis tabouensis* [22], *Stauranthus perforatus* [35]
	III: 3′,4′-diOH (*trans*), 3′,4′-dihydro, 7-OCH_2O-8, 1-Me	*Stauranthus perforatus* [37]
Zanthophylline	**III**: 8-OMe, 1-CH_2OAc	*Andreadoxa flava* [38], *Zanthoxylum monophyllum* [44]
–	**III**: 7-OMe, 8-$OCH_2CH = CMe_2$, 1-Me	*Vepris stolzii* [44]
–	**III**: 7-$OCH_2CH = CMe_2$	*Vepris bilocularis* [30]
–	**III**: 7-$OCH_2CH = CMe_2$, 1-Me	*Vepris bilocularis* [30]
–	**III**: 1-Me, 7-OMe, 	*Vepris stolzii* [44]
		
7-OCH_2O-8		*Stauranthus perforatus* [37]
7,8-diOMe		*Stauranthus perforatus* [37]
7-OCH_2O-8, 3′- = O		*Stauranthus perforatus* [37]
7,8-diOMe, 3′- = O		*Stauranthus perforatus* [37]

(*continued*)

Table 24.8 (continued)

Alkaloids	Substituents	Occurrence
Phenaglydone		*Glycosmis cyanocarpa* [28]
		
–	3″,4″-epoxide, R = Me	*Zanthoxylum simulans* [44]
Huajiaosimuline	1-Me, 3″ = O	*Zanthoxylum simulans* [28]
(+)-Zanthosimuline	1-Me, 3″,4″-dihydro	*Zanthoxylum simulans* [28]
Simulansine	1-Me, 3″-OH	*Zanthoxylum simulans* [30]
Simulenosine	1-Me, 2″,3″-dihydro, 4″-OH	*Zanthoxylum simulans* [32]
Peroxysimulenosine	1-Me, 2″,3″-dihydro, 4″-OOH	*Zanthoxylum simulans* [32]
		
Cis-Erioaustralasine R = β-H, R_1 = CH_2OAc		*Eriostemon australasius* subsp. *banskii* [28]
Trans-Erioaustralasine R = α-H, R_1 = CH_2OAc		*Eriostemon australasius* subsp. *banskii* [28], *Halfordia kendack* [36]
(+)-*trans*-Deacetoxyerioaustralasine R = α-H, R_1 = Me		*Halfordia kendack* [36]
(+)-*trans*-Deacetoxyerioaustralasine hydrate R = Me *Trans*-Erioaustralasine hydrate R = CH_2OAc		*Halfordia kendack* [36]

(*continued*)

Table 24.8 (continued)

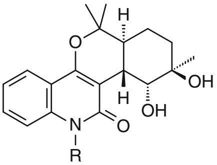
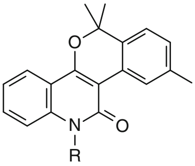
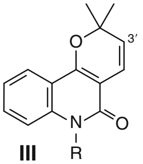
Alkaloids	Substituents	Occurrence
(+)-*trans*-1′-Epideacetoxyerioaustralasine hydrate R = Me		*Halfordia kendack* [36]
Benzosimuline		*Zanthoxylum simulans* [32]

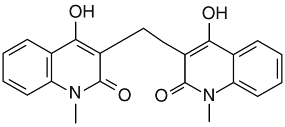
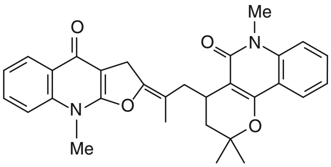
Table 24.9 Occurrence of angular pyranoquinolin-2(1*H*)-one alkaloids in non-rutaceous species



III

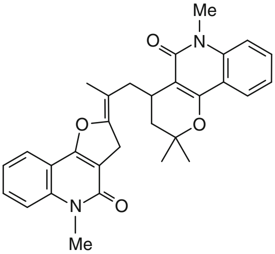
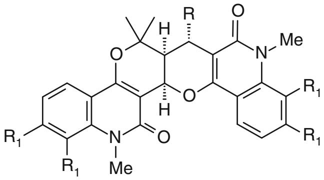
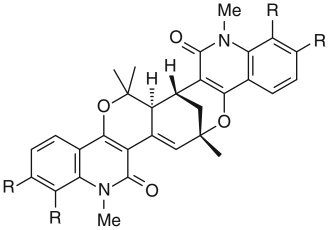
Alkaloids	Substituents	Occurrence
Flindersine	**III**	*Acalypha indica* (Euphorbiaceae) [34]
N-Methylflindersine	**III**: 1-Me	*Xylocarpus granatum* (Meliaceae) [31]

Table 24.10 Occurrence of dimeric quinolin-2(1*H*)-one/4(1*H*)-one alkaloids in Rutaceae species

Alkaloids	Substituents	Occurrence
Zanthobisquinolone		*Zanthoxylum simulans* [28]
teledimerine		*telea trifoliata* [44]

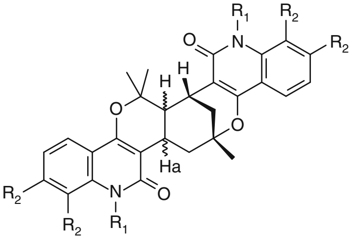
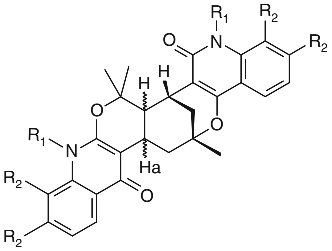
(*continued*)

Table 24.10 (continued)

Alkaloids	Substituents	Occurrence
Pteledimeridine		*Ptelea trifoliata* [44]
		
Paraensidimerin D (R = CH = CMe$_2$, R$_1$ = H)		*Euxylophora paraensis* [17, 44]
Paraensidimerin B (R = CH$_2$C(OH) Me$_2$, R$_1$ = H)		*Euxylophora paraensis* [17, 44]
Araliopdimerine A (R = CH = CMe$_2$, R$_1$ = OMe)		*Araliopsis tabouensis* [22]
Araliopdimerine B (R = CH$_2$C(OH) Me$_2$, R$_1$ = OMe)		*Araliopsis tabouensis* [22]
		
Paraensidimerin G (R = H)		*Euxylophora paraensis* [17]
Araliopdimerine C (R = OMe)		*Araliopsis tabouensis* [22]

(*continued*)

Table 24.10 (continued)

Alkaloids	Substituents	Occurrence
	 R1 R2 R2 O N H H O O Ha R2 N O R2 R1	
Paraensidimerin A $R_1 = Me$, $R_2 = H$, (α-H, α-Ha)		*Euxylophora paraensis* [17]
Paraensidimerin C $R_1 = Me$, $R_2 = H$, (α-H, β-Ha)		*Euxylophora paraensis* [17]
Paraensidimerin E $R_1 = Me$, $R_2 = H$, (β-H, β-Ha)		*Euxylophora paraensis* [17]
Paraensidimerin F $R_1 = Me$, $R_2 = H$, (β-H, α-Ha)		*Euxylophora paraensis* [17]
Vepridimerine A $R_1 = Me$, $R_2 = OMe$, (α-H, α-Ha)		*Araliopsis tabouensis* (*A. soyauxii*) [23], *Vepris louisii* [44]
Vepridimerine B $R_1 = Me$, $R_2 = OMe$, (α-H, β-Ha)		*Araliopsis tabouensis* (*A. soyauxii*) [23], *Oricia renieri* [44], *Vepris louisii* [44]
	 R1 R2 R2 O N H H O O R1 N Ha R2 O R2	
Geijedimerine $R_1 = H$, $R_2 = H$, (α-Ha, β-H)		*Geijera balansae* [21]
Vepridimerine C $R_1 = Me$, $R_2 = OMe$, (α-H, α-Ha)		*Araliopsis tabouensis* (*A. soyauxii*) [23], *Oricia renieri* [44], *Vepris louisii* [44]
Vepridimerine D $R_1 = Me$, $R_2 = OMe$, (α-H, β-Ha)		*Araliopsis tabouensis* (*A. soyauxii*) [23], *Oricia renieri* [44]

(*continued*)

Table 24.10 (continued)

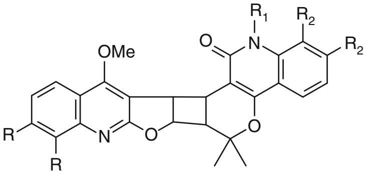
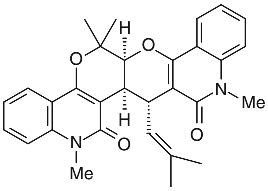
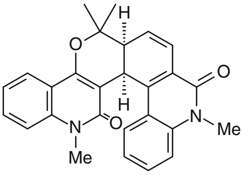
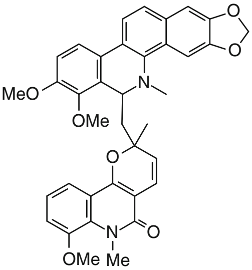
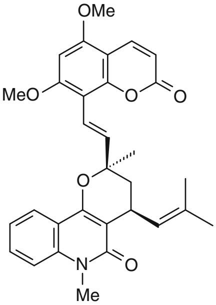
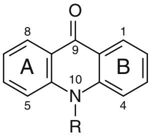
Alkaloids	Substituents	Occurrence
Haplodimerine ($R = OMe, R_1, R_2 = H$)		*Haplophyllum foliosum* [23]
Melicobisquinolinone A		*Melicope ptelefolia* (*Euodia lepta*) [33]
Melicobisquinolinone B		*Melicope ptelefolia* (*Euodia lepta*) [33]
Simulanoquinoline		*Zanthoxylum simulans* [28]
Toddacoumalone		*Toddalia asiatica* [26]

Table 24.11 Occurrence of acridone alkaloids in Rutaceae species



Alkaloids	Substituents	Occurrence
Acridone	10-H	*Thamnosma montana* [44]
–	10-Me	*Thamnosma montana* [42, 44], *T. montana* (cell suspension cultures) [28]
–	1-OH, 10-H	*Boenninghausenia albiflora* [44], *Boronia bowmanii* [28], *B. lanceolata* [28]
–	1-OH, 10-Me	*Boenninghausenia albiflora* [44], *Boronia lanceolata* [28], *Glycosmis mauritiana* [43], *Ruta chalepensis* [25], *R. chalepensis* var. *latifolia* [23], *R. graveolens* [42], *Thamnosma rhodesica* [38]
–	1-OH, 7-OMe, 10-H	*Boenninghausenia albiflora* [44]
–	1,3-diOH, 10-Me	*Citrus hybrids* [22], *C. hybrid* "*Yalaha*"(*C. paradisi* x *C. tangerina*) [30]
–	1,3-diOMe, 10-H	*Bauerella simplicifolia* [44], *B. simplicifolia* ssp. *neo-scotica* [15]
–	1-OH, 3-OMe	*Zanthoxylum macrophylla* (*F. macrophylla*, = *Z. gillettii*) [31]
Tegerrardin A	1-OMe, 3-OH, 10-Me	*Teclea gerrardii* [66], *Zanthoxylum leprieurii* [70]
–	1-OH, 3-OMe, 10-Me	*Almeidea rubra* [39], *Boenninghausenia albiflora* [44], *Esenbeckia febrifuga* [62], *E. litoralis* [44], *E. pentaphylla* [40], *E. pilocarpoides* [19], *Feronia limonia* (*F. elephantum*) [36], *Glycosmis arborea* [39], *Helietta parvifolia* [21], *Ruta graveolens* [44], *Ruta* callus cultures (*R. bracteosa*, *R. chalepensis*, *R. macrophylla*) [26], *Thamnosma montana* (cell suspension cultures) [28], *Vepris pilosa* [44], *Zanthoxylum leprieurii* (*Fagara leprieurii*), *Z. macrophylla* (*F. macrophylla*, = *Z. gillettii*) [31], *Z. rubescens* (*F. rubescens*) [42], *Z. leprieurii* [44]

(*continued*)

Table 24.11 (continued)

Alkaloids	Substituents	Occurrence
–	1,3-diOMe, 10-Me	*Acronychia baueri* [42], *Bauerella simplicifolia* [44], *B. simplicifolia* ssp. *neo-scotica* [15], *Boronia lanceolata* [28], *Diphasia angolensis* [44], *Euodia triphylla* [44], *Oricia suaveolens* [44], *Teclea gerrardii* [66], *T. verdoorniana* [44], *Vepris ampody*, *V. biloculares* [42], *V. macrophylla* [22], *V. pilosa* [44]
Vebilocine	1-OH, 3-$OCH_2CH = CMe_2$, 10-Me	*Vepris bilocularis* [30]
Tegerrardin B	1-$OCH_2CH = CMe_2$, 3-OH, 10-Me	*Teclea gerrardii* [66]
–	1,7-diOH	*Boronia lanceolata* [28]
–	1,8-diOH	*Boronia bowmanii* [28], *B. lanceolata* [28]
–	1,8-diOH, 10-Me	*Boronia lanceolata* [28]
–	1-OH, 2-$CH_2CH = CMe_2$, 3-OMe, 10-Me	*Glycosmis mauritiana* [44]
Glycocitrine-II	1,3-diOH, 4-$CH_2CH = CMe_2$, 10-Me	*Glycosmis citrifolia* [16]
–	1,3-diOH, 4-$CH = CHC(OH)$ Me_2, 10-Me	*Boenninghausenia japonica* [39]
–	1,3-diOH, 2,4-di$CH_2CH = CMe_2$, 10-H	*Severinia buxifolia* [71]
–	1,3-diOH, 4-$CH_2CH(OH)C$ (epoxideCH_2)CH_2OH, 10-Me	*Boenninghausenia japonica* [39]
O-Methylglycocitrine-II	1-OH, 3-OMe, 4-$CH_2CH = CMe_2$, 10-Me	*Glycosmis citrifolia* [16]
–	1,3,5-triOH, 10-Me	*Citrus hybrid* "*Yalaha*"(*C. paradisi* x *C. tangerina*) [30]
Junosine	1,3,5-triOH, 2-$CH_2CH = CMe_2$, 10-Me	*Atalantia monophylla* [72], *Bosistoa selwynii* [31], *B. transversa* [30], *Citrus junos* [21], *Glycosmis trichanthera* (*G. calcicola*) [33], *Swinglea glutinosa* [73]
Buxifoliadine-C	1,5-diOH, 2-$CH_2CH = CMe_2$, 3-OMe, 10-H	*Severinia buxifolia* [34]
(−)-Bosistidine	1,3,5-triOH, 2-$CH_2CH(OH)C$ (=CH_2)Me, 10-Me	*Bosistoa transversa* [30]
Glycocitrine-III	1,3,5-triOH, 2-$CH_2CH = C(Me)$ $CH_2CH_2CH = CMe_2$, 10-Me	*Glycosmis citrifolia* [34], *G. pentaphylla* [34]
–	1,3,5-triOH, 4-$CH_2CH = C(Me)$, 10-H	*Oriciopsis glaberrima* [40]

(*continued*)

Table 24.11 (continued)

Alkaloids	Substituents	Occurrence
Oriciacridone E	1,3,5-triOH, 4-$CH_2CH = C(Me)$, 10-Me	*Oriciopsis glaberrima* [40]
Glycocitrine-IV	1,3,5-triOH, 2-$CH_2CH = C(Me)$, 4-OMe, 10-Me	*Glycosmis citrifolia* [34], *Swinglea glutinosa* [36]
Yukomine	1,3-diOH, 2-$CH_2CH = CMe_2$, 5-OMe, 10-Me	*Citrus yuko* [26]
Evoxanthidine	1-OMe, 2-OCH_2O-3, 10-H	*Euodia xanthoxyloides* [42], *Teclea amanuensis* [64]
Norevoxanthine	1-OH, 2-OCH_2O-3, 10-Me	*Teclea grandifolia* [42]
Xanthoxoline	1-OH, 2,3-diOMe, 10-H	*Euodia xanthoxyloides* [42], *Vepris dainelli* [23], *Zanthoxylum leprieurii* [21], *Z. macrophylla* (*F. macrophylla*, = *Z. gillettii*) [31]
–	1,3-diOH, 2-OMe, 10-Me	*Ruta graveolens* [44], *Zanthoxylum leprieurii* [74]
–	1,3-diOH, 4-OMe, 10-Me	*Micromelum integerrimum* [75]
–	1,3-diOMe, 2-OH, 10-Me	*Teclea amanuensis* [64]
Yukodine	1,3-diOH, 5-OMe, 10-Me	*Citrus yuko* [27]
Evoxanthine	1-OMe, 2-OCH_2O-3, 10-Me	*Balfourodendron riedelianum* [42], *Dictamnus albus* [44], *Euodia alata* [42], *E. triphylla* [44], *E. xanthoxyloides* [42], *Oricia gabonensis*, *O. renieri* [44], *O. suaveolens* [44], *Teclea boiviniana* [44], *T. gerrardii* [66], *T. grandifolia*, *T. natalensis* [42], *T. verdoorniana* [44], *Vepris ampody*, *V. biloculares* [42], *V. macrophylla* [22], *V. sclerophylla* [34]
Arborinine	1-OH, 2,3-diOMe, 10-Me	*Acronychia haplophylla* [42], *Almeidea coerulia* [32], *A. rubra* [32], *Araliopsis tabouensis* (*A.* soyauxii) [23], Diphasia *klaineana* [44], *Erthela bahiensis* [40], *Ertela* (*Monnieria*) *trifolia* [61], *Esenbeckia litoralis* [37], *Euodia alata*, *E. xanthoxyloides* [42], *Glycosmis arborea* [42], *G. bilocularis* [44], *G. mauritiana* [44], *G. parva* [76], *G. pentaphylla* [34], *Melicope micrococca* [33], *Monnieria trifolia* [42, 44], *Oricia renieri* [44], *Ravenia spectabilis* [42], *Ruta chalepensis* [42, 44], *R. graveolens*, *R. montana* [42],

(*continued*)

Table 24.11 (continued)

Alkaloids	Substituents	Occurrence
		Ruta callus cultures (*R. bracteosa*, *R. chalepensis*, *R. macrophylla*) [26], *Sarcomelicope leiocarpa* [19], *Teclea boiviniana* [44], *T. borenensis* [23], *T. gerrardii* [66], *T. natalensis* [42], *T. nobilis* [38], *T. trichocarpa* [37], *Vepris biloculares* [42], *V. bitoravina* [22], *V. pilosa* [44], *Zanthoxylum leprieurii* (*Fagara leprieurii*), *Z. macrophylla* (*F. macrophylla*) [42], *Z. macrophylla* (*F. macrophylla*, = *Z. gillettii*) [31], *Z. rubescens* (*F. rubescens*) [42], *Z. leprieurii* [44], *Z. simulans* [32]
Toddaliopsin C	1-OH, 2,3-diOMe, 10-CH_2OAc	*Toddaliopsis bremekampii* [40]
–	1-OH, 3,4-diOMe, 10-Me	*Sarcomelicope leiocarpa* [19], *Esenbeckia litoralis* [37], *Zanthoxylum leprieurii* [70]
–	1,5-diOH, 3-OMe, 10-Me	*Atalantia ceylanica* [21]
Yukodinine	1-OH, 3,5-diOMe, 10-Me	*Citrus hybrids* [27]
Pummeline	1,6-diOH, 3-OMe, 10-Me	*Citrus hybrids* [27], *C. hybrid* "*Yalaha*"(*C. paradisi* x *C. tangerina*) [30]
–	1,8-diOH, 3-OMe	*Boronia bowmanii* [28]
–	1,3,8-triOMe, 10-Me	*Boronia lanceolata* [28]
Citropremide	1-OMe, 3,8-diOH, 10-Me	*Citropsis gabunensis* [53]
–	1,2,3-triOH, 10-H	*Severinia buxifolia* [34]
Toddaliopsin A	1,2,3-triOMe, 10-H	*Toddaliopsis bremekampii* [40]
–	1,2,3-triOMe, 10-Me	*Almeidea rubra* [32], *Bauerella simplicifolia* ssp. *neo-scotica* [44], *Euodia alata* [42], *Melicope leratii* [44], *Sarcomelicope argirophylla* [21], *S. dogniensis* [24], *S. megistophylla* [29], *Teclea gerrardii* [65], *Vepris biloculares* [42]
Toddaliopsin B	1,2,3-triOMe, 10-CH_2OAc	*Toddaliopsis bremekampii* [40]
Toddaliopsin D	1,2,3-triOMe, 10-CH_2OMe	*Toddaliopsis bremekampii* [40]
–	1,3,4-triOMe, 10-Me	*Acronychia baueri* [18], *Medicosma fareana* [31], *Teclea boiviniana* [44]
–	1,3,5-triOMe, 10-Me	*Teclea boiviniana* [44]
Helebelicine A	1,4-diOMe, 3-OH, 10-Me	*Zanthoxylum leprieurii* [74]
Helebelicine B	1,2-diOMe, 3-OH, 10-Me	*Zanthoxylum leprieurii* [74]
–	1,2-diOH, 3-OMe, 10-Me	*Zanthoxylum leprieurii* [74]
Oligophylidine	1,8-diOH, 3-OMe, 10-Me	*Acronychia oligophylebia* [19]

(*continued*)

Table 24.11 (continued)

Alkaloids	Substituents	Occurrence
Evoprenine	1-OH, 2-OMe, 3-$OCH_2CH = CMe_2$, 10-Me	*Euodia alata* [42]
Glycocitrine-I	1,5-diOH, 3-OMe, 4-$CH_2CH = CMe_2$	*Citrus grandis* [22], *C. hybrid* "*Yalaha*"(*C. paradisi* x *C. tangerina*) [30], *C. maxima* [40], *C. paradisi* [32], *Glycosmis citrifolia* [16], *Severinia buxifolia* [34]
(−)-Marshmine	1,5-diOH, 3-OMe, 4-$CH_2CH(OH)C(=CH_2)Me$, 10-Me	*Citrus hybrid* "*Yalaha*"(*C. paradisi* x *C. tangerina*) [30], *C. paradisi* [29]
–	1-OH, 4-OMe, 3-$OCH_2CH = C(Me)CH_2CH_2CH = CMe_2$, 10-Me	*Sarcomelicope leiocarpa* [19]
–	1,3,5-triOH, 2,8-di$CH_2CH = CMe_2$	*Swinglea glutinosa* [36]
–	1,3,5-triOH, 4-OMe, 2,8-di$CH_2CH = CMe_2$	*Swinglea glutinosa* [77]
Xanthoevodine	1,4-diOMe, 2-OCH_2O-3, 10-H	*Acronychia baueri* [42], *Bauerella simplicifolia* ssp. *neo-scotica* [44], *Euodia xanthoxyloides* [42], *Melicope leratii* [44], *Sarcomelicope leiocarpa* [19]
Normelicopine	1-OH, 2-OMe, 3-OCH_2O-4, 10-Me	*Acronychia baueri* [44], *Medicosma subsessilis* [38], *Sarcomelicope megistophylla* [34]
Normelicopidine	1-OH, 4-OMe, 2-OCH_2O-3, 10-Me	*Acronychia baueri* [44], *Medicosma subsessilis* [38], *Sarcomelicope dogniensis* [24], *S. follicularis* [38], *S. megistophylla* [34]
5-Hydroxyarborinine	1,5-diOH, 2,3-diOMe, 10-Me	*Citropsis articulata* [45], *Glycosmis bilocularis* [44], *G. pentaphylla* [34], *Luvunga angustifolia* [27], *Pleiospermium alatum* [21]
Citrusamine	1,5-diOH, 3,4-diOMe, 10-Me	*Bosistoa selwynii* [31], *B. transversa* [30], *Citrus hybrids* [22], *C. hybrid* "*Yalaha*"(*C. paradisi* x *C. tangerina*) [30]
Melicopine	1,2-diOMe, 3-OCH_2O-4, 10-Me	*Acronychia acidula*, *A. baueri* [42], *Bauerella simplicifolia* ssp. *neo-scotica* [44], *Medicosma subsessilis* [38], *Melicope fareana* [42], *Sarcomelicope follicularis* [38], *S. leiocarpa* [19], *S. megistophylla* [34]

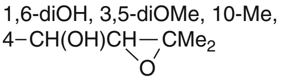
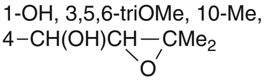
(*continued*)

Table 24.11 (continued)

Alkaloids	Substituents	Occurrence
Melicopidine	1,4-diOMe, 2-OCH_2O-3, 10-Me	*Acronychia baueri* [42], *Bauerella simplicifolia* ssp. *neo-scotica* [44], *Euodia alata*, *E. xanthoxyloides* [42], *Medicosma fareana* [31], *M. subsessilis* [38], *Melicope fareana* [42], *M. leptococca* [18], *M. leratii* [44], *Sarcomelicope argirophylla* [21], *S. dogniensis* [24], *S. follicularis* [38], *S. glauca* [21], *S. megistophylla* [29]
Tecleanthine	1,5-diOMe, 2-OCH_2O-3, 10-Me	*Diphasia angolensis* [44], *Oricia suaveolens* [44], *Sarcomelicope leiocarpa* [19], *Teclea boiviniana* [44], *T. natalensis* [42], *T. trichocarpa* [18], *T. verdoorniana* [44], *Vepris sclerophylla* [34]
Citpressine-I	1,6-diOH, 3,5-diOMe, 10-Me	*Citrus deliciosa* [33], *C. depressa* [16], *C. grandis* [17], *C. hybrid "Yalaha"(C. paradisi* x *C. tangerina)* [30], *C. paradisi* [32], *C. sulcata* [26]
Natsucitrine-I	1,6-diOH, 3,5-diOMe, 10-H	*Citrus hybrid "Yalaha"(C. paradisi* x *C. tangerina)* [30], *C. natsudaidai* [19], *C. sulcata* [26]
Citpressine-II	1-OH, 3,5,6-triOMe, 10-Me	*Citrus depressa* [16], *C. grandis* [17], *C. hybrid "Yalaha"(C. paradisi* x *C. tangerina)* [30], *C. paradisi* [32]
Natsucitrine-II	1-OH, 3,5,6-triOMe, 10-H	*Citrus funadoka* [23], *C. grandis* [22], *C. hybrid "Yalaha"(C. paradisi* x *C. tangerina)* [30], *C. maxima* [40], *C. paradisi* [32], *C. natsudaidai* [19]
Grandisine-I	1,5-diOH, 3,6-diOMe, 10-Me	*Citrus grandis* [17], *C. maxima* [40]
Grandisine-II	1,3-diOH, 5,6-diOMe, 10-Me	*Citrus funadoka* [23], *C. grandis* [17]
Marshdine	1-OH, 3-OMe, 5-OCH_2O-6, 10-Me	*Citrus paradisi* [29]
Grandisine-III	1,3,6-triOH, 5-OMe, 10-Me	*Citrus hybrids* [27]
Citrusinine-I	1,5-diOH, 3,4-diOMe, 10-Me	*Atalantia monophylla* [72], *A. wightii* [78], *Citrus grandis* [17], *C. hybrid "Yalaha"(C. paradisi* x *C. tangerina)* [30], *C. limonia* [24], *C. maxima* [40], *C. nobilis* [23], *C. sinensis* [17], *Severinia buxifolia* [34], *Swinglea glutinosa* [73]

(*continued*)

Table 24.11 (continued)

Alkaloids	Substituents	Occurrence
Glycosparvarine	1,3,5-triOH, 2-OMe, 10-Me	*Glycosmis parva* [76]
Citrusinine-II	1,3,5-triOH, 4-OMe, 10-Me	*Atalantia wightii* [78], *Citrus hybrid "Yalaha"*(*C. paradisi* x *C. tangerina*) [30], *C. sinensis* [17], *Severinia buxifolia* [34], *Swinglea glutinosa* [36]
Prenylcitpressine	1,6-diOH, 3,5-diOMe, 4-$CH_2CH = CMe_2$ 10-Me	*Citrus decumana* [27], *C. depressa* [16], *C. grandis* [17]
Buntanmine A	1,6-diOH, 3,5-diOMe, 4-CH_2CH (OH) $C(=CH_2)Me$, 10-Me	*Citrus grandis* [24]
(−)-Margrapine-A	 1,6-diOH, 3,5-diOMe, 10-Me, 4−CH(OH)CH—CMe_2 O	*Citrus paradisi* [31]
(−)-Margrapine-B	 1-OH, 3,5,6-triOMe, 10-Me, 4−CH(OH)CH—CMe_2 O	*Citrus paradisi* [31]
Grandisinine	1,3,6-triOH, 5-OMe, 4-$CH_2CH = CMe_2$ 10-Me	*Citrus decumana* [27], *C. grandis* [17], *C. hybrid "Yalaha"*(*C. paradisi* x *C. tangerina*) [30], *C. paradisi* [32]
Buntanine	1,3,6-triOH, 5-OMe, 3-$CH_2CH = CMe_2$ 10-Me	*Citrus grandis* [23]
Baiyumine-B	1-OH, 3,5,6-triOMe, 4-$CH_2CH = CMe_2$ 10-Me	*Citrus grandis* [21]
–	1,2,3,4-tetraOMe, 10-H	*Bauerella simplicifolia* ssp. *neo-scotica* [44], *Sarcomelicope leiocarpa* [19]
Normelicopicine	1-OH, 2,3,4-triOMe, 10-Me	*Acronychia baueri* [44], *Medicosma fareana* [31], *Sarcomelicope follicularis* [39], *S. leiocarpa* [19], *S. pembaiensis* [23], *Teclea trichocarpa* [37]
–	1-OH, 2,3,4-triOMe, 10-H	*Vepris macrophylla* [22]
5-Methoxyarborinine	1-OH, 2,3,5-triOMe, 10-Me	*Luvunga angustifolia* [27]
–	2-OH, 1,3,4-triOMe, 10-Me	*Sarcomelicope leiocarpa* [19]
Melicopicine	1,2,3,4-tetraOMe, 10-Me	*Acronychia baueri* [42], *Bauerella simplicifolia* ssp. *neo-scotica* [44], *Melicope fareana* [42], *M. leptococca* [18], *Sarcomelicope argirophylla* [21], *S. follicularis* [38], *S. glauca* [21], *S. leiocarpa* [19], *S. megistophylla* [34], *Teclea boiviniana* [44], *T. gerrardii* [65], *T. trichocarpa* [18], *Vepris sclerophylla* [34]

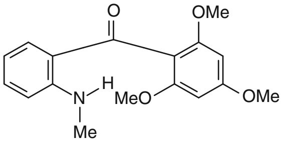
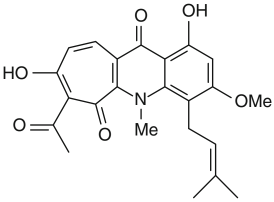
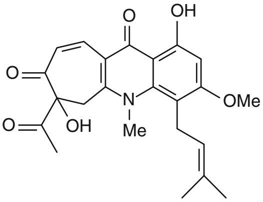
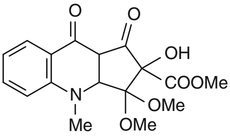
(*continued*)

Table 24.11 (continued)

Alkaloids	Substituents	Occurrence
Buxifoliadine-H	1,3,6-triOH, 4,5-OMe, 10-Me	*Severinia buxifolia* [34]
6-Methoxytecleanthine	1,5,6-triOMe, 2-OCH_2O-3, 10-Me	*Teclea boiviniana* [44], *T. trichocarpa* [18], *Vepris sclerophylla* [34]
Citbrasine	1,5-diOH, 2,3,4-triOMe, 10-Me	*Citrus sinensis* [17], *Swinglea glutinosa* [73]
Atalafoline	1,3-diOH, 2,5,6-triOMe, 10-Me	*Atalantia buxifolia* [21], *Citrus grandis* [24]
–	1,6-diOH, 2,3,5-triOMe, 10-Me	*Pleiospermium alatum* [21]
Glycofolinine	1,6-diOH, 3,4,5-triOMe, 10-Me	*Glycosmis citrifolia* [30], *G. parva* [76]
–	1,5,6-triOH, 2,3-diOMe, 10-Me	*Pleiospermum alatum* [24]
Cuspanine	1-OH, 2,3,5,6-tetraOMe	*Angostura paniculata* [27]
–	1-OH, 2,3,5,6-tetraOMe, 10-Me	*Pleiospermum alatum* [24]
Cusculine	1,2,3,5,6-pentaOMe	*Angostura paniculata* [27]
Glyfoline	1,6-diOH, 2,3,4,5-tetraOMe	*Glycosmis citrifolia* [16]
Citramine	1,3,6-triOH, 2,5-diOMe, 10-Me	*Glycosmis parva* [76], *Citrus hybrids* [22], *C. hybrid “Yalaha”* (*C. paradisi* x *C. tangerina*) [30]
Atalafoline B	1,4,5-triOH, 3,6-diOMe, 10-Me	*Atalantia buxifolia* [23]
Atalaphylline	1,3,5-triOH, 2,4-di$CH_2CH = CMe_2$, 10-H	*Atalantia monophylla* [42], *A. wightii* [78], *Severinia* (= *Atalantia*) *buxifolia* [33]
Bosistine	1,3,5-triOH, 2-$CH_2CH(OH)C(=CH_2)Me$, 4-$CH_2CH = CMe_2$, 10-Me	*Bosistoa selwynii* [31], *B. transversa* [30]
Atalaphyllidine A	1,3,8-triOH, 2,7-di$CH_2CH = CMe_2$, 10-H	*Atalantia monophylla* [44]
N-Methylatalaphylline	1,3,5-triOH, 2,4-di$CH_2CH = CMe_2$, 10-Me	*Atalantia monophylla* [42], *A. wightii* [16], *Bosistoa selwynii* [31], *B. transversa* [30], *Glycosmis parva* [76], *G. trichanthera* (*G. calcicola*) [33]
Buxifoliadine-A	1,5-diOH, 2,4-di$CH_2CH = CMe_2$, 3-OMe, 10-Me	*Atalantia monophylla* [72], *Severinia buxifolia* [34]
Buxifoliadine-B	1,5-diOH, 2,10-di$CH_2CH = CMe_2$, 3-OMe	*Severinia buxifolia* [34]
–	1-OH, 2,4-di$CH_2CH = CMe_2$, 3,5-diOMe, 10-H	*Atalantia monophylla* [44]
–	1-OMe, 3-$OC(Me_2)CHO$, 4-CHO, 10-H	*Sarcomelicope dogniensis* [22]
(+)-Megistophylline I	1-OH, 2-OMe, 3- = O, 4-OMe, 4-$CH_2CH = CMe_2$, 10-Me, 4,5-dihydro	*Sarcomelicope megistophylla* [34]

(*continued*)

Table 24.11 (continued)

Alkaloids	Substituents	Occurrence
(+)-Megistophylline II	1-OH, 2-OMe, 3- = O, 4-OMe, 4-CH_2CH = C(Me) CH_2CH_2CH = CMe_2, 10-Me, 4,5-dihydro	*Sarcomelicope megistophylla* [34]
Glycocitrine-VI	1-OH, 3- = O, 4-diCH_2CH = CMe_2, 10-Me, 4,5-dihydro	*Glycosmis citrifolia* [34]
Tecleanone		*Diphasia klaineana* [44], *Oricia renieri* [44], *O. suaveolens* [44], *Teclea gerrardii* [66], *T. grandifolia* [44], *T. verdoorniana* [44]
Citropone-B		*Citrus natsudaidai* [19]
Citropone-C		*Citrus* hybrid dunkan x hamlin [25], *C.* hybrid kiyomo x ito [25], *C.* hybrid ogonkan x hyuganatsu [25], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30], *C. paradisi* [25]
Fareanine		*Medicosma fareana* [31], *Sarcomelicope megistophylla* [34]
1-Methyl-2,3-dicarbomethoxyquinolin-4(1*H*)-one	1-Me, 2-COOMe, 3-COOMe	*Sarcomelicope dogniensis* [41]
(+)-Sarcomejine B	1-Me, 2-CHOMeCOOMe, 3-COOMe	*Sarcomelicope megistophylla* [41]
Megistonine II	1-Me, 2-COOMe, 3-OMe, 7-OH, 8-OMe	*Sarcomelicope megistophylla* [41]

(*continued*)

Table 24.11 (continued)

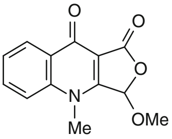
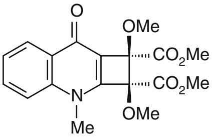
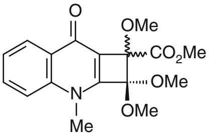
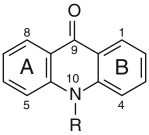
Alkaloids	Substituents	Occurrence
Megistonine I	1-Me, 2-COOMe, 3-OMe, 7-OH, 8-Pr	*Sarcomelicope megistophylla* [41]
(−)-Megistolactone		*Sarcomelicope folicularis* [41], *S. megistophylla* [41]
Cyclomegistine		*Sarcomelicope folicularis* [41], *S. megistophylla* [41], *Teclea gerrardii* [65]
Cyclomegistine B		*Sarcomelicope megistophylla* [65]

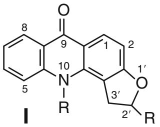
Table 24.12 Occurrence of acridone alkaloids in non-rutaceous species



Alkaloids	Substituents	Occurrence
–	1-OMe, 10-Me	aff. *Samadera* SAC-2825 (Simaroubaceae) [31]
–	1,8-diOH, 10-Me	aff. *Samadera* SAC-2825 (Simaroubaceae) [31]
Arborinine	1-OH, 2,3-diOMe, 10-Me	*Saussurea nepalensis* (Asteraceae) [23]

Frequently a prenyl at aromatic ring is present either as an alkyl side chain or in the form of a pyran or furan ring, e.g., tecleaverdoornine (**7**, R = Me, 6-OCH_2O-7, 8-OH, 5-CH_2CH = CMe_2), medicosmine, and choisyne (Table 24.7), isolated from *Teclea ouabanguiensis* [44], *Medicosma cunninghamii* [42], and *Choisya arizonica* [42], respectively.

Table 24.13 Occurrence of dihydro- and furoacridone alkaloids in Rutaceae species



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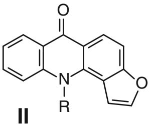

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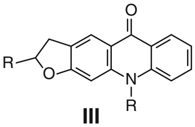
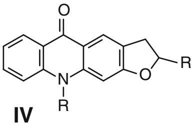


III



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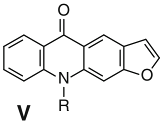
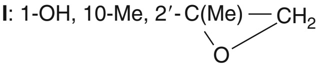
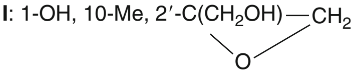


V

Alkaloids	Substituents	Occurrence
Rutacridone	**I**: 2′-C(=CH_2)Me, 1-OH, 10-Me	*Boenninghausenia albiflora* [44], *Ruta chalepensis* [44], *R. graveolens* [42], *Ruta* callus cultures (*R. bracteosa, R. chalepensis*, *R. macrophylla*) [26], *Thamnosma montana* (cell suspension cultures) [28], *T. rhodesica* [38]
Rutacridone epoxide		*Boenninghausenia albiflora* [25], *Ruta graveolens* [44], *Ruta* callus cultures (*R. bracteosa, R. chalepensis*, *R. macrophylla*) [26], *Thamnosma montana* (cell suspension cultures) [28]
Gravacridonol	**I**: 2′-C(=CH_2)CH_2OH, 1-OH, 10-Me	*Ruta graveolens* [44], *Ruta* callus cultures (*R. bracteosa, R. chalepensis*, *R. macrophylla*) [26], *Thamnosma montana* (cell suspension cultures) [28]
–	**I**: 2′-C(=CH_2)CH_2Cl, 1-OH, 10-Me	*Ruta* callus cultures (*R. bracteosa, R. chalepensis*, *R. macrophylla*) [26]
Hydroxyrutacridone epoxide		*Ruta graveolens* [44], *Ruta* callus cultures (*R. bracteosa, R. chalepensis*, *R. macrophylla*) [26]
Gravacridondiol	**I**: 2′-C(OH)(CH_2OH)Me, 1-OH, 10-Me	*Ruta graveolens* [42], *Thamnosma montana* (cell suspension cultures) [28], *T. rhodesica* [38]
(+)-Gravacridondiol acetate	**I**: 2′-C(OH)(CH_2OAc)Me, 1-OH, 10-Me	*Ruta graveolens* [25]
Gravacridondiol monomethyl ether	**I**: 2′-C(OH)(CH_2OMe)Me, 1-OH, 10-Me	*Ruta graveolens* [42]
Gravacridontriol	**I**: 2′-C(OH)$(CH_2OH)_2$, 1-OH, 10-Me	*Ruta graveolens* [44], *Thamnosma montana* (cell suspension cultures) [28]
Gravacridonchlorine	**I**: 2′-C(Cl)(CH_2OH)Me, 1-OH, 10-Me	*Ruta graveolens* [42], *Ruta* callus cultures (*R. bracteosa, R. chalepensis*, *R. macrophylla*) [26]

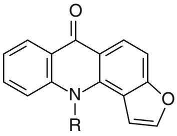
(*continued*)

Table 24.13 (continued)

Alkaloids	Substituents	Occurrence
Isogravacridonchlorine	**I**: 2′-C(OH)(CH_2Cl)Me, 1-OH, 10-Me	*Ruta chalepensis* [35], *R. graveolens* [44], *Thamnosma montana* (cell suspension cultures) [28]
Gravacridonolchlorine	**I**: 2′-C(Cl)(CH_2OH)$_2$, 1-OH, 10-Me	*Ruta graveolens* [42]
Gravacridonol glucoside	**I**: 2′-C(=CH_2)CH_2Oglucose, 1-OH, 10-Me	*Thamnosma montana* (cell suspension cultures) [28]
Gravacridondiol glucoside[a]	**I**: 2′-C(O-glucose)CH_2(OH)Me, 1-OH, 10-Me	*Ruta graveolens* [44], *Thamnosma montana* (cell suspension cultures) [28]
Gravacridontriol glucoside[a]	**I**: 2′-C(O-glucose)CH_2(OH)CH_2OH, 1-OH, 10-Me	*Ruta graveolens* [44], *Thamnosma montana* (cell suspension cultures) [28]
(−)-Rhodesiacridone	**I**: 2′-C(OH)CH_2(OH)COOMe, 1-OH, 10-Me	*Thamnosma rhodesica* [38]
(+)-Oriciacridone C	**I**: 2′-C(=CH_2)Me, 1,5-diOH, 10-H	*Oriciopsis glaberrima* [40]
1-Hydroxy-*N*-methylfuracridone	**II**: 1-OH, 10-Me	*Ruta graveolens* [44], *R. chalepensis* var. *latifolia* [23], *Glycosmis citrifolia* [16]
Chaloridone	**II**: 1-OCH$_2$O-2, 10-Me	*Ruta chalepensis* [22]
Furofoline-II	**II**: 1-OH, 10-Me, 2′-C(OH)Me$_2$	*Glycosmis citrifolia* [16]
Hallacridone	**II**:, 1-OH, 10-Me, 2′-COMe	*Ruta* callus cultures (*R. bracteosa*, *R. chalepensis*, *R. macrophylla*) [26], *Thamnosma montana* (cell suspension cultures) [28]
Thehaplosine	**II**: 1,2-diOMe, 10-Me	*Haplophyllum thesioides* [28]
Buxifoliadine-F	**II**: 1-OMe, 5-OH, 10-H	*Severinia buxifolia* [34]
Furoparadine	**II**: 1,6-diOH, 5-OMe, 10-Me	*Citrus paradisi* [29]
Glycocitrine-V	**III**: 1-OH, 3,4,5-OMe, 2′-α-C(OH)Me$_2$, 3′-β-OH, 10-Me	*Glycosmis citrifolia* [34]
–	**IV**: 1,5-diOH, 4-OMe, 10-Me, 2′-C(OH)Me$_2$	*Swinglea glutinosa* [79]
Buxifoliadine-E	**IV**: 1-OH, 4-CH_2CH = CMe$_2$, 5-OH, 10-H, 2′-C(OH)Me$_2$	*Atalantia monophylla* [72], *Severinia buxifolia* [34]
N-Methylbuxifoliadine-E	**IV**: 1-OH, 4-CH_2CH = CMe$_2$, 5-OH, 10-Me, 2′-C(OH)Me$_2$	*Atalantia monophylla* [72]
(+)-Oriciacridone D	**IV**: 2′-C(=CH_2)Me, 1,5-diOH, 10-H	*Oriciopsis glaberrima* [40]
Buxifoliadine-G	**V**: 1-OMe, 4-CH_2CH = CMe$_2$, 5-OH, 10-Me	*Severinia buxifolia* [34]

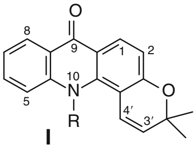
[a] (in Ref. [38] the authors showed that sugar was attached to the tertiary alcohol)

Table 24.14 Occurrence of furoacridone alkaloids in non-rutaceous species



Alkaloids	Substituents	Occurrence
1-Hydroxy-*N*-methylfuracridone	1-OH, 10-Me	*Piper pedicellosum* (Piperaceae) [33]

Table 24.15 Occurrence of dihydro- and pyranoacridone alkaloids in Rutaceae species



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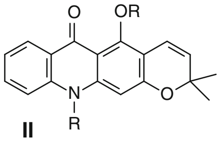

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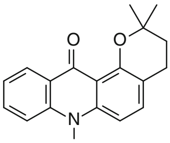

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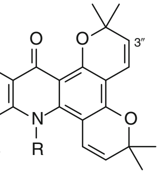

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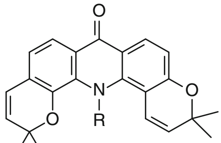

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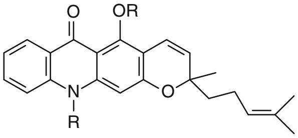


VI

Alkaloids	Substituents	Occurrence
Des-*N*-methyl-Noracronycine	**I**: 1-OH, 10-H	*Glycosmis citrifolia* [18], *G. trichanthera* (*G. calcicola*) [33], *Murraya paniculata* [44]
Des-*N*-methyl-Acronycine	**I**: 1-OMe, 10-H	*Acronychia baueri* [18], *Glycosmis citrifolia* [18], *G. mauritiana* [24], *G. pentalhylla* [42], *Medicosma subsessilis* [38], *Murraya paniculata* [44], *Sacomelicope dogniensis* [22], *S. follicularis* [38], *S. megistophylla* [34]
Noracronycine	**I**: 1-OH, 10-Me	*Acronychia baueri* [18], *Boenninghausenia albiflora* [44], *Glycosmis citrifolia* [18], *G. mauritiana* [24], *G. pentalhylla* [42], *Medicosma subsessilis* [38], *Murraya paniculata* [44], *Sarcomelicope megistophylla* [34]
Acronycine	**I**: 1-OMe, 10-Me	*Acronychia baueri* [42, 44], *Bauerella simplicifolia* ssp. *neo-scotica* [44], *Medicosma subsessilis* [38], *Melicope leptococca* [18], *Sarcomelicope argyrophylla* [21], *S. dogniensis* [22], *S. follicularis* [38], *S. glauca* [21], *S. megistophylla* [34], *S. pembaiensis* [23]

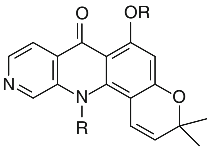
(*continued*)

Table 24.15 (continued)

Alkaloids	Substituents	Occurrence
Severifoline	**I**: 1-OH, 2-$CH_2CH = CMe_2$, 10-H	*Severinia buxifolia* [16]
N-mehtylseverifoline	**I**: 1-OH, 2-$CH_2CH = CMe_2$, 10-Me	*Glycosmis citrifolia* [18], *Severinia buxifolia* [16]
Atalaphyllidine	**I**: 1,5-diOH, 10-H	*Atalantia macrophylla* [44], *Citrus sinensis* grafted on *C. limonia* [61], *Glycosmis citrifolia* [18], *G. macranta* [80], *Oriciopsis glaberrima* [40], *Severinia buxifolia* [34]
5-Hydroxynoracronycine	**I**: 1,5-diOH, 10-Me	*Atalantia ceylanica* [44], *A. wightii* [78], *Balsamocitrus paniculata* [58], *Citropsis articulata* [45], *Citrus depressa*, *C. grandis* [17], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30], *C. limonia* [24], *C. natsudaidai* [21], *C. maxima* [40], *C. nobilis* [23], *C. sulcata* [26], *Glycosmis citrifolia* [18], *G. trichanthera* (*G. calcicola*) [33], *Pleiospermum alatum* [24], *Poncirus trifoliata* [21], *Swinglea citrumelo* [36], *S. glutinosa* [36]
7-Hydroxynoracronycine	**I**: 1,7-diOH, 10-Me	*Glycosmis macranta* [80]
Baiyemine-A	**I**: 1-OH, 5-OMe, 10-Me	*Citrus depressa* [16], *C. grandis* [21], *C. junos* [21]
Citracridone-I	**I**: 1,6-diOH, 5-OMe, 10-Me	*Balsamocitrus paniculata* [58], *Citrus depressa* [16], *C. grandis* [17], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30], *C. limonia* [24], *C. nobilis* [23], *C. paradisi* [32], *C. sinensis* [17], *C. sulcata* [26], *Glycosmis citrifolia* [18], *G. pentaphylla* [34]
Acrifoline	**I**: 1,6-diOH, 5-OMe, 10-H	*Glycosmis citrifolia* [30], *G. pentaphylla* [34]
Citracridone-III	**I**: 1,5,6-triOH, 10-Me	*Balsamocitrus paniculata* [58], *Citrus C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30], *C. maxima* [40], *C. yuko* [26]
–	**I**: 1,5-diOH, 6-OMe, 10-Me	*Citrus decumana* [18], *C. deliciosa* [33]
Citracridone-II	**I**: 1-OH, 5,6-diOMe, 10-Me	*Citrus decumana* [18], *C. depressa* [16], *C. grandis* [17], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30]
Balsacridone A	**I**: 1-OH, 5,6-di$OCH_2CH = CMe_2$, 10-Me	*Balsamocitrus paniculata* [58]
Balsacridone B	**I**: 1-OH, 6-$OCH_2CH = CMe_2$, 10-Me	*Balsamocitrus paniculata* [58]

(*continued*)

Table 24.15 (continued)

Alkaloids	Substituents	Occurrence
Atalaphyllinine	**I**: 1,5-diOH, 2-$CH_2CH = CMe_2$, 10-H	*Atalantia monophylla* [44], *Severinia buxifolia* [16]
N-Methylatalaphyllinine	**I**: 1,5-diOH, 2-$CH_2CH = CMe_2$, 10-Me	*Atalantia ceylanica* [44], *A. monophylla* [16], *A. wightii* [16], *Bosistoa selwynii* [31], *B. transversa* [30], *Glycosmis citrifolia* [18], *G. trichanthera* (*G. calcicola*) [33], *Severinia buxifolia* [16]
5-Hydroxynoracronycine alcohol	**I**: 1,5-diOH, 10-Me, 2′-CH_2OH	*Citrus maxima* [40]
- (+)	**I**: 4′-OH, 1-OMe, 10-H, 3′,4′-dihydro	*Sacomelicope dogniensis* [22]
–	**I**: 4′ = O, 1-OMe, 10-H, 3′,4′-dihydro	*Sacomelicope dogniensis* [22]
–	**I**: 4′ = O, 1-OMe, 10-OH, 3′,4′-dihydro	*Sacomelicope dogniensis* [22]
–	**I**: 3′-OH, 4′ = O, 1-OH, 10-H, 3′,4′-dihydro	*Medicosma subsessilis* [38]
–	**I**: 3′,4′-diOH (*cis*), 1-OMe, 10-Me, 3′,4′-dihydro	*Medicosma subsessilis* [38], *Sarcomelicope dogniensis* [24], *S. glauca* [21]
–	**I**: 3′,4′-diOH (*trans*), 1-OMe, 10-Me, 3′,4′-dihydro	*Medicosma subsessilis* [38], *Sarcomelicope dogniensis* [24], *S. glauca* [21]
- (−)	**I**: 3′,4′-diOH (*trans*), 1-OMe, 10-H, 3′,4′-dihydro	*Sacomelicope dogniensis* [22]
-(+)-	**I**: 3′-β-OH, 4′-α-OH, 1,6-diOH 5-OMe, 10-Me, 3′,4′-dihydro	*Citrus paradisi* [29]
(+)-Acronycine epoxide	**I**: 3′,4′-epoxide, 1-OMe, 10-Me, 3′,4′-dihydro	*Sacomelicope argyrophylla* [23], *S. simplicifolia* [23]
Azaacridone-A		*Citrus paradisi* [27]
Junosidine	**II**: 1-OH, 10-Me	*Citrus junos* [22]
Buxifoliadine-D	**II**: 1-OH, 4-$CH_2CH = CMe_2$, 10-H	*Severinia buxifolia* [34]
Yukocitrine	**II**: 1,5-diOH, 10-Me	*Atalantia monophylla* [72], *Bosistoa selwynii* [31], *B. transversa* [30], *Citrus yuko* [27]
Cycloatalaphylline-A	**II**: 1,5-diOH, 4-$CH_2CH = CMe_2$, 10-H	*Atalantia monophylla* [72]
N-Methylcyclo-atalaphylline A	**II**: 1,5-diOH, 4-$CH_2CH = CMe_2$, 10-Me	*Atalantia monophylla* [72], *Glycosmis parva* [76]

(*continued*)

Table 24.15 (continued)

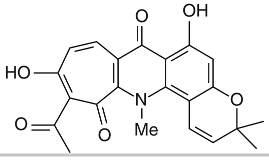
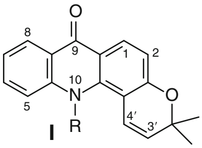
Alkaloids	Substituents	Occurrence
–	**II**: 1,5-diOH, 4-CH_2CH(OH)C(=CH_2)Me, 10-Me	*Bosistoa transversa* [30], *Glycosmis trichanthera* (*G. calcicola*) [33]
Pyranofoline	**II**: 1,5-diOH, 4-OMe	*Glycosmis citrifolia* [16], *Swinglea glutinosa* [73]
Honyumine	**II**: 1,6-diOH, 5-OMe	*Citrus grandis* [21]
–	**III**: 3,5-diOH, 4-OMe, 3′-OH, 10-Me	*Swinglea glutinosa* [77]
–	**IV**: 5-OH, 10-Me, 3′,4′,3″,4″-terahydro	*Atalantia monophylla* [42]
Macranthanine	**V**	*Glycosmis macranta* [80]
–	**VI**: 1-OH, 10-Me	*Glycosmis citrifolia* [16]
Citropone-A		*Citrus grandis*, *C. natsudaidai* [19], *C. nobilis* [23]

Table 24.16 Occurrence of pyranoacridone alkaloids in non-rutaceous species



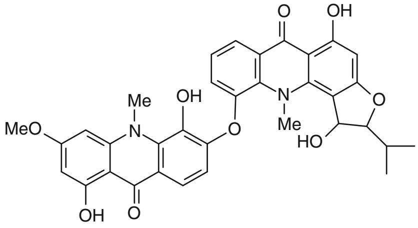
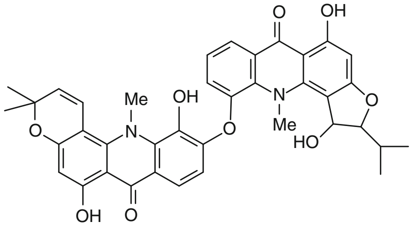
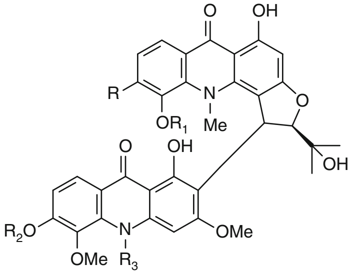
I

Alkaloids	Substituents	Occurrence
7-Hydroxynoracronycine	1,7-diOH, 10-Me	*Rauvolfia verticillata* (Apocynaceae) [82]

The anellation of the three-ring system is generally linear, such as in dictamnine (**7**, R = Me), but a few representatives with angular anellation have been found, e.g., 2′,3′-dihydrofuroquinolin-2-one araliopsine (**9**, N-Me) isolated from *Araliopsis soyauxii* [44]. However, angular furoquinoline alkaloids are not known.

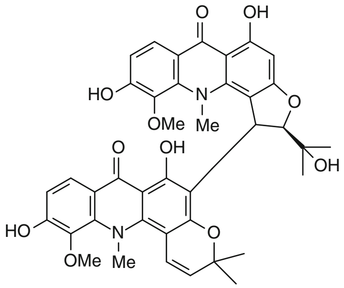
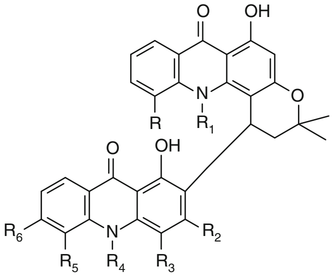
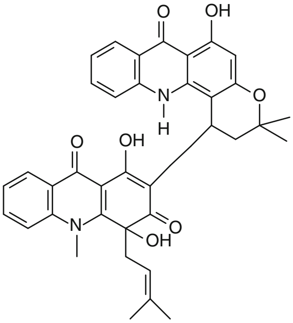
2′,2′-Dimethyl-3′-hydroxydihydropyranoquinolin/4-ones (**10**, **11**) have been obtained from several rutaceous species. Although tracer feeding experiments have not been reported, the co-occurrence of furoquinolin/one and these pyrano derivatives suggest that the biosynthesis of both groups involve oxidative cyclization of a common 3-(2,3-epoxy-3-methybutenyl)-4-hydroxyquinolin-2-one precursor.

Table 24.17 Occurrence of dimeric acridone alkaloids in Rutaceae species

Alkaloids	Substituents	Occurrence
Atalanine		*Atalantia ceylanica* [44]
Ataline		*Atalantia ceylanica* [44]
		
Citbismine-A (R, R_1, R_3 = H, R_2 = Me)		*Citrus grandis* [30], *C. paradisi* [29]
Citbismine-B (R = OH, R_1, R_2 = Me, R_3 = H)		*Citrus paradisi* [30]
Citbismine-C (R = OH, R_1, R_2, R_3 = Me)		*Citrus paradisi* [30]
Citbismine-E (R = OH, R_2 = H, R_1, R_3 = Me)		*Citrus paradisi* [30]
Citbismine-F (R = H, R_1 = H, R_2, R_3 = Me)		*Citrus paradisi* [33]

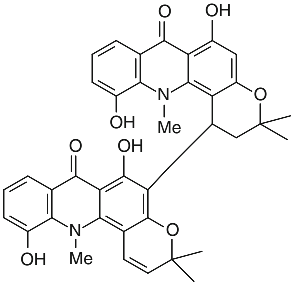
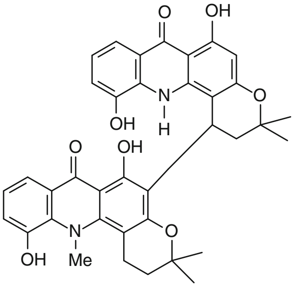
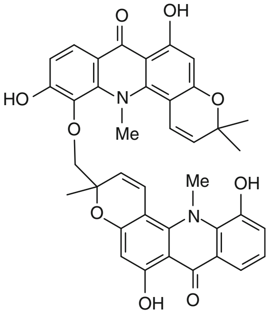
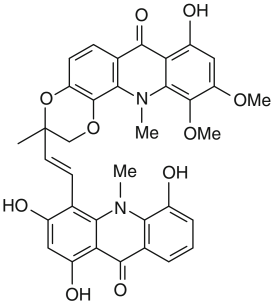
(*continued*)

Table 24.17 (continued)

Alkaloids	Substituents	Occurrence
Citbismine-D	 O OH HO N OMe Me O OH O OH HO N OMe Me O	*Citrus paradisi* [30]
	 O OH N R R_1 O O OH R_6 N R_2 R_5 R_4 R_3	
Glycobismine-A (R, R_1, R_5, R_6 = H, R_2 = OH, R_3 = $CH_2CH = CMe_2$, R_4 = Me)		*Glycosmis citrifolia* [18]
Buntanbismine (R = OH, R_1 = Me, R_2 = OMe, R_3, R_4 = H, R_5, R6 = OMe)		*Citrus grandis* f. *buntan* [30]
(±)-Glycobismine-B (diastereoisomer)	 O OH N H O O OH N O OH	*Glycosmis citrifolia* [27]
(±)-Glycobismine-C (diastereoisomer)		*Glycosmis citrifolia* [27]

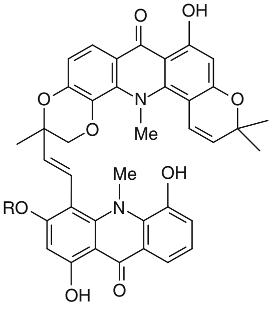
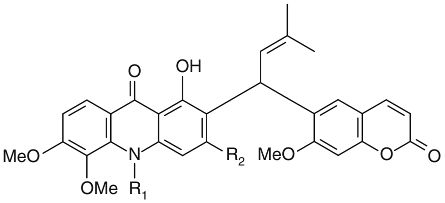
(*continued*)

Table 24.17 (continued)

Alkaloids	Substituents	Occurrence
Bis-5-hydroxynoracronycine	 O OH OH N Me O O OH OH N Me O	*Citris paradisi* [32], *Swinglea glutinosa* [77]
Oriciacridone F	 O OH OH N H O O OH OH N Me O	*Oriciopsis glaberrima* [40]
Glycobismine-G	 O OH HO N Me O O Me OH O N OH O	*Glycosmis citrifolia* [38]
Glycobismine-D	 O OH O N OMe O Me OMe Me OH HO N OH O	*Glycosmis citrifolia* [34]

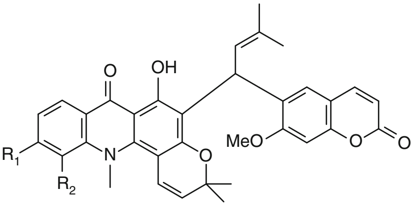
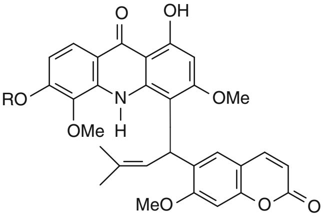
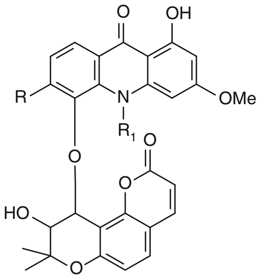
(*continued*)

Table 24.17 (continued)

Alkaloids	Substituents	Occurrence
Glycobismine-E R = Me	 O OH N Me O O O Me OH RO N OH O	*Glycosmis citrifolia* [34, 38]
Glycobismine-F R = H		*Glycosmis citrifolia* [34, 38]
		
Acrimarine H (R_1 = Me, R_2 = OMe, R_3 = OH, R_4 = H)		*Citrus* hybrid kiyomo x ito [25], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30]
(−)-Acrimarine A (R_1 = Me, R_2 = OH, R_3, R_4 = OMe)		*Citrus funadoka* [23]
(−)-Acrimarine B (R_1 = H, R_2, R_3, R_4 = OMe)		*Citrus funadoka* [23], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30]
Acrimarine **N** (R_1 = Me, R_2, R_3, R_4 = OMe)		*Citrus hybrid "Yalaha"* [29]
(±)-Acrimarine K (R_1 = Me, R_2 = OH, R_3 = OMe, R_4 = OH)		*Citrus funadoka* [27]
(±)-Acrimarine M (R_1 = Me, R_2 = OH, R_3, R_4 = H)		*Citrus funadoka* [27]
(+)-Acrimarine E (R_1 = H, R_2, R_3 = OMe, R_4 = OH)		*Citrus funadoka* [24], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30]

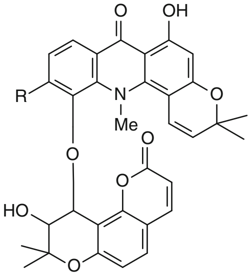
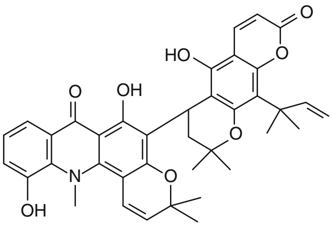
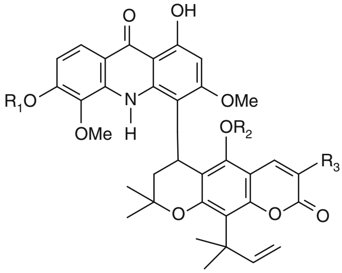
(*continued*)

Table 24.17 (continued)

Alkaloids	Substituents	Occurrence
Acrimarine F (R_1 = Me, R_2, R_3 = OMe, R_4 = OH)		*Citrus funadoka* [24], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30]
Acrimarine G (R_1 = Me, R_2, R_3 = OH, R_4 = H)		*Citrus funadoka* [24]
(±)-Acrimarine-I R_1 = H, R_2 = OH		*Citrus hybrids* [27]
(±)-Acrimarine-J R_1 = OH, R_2 = OMe		*Citrus hybrids* [27]
		
(−)-Acrimarine C (= (±)-Acrimarine L) (R = H)		*Citrus funadoka* [23, 27], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30]
(−)-Acrimarine D (R = Me)		*Citrus funadoka* [24], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30]
(+)-Neoacrimarine G (R = H, R_1 = Me)		*Citrus paradisi* [32]

(*continued*)

Table 24.17 (continued)

Alkaloids	Substituents	Occurrence
(+)-Neoacrimarine F (R = OH, R_1 = Me)		*Citrus hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [32]
(−)-Neoacrimarine J (R = OH, R_1 = H)		*Citrus paradisi* [33]
(−)-Neoacrimarine I (R = OMe, R_1 = H)		*Citrus paradisi* [33]
		
(+)-Neoacrimarine-H R = H		*Citrus paradisi* [33]
Neoacrimarine-C R = OH		*Citrus hassaku* [28], *C. hybrid "Yalaha"* (*C. paradisi* x *C. tangerina*) [30]
Neoacrimarine-D		*Citrus hassaku* [28]
	 O OH R$_1$O N H OMe OMe OR$_2$ R$_3$ O O O	
(±)-Neoacrimarine-A R_1 = Me, R_2 = H, R_3 = C(Me_2) CH = CH_2, 3′,4′-dehydro		*Citrus hybrids* [27]

(*continued*)

Table 24.17 (continued)

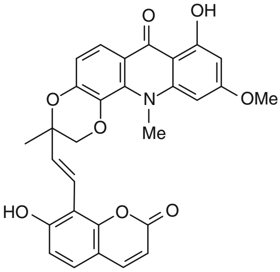
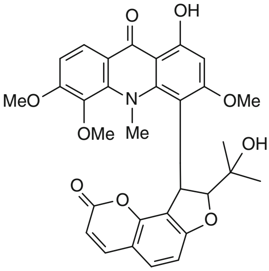
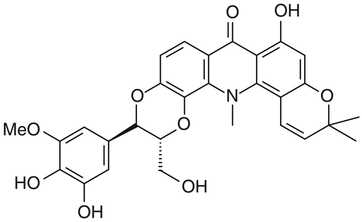
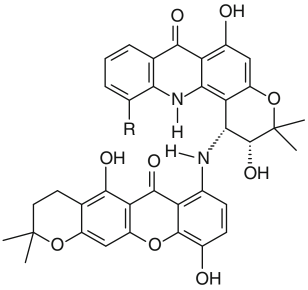
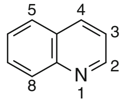
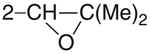
Alkaloids	Substituents	Occurrence
(±)-Neoacrimarine-B R_1, R_2 = H, R_3 = C(Me$_2$)CH = CH$_2$, 3′,4′-dehydro		*Citrus hybrids* [27]
(−)-Neoacrimarine-E R_1 = Me, R_2, R_3 = H,		*Citrus hybrids* [27]
(+)-Dioxinoacrimarine-A		*Citrus hybrid* "Yalaha" [29]
Neoacrimarine-K		*Citrus paradisi* [33]
Acrignine-A		*Citrus grandis* [27], *C. hybrid* *"Yalaha"* (*C. paradisi* x *C. tangerina*) [30], *C. yuko* [27]
(−)-Oriciacridone A R = H		*Oriciopsis glaberrima* [40]
(−)-Oriciacridone B R = OH		*Oriciopsis glaberrima* [40]

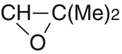
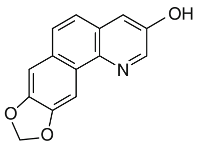
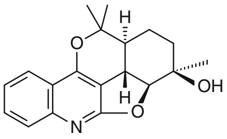
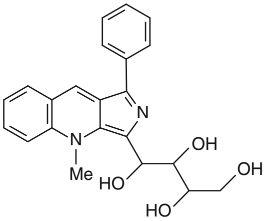
Table 24.18 Occurrence of quinoline alkaloids in the Rutaceae species



Alkaloid	Substituents	Occurrence
Quinoline		*Angostura trifoliata*[a] [1], *Citrus aurantium* [42], *Galipea officinalis*[a] [42]
3-Hydroxyquinoline (3-quinolol)	3-OH	*Ruta montana* [41]
Quinaldine	2-Me,	*Angostura trifoliata*[a] [1], *Galipea officinalis*[a] [44]
2-*n*-Propylquinoline	2-*n*-C_3H_7	*Galipea bracteata*[a] [41], *G. longiflora*[a] [41]
Chimanine B	2-(E) CH = $CHCH_3$	*Galipea longiflora*[a] [41]
Chimanine D		*Galipea longiflora*[a] [41]
2-*n*-Pentyl-quinoline (2-*n*-amylquinoline)	2-*n*-C_5H_{11}	*Angostura trifoliata*[a] [1], *Galipea bracteata*[a] [41], *G. longiflora*[a] [41], *G. officinalis*[a] [41]
2-(Pent-1-enyl)-quinoline	2-CH = CHC_3H_7	*Galipea bracteata*[a] [41]
4-Methoxyquinaldine	2-Me, 4-OMe	*Galipea officinalis*[a] [41], *Ruta montana* [41]
Chimanine A	2-*n*-C_3H_7, 4-OMe	*Galipea longiflora*[a] [41]
Chimanine C	2-(E) CH = $CHCH_3$, 4-OMe	*Galipea longiflora*[a] [41]
4-Methoxy-2-*n*-pentylquinoline (quinoleine)	2-*n*-C_5H_{11}, 4-OMe	*Angostura trifoliata*[a] [1], *Galipea longiflora*[a] [24, 41], *G. officinalis*[a] [41], *Zanthoxylum avicennae* [41]
4-Methoxy-2-*n*-heptylquinoline	2-*n*-C_7H_{15}, 4-OMe	*Zanthoxylum avicennae* [41]
4-Methoxy-2-(pent-1-enyl)quinoline	2-CH = CHC_3H_7, 4-OMe	*Galipea longiflora*[a] [24]
4-Methoxy-2-(1-ethylpropyl) quinolone	2-$CH(CH_2CH_3)CH_2CH_3$, 4-OMe	*Esenbeckia leiocarpa* [41]
4-Methoxy-2-(8-oxononyl)quinolone	2-$(CH_2)_7COCH_3$, 4-OMe	*Ruta montana* [41]
1-Methyl-2-propyl-1,2,3,4-tetrahydroquinoline	1-Me, 2-H, *n*-C_3H_7, 3-H,H, 4-H,H	*Galipea officinalis*[a] [41]

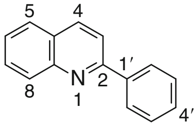
(*continued*)

Table 24.18 (continued)

Alkaloid	Substituents	Occurrence
2-*n*Pentyl-1,2,3,4-tetrahydroquinoline	1-H, 2-H, *n*-C_5H_{11}, 3-H,H, 4-H,H	*Galipea officinalis*[a] [41]
(−)-Angustureine	1-Me, 2-H, *n*-C_5H_{11}, 3-H,H, 4-H,H	*Galipea officinalis*[a] [41]
–	2-OCH_2O-3, 4,7-diOMe	*Acronychia laurifolia* (*A. pedunculata*) [34]
Montanine	2,4-diOMe	*Aegle marmelos* [38], *Hortia colombiana* [32], *Ruta montana* [24]
Isopteleprenine	2,4-diOMe, 7-OCH_2O-8, 3-$CH_2CH = CMe_2$,	*Orixa japonica* [28]
(−)-Preorixine	2,4-diOMe, 7-OCH_2O-8, 3−CH_2CH−C(Me)$_2$ 	*Orixa japonica* [27]
(+)-Isoptelefolidine	2,4-diOMe, 7-OCH_2O-8, 3-$CH_2CH(OH)C(=CH_2)Me$,	*Orixa japonica* [28]
Toddaquinoline		*Toddalia asiatica* [28], *Zanthoxylum beecheyanum* (*Z. arnottianum*) [39], *Z. nitidum* [34], *Z. simulans* [29]
Furoerioaustralasine		*Eriostemon australasius* subsp. *banskii* [28]
(−)-Folipidine		*Haplophyllum foliosum* [39], *H. pedicellatum* [39]

[a] *Angostura trifoliata* (syn., *Galipea officinalis*, see item III); *A. longiflora* (syn., *G. longiflora* K. Krause); *A. bracteata* (syn., *G. bracteata*); [41].

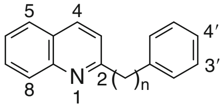
Table 24.19 Occurrence of 2-arylquinoline alkaloids in the Rutaceae species



Alkaloids	Substituents	Occurrence
2-Phenylquinoline		*Galipea longiflorae*[a] [41]
4-Methoxy-2-phenylquinoline	4-OMe,	*Lunasia amara* [2, 41], *Galipea longiflora*[a] [41]
Dubamine	3′-OCH_2O-4′	*Haplophyllum dubium* [3, 41], *H. latifolium* [59], *Dictamnus albus* [42]
Graveolinine	4-OMe, 3′-OCH_2O-4′	*Lunasia amara* [3, 41], *Ruta graveolens* [3, 41], *R. chalepensis* [41], *R. chalepensis* var. *latifolia* [41]

[a] *Angostura longiflora* (syn., *G. longiflora* K. Krause); [41].

Table 24.20 Occurrence of 2-alkylarylquinoline alkaloids in the Rutaceae species



Alkaloids	Substituents	Occurrence
2-(3,4-Dimethoxyphenylethyl) quinoline	n = 2, 3′-OMe, 4′-OMe	*Galipea longiflora*[a] [41], *G. officinalis*[a] [41]
Demethoxycusparine [2-(3,4-methylenedioxyphenylethyl) quinoline]	n = 2, 3′-OCH_2O-4′	*Galipea longiflora* [41], *G. bracteata*[a] [41], *G. officinalis*[a] [41]
2-(3-Hydroxy-4-methoxyphenylethyl)-4-methoxyquinoline or [(3-methoxy-4-hydroxyphenylethyl]-4-methoxyquinoline isomer	n = 2, 4-OMe, 3′-OH, 4′-OMe	*Galipea officinalis*[a] [41]
Cusparine	n = 2, 4-OMe, 3′-OCH_2O-4′	*Angostura trifoliata*[a] [1], *Galipea longiflora*[a] [41], *G. officinalis*[a] [41]
Galipoline	n = 2, 4-OH, 3′-OMe, 4′-OMe	*Angostura trifoliata*[a] [1], *Galipea officinalis*[a] [42]
Galipine	n = 2, 4-OMe, 3′-OMe, 4′-OMe	*Angostura trifoliata*[a] [1] *Galipea officinalis*[a] [41]
2-(3,4-Methylenedioxystyryl)-4-methoxyquinoline	n = CH = CH, 4-OMe, 3′-OCH_2O-4′	*Galipea longiflora*[a] [41]
2-(3,4-Dimethoxyphenylethyl)-1,2,3,4-tetrahydroquinoline	n = 2, 1-H, 2-H, 3-H,H, 4-H,H, 3′-OMe, 4′-OMe	*Galipea officinalis*[a] [41]

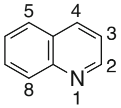
(*continued*)

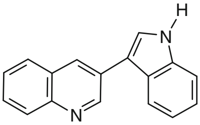
Table 24.20 (continued)

Alkaloids	Substituents	Occurrence
2-(3,4-Methylenedioxyphenylethyl)-1,2,3,4-tetrahydroquinoline	n = 2, 1-H, 2-H, 3-H,H, 4-H,H, 3′-OCH_2O-4′	*Galipea officinalis*[a] [41]
(−)-Galipeine	n = 2, 1-Me, 2-H, 3-H, H, 4-H,H, 3′-OH, 4′-OMe	*Galipea officinalis*[a] [41]
3,4-Methylenedioxicuspareine or (−)-Galipinine (Allocuspareine)	n = 2, 1-Me, 2-H, 3-H, H, 4-H,H, 3′-OCH_2O-4′	*Galipea officinalis*[a] [41]
Cuspareine	n = 2, 1-Me, 2-H, 3-H, H, 4-H,H, 3′-OMe, 4′-OMe	*Angostura trifoliata* [1], *Galipea officinalis*[a] [41]
2-[6-(3,4-Methylenedioxyphenyl) hexyl]-4-methoxyquinoline	n = 6, 4-OMe, 3′-OCH_2O-4′	*Ruta chalepensis* [41]

[a] *Angostura trifoliata* (syn., *Galipea officinalis*, see item III); *A. longiflora* (syn., *G. longiflora* K. Krause); *A. bracteata* (syn., *G. bracteata*); [41].

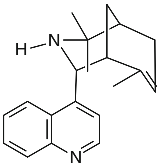
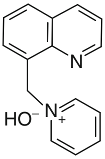
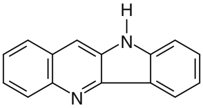
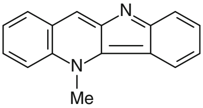
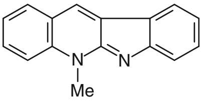
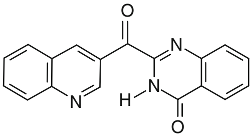
Table 24.21 Occurrence of quinoline alkaloids in non-rutaceous species



Alkaloids	Substituents	Occurrence
Quinoline		*Peganum harmala* (Zygophyllaceae) [41]
Quinaldine	2-Me	*Peganum harmala* [41]
3-Phenylquinoline	3-Ph	*Peganum nigellastrum* [41]
3-(4-Hydroxyphenyl) quinoline	3-*p*-OH-Ph	*Peganum nigellastrum* [41]
Quinoline-3-carboxamide	3-$CONH_2$	*Peganum nigellastrum* [41]
–	2-OCH_2O-3, 4,8-diOMe	*Sebastiania corniculata* (Euphorbiaceae) [39]
–	2-OCH_2O-3, 4,7,8-triOMe	*Acanthosyris paulo-alvinii* (Santalaceae) [32], *Sebastiania corniculata* [39]
Echinorine	1-Me, 4-OMe	*Echinops sphaerocephalus* (Asteraceae) [89]
–	2-CH = CMe_2, 4,6-diMe, 7-OH	*Curcuma longa* (Zingiberaceae) [97]
3-(1*H*-Indol-3-yl)quinoline		*Peganum nigellastrum* [41]

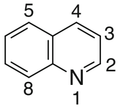
(*continued*)

Table 24.21 (continued)

Alkaloids	Substituents	Occurrence
–		*Aristotelia chilensis* (Elaeocarpaceae) [28]
Sibiridine		*Nitraria schoberi* [37], *N. sibirica* (Nitrareaceae) [37]
–		*Justicia betonica* (Acanthaceae) [98]
Cryptolepine		*Cryptolepis sanguinolenta* (Periplocaceae) [100], *Isatis indigotica* (Brassicaceae) [99]
NeoCryptolepine		*Cryptolepis sanguinolenta* (Periplocaceae) [100]
	Quinoline + Quinazoline	
Luotonin F		*Peganum nigellastrum* [41]

Thus, the isolation of (+)-(R)-isoplatydesmine and (−)-(S)-ribalinine from *Araliopsis soyauxii* suggests that the biosynthesis of these compounds via an 3-[(2-S)-2,3-epoxy-3-methybutenyl)-4-hydroxyquinolin-2-one occurs by cyclization with stereochemical inversion furnishing the dihydrofuroquinolin/4-one (**4**, **5**), and reaction at the tertiary carbon atom of the epoxide ring giving the dihydropyranoquinolin/4-one (**10**, **11**), without affecting the chiral center (Scheme 24.1).

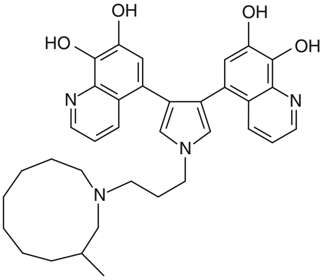
Table 24.22 Occurrence of quinoline alkaloids in fungi, bacteria, marine organisms, and animal



Alkaloids	Substituents	Occurrence
Quinoline		*Oreophoetes peruana* (insect) [32]
Quinaldine	2-Me	*Copenatus mesoleucus* (skunk) [27]
2-Quinolinemethanethiol	2-CH_2SH	*Copenatus mesoleucus* (skunk) [27]
Jineol	3,8-diOH	*Scolopendra subspinipes mutilans* (centipede) [31]
–	2,8-diOH, 3,4-diOMe	*Scolopendra subspinipes mutilans* (centipede) [83]
Scolopendrine	2-OH, 3-OMe, 7-CH_2(3′-OMe, 4′-OH)Ph, 8-OSO_3^-	*Scolopendra subspinipes mutilans* (centipede) [36]
2-Pentyl-4-hydroxyquinoline	2-*n*-C_5H_{11}, 4-OH	*Alteromonas sp.*(marine bacterium), [41]
2-Heptyl-4-hydroxyquinoline	2-*n*-C_7H_{15}, 4-OH	*Alteromonas sp.*(marine bacterium), [41], *Pseudomonas aeruginosa* [41]
2-Nonyl-4-hydroxyquinoline	2-*n*-C_9H_{19}, 4-OH	*Pseudomonas aeruginosa* [41], *Pseudomonas* (strain 1531-E7 – associated with sponge *Homophymia* sp.) [41]
2-Undecyl-4-hydroxyquinoline	2-*n*-$C_{11}H_{23}$, 4-OH	*Pseudomonas aeruginosa* [41]
2-(Undec-1-enyl)-4-hydroxyquinoline	2-CH = CH$(CH_2)_8CH_3$, 4-OH	*Pseudomonas aeruginosa* [41]
Helquinoline	2-H, Me, 3-H_2, 4-H, OH, 8-CO_2H	*Janibacter limosus* [41]
Aurachin A (see Scheme 24.15)		*Stigmatella aurantiaca* (gram-negative bacterium) [41]
Aurachin B	1-O^-, 2-Me, 3-OH, 4-Frn	*Stigmatella aurantiaca* (gram-negative bacterium) [41]
Aurachin F – I, K, L, P (see Scheme 24.15)		*Stigmatella aurantiaca* (gram-negative bacterium) [41, 84]
2-Heptyl-4-hydroxyquinoline *N*-oxide	1-O^-, 2-*n*-C_7H_{15}, 4-OH	*Pseudomonas aeruginosa* [41]
2-Nonyl-4-hydroxyquinoline *N*-Oxide	1-O^-, 2-*n*-C_9H_{19}, 4-OH	*Pseudomonas* (strain 1531-E7 – associated with sponge *Homophymia* sp.) [41], *Pseudomonas aeruginosa* [41]
2-(Hept-1-enyl)-4-hydroxyquinoline *N*-oxide	1-O^-, 2-CH = CH$(CH_2)_4CH_3$, 4-OH	*Pseudomonas aeruginosa* [41]
2-(Non-1-enyl)-4-hydroxyquinoline *N*-oxide	1-O^-, 2-CH = CH$(CH_2)_6CH_3$, 4-OH	*Pseudomonas aeruginosa* [41]

(*continued*)

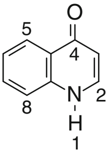
Table 24.22 (continued)

Alkaloids	Substituents	Occurrence
2-(Undec-1-enyl)-4-hydroxyquinoline *N*-oxide	1-O^-, 2-CH = $CH(CH_2)_8CH_3$, 4-OH	*Pseudomonas aeruginosa* [41]
–	6-OH, 8-COOH	*Cortinarius subtortus* [85]
–	4-NH_2, 6-OH, 8-COOH	*Cortinarius subtortus* [85]
Quinocitrinine A (see Scheme 24.23)		*Penicillium citrinum* (VKM FW-800), [41]
Quinocitrinine B (see Scheme 24.23)		*Penicillium citrinum* (VKM FW-800) [41]
(+)-Halitulin		*Haliclona tulearensis* (marine sponge) [33]

Frn Farnesyl group

However, 2′,2′-dimethyl-3′-hydroxydihydropyranoquinolin/4-ones and dihydrofuroquinolin/4-ones present in other rutaceous species do not have the same stereochemical relationship. Isoplatydesmine and ribalinine isolated from *A. tabouensis* had both R-configuration, 2′-R- and 3′-R, respectively. (+)-(R)-Balfourodine and (+)-(R)-isobalfourodine of *Balfourodendron riedelianun* are other examples, suggesting that the biosynthetic route given in Scheme 24.1 does not apply in these cases. The in vitro rearrangement of balfourodine to isobalfourodine in acetic anhydride and pyridine showed in Scheme 24.2 [41, 109] may well be a model for the biosynthetic process in the Rutaceae, rather than those showed in Scheme 24.1. However, a similar rearrangement of (+)-(R)-isoplatydesmine to (+)-(S)-ribalinine acetate, which was hydrolysed to (−)-(S)-ribalinine must occur by inversion of configuration at the chiral center [110]. A proposal is that a dual mechanism is involved, inversion of configuration predominating with isoplatydesmine and retention with balfourodine [41, 110, 111]. Few linear pyranoquinolines (**12**) have been found, only 4-methoxypyranoquinoline, 4,7,8-trimethoxypyranoquinoline isolated from *Euodia pilulifera* [24], and 4,8-dimethoxypyranoquinoline from *Dictamnus dasycarpus* [52] (Table 24.8). The formation of the pyrano ring can be explained by the loss of the hydroxyl substituent at C-3′ (Scheme 24.1). Linear pyranoquinolin-4(1*H*)-ones have not yet been detected in rutaceous species.

Table 24.23 Occurrence of Alkylquinolin-4(1*H*)-one alkaloids in the Rutaceae Species



Alkaloid	Substituents	Occurrence
1,2-Dimethylquinolin-4(1*H*)-one	1-Me, 2-Me	*Acronychia baueri* [42], *Glycosmis citrifolia* [30], *Platydesma campanulata* [41], *Ruta Montana* [41]
1-Acetoxymethyl-2-methylquinolin-4(1*H*)-one	1-CH_2OAc, 2-Me	*Boronia bowmanii* [41], *B. lanceolada* [41]
Leptomerine	1-Me, 2-*n*-C_3H_7	*Haplophyllum leptomerum* [41]
1-Acetoxymethyl-2-*n*-propylquinolin-4(1*H*)-one	1-CH_2OAc, 2-*n*-C_3H_7	*Boronia ternata* [41]
Schinifoline[a]	1-Me, 2-*n*-C_7H_{15}	*Zanthoxylum schinifolium* [41]
1-Methyl-2-*n*-nonylquinolin-4(1*H*)-one	1-Me, 2-*n*-C_9H_{19}	*Boronia bowmanii* [41], *Euodia rutaecarpa* [41], *E. sutchuenensis* [41], *Haplophyllum acutifolium* [41], *H. tuberculatum* [41], *Raulinoa echinata* [41], *Ruta graveolens* [12, 41]
1-Acetoxymethyl-2-*n*-nonylquinolin-4(1*H*)-one	1-CH_2OAc, 2-*n*-C_9H_{19}	*Boronia bowmanii* [41]
1-Methyl-2-(8-oxononyl)quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_7COCH_3$	*Ruta montana* [41]
1-Methyl-2-*n*-decylquinolin-4(1*H*)-one	1-Me, 2-*n*-$C_{10}H_{21}$	*Euodia rutaecarpa* [41], *Ruta graveolens* [41]
1-Methyl-2-(9-oxodecyl)quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_8COCH_3$	*Ruta montana* [41]
1-Methyl-2-(10-oxoundecyl)quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_9COCH_3$	*Euodia rutaecarpa* [86]
1-Methyl-2-*n*-undecylquinolin-4(1*H*)-one	1-Me, 2-*n*-$C_{11}H_{23}$	*Euodia rutaecarpa* [41], *Ruta graveolens* [41]

(*continued*)

Table 24.23 (continued)

Alkaloid	Substituents	Occurrence
1-Methyl-2-*n*-dodecylquinolin-4(1*H*)-one	1-Me, 2-*n*-$C_{12}H_{25}$	*Euodia rutaecarpa* [41]
Dihydroevocarpine	1-Me, 2-*n*-$C_{13}H_{27}$	*Euodia officinalis* [41], *E. rutaecarpa* [41]
1-Methyl-2-*n*-tetradecylquinolin-4(1*H*)-one	1-Me, 2-*n*-$C_{14}H_{29}$	*Euodia rutaecarpa* [41]
1-Methyl-2-*n*-pentadecylquinolin-4(1*H*)-one	1-Me, 2-*n*-$C_{15}H_{31}$	*Boronia algida* [41], *Euodia rutaecarpa* [41]
1-Methyl-2-[(Z)-undec-5-enyl] quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_4CH = CH(CH_2)_4CH_3$	*Euodia rutaecarpa* [41]
1-Methyl-2-[(Z)-undec-6-enyl] quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_5CH = CH(CH_2)_3CH_3$	*Euodia officinalis* [41], *E. rutaecarpa* [41]
1-Methyl-2-[(Z)-tridec-7-enyl]quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_6CH = CH(CH_2)_4CH_3$	*Euodia rutaecarpa* [41]
1-Methyl-2-[(4Z)-tridec-4-enyl]quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_3CH = CH(CH_2)_7CH_3$	*Euodia rutaecarpa* [41]
Evocarpine	1-Me, 2-$(CH_2)_7CH = CH(CH_2)_3CH_3$	*Euodia officinalis* [41], *E. rutaecarpa* [41]
1-Methyl-2-[(Z)-pentadec-10-enyl] quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_9CH = CH(CH_2)_3CH_3$	*Euodia rutaecarpa* [41]
1-Methyl-2-[(Z)-pentadec-9-enyl] quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_8CH = CH(CH_2)_4CH_3$	*Euodia rutaecarpa* [41]
1-Methyl-2-[(Z)-pentadec-6-enyl] quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_5CH = CH(CH_2)_7CH_3$	*Euodia rutaecarpa* [41]
1-Methyl-2-[(4Z,7Z)-tridec-4,7-dienyl] quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_3CH = CHCH_2CH = CH(CH_2)_4CH_3$	*Euodia rutaecarpa* [41]
1-Methyl-2-[(6Z,9Z)-pentadec-6,9-dienyl] quinolin-4(1*H*)-one	1-Me, 2-$(CH_2)_5CH = CHCH_2CH = CH(CH_2)_4CH_3$	*Euodia rutaecarpa* [41]
Leiokinine B [1-Methyl-2-(1-ethylpropyl)quinolin-4(1*H*)-one]	1-Me, 2-$CH(CH_2CH_3)CH_2CH_3$	*Esenbeckia leiocarpa* [41]
2-*n*-Propylquinolin-4(1*H*)-one	2-*n*-C_3H_7	*Boronia ternata* [42]

(*continued*)

Table 24.23 (continued)

Alkaloid	Substituents	Occurrence
2-*n*-Heptylquinolin-4(1*H*)-one	2-*n*-C_7H_{15}	*Ruta graveolens* [41]
2-*n*-Octylquinolin-4(1*H*)-one	2-*n*-C_8H_{17}	*Ruta graveolens* [41]
2-(8-Oxononyl) quinolin-4(1*H*)-one	2-$(CH_2)_7COCH_3$	*Ruta montana* [41]
2-*n*-Nonylquinolin-4(1*H*)-one	2-*n*-C_9H_{19}	*Boronia bowmanii* [41], *Euodia rutaecarpa* [41], *E. sutchuenensis* [41], *Haplophullum acutifolium* [87], *Raulinoa echinata* [41], *Ruta graveolens* [41]
2-*n*-Decylquinolin-4(1*H*)-one	2-*n*-$C_{10}H_{21}$	*Ruta graveolens* [41]
2-*n*-Undecylquinolin-4(1*H*)-one	2-*n*-$C_{11}H_{23}$	*Euodia rutaecarpa* [41], *Ptelea trifoliata* [41], *Ruta graveolens* [41]
2-*n*-Tridecylquinolin-4(1*H*)-one	2-*n*-$C_{13}H_{27}$	*Euodia rutaecarpa* [41]
Acutine	2-$(CH_2)_3CH = CHCH_2CH_3$	*Haplophullum acutifolium* [41]
Haplacutine F (3-*Z*)	2-$(CH_2)_2CH = CH(CH_2)_4CH_3$	*Haplophullum acutifolium* [87]
2-(*n*-Nona-3,6-dienyl) quinolin-4(1*H*)-one (Haplacutine E, 3-*Z*, 6-*Z*)	2-$(CH_2)_2CH = CHCH_2CH = CHCH_2CH_3$	*Haplophullum acutifolium* [87], *Vepris ampody* [41]
Haplacutine A (3*Z*, 5*E*)	2-$(CH_2)_2CH = CHCH = CHCH(OH)CH_2CH_3$	*Haplophullum acutifolium* [87]
Haplacutine B (4*E*, 5*Z*)	2-$(CH_2)_2CH(OH)CH = CHCH = CHCH_2CH_3$	*Haplophullum acutifolium* [87]
Haplacutine C (4*E*, 5*E*)	2-$(CH_2)_2CH(OH)CH = CHCH = CHCH_2CH_3$	*Haplophullum acutifolium* [87]
Haplacutine D (4*E*, 5*Z*)	2-$(CH_2)_2COCH = CHCH = CHCH_2CH_3$	*Haplophullum acutifolium* [87]
Hapovine	2-$(CH_2)_5(CH = CH)_3CH_3$	*Haplophyllum popovii* [41]
2-(*n*-9-Hydroxynonyl) quinolin-4(1*H*)-one	2-$(CH_2)_8CH_2OH$	*Vepris ampody* [41]
2-(*n*-10-Oxoundecyl) quinolin-4(1*H*)-one	2-$(CH_2)_9COCH_3$	*Euodia rutaecarpa* [86], *Vepris ampody* [41]
Malatyamine ethyl ester	2-$(CH_2)_5CO_2Et$ (or COOH)	*Haplophyllum carpadocicum* [41]
2-(12-Hydroxy-12-methyltridecyl) quinolin-4(1*H*)-one	2-$(CH_2)_{11}COH(CH_3)_2$	*Dictyoloma vandellianum* (syn. *D. incanescens)* [41]

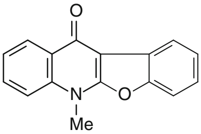
(*continued*)

Table 24.23 (continued)

Alkaloid	Substituents	Occurrence
2-(14-Hydroxy-14,15-dimethylhexadecyl) quinolin-4(1*H*)-one	2-$(CH_2)_{13}COHCH_3CH(CH_3)_2$	*Dictyoloma vandellianum* (syn. *D. incanescens*) [41]
2,3-Dimethylquinolin-4(1*H*)-one	2-Me, 3-Me	*Boronia bowmanii* [41], *B. lanceolada* [41]
1-Acetoxymethyl-2,3-dimethylquinolin-4(1*H*)-one	1-CH_2OAc, 2-Me, 3-Me	*Boronia bowmanii* [41], *B. lanceolada* [41]
Leiokinine A	1-Me, 2-*n*-C_3H_7, 3-OMe	*Esenbeckia leiocarpa* [41]
2-(10-Hydroxy-10-methyldodecyl)-3-methoxyquinolin-4(1*H*)-one	2-$(CH_2)_9COH(CH_3)CH_2CH_3$, 3-OMe	*Spathelia excelsa* (syn. *Sohnreyia*) [41]
2-(11-Hydroxy-11-methyldodecyl)-3-methoxyquinolin-4(1*H*)-one	2-$(CH_2)_{10}COH(CH_3)_2$, 3-OMe	*Spathelia excelsa* (syn. *Sohnreyia*) [41]
2-(12-Hydroxytridecyl)-3-methoxyquinolin-4(1*H*)-one	2-$(CH_2)_{11}COH(CH_3)$, 3-OMe	*Spathelia excelsa* (syn. *Sohnreyia*) [41]
2-(12-Hydroxy-12-methyltridecyl)-3-methoxyquinolin-4(1*H*)-one	2-$(CH_2)_{11}COH(CH_3)_2$, 3-OMe	*Dictyoloma vandellianum* (syn. *D. incanescens*) [41]
2-(12-Oxotridecyl)-3-methoxyquinolin-4(1*H*)-one	2-$(CH_2)_{11}CO(CH_3)$, 3-OMe	*Spathelia excelsa* (syn. *Sohnreyia*) [41]
2-(14-Hydroxy-14,15-dimethylhexadecyl)-3-methoxyquinolin-4(1*H*)-one	2-$(CH_2)_{13}COHCH_3CH(CH_3)_2$, 3-OMe	*Dictyoloma vandellianum* (syn. *D. incanescens*) [41]
6-Hydroxy-2-(3-hydroxy-3-methylbutyl)quinolin-4(1*H*)-one	2-$(CH_2)_2COH(CH_3)_2$, 6-OH	*Spathelia excelsa* (syn. *Sohnreyia*) [41]
7-Hydroxy-2-(3-hydroxy-3-methylbutyl)quinolin-4(1*H*)-one	2-$(CH_2)_2COH(CH_3)_2$, 7-OH	*Spathelia excelsa* (syn. *Sohnreyia*) [41]
1-Methyl-2-*n*-pentyl-8-methoxyquinolin-4(1*H*)-one	1-Me, 2-*n*- C_5H_{11}, 8-OMe	*Esenbeckia almawillia* [41]
1-Methyl-2-*n*-hexyl-8-methoxyquinolin-4(1*H*)-one	1-Me, 2-*n*- C_6H_{15}, 8-OMe	*Esenbeckia almawillia* [41]

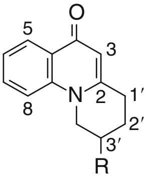
(*continued*)

Table 24.23 (continued)

Alkaloid	Substituents	Occurrence
1-Methyl-2-*n*-heptyl-8-methoxyquinolin-4(1*H*)-one	1-Me, 2-*n*- C_7H_{15}, 8-OMe	*Esenbeckia almawillia* [41]
8-Methoxy-1-methyl-2-tridecylquinolin-4(1*H*)-one	1-Me, 2-*n*-$C_{13}H_{27}$, 8-OMe	*Esenbeckia leiocarpa* [41]
Heptaphyllone A	1-Me, 2-OPh	*Raputia heptaphylla* [56]
Heptaphyllone B		*Raputia heptaphylla* [56]

Pr prenyl group.
[a]The name Schinifoline has been used for a different alkaloid [27].

Table 24.24 Occurrence of 2,4′-piperidine-3′-alkylquinolin-4(1*H*)-one alkaloids in the Rutaceae species

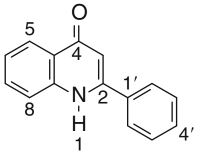


Alkaloid	Substituents	Occurrence
Dictyolomide A	3′-$(CH_2)_2CH = CHCH_2CH_3$ (Z)	*Dictyoloma peruviana* [41], *D. vandellianum* (syn. *D. incanescens* DC) [41]
Dictyolomide B	3′-$CHOH(CH_2)_4CH_3$	*Dictyoloma peruviana* [41]
(+)-6-Methoxydictyolomida A	3′-$(CH_2)_2CH = CHCH_2CH_3$ (Z), 6-OMe	*Dictyoloma vandellianum* (syn. *D. incanescens* DC) [41]

2′,2′-Dimethyldihydropyranoquinolin/4-ones (**13**, **14**) have also been obtained from rutaceous species, but they can be artifacts formed from 3-(3,3-dimethylallyl)-4-hydroxyquinolin-2-one (**2**) during isolation, by an electrophilic attack involving transfer of a proton to the alkene and the oxygen would be added to the more substituted carbon.

Angular pyranoquinolin-2(1*H*)-one are more widely distributed in the rutaceous species, however, the possible precursor angular 2′,2′-dimethyl-3′-hydroxydihydropyranoquinolin-2-one had not been reported until 2006. Thus, Grundon suggested that these alkaloids might be biogenetically derived from an alternative mechanism involving a quinone methide intermediate, as illustrated by a synthesis of *N*-methylflindersine in Scheme 24.3 [112].

Table 24.25 Occurrence of 2-arylquinolin-4(1*H*)-one alkaloids in the Rutaceae species



Alkaloids	Substituents	Occurrence
a	1-Me	*Balfourodendron riedelianum* [3, 41], *Casimiroa edulis* [41], *Flindersia fournieri* [41]
		Haplophyllum foliosum [41], *H. perforatum* [41], *H. leptomerum* [41], *Raulinoa echinata* [41]
Reevesianine-A	1-Me, 4′-OH	*Skimmia reevesiana* [41]
Folimidine	1-Me, 3′-OH, 4′-OH	*Haplophyllum foliosum* [41]
Norgraveoline	3′-OCH_2O-4′	*Haplophyllum dubium* [41], *H. foliosum* [41]
Graveoline (Rutamine)	1-Me, 3′-OCH_2O-4′	*Haplophyllum perforatum* [41], *H. dubium* [41], *H. foliosum* [41], *Ruta graveolens* [3, 41]
		R. chalepensis [41], *R. bracteosa* [41], *R. angustifolia* [41], *R. chalepensis* var. *latifolia* [41]
5-Hydroxygraveoline	1-Me, 5-OH, 3′-OCH_2O-4′	*Lunasia mara* [50, 54]
3′-Hydroxygraveoline	1-Me, 3′-OH, 4′-OCH_2O-5′	*Ruta chalepensis* [41]
b	1-Me, 3′-OMe, 4′-OCH_2O-5′	*Esenbeckia grandiflora* [41]
c	1-Me, 8-OMe, 3′-OMe, 4′-OCH_2O-5′	*Esenbeckia grandiflora* [41]
Eduleine	1-Me, 7-OMe	*Casimiroa edulis* [41], *Lunasia amara* [2, 41], *L. quercifolia* [3, 41]
Lunamarine	1-Me, 7-OMe, 3′-OCH_2O-4′	*Lunasia amara* [3, 41]
Eduline	1-Me, 6-OMe	*Casimiroa edulis* [41], *Esenbeckia pentaphylla* [41], *Orixa japonica* [41], *Skimmia japonica* [41]
Reevesianine-B	1-Me, 6-OMe, 4′-OH	*Skimmia reevesiana* [41]
Lunasia I	1-Me, 6-OMe, 3′-OCH_2O-4′	*Lunasia amara* [41]
d	1-Me, 5-OH	*Casimiroa edulis* [41], *Lunasia quercifolia* [41], *Skimmia japonica* [41]
Japonine	1-Me, 3-OMe, 6-OMe	*Orixa japonica* [41]
e	5-OMe, 6-OMe, 3′-OMe	*Casimiroa edulis* [41]
f	5-OMe, 6-OMe, 3′-OMe, 4′-OMe	*Casimiroa edulis* [41]
g	5-OMe, 6-OMe, 2′-OMe, 3′-OMe, 6′-OMe	*Casimiroa edulis* [41]

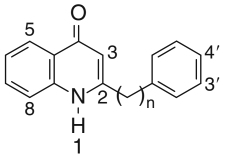
(*continued*)

Table 24.25 (continued)

Alkaloids	Substituents	Occurrence
–	3-*n*-C_3H_7, 5,8-diOMe, 2′-OH, 4′-OMe	*Casimiroa edulis* [88]
–	3-*n*-C_3H_7, 5,8-diOMe, 3′-OMe	*Casimiroa edulis* [88]
–	3-*n*-C_3H_7, 5,8-diOMe, 2′,3′-diOMe	*Casimiroa edulis* [88]

a: 1-Methyl-2-phenylquinolin-4(1*H*)-one; **b**: 1-Methyl-2-(3-methoxy-4,5-methylenedioxyphenyl) quinolin-4(1*H*)-one; **c**: 8-Methoxy-1-methyl-2-(3-methoxy-4,5-methylenedioxyphenyl)quinolin-4(1*H*)-one; **d**: 5-Hydroxy-1-methyl-2-phenylquinolin-4(1*H*)-one; **e**: 5,6-Dimethoxy-2-(3-methoxyphenyl)quinolin-4(1*H*)-one; **f**: 5,6-Dimethoxy-2-(3,4-dimethoxyphenyl)quinolin-4(1*H*)-one; **g**: 5,6-Dimethoxy-2-(2,3,6-trimethoxyphenyl)quinolin-4(1*H*)-one.

Table 24.26 Occurrence of 2-alkylarylquinolin-4(1*H*)-one alkaloids in the Rutaceae species

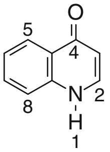


Alkaloids	Substituents	Occurrence
2-[4-(3,4-Methylenedioxyphenyl)butyl]quinolin-4(1*H*)-one (Rutaverine)	n = 4, 3′-OCH_2O-4′	*Ruta graveolens* [41]
2-[6-(3,4-Methylenedioxyphenyl)hexyl]quinolin-4(1*H*)-one	n = 6, 3′-OCH_2O-4′	*Ruta chalepensis* [41]
1-Methyl-2-[6-(3,4-methylenedioxyphenyl)hexyl]-] quinolin-4(1*H*)-one	n = 6, 1-Me, 3′-OCH_2O-4′	*Ruta graveolens* [41]

More recently, angular 2′,2′-dimethyl-3′-hydroxydihydropyranoquinolin-2-one (**15**) was isolated from *Skimmia laureolea* [69], which provided substantial indirect evidence that the loss of the hydroxyl substituent at C-3′ may be involved in the formation of pyranoquinolin-2-one (**16**, Scheme 24.1). This probably represents the natural process for all pyrano rings in quinoline alkaloids.

Quinoline alkaloid containing dihydrofuran ring with germinal dimethyl substituents have been found in Rutaceae, and one proposal for its biosynthesis involves Claisen rearrangement of the 4-(3,3-dimethylallyloxy)-quinolin-2-one giving 1,1-dimethylallyl derivative **17** (Scheme 24.4). This later can also suffer an abnormal Claisen rearrangement yielding 3-(1,2-dimethylprop-2-enyl)-4-hydroxyquinolin-2-one (**18**). Their cyclization products as 2′,3′,3′-trimethylquinolin-4-one (**19**) and 2′,2′,3′-trimethylquinolin-4-one (**20**) are also constituents of Rutaceae.

Table 24.27 Occurrence of quinolin-4(1*H*)-ones in non-rutaceous species



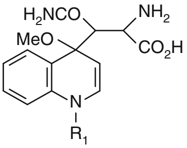
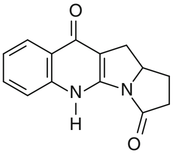
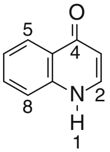
Alkaloids	Substituents	Occurrence
Echinopsine	1-Me	*Echinops sphaerocephalus* (Asteraceae) [89]
1-Acetoxymethyl-2-(10-acetoxyundecyl) quinolin-4(1*H*)-one	1-CH_2Oac, 2-$(CH_2)_9CHOHCH_3$	Aff. *Samadera* SAC-2825 (Simaroubaceae) [41]
Echinoramine-I		*Echinops ritro* (Asteraceae), [26]
(−)-Chestnutamide		*Castanea mollissima* (Fagaceae) [35]

Table 24.28 Occurrence of quinolin-4(1*H*)-one alkaloids in fungi, bacteria, and marine organisms



Alkaloids	Substituents	Occurrence
2-*n*-Nonylquinolin-4(1*H*)-one	2-*n*-C_9H_{19}	*Pseudomonas* (strain 1531-E7 – associated with sponge *Homophymia* sp.) [41]
2-*n*-Undecylquinolin-4(1*H*)-one	2-*n*-$C_{11}H_{23}$	*Pseudomonas* (strain 1531-E7 – associated with sponge *Homophymia* sp.) [41]

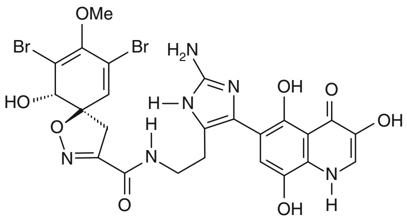
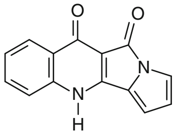
(*continued*)

Table 24.28 (continued)

Alkaloids	Substituents	Occurrence
2-(Hept-1-enyl) quinolin-4(1*H*)-one	2-CH = $CH(CH_2)_4CH_3$	*Pseudomonas aeruginosa* [41]
2-[(1E)-Undec-1-enyl] quinolin-4(1*H*)-one	2-CH = $CH(CH_2)_8CH_3$	*Pseudomonas* (strain 1531-E7 – associated with sponge *Homophymia* sp.) [41]
2-(Undec-3-enyl) quinolin-4(1*H*)-one	2-$(CH_2)_2CH$ = $CH(CH_2)_6CH_3$	*Pseudomonas aeruginosa* [41]
3-Methyl-2-*n*-pentylquinolin-4(1*H*)-one	2-*n*-C_5H_{11}, 3-Me	*Pseudomonas cepacia* (strain PC-II) [41]
3-Methyl-2-*n*-heptylquinolin-4(1*H*)-one	2-*n*-C_7H_{15}, 3-Me	*Pseudomonas cepacia* (strain PC-II) [41]
3-Methyl-2-*n*-nonylquinolin-4(1*H*)-one	2-*n*-C_9H_{19}, 3-Me	*Pseudomonas cepacia* (strain PC-II) [41]
3-Methyl-2-(hept-2-enyl)quinolin-4(1*H*)-one	2-CH_2CH = $CH(CH_2)_3CH_3$, 3-Me	*Pseudomonas cepacia* [41] (strain PC-II) [41], *Pseudomonas cells* [41]
3-Methyl-2-(non-2-enyl)quinolin-4(1*H*)-one	2-CH_2CH = $CH(CH_2)_5CH_3$, 3-Me	*Pseudomonas cepacia* [41] (strain PC-II) [41]
1-Hydroxy-2-(non-2-enyl)-3-methylquinolin-4(1*H*)-one	1-OH, 2-CH_2CH = $CH(CH_2)_5CH_3$, 3-Me	*Arthrobacter sp.* (strain YL-02729S) [41]
1-Methyl-2-(1-hydroxygeranyl) quinolin-4(1*H*)-one	1-Me, 2-CHOHCH = $CHCH_3(CH_2)_2CH$ = $C(CH_3)_2$	*Pseudonocardia sp.* (CL38489) [41]
1-Methyl-2-(3-hydroxy-3,7-dimethyl-octa-1,6-dienyl) quinolin-4(1*H*)-one	1-Me, 2-CH = $CHCOHCH_3(CH_2)_2CH$ = C $(CH_3)_2$	*Pseudonocardia sp.* (CL38489) [41]
2-Geranylquinolin-4(1*H*)-one	2-Ger	*Pseudonocardia sp.* (CL38489) [41]
1-Methyl-2-geranylquinolin-4(1*H*)-one	1-Me, 2-Ger,	*Pseudonocardia sp.* (CL38489) [41]
2-Geranyl-3-methylquinolin-4(1*H*)-one	2-Ger, 3-Me	*Pseudonocardia sp.* (CL38489) [41]
1-Methyl-2-geranyl-3-methylquinolin-4(1*H*)-one	1-Me, 2-Ger, 3-Me	*Pseudonocardia sp.* (CL38489) [41]

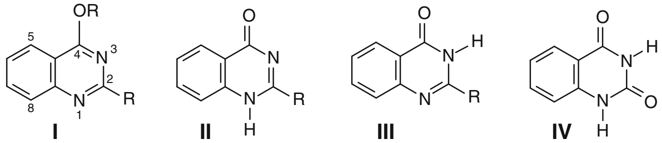
(*continued*)

Table 24.28 (continued)

Alkaloids	Substituents	Occurrence
1-Methyl-2-(6,7-epoxygeranyl)quinolin-4(1*H*)-one	1-Me, 2-Ger (6,7-epoxy), 3-Me	*Pseudonocardia sp.* (CL38489) [41]
1-Methylthiolmethyl-2-geranyl-3-methylquinolin-4(1*H*)-one	1-CH_2SMe, 2-Ger, 3-Me	*Pseudonocardia sp.* (CL38489) [41]
Aurachin C	1-OH, 2-Me, 3-Frn	*Stigmatella aurantiaca* (gram-negative bacterium) [41]
Aurachin D	2-Me, 3-Frn	*Stigmatella aurantiaca* (gram-negative bacterium) [41]
Aurachin E (see Scheme 24.15)		*Stigmatella aurantiaca* (gram-negative bacterium) [41]
(S)-(−)-Quinolactacin B (see Scheme 24.22)		*Penicillium sp.* (EPF-6) [41]
(9S, 1'R)-Quinolactacin A1 (see Scheme 24.23)		*Penicillium sp.* (EPF-6) [41], *Penicillium citrinum* (90648), [41]
(9S, 1'S)-Quinolactacin A2 (see Scheme 24.23)		*Penicillium sp.* (EPF-6) [41], *Penicillium citrinum* (90648) [41]
Quinolactacin C (see Scheme 24.23)		*Penicillium* sp. (EPF-6) [41]
Quinolactacide (see Scheme 24.23)		*Penicillium citrinum* Thom (F 1539) [41]
Uranidine	3,5,8-triOH	*Oceanapia* sp. (sponge) [35]
Uranidine derivative	 OMe Br Br HO O N O H N H₂N H—N N OH O OH N H OH	*Oceanapia* sp. (sponge) [35]
Quinolactacide		*Penicillium citrinum* [39]

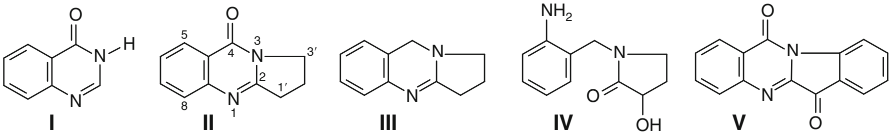
Frn Farnesyl group

Table 24.29 Occurrence of quinazoline alkaloids in Rutaceae species



Alkaloids	Substituents	Occurrence
Glycophymoline	**I**: 2-CH_2Ph, 4-OMe	*Glycosmis arborea* [14], *Gl. pentaphylla* [44]
Glycorine	**II**: 1-Me	*Glycosmis arborea* [42], *G. bilocularis* [44]
–	**II**: CH = C(Me)CH_2CH (Me)$CH_2$$CHMe_2$	*Zanthoxylum budrunga* [37]
Glycophymine	**II**: 2-CH_2Ph	*Glycosmis pentaphylla* [13]
Arborine	**II**: 1-Me, 2-CH_2Ph	*Glycosmis arborea* [42], *G. bilocularis* [44], *G. cochinchinensis* [34], *G. pentaphylla* [44]
Glycozolone-A	**II**: 1-Me, 2-CH_2Ph, 2,3 dihydro	*Glycosmis cochinchinensis* [34]
Glycozolone-B	**II**: 1-Me, 2-CH2-*p*-OMePh, 2,3 dihydro	*Glycosmis cochinchinensis* [34]
Glycosminine	**III**: 2-CH_2Ph	*Glycosmis arborea* [42], *G. pentaphylla* [44]
(Z)-Bogorin (E)-Bogorin	**III**: 2-CH = CHPh	*Glycosmis* cf. *chlorosperma* [33]
Glycosmicine	**IV**: 1-Me	*Glycosmis arborea* [42]
–	**IV**: 1-Me, 3-CH_2CH_2Ph	*Zanthoxylum arborescens* [44]
–	**IV**: 1-Me, 3-[CH_2CH_2-(4′-OMe-Ph)]	*Zanthoxylum arborescens* [44]

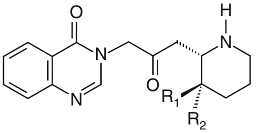
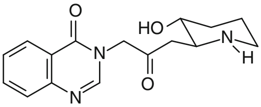
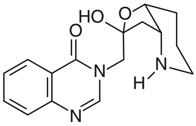
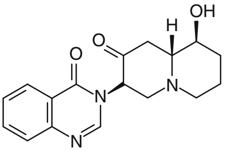
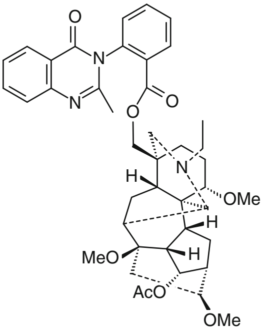
Table 24.30 Occurrence of quinazoline alkaloids in non-rutaceous species



Alkaloids	Substituents	Occurrence
–	**I**	*Hydrangea chinensis* (Saxifragaceae) [35], *Strobilanthes cusia* (Acanthaceae) [27]
–	**I**: 2-Me, 3-*o*-COOMe-Ph	*Aconitum pseudo-laeve* var. *erectum* (Ranunculaceae) [40]
Echinozolinone	**I**: 3-CH_2CH_2OH	*Echinops echinatus* (Asteraceae) [21]
Pegamine	**I**: 3-$CH_2CH_2CH_2$OH	*Peganum nigellastrum* (Zygophyllaceae) [34]

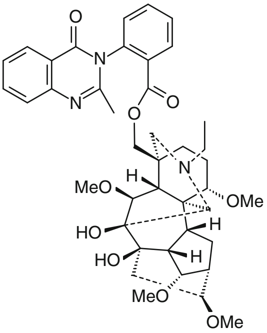
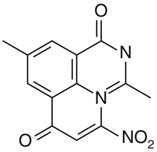
(*continued*)

Table 24.30 (continued)

Alkaloids	Substituents	Occurrence
–	**I**: 3-$CH_2CH_2CH_2CH_2OH$	*Dichroa febrifuga* (Hydrangeaceae) [35]
7-Hydroxyechinozolinone	**I**: 3-CH_2CH_2OH, 7-OH	*Echinops echinatus* (Asteraceae) [26]
–	**I**: 3-*o*-OH-Ph	*Isatis indigotica* (Brassicaceae) [31]
–	**I**: 3-*o*-CO_2H-Ph	*Isatis indigotica* (Brassicaceae) [31], *I. tinctoria* [90]
	I: 2- = O, 1,2-dihydro	*Isatis tinctoria* (Brassicaceae) [31], *Strobilanthes cusia* (Acanthaceae) [27]
cis-Febrifugine $R_1 = H$, $R_2 = OH$ *trans*-Febrifugine $R_1 = OH$, $R_2 = H$		*Hydrangea macrophylla* (Saxifragaceae) [25]
Trans-Febrifugine		*Hydrangea macrophylla* (Saxifragaceae) [32]
Isofebrifugine		*Hydrangea macrophylla* (Saxifragaceae) [32]
(+)-Neodichroine		*Dichroa febrifuga* (Hydrangeaceae) [35], *Hydrangea chinensis* (Saxifragaceae) [35, 38][a]
(−)-14-*O*-acetyl-8-*O*-methylcammaconine ester		*Aconitum pseudo-laeve* var. *erectum* (Ranunculaceae) [40]

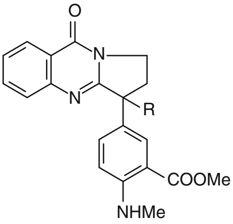
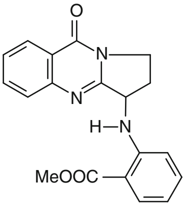
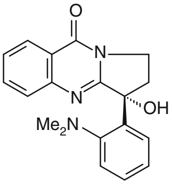
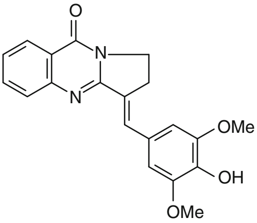
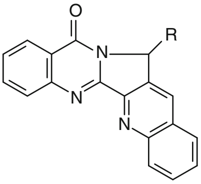
(*continued*)

Table 24.30 (continued)

Alkaloids	Substituents	Occurrence
(+)-Lycoctonine ester		*Aconitum pseudo-laeve* var. *erectum* (Ranunculaceae) [40]
Liguducimine		*Ligularia duciformis* (Asteraceae) [73]
Deoxyvasicinone	**II**	*Adhotoda vasica* (Acanthaceae) [15], *Isatis indigotica* (Brassicaceae) [32], *I. tinctoria* [90], *Nitraria schoberi* (Zygophyllaceae) [35], *Peganum harmala* (Zygophyllaceae) [17], *P. multisectum* [32]
Deoxyvasicinone *N*-oxide	**II**: 1-O	*Nitraria komarovii* (Zygophyllaceae) [28]
Vasicinone	**II**: 1′-OH	*Adhotoda beddomein* (Acanthaceae) [21], *A. vasica* [15], *A. zeylanica* [26], *Anisotes trisulcus* (Acanthaceae) [32], *Galium aparine* (Rubiaceae) [23], *Nitraria schoberi* (Zygophyllaceae) [35], *N. sibirica* [17], *Peganum harmala* [91], *P. multisectum* (Zygophyllaceae) [32], *Sida acuta*, *S. humilis*, *S. rhombifolia*, *S. spinosa* (Malvaceae) [17]
Vasicinone *N*-oxide	**II**: 1′-OH, 1-O	*Nitraria komarovii* (Zygophyllaceae) [28]
–	**II**: 1-H, 2-OMe, 1′-OH, 1,2-dihydro	*Eranthemum nervosum* (Acanthaceae) [36]
Vasicinolone	**II**: 6,1′-diOH	*Adhatoda vasica* (Acanthaceae) [17]
	II: 6-OMe, 1′-diOH	*Adhatoda vasica* (Acanthaceae) [31]
–	**II**: 8-OMe, 1′-diOH	*Adhatoda vasica* (Acanthaceae) [21]

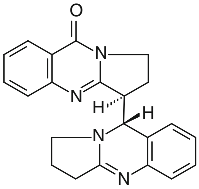
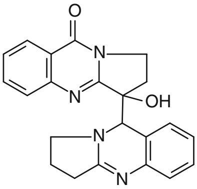
(*continued*)

Table 24.30 (continued)

Alkaloids	Substituents	Occurrence
Anisotine R = H		*Adhotoda vasica* (Acanthaceae) [29], *Anisotes trisulcus* (Acanthaceae) [32]
1′-Hydroxyanisotine R = OH		
Vasnetine		*Adhotoda vasica* (Acanthaceae) [29]
Desmethoxyaniflorine		*Adhotoda vasica* (Acanthaceae) [31]
Isaindigotone		*Isatis indigotica* (Brassicaceae) [31], *I. tinctoria* [36]
Luotonin A R = H		*Peganum nigellastrum* (Zygophyllaceae) [32, 34]
Luotonin B R = OH		*Peganum nigellastrum* [32, 34]
Luotonin E R = OMe		*Peganum nigellastrum* [32, 34]

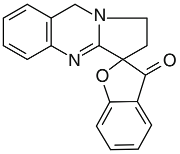
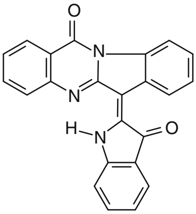
(*continued*)

Table 24.30 (continued)

Alkaloids	Substituents	Occurrence
Dipegine		*Peganum harmala* (Zygophyllaceae) [35]
Dipeginol		*Peganum harmala* (Zygophyllaceae) [35]
Deoxyvasicine	**III**	*Adhotoda vasica* (Acanthaceae) [18], *Mackinlaya macrosciadea* (Araliaceae) [19], *Nitraria schoberi* (Zygophyllaceae) [35], *Peganum harmala* (Zygophyllaceae) [40], *P. multisectum* [32], *P. nigellastrum* [32]
Deoxypeganine *N*-oxide	**III**: 1-O	*Nitraria komarovii* (Zygophyllaceae) [29]
Vasicine (peganine)	**III**: 1′-OH	*Adhatoda beddomein* (Acanthaceae) [21], *A. vasica* [23], *A. zeylanica* (callus cultures) [40], *Anisotes trisulcus* (Acanthaceae) [32], *Galega orientalis* [24], *G. battiscombei* [37], *G. lindblom* (Fabaceae) [37], *Peganum harmala* (Zygophyllaceae) [17], *P. multisectum* [32], *Sida acuta*, *S. humilis*, *S. rhombifolia*, *S. spinosa* (Malvaceae) [17]
Peganine *N*-oxide	**III**: 1-O, 1′-OH	*Nitraria komarovii* (Zygophyllaceae) [29]
Peganol	**III**: 4-OH	*Nitraria schoberi* (Zygophyllaceae) [40]
Peganol *N*-oxide	**III**: 1-O, 4-OH	*Nitraria komarovii* (Zygophyllaceae) [35]
- (−)	**III**: 3′-OH	*Galium aparine* (Rubiaceae) [23]
Linaric acid	**III**: 3′-COOH	*Linaria vulgaris* (Scrophulariaceae) [37]
Vasicinol	**III**: 6,1′-diOH	*Linaria vulgaris* (Scrophulariaceae) [37], *Sida acuta*, *S. humilis*, *S. rhombifolia*, *S. spinosa* (Malvaceae) [17]
–	**III**: 8-OMe, 1′-OH	*Adhatoda vasica* (Acanthaceae) [21], *Anisotes trisulcus* (Acanthaceae) [37]
- (−)	**III**: 5-OH, 1′,2′-dehydro	*Galium aparine* (Rubiaceae) [23]
(−)-Trisulcusine		*Anisotes trisulcus* (Acanthaceae) [37]

(*continued*)

Table 24.30 (continued)

Alkaloids	Substituents	Occurrence
		
Vasicol	**IV**	*Adhotoda vasica* (Acanthaceae) [15], [23, correct structure]
Tryptanthrin	**V**	*Baphicacanthus cusia* (Acanthaceae) [19], *Calanthe aristurifera* (Orchidaceae) [33], *C. discolor* [33], *C. liukiuensis* [35], *C. reflexa* [33], *Isatis indigotica* (Brassicaceae) [35], *I. tinctoria* [28], *Polygonum tinctorium* (Polygonaceae), [58], *Strobilanthes cusia* (Acanthaceae) [14], *Wrightia tinctoria* (Apocynaceae) [31]
Candidine (Qingdainone)		*Baphicacanthus cusia* (Acanthaceae) [19, 24], *Isatis indigotica* (Brassicaceae) [31]

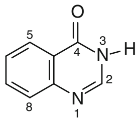
[a]Chang and coworkers showed that the structure of [a](+)-Hydrachine A was identical to (+)-Neodichroine [142].

The synthesis of ifflaiamine (**19**, R = Me) and lemobiline (**20**, R = Me) by means of the rearrangement reactions provided circumstantial support for the biogenetic pathway summarized in Scheme 24.4 [112].

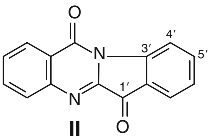
The geranylquinoline alkaloids formed probably by normal and abnormal Claisen rearrangement are also common in Rutaceae, as bucharaminol (**23**) and bucharidine (**25**) (Scheme 24.5).

No data is available on the biosynthesis of dimeric quinoline alkaloids, which have been found in Rutaceae (Table 24.10); the proposed biogenetic pathway represents modes of Diels-Alder addition of prenyl dienes, as summarized for vepridimerine A (**26**, α-Ha) and B (**26**, β-Ha) in Scheme 24.6 [112]. These isomers suggest that dimerization of a quinoline diene takes place during isolation or that dimerization occurs nonenzymatically in the plant tissue.

Table 24.31 Occurrence of quinazoline alkaloids in fungi, bacteria, marine organisms, and animal



I

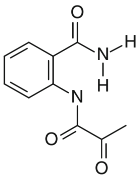
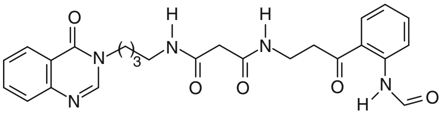
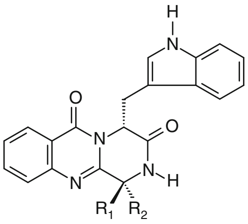


II

Alkaloids	Substituents	Occurrence
–	**I**: 2-Me	*Bacillus cereus* [25], *Streptomyces* strain GW23/1540 [39]
–	**I**: 2-COMe	*Cladobotryum varium* [29], *Fusarium langsethiae* [39], *F. lateritium* [28], *F. sambucinum* [27], *F. sporotrichioides* [39], *Penicillium persicinum* sp. nov. [39]
(−)-Chrysogine	**I**: 2-CH(OH)Me	*Fusarium culmorum* [35], *F. equiseti* [37], *F. langsethiae* [39], *F. lateritium* [28], *F. sambucinum* [27], *F. sporotrichioides* [39], *Penicillium notatum* [39], *P. persicinum* sp. nov. [39]
–	**I**: 2-CH(OH)Me, 2-α-Me	*Streptomyces* strain GW23/1540 [39]
–	**I**: 2-CH(OH)Me, 2-β-Me	*Streptomyces* strain GW23/1540 [39]
Dictyoquinazol A	**I**: 3-*o*-CH_2OH-*p*-OMe-Ph, 6-OMe	*Dictyophora indusiata* (mashroom) [37]
Dictyoquinazol C	**I**: 1-CHO, 3-*o*-CH_2OH-*p*-OMe-Ph, 6-OMe	*Dictyophora indusiata* (mashroom) [37]
Dictyoquinazol B	**I**: 1-CHO, 3-*o*-CH_2OH-*p*-OMe-Ph, 6-OMe, 4-deoxi, 4-2H	*Dictyophora indusiata* (mashroom) [37]
–	**I**: 2- = O, 1,2-dihydro	*Streptomyces* strain GW23/1540 [39]
–	**I**: 1,3-diMe, 2- = O, 1,2-dihydro	*Phyllopertha diversa* (sex pheromone of beetle) [31]
–	**I**: 1,3-diMe, 2- = O, 1,2-dihydro, 5-Me	*Streptomyces* strain GW2/577 [39]

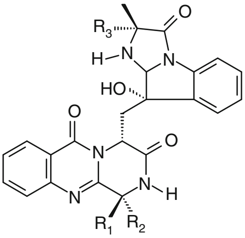
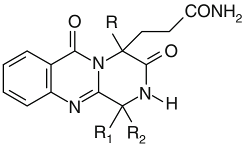
(*continued*)

Table 24.31 (continued)

Alkaloids	Substituents	Occurrence
Tryptanthrin	**II**	*Candida lipolytica* (microrganismo) [14], *Elephas maximus* (sex pheromones of the Asian elephant) [28], *Oceanibulbus idolifex* gen. nov., sp. nov. (bacteria) [39], *Schizophyllum commune* (mushroom) [34]
Ophiuroidine	**II**: 8,5′,6′-triOH	*Ophiocoma riisei* (ophiuroids, brittle stars) [58]
–		*Fusarium sambucinum* [27]
Monodontamide F		*Monodonta lábio* (mollusc) [28]
		
Glyantrypine R_1, $R_2 = H$		*Aspergillus clavatus* [26]
Fiscalin B $R_1 = CHMe_2$, $R_2 = H$		*Neosartorya fischeri* [27], *Corynascus setosus* [31]
Fumiquinazoline F ($R_1 = Me$, $R_2 = H$)		*Aspergillus fumigatus* [30], *Penicillium thymicola* [33]

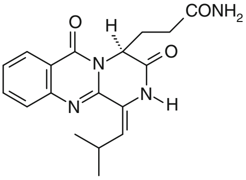
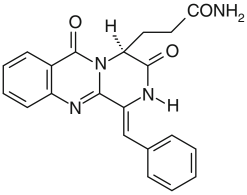
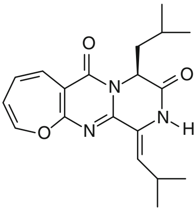
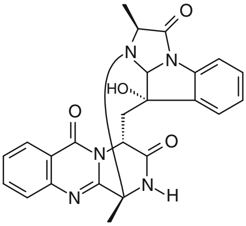
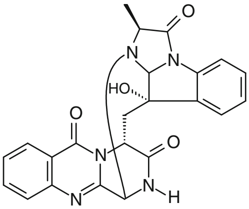
(*continued*)

Table 24.31 (continued)

Alkaloids	Substituents	Occurrence
Fumiquinazoline G ($R_1 = H$, $R_2 = Me$)		*Aspergillus fumigatus* [30]
		
Fumiquinazoline A $R_1 = Me$, $R_2, R_3 = H$		*Aspergillus fumigatus* [26, 30]
Fumiquinazoline B R_1, $R_3 = H$, $R_2 = Me$		*Aspergillus fumigatus* [26, 30]
Fumiquinazoline E $R_1 = Me$, $R_3 = H$, $R_2 = OMe$		*Aspergillus fumigatus* [26, 30]
Fiscalin A $R_1 = CHMe_2$, $R_3 = H$, $R_2 = H$		*Neosartorya fischeri* [27]
Fiscalin C $R_1 = CHMe_2$, $R_3 = Me$, $R_2 = H$		*Neosartorya fischeri* [27]
		
(+)-Verrucine A R, $R_1 = \alpha\text{-H}$, $R_2 = CH_2Ph$		*Penicillium verrucosum* [34]
(+)-Verrucine B $R_1 = \alpha\text{-H}$, $R = \beta\text{-H}$, $R_2 = CH_2Ph$		*Penicillium verrucosum* [34]
Anacine R, $R_1 = \alpha\text{-H}$, $R_2 = CH_2CHMe_2$		*Penicillium aurantiogriseum* SPO-19, [92], *P. nordicum* [37]
Aurantiomide B $R = \alpha\text{-H}$, $R_1 = CH_2CHMe_2$, $R_2 = \alpha\text{-OH}$		*Penicillium aurantiogriseum* SPO-19 [92]
Aurantiomide A $R = \alpha\text{-H}$, $R_1 = CH_2CHMe_2$, $R_2 = \alpha\text{-OMe}$		*Penicillium aurantiogriseum* SPO-19 [92]

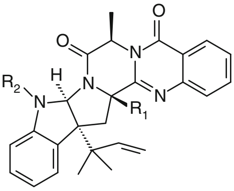
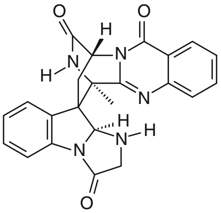
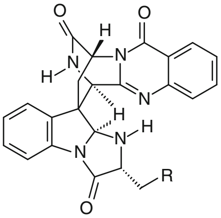
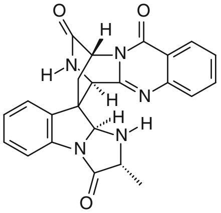
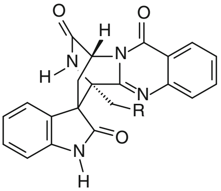
(*continued*)

Table 24.31 (continued)

Alkaloids	Substituents	Occurrence
Aurantiomide C	 CONH$_2$ O H N O N H N	*Penicillium aurantiogriseum* SPO-19 [92]
Verrucine F	 CONH$_2$ O H N O N H N	*Penicillium verrucosum* [93]
(−)-Janoxepin		*Aspergillus janus* [40]
Fumiquinazoline D	 O N N HO O N O N H N	*Aspergillus fumigatus* [30]
Cottoquinazoline A	 O N N HO O N O N H N	*Aspergillus versicolor* [94]

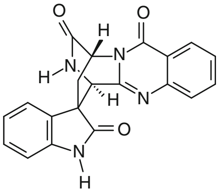
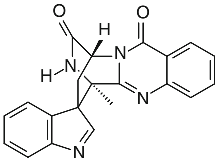
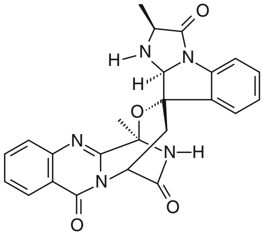
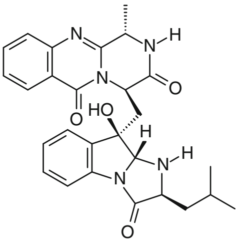
(*continued*)

Table 24.31 (continued)

Alkaloids	Substituents	Occurrence
(−)-Ardeemin R_1, $R_2 = H$	 O O N N N H R_2 N R_1	*Aspergillus fischeri* var. *brasiliensis* [28]
(−)-5-*N*-Acetylardeemin $R_1 = H$, $R_2 = Ac$		*Aspergillus fischeri* var. *brasiliensis* [28]
(−)-15b-β-Hydrox-5-*N*-acetylardeemin $R_1 = OH$, $R_2 = Ac$		*Aspergillus fischeri* var. *brasiliensis* [28]
Spiroquinazoline	 O O H N H–N N H N–H N O	*Aspergillus flavipes* [28]
Alanditrypinone R = 3-indolyl	 O O H N H–N N H N–H N R O	*Eupenicillium* sp. [39]
(−)-Alantryphenone R = Ph		*Eupenicillium* sp. [39]
(−)-Alantryleunone R = $CHMe_2$		*Eupenicillium* sp. [39]
Lapatin A	 O O H N H–N H N H N–H N O	*Penicillium lapatayae* [39]
	 O O H N H–N N R O N H	

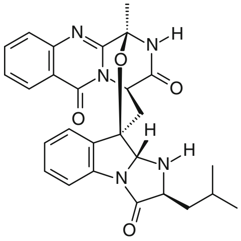
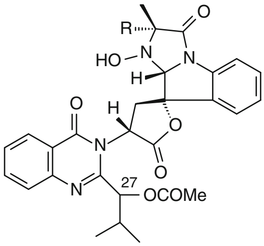
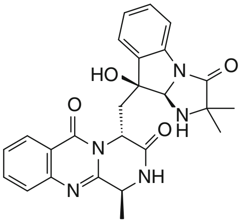
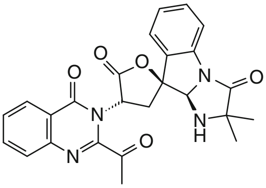
(*continued*)

Table 24.31 (continued)

Alkaloids	Substituents	Occurrence
(+)-Alantrypinone R = H		*Penicillium thymicola* [33]
(−)-Serantrypinone R = OH		*Penicillium thymicola* IBT 5891 [36]
Lapatin B		*Penicillium lapatayae* [39]
Alantrypinene B		*Eupenicillium* sp. [39]
Fumiquinazoline C		*Aspergillus fumigatus* [26]
(−)-Fumiquinazoline I		*Acremonium* sp. [34]

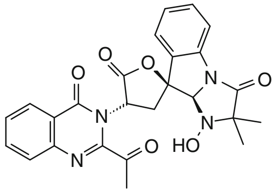
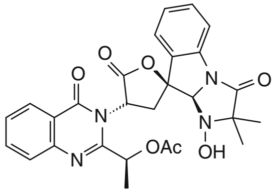
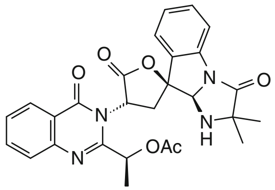
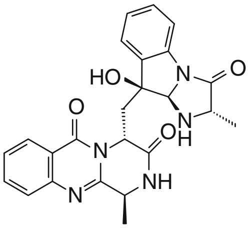
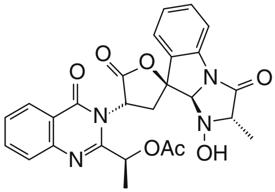
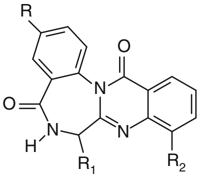
(*continued*)

Table 24.31 (continued)

Alkaloids	Substituents	Occurrence
(−)-Fumiquinazoline H		*Acremonium* sp. [34]
		
Tryptoquivaline R = Me (27S)		*Aspergillus* [31]
Nortryptoquivaline R = H (27S)		*Aspergillus* [31]
27-*epi*-Tryptoquivaline R = Me (27 R)		*Corynascus setosus* [31]
27-*epi*-Nortryptoquivaline R = H (27R)		*Corynascus setosus* [31]
15-Dimethyl-2-*epi*-fumiquinazoline A		*Penicillium aethiopicum* [95]
Deoxytryptoquialanone		*Penicillium aethiopicum* [95]

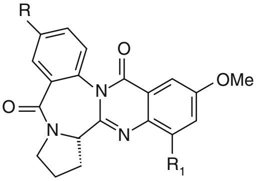
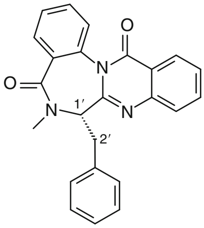
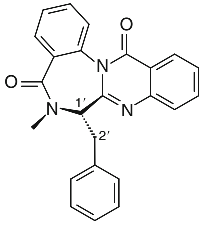
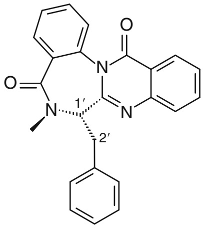
(*continued*)

Table 24.31 (continued)

Alkaloids	Substituents	Occurrence
Tryptoquialanone		*Penicillium aethiopicum* [95]
Tryptoquialanine		*Penicillium aethiopicum* [95]
Deoxytryptoquialanine		*Penicillium aethiopicum* [95]
2-*Epi*-fumiquinazoline A		*Penicillium aethiopicum* [95]
Nortryptoquialanine		*Penicillium aethiopicum* [95]
		

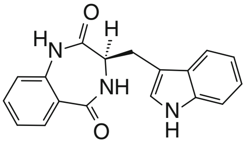
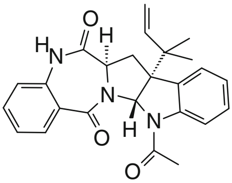
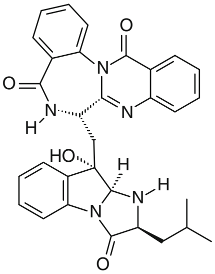
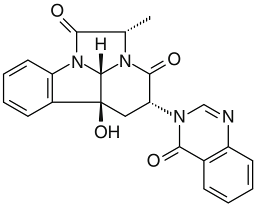
(*continued*)

Table 24.31 (continued)

Alkaloids	Substituents	Occurrence
Sclerotigenin R, R_1, R_2 = H		*Penicillium nordicum* [37], *P. sclerotigenum* [33]
(−)-Circumdatin F R, R_2 = H, R_1 = Me		*Aspergillus ochraceus* [33]
(−)-Circumdatin C R = OH, R_1 = Me, R_2 = H		*Aspergillus ochraceus* [33]
(−)-Circumdatin G R = H, R_1 = Me, R_2 = OH		*Aspergillus ochraceus* [35]
(−)-Circumdatin E R = H, R_1 = OH		*Aspergillus ochraceus* [33, 39]
(−)-Circumdatin D R = OMe, R_1 = OH		*Aspergillus ochraceus* [33, 39]
(−)-Circumdatin H R = OMe, R_1 = H		*Aspergillus ochraceus* [33, 39]
Benzomalvin B (1′,2′-duhydro) Benzomalvin C (1′,2′-β-epoxide)		*Penicillium* sp. [28]
Benzomalvin A		*Penicillium* sp. [28, 30]
Benzomalvin D		*Penicillium* sp. [30]

(*continued*)

Table 24.31 (continued)

Alkaloids	Substituents	Occurrence
(R)-Benzodiazepinedione		*Neosartorya fischeri* [96]
Acetylaszonalenin		*Neosartorya fischeri* [96]
Asperlicin		*Aspergillus alliaceus* [94]
Chaetominine		*Chaetomium* sp. [94]

A second type of quinoline dimer appears to be formed by acid-catalyzed reaction of the two alkaloids, as exemplified for pteledimerine (**27**, R = Me) in Scheme 24.7 [44].

Zanthoxyllum rhoifolium, *Metrodoria nigra*, and *Sarcomelicope megistophylla* yielded intriguing range of unprecedented *seco*-furoquinoline alkaloids dimethylrhoifolinate (**29**, R = Me, R_1 = H), furomegistine I (**29**, R = Me, R_1 = OMe) and furomegistine II (**30**, R = Me, R_1 = OMe) [26, 30, 35] formed from highly oxygenated quinolones by oxidative cleavage of ring A, (Scheme 24.8, Table 24.5).

Table 24.32 Biological activities of quinoline/one, quinazoline, and acridones alkaloids

Activity	Compounds
Antiprotozoal activity	
Leishmaniasis	1,5-Dihydroxy-2,3-dimethoxy-10-methyl-9-acridone, 2-phenylquinoline, 4-methoxy-2-phenylquinoline, 2-(3,4-methylenedioxyphenylethyl) quinoline, cusparine, 2-(3,4-dimethoxyphenylethyl)quinoline, 2-(pent-1-enyl)quinoline, 2-*n*-pentylquinoline, 2-*n*-propylquinoline, 5-hydroxynoracronycine, arborinine, chimanines A-D, dictyolomides A and B, melicopicine, N-nethyl-8-methoxyflindersine, normelicopicine, skimmianine [28, 30, 31, 38, 41, 45, 56].
Chagas	1,3,5-Trihydroxy-2,8-*bis*(3-methylbut-2-enyl)-10-methyl-9-acridone, 1,3,5-Trihydroxy-4-methoxy-10-methyl-2,8-*bis*(3-methylbut-2-enyl) acridin-9(10*H*)-one, 1,5-dihydroxy-2,3-dimethoxy-10-methyl-9-acridone, 2,3-Dihydro-4,9-dihydroxy-2-(2-hydroxypropan-2-yl)-11-methoxy-10-methylfuro[3,2-*b*]acridin-5(10*H*)-one, 3,4-Dihydro-3,5,8-trihydroxy-6-methoxy-2,2,7-trimethyl-2*H*-pyrano[2,3-*a*]acridin-12(7*H*)-one, 1-methyl-2-nonylquinolin-4(1*H*)-one, 2-(pent-1-enyl)quinoline, 2-nonylquinolin-4(1*H*)-one, 2-*n*-pentylquinoline, 2-*n*-propylquinoline, 5-hydroxynoracronycine, *bis*-5-hydroxynoracronycine, arborinine, citibrasine, citrusinine-I, citrusinine-II, chimanine B, chimanine D, glycocitrine IV, isodictamnine, iso-γ-fagarine, kokusagine, melicopicine, *N*-methylacridone, normelicopicine, pyranofoline, (7*R*,8*R*)-7-Acetoxy-8-[(*E*)-3-hydroxy-3-methylbut-1-enyl]-4,8-dimethoxy-5,6,7,8-tetrahydrofuro[2,3-*b*]quinoline, skimmianine [39, 41, 45, 56, 77].
Malaria	1,3,5-Trihydroxy-2,8-*bis*(3-methylbut-2-enyl)-10-methyl-9-acridone, 1,3,5-Trihydroxy-4-methoxy-10-methyl-2,8-*bis*(3-methylbut-2-enyl) acridin-9(10*H*)-one, 1,5-dihydroxy-2,3-dimethoxy-10-methyl-9-acridone, 2,3-Dihydro-4,9-dihydroxy-2-(2-hydroxypropan-2-yl)-11-methoxy-10-methylfuro[3,2-*b*]acridin-5(10*H*)-one, 3,4-Dihydro-3,5,8-trihydroxy-6-methoxy-2,2,7-trimethyl-2*H*-pyrano[2,3-*a*]acridin-12(7*H*)-one, 2-(3,4-methylenedioxyphenylethyl)quinoline, 2-*n*-nonylquinolin-4(1*H*)-one, 2-nonylquinolin-4-ol *N*-oxide, 2-[(1E)-undec-1-enyl]quinolin-4(1*H*)-one, 2-*n*-undecylquinolin-4(1*H*)-one, 2-pentylquinoline, 2-propylquinoline, 4-methoxy-2-phenylquinoline, 5-hydroxynoracronycine, *bis*-5-hydroxynoracronycine, acridone, acronycidine, acronycine, acronydine, acutine, angustureine, arborinine, artemisinin, atalaphyllinine, buxifoliadine-H, citibrasine, chimanines B, chimanines D, chloroquine, citrusinine-I, citrusinine-II, cryptolepine, cuspareine, γ-fagarine, febrifugine, flindersiamine, galipeine, galipinine, glycocitrine-IV, (−)-gravacridonediol, haplacutine E, haplopine, isofebrifugine, (−)-janoxepin, kokusaginine, mefloquine, melicopicine, *N*-methyl-2-nonylquinolin-4-one, neocryptolepine, normelicopicine, pyranofoline, quinazolin-4(3*H*)-one, quinine, (−)-rhodesiacridone, (−)-rutacridone, severifoline, skimmianine, tryptanthrin [23, 28, 30, 32, 34,36–38, 40, 41, 45, 56, 62, 66, 77, 87, 100, 153, 154].
Molluscicidal activity	2-(3,4-Methylenedioxyphenylethyl)quinoline, 2-*n*-pentylquinoline, 2-(pent-1-enyl)quinoline, 2-propylquinoline, cusculine [24, 27, 41].
Antimicrobial activity	1-Hydroxy-2-(non-2-enyl)-3-methylquinolin-4(1*H*)-one, 1-methyl-2-[(Z)-tridec-7-enyl]quinolin-4(1*H*)-one, 1-methyl-2-(1-hydroxygeranyl) quinolin-4(1*H*)-one, 1-methyl-2-(3-hydroxy-3,7-dimethyl-octa-1,6-dienyl)quinolin-4(1*H*)-one, 1-methyl-2-[(4Z)-tridec-4-enyl] quinolin-4-one, 1-methyl-2-[(4Z,7Z)-tridec-4,7-dienyl]quinolin-4(1*H*)-one,

(*continued*)

Table 24.32 (continued)

Activity	Compounds
	1-methyl-2-geranylquinolin-4(1*H*)-one, 1-methyl-2-*n*-nonylquinolin-4(1*H*)-one, 1-methyl-2-*n*-undecylquinolin-4(1*H*)-one, 1-methyl-2-pentadecylquinolin-4(1*H*)-one, 1-methylthiolmethyl-2-geranyl-3-methylquinolin-4(1*H*)-one, 1,2-dimethylquinolin-4(1*H*)-one, 1,3-dimethyl-2-(6,7-epoxygeranyl)quinolin-4(1*H*)-one, 1,3-dimethyl-2-geranylquinolin-4(1*H*)-one, 1,3-dimethylquinolin-4(1*H*)-one, 1,7-dihydroxyacridone, 1,8-dihydroxy-3-methoxyacridone, (+)-1,3,5-trihydroxy-4-prenylacridone, 2-(hept-2-enyl)-3-methylquinolin-4-one, 2-alkylquijnolin-4-one, 2-decyl-1-methylquinolin-4(1*H*)-one, 2-geranyl-3-methylquinolin-4(1*H*)-one, 2-geranylquinolin-4(1*H*)-one, 2-heptyl-4-hydroxyquinoline, 2-heptylquinolin-4-ol, 2-heptyl-4-hydroxyquinoline N-oxide, 2-*n*-heptyl-4-hydroxyquinoline, 2-*n*-pentyl-4-hydroxyquinoline, 2-*n*-nonylquinolin-4(1*H*)-one , 2-nonyl-4-hydroxyquinoline *N*-oxide, 2-pentylquinolin-4-ol, 4-methoxy-2-pentylquinoline, 8-bromotryptanthrin, 8-chlorotryptanthrin, 8-methoxy-4(1*H*)-quinolone-2-carboxylic acid, 8-methyltryptanthrin, atanine, aurachins A-D, citrusinine I, cuspareine, cusparine, demethoxycusparine , dihydroevocarpine, evocarpine, flindersiamine, flindersine, galipine, haplopine, helquinoline, kokusaginine, lagunamycin, *N*-methyl-3-(2,3-dihydroxy-3-methylbutyl)-4,7-dimethoxyquinolin-2-one, *N*-methyl-3-(2,3-epoxy-3-methylbutyl)-4-hydroxy-7-methoxyquinolin-2-one, *N*-methylquinolin-2-one, (−)-oriciacridone A, (+)-oriciacridone F, pteleatinium chloride, pteleine, quinocitrines A and B, quinolactacin A2, quinolactacins A, B and C, quinoline-4-carbaldehyde, skimmianine, transtorine, tryptanthrin, 4-methoxy-2-phenylquinoline, dictamnine, γ-fagarine, graveolinine (4-methoxy-2-(3′,4′-methylenedioxy)phenyl quinoline), kokusagine (7,8-methylenedioxydictamine) [22–24, 27, 29, 32–35, 37, 38, 40, 41, 59, 155, 161].
Antimicrobial activity against plant pathogens	1-Methyl-2-[6-(3,4-methylenedioxyphenylhexyl]quinolin-4(1*H*)-one, 2-(hept-2-enyl)-3-methylquinolin-4(1*H*)-one, 2-(non-2-enyl)-3-methylquinolin-4(1*H*)-one, 2-[4-(3,4-methylenedioxyphenylbutyl] quinolin-4(1*H*)-one, 2-heptyl-3-methylquinolin-4(1*H*)-one, 2-*n*-nonylquinolin-4(1*H*)-one, 2-nonyl-3-methylquinolin-4(1*H*)-one, 2-pentyl-3-methylquinolin-4(1*H*)-one, 3-methyl-2-(hept-2-enyl)quinolin-4(1*H*)-one, 3-methyl-2-(non-2-enyl)quinolin-4(1*H*)-one, evocarpine, graveoline, rutamine [41, 155]
Activity against fungus, symbiotic fungus and algicide	1-Methyl-2-[6-(3,4-methylenedioxyphenyl)-hexyl]quinolin-4-one, 2-(hept-2-enyl)-3-methylquinolin-4-one, 2-(heptyl)-3-methylquinolin-4-one, 2-(nonyl)-3-methylquinolin-4-one, 2-nonylquinolin-4(1*H*)-one, 2-(oct-2-enyl)-3-methylquinolin-4-one, 2-(pentyl)-3-methylquinolin-4-one, 5-methoxydictamnine, 6-hydroxyquinoline-8-carboxylic acid, (*Z*)-bogorin, dictamnine, γ –fagarine, flindersiamine, flindersine, graveoline, haplamine, haplopine, kokusaginine, maculine, melicobisquinolinones A and B, platydesmine, rutacridone, rutacridone epoxide, rutavarin, skimmianine, Atalaphyllidine, citbrasine, citrusinine I, citrusinine II, glycocitrine IV, junosine [30, 32, 33, 36, 37, 40, 41, 60, 73, 85].
Antitumor activity	1,3,5- Trihydroxy-2,8-*bis*(3-methylbut-2-enyl)-10-methyl-9-acridone, 1,3,5-Trihydroxy-4-methoxy-10-methyl-2,8-bis(3-methylbut-2-enyl) acridin-9(10*H*)-one, (−)-1,3-Dihydroxy-4-(2-hydroxy-3-hydroxymethyl-

(*continued*)

Table 24.32 (continued)

Activity	Compounds
	3,4-epoxybutyl)-*N*-methylacridone, (−)-5-*N*-acetylardeemin, 2,3-dihydro-4,9-dihydroxy-2-(2-hydroxy-propan-2-yl)-11-methoxy-10-methylfuro[3,2-b]acridin-5(10*H*)-one, 5-dihydroxyacronycine, 1-hydroxy-3-methoxy-10-methyl-9-acridone, 1-hydroxy-2,3-dimethoxy-10-methyl-9-acridone, 3-hydroxy-1-methoxy-10-methyl-9-acridone, 5-hydroxy-*N*-methylseverifoline (*N*-methylatalaphyllinine), 1-methyl-2-undecyl-4-quinolone, 5-methoxymaculine, 6-(2′-Hydroxy-3′-chloroprenyloxy)-4,7-dimethoxyfuroquinoline, 7-hydroy-8-methoxydictamine, 7-Isopentenyloxy-γ-fagarine, 7-(2′-hydroxy-3′-chloroprenyloxy)-4,8-dimethoxyfuroquinoline, 9-hydroxynoracronycine, acronycine, arborinine, aspoquinolone A, aspoquinolone B, atalaphyllidine, atalaphyllinine, atanine, aurantiomide B, aurantiomide C, *bis*-5-hydroxyacronycine, casimiroine, chloroevellerine, citbrasine, des-*N*-methylnoracronycine, dihydroevocarpine, epoxievellerine, evocarpine, evodiamine, γ-fagarine, flindersiamine, flindersine, glyfoline, haplamine, haplopine, haplophytin-A (10-methoxy-2,2-dimethyl-2,6-dihydro-pyrano[3,2-c]quinolin-5-one), helebelicine B (3-Hydroxy-1,2-dimethoxy-10-methyl-9-acridone), isodictaminnine, iso-γ-fagarine, kokusagine, maculine, methylacridin-9(10H)-one, methylnkolbisine, orixalone A, orixinone, perfamine, preorixine, (−)-spiroquinazoline, rutaecarpine, skimmianine, tecleanatalesine B, tryptanthrin [23, 25, 27–29, 32–34, 39–41, 47, 51, 56, 61, 63, 74, 79, 82, 92, 155–157].
Toxicity in the brine shrimp	3-Hydroxy-1-methyl-3-(2-oxopropyl)quinoline-2,4(1*H*,3*H*)-dione, 4-hydroxy-3-methoxy-1-methyl-2(1*H*)-quinolinone, methyl 2-(3-hydroxy-1-methyl-2,4-dioxo-1,2,3,4-tetrahydroquinolin-3-yl)-acetate, 10*H*-Indolo[3,2-b]quinoline, asimicilone, (*Z*)-bogorin, dihydroevocarpine, evocarpine, kokusagainine, *N*-methylflindersine, *N*-methylswietenidine-B [27, 32, 33, 41, 46, 98].
Inhibitory effect on tumor necrosis factor (TNF) production	Quinolactacins A–C [41]
Nuclear Factor of Activated T Cells – NFAT inhibitor	1-Methyl-2-[(4Z,7Z)-tridec-4,7-dienyl]quinolin-4(1*H*)-one, 1-methyl-2-[(6Z,9Z)-pentadec-6,9-dienyl]quinolin-4(1*H*)-one, 1-methyl-2-*n*-nonylquinolin-4(1*H*)-one, 1-methyl-2-pentadecylquinolin-4(1*H*)-one, 1-methyl-2-tridecylquinolin-4(1*H*)-one, 1-methyl-2-undecylquinolin-4(1*H*)-one, 2-*n*-nonylquinolin-4(1*H*)-one , 2-n-undecylquinolin-4(1*H*)-one, dihydroevocarpine, evocarpine [38, 41].
Cytotoxic activity	1,3,5-Trihydroxy-2,8-*bis*(3-methylbut-2-enyl)-10-methyl-9-acridone, 1,3,5-Trihydroxy-4-methoxy-10-methyl-2,8-*bis*(3-methylbut-2-enyl) acridin-9(10*H*)-one, 1,5-dihydroxy-2,3-dimethoxy-10-methyl-9-acridone, 2,3-dihydro-4,9-dihydroxy-2-(2-hydroxy-propan-2-yl)-11-methoxy-10-methylfuro[3,2-b]acridin-5(10*H*)-one, 3,4-Dihydro-3,5,8-trihydroxy-6-methoxy-2,2,7-trimethyl-2*H*-pyrano[2,3-*a*]acridin-12(7*H*)-one, 3,4-Dihydro-3,5,8-trihydroxy-6-methoxy-2,2,7-trimethyl-2*H*-pyrano[2,3-a]acridin-12(7*H*)-one, 1-hydroxy-3-methoxy-10-methyl-9-acridone, 1-hydroxy-2,3-dimethoxy-10-methyl-9-acridone, 1-hydroxy-*N*-methylacridone, 1-methyl-2-[(Z)-pentadec-9-enyl]quinolin-4(1*H*)-one, 1-methyl-2-[(Z)-pentadec-10-enyl]quinolin-4(1*H*)-one, 2-hydroxy-4-methoxy-3-prenyl-quinoline, 2-(3,4-methylenedioxyphenyletyl) quinoline, 2-[2-(3,4-methylenedioxyphenyl)ethyl]quinol, 2-nonyl-4-

(*continued*)

Table 24.32 (continued)

Activity	Compounds
	hydroxyquinoline *N*-oxide, 3,8-diacetoxyquinoline, 3,8-dimethoxyquinoline, 3-hydroxy-1-methoxy-10-methyl-9-acridone, 5-dihydroxyacronycine, *bis*-5-hydroxyacronycine, 5-hydroxynoracronycine, acridone, acrimarine-F, acronycine, angustureine, angustureine, arborinine, artemisinin, aspoquinolone A, aspoquinolone B, atalaphyllinine, atanine, balsacridone A, balsacridone B, (*Z*)-bogorin, buxifoliadine-B, buxifoliadine-D, buxifoliadine-H, casimiroin, chloroquine, citbrasine, citracridone-III, citrusinine-I, citrusinine-II, cryptolepine, cuspareine, cuspareine, dictamnine, dihydroevocarpine, epoxide acronycine, evocarpine, evolitrine, γ-fagarine, febrifugine, flindersine, fumiquinazolines A-G, furomegistine I and II, galipeine, galipeine, galipinine, glycocitrine-I, glycocitrine-II, glycocitrine-IV, glyfoline, grandisine-I, 5-hydroxynoracronycine, 5-hydroxynoracronycine alcohol, *bis*-5-hydroxynoracronycine, gravacridonediol, gavacridonediol monomethyl ether, gravacridonetriol, graveoline, graveoline, halitulin, haplamine, haplopine, helebelicine B (3-Hydroxy-1,2-dimethoxy-10-methyl-9-acridone), huajiaosimuline, isogravacridone chlorine, jineol (3,8-dihydroxyquinoline), kokusaginine, luotonin A (quino[2′,3′ : 3,4]pyrrolo[2,1-*b*]quinazolinone), maculosidine, natsucitrine-II, ngustureine, normelicopicine, mefloquine, *O*-methylglycocitrine-II, neocryptolepine, pyranofoline, quinine, quinocitrinines A and B, rutacridone, severibuxine, severifoline, skimmianine, tryptanthrin, zanthosimuline [29–41, 45, 51, 56, 74, 77, 79, 81, 100, 154, 156, 158, 159].
Antimutagenic	5-Hydroxy-1-methyl-2-phenylquinolin-4(1*H*)-one, (–)-isogravacridonchlorine, rutacridone, rutacridone epoxide [23, 25, 41].
Enzyme inhibitors	1,3,5-Trihydroxy-4-methoxy-10-methyl-2,8-bis(3-methylbut-2-enyl) acridin-9(10H)-one, 1,3,5-trihydroxy-2,8-bis(3-methylbut-2-enyl)-10-methyl-9-acridone, 2,3-dihydro-4,9-dihydroxy-2-(2-hydroxy-propan-2-yl)-11-methoxy-10-methylfuro[3,2-b]acridin-5(10H)-one, 3,4-dihydro-3,5,8-trihydroxy-6-methoxy-2,2,7-trimethyl-2Hpyrano[2,3-a]acridin-12(7H)-one, 1-Hydroxy-2-(non-2-enyl)-3-methylquinolin-4(1*H*)-one, 1-methyl-2-[(4Z,7Z)-tridec-4,7-dienyl]quinolin-4(1*H*)-one, 1-methyl-2-[(4Z,7Z)-tridec-4,7-dienyl]quinolin-4(1*H*)-one, 1-methyl-2-[(6Z,9Z)-pentadec-6,9-dienyl]quinolin-4(1*H*)-one, 1-methyl-2-[(6Z,9Z)-pentadec-6,9-dienyl]quinolin-4(1*H*)-one, 1-methyl-2-[(Z)-undec-6-enyl]quinolin-4(1*H*)-one, 1-methyl-2-*n*-nonylquinolin-4(1*H*)-one, 1-methyl-2-tetradecylquinolin-4(1*H*)-one, 1-methyl-2-undecylquinolin-4(1*H*)-one, 2-heptyl-4-hydroxyquinoline *N*-oxide, 2-methyl-4(3*H*)-quinazolinone, 2′-hydroxylunidonine, 3-Hydroxy-2,2,6-trimethyl-3, 4, 5, 6-tetrahydro-2H-pyrano[3,2-c] quinoline 5-one, 5-hydroxygraveroline, 5-hydroxynoracronycine, 8-methoxy-ifflaiamine, acronycine, aurachin A, aurachin B, aurachin C, aurachin D, balfourodine, balfourodinium, casimiroin, (−)-circumdatin, circumdatin E, citibrasine, citrusinine-I, citrusinine-II, confusameline, deoxypeganine, deoxyvasicine, dictamnine, evocarpine, fiscalins A-C, glycocitrine-I, glycocitrine-IV, ifflaiamine, isaindigotone, isoacronycine, kokusaginine, (−)-lagunamycin, lunacridine, lunacrine, lunamarine, lunasine, lunidine, lunidonine, lunine, methyl isoplatydesmine, N-methyl-flindersine, orixalone A, pyranofoline, quinolactacin A1, quinolactacin A2,

(*continued*)

Table 24.32 (continued)

Activity	Compounds
	ribalinine, rutacridone, skimmianine, tryptanthrin, 1-Methyl-2-undecyl-4-quinolone, dihydroevocarpine, evodiamine, rutaecarpine [25–30, 32, 35–39, 41, 50, 53, 54, 69, 155, 158, 160, 162].
Antiplatelet activity	2-Phenylquinolin-4(1*H*)-one, 4methoxy-1-methylquinolin-2-one, atanine, confusadine, confusameline, dictamnine, dutadrupine, evolitrine, γ-fagarine, graveoline [1-methyl-2-(3,4-methylenedioxyphenyl)quinolin-4(1*H*)-one], graveolinine [4-methoxy-2-(3,4-methylenedioxyphenyl) quinoline], haplopine, kokusaginine, robustine, skimmianine [28, 33, 34, 37, 41].
Insecticide, antifeedant, nematicide activities	1-Hydroxy-3-methoxy-*N*-methylacridone, *rel*-(3*R*,4*R*)-(-)-3-Methoxy-4,5-dihydroxy-4-(4-methoxyphenyl)-3,4-dihydroquinolin-2-one, *rel*-(3*R*,4*R*)-(-)-3-Methoxy-4-hydroxy-4-(4-methoxyphenyl)-3,4-dihydroquinolin-2-one, arborinine, atanine, dictamnine, evolitrine, leiokinine A and B, *N*-methylquinolinium-2-carboxylate, penigequinolone A and B, (-)-peniprequinolone, quinolactacide, vasicine, vasicinol, vasicinone, xanthoxoline [22, 25, 29, 33–36, 39, 41].
Miscellaneous biological activities	
Inhibitory effect on germination	2-(Pent-1-enyl)quinoline, 2-*n*-pentylquinoline, 2-*n*-propylquinoline, graveoline [38, 41].
Anti-HIV activity	1-Hydroxy-*N*-methylacridone, 2-(pent-1-enyl)quinoline, 2-(pent-2-en) quinolin, 2-acetyl-4(3H)-quinazolinone, 2-*n*-pentylquinoline, 3-prenyl-4-prenyloxyquinolin-2-one, 4-methoxy-1-methylquinolin-2-one, *N*-methyl-3,3-diprenylquinoline-2,4-dione, buchapine, γ-fagarine, haplopine, (+)-platydesmine, uranidine [28, 30, 34, 38, 40, 41].
Muscle relaxant	Eduline, japonine [41, 43].
Estrogenic activity	1-Methyl-2-phenylquinolin-4(1*H*)-one, acutine [41].
Anti-inflammatory activity	Toddaliopsins A–D, tryptanthrin [40, 155].
Neurodegenerative diseases	Dictyoquinazols A–C [37].
Phtotoxicity (anti-psoriasis activity)	7-Desmethylskimmianine, 8-methoxypsoralen, dictamnine, isodictamnine, maculine, maculosidine [152].
Potassium channel blockers	Dictamnine, γ-fagarine, kokusaginine, *ent*-(-)-platydesmine, robustine, skimmianine [35].
Antiarrythmic activity	Acrophyllidine [34].
Vasorelaxing activity	Atanine, vasicinone [33, 91].
Pollen-growth inhibitors	Penigequinolones A and B [31].
Anti-inflammatory and antihypertensive	2-(2-Hydroxy-4-methoxyphenyl)-5,8-dimethoxy-3-propylquinoline-4(1*H*)-one, 5,6-Dimethoxy-2-(2,5,6-trimethoxyphenil)-1*H*-quinoline-4(1*H*)-one, 5,8-Dimethoxy-2-(2,3-dimethoxyphenil)-3-propylquinoline-4(1*H*)-one, 5,8-dimethoxy-2-(3-methoxyphenyl)-3-propylquinoline-4(1*H*)-one, (−)-Benzomalvin A, quinazoline-2,4-dione [28, 31, 88].
Endotoxic to limulus amoebocyte lysate test	3-(2-Carboxyphenyl)-4(3*H*)-quinazolinone [31].

(*continued*)

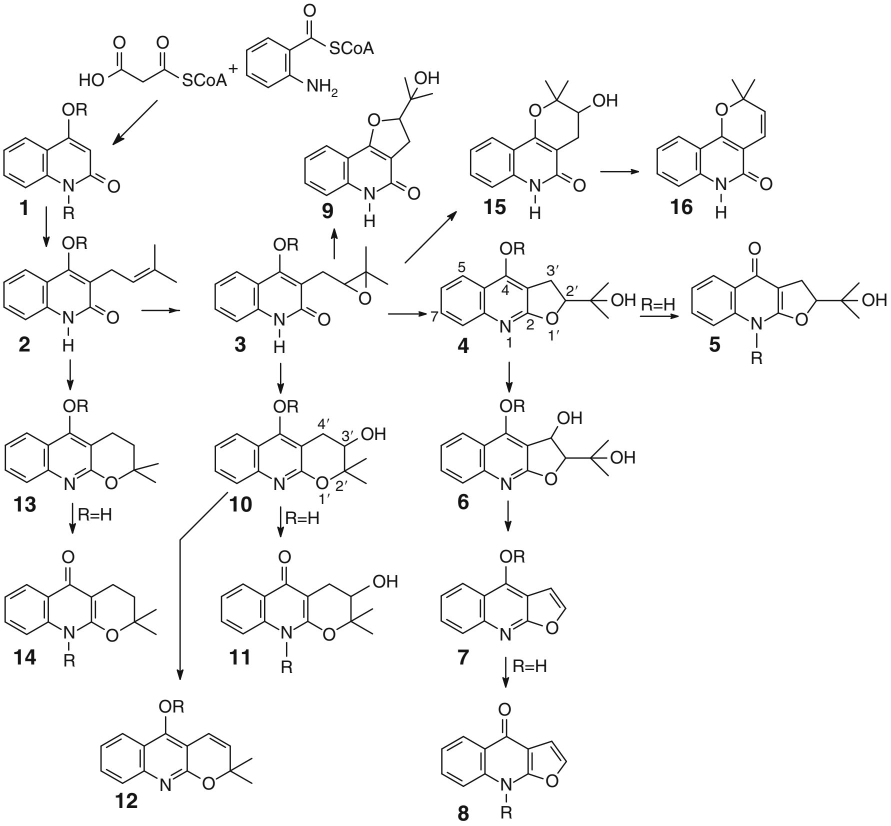
Table 24.32 (continued)

Activity	Compounds
Tremor and paralysis effects	27-*Epi*-nortryptoquivaline, 27-*epi*-tryptoquivaline, tryptoquivaline [31].
Pharmacokinetic	Dihydroevocarpine, evocarpine [26].
Raising body temperature	Evocarpine, evodiamine [25].
Anticoccidial	*Cis* and *trans* febrifugine [25].
Oxytocin effect and stimulation of muscular contraction	Vasicine [24].
Antispasmodic activity	Citracridone-I [22].
Antioxidant activity	4-Amino-6-hydroxyquinoline-8-carboxylic acid, 6-hydroxyquinoline-8-carboxylic acid, 7-hydroxy-1-oxo-1,2-dihydroisoquinoline-5-carboxylic acid, kokusaginine, Ophiuroidine (4,8,9-trihydroxyindolo[2,1-b] quinazoline-6,12-dione), quinosuaveoline B, skimmianine, tecleaverdoornine [57, 58, 63, 85].
Antiviral activity	5-Methoxydictamnine, dictamnine, glycofolinine, glycosparvarine [60,76].
Antiallergic activity	Buxifoliadine-E, citrusinine-I, *N*-methylcyclo-atalaphylline-A [72].
Phagocyte oxidative burst	Balsacridone A, balsacridone B, citracridone I, citracridone III, 5-hydroxynoracronycine [81].

A group of polycyclic quinoline alkaloids formally incorporating a geranyl group at C-3 form a tetracyclic skeleton by acid-catalyzed reaction (Scheme 24.9). Four of these compounds were isolated from *Halfordia kendak* (Rutaceae), *trans*-erioaustralasine (**31**, R = CH_2OAc), *trans*-deacetoxyerioaustralasine (**31**, R = Me), *trans*-deacetoxyerioaustralasine hydrate (**32**, α-tertiary hydroxyl, R = Me), *trans*-erioaustralasine hydrate (**32**, α-tertiary hydroxyl, R = CH_2OAc), *trans*-epideacetoxyerioaustralasine hydrate (**32**, β-tertiary hydroxyl, R = Me) (Table 24.8).

Acridone alkaloids are unique metabolites of the Rutaceae plant family (Table 24.11), with only few exceptions reported from single genera of the Asteraceae (Table 24.12), Simaroubaceae (Sapindales, Table 24.12), Piperaceae (Table 24.14), and Apocynaceae (Table 24.16). *Ruta graveolens* accumulates *N*-methylated acridones exclusively (Tables 24.11 and 24.13), whereas *N*-acetoxymethyl- and *N*-methoxymethylacridones were isolated recently from the south-central African plant *Toddaliopsis bremekampii* (Table 24.11), and a few unmethylated acridones have been reported from other genera (Tables 24.11, 24.13, and 24.15). The latter compounds conceivably derive from *N*-methylacridones, analogous to the formation of nornicotine from nicotine [113, 114].

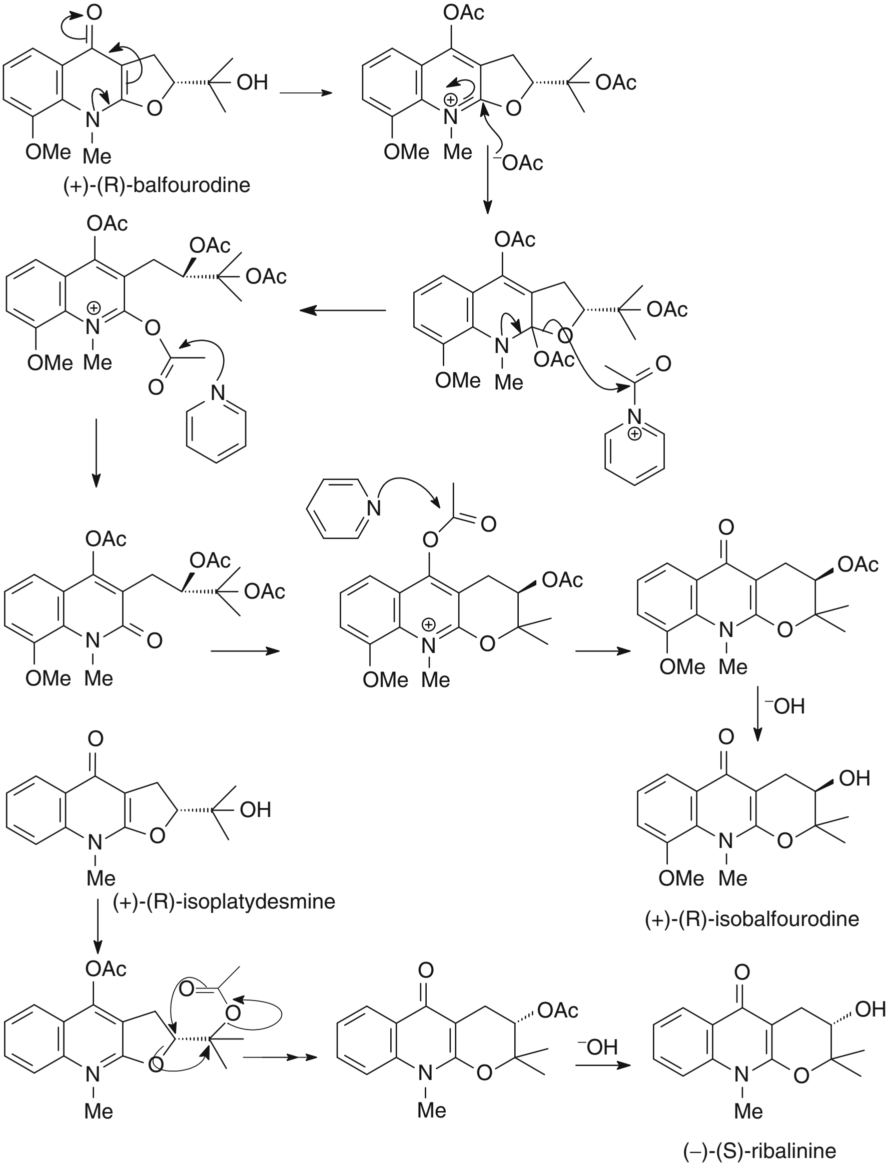
An acridone synthase responsible for the conversion of *N*-methylanthraniloyl CoA and malonyl CoA into 1,3-dihydroxy-*N*-methylacridone (**33**, Scheme 24.10) has been purified from cell suspension cultures of *Ruta graveolens* and characterized [115, 116]. The essential *N*-methylation of the starter substrate was supported also by the anthranilate *N*-methyltransferase activity detected in crude extracts of *R*. *graveolens* cells [117].



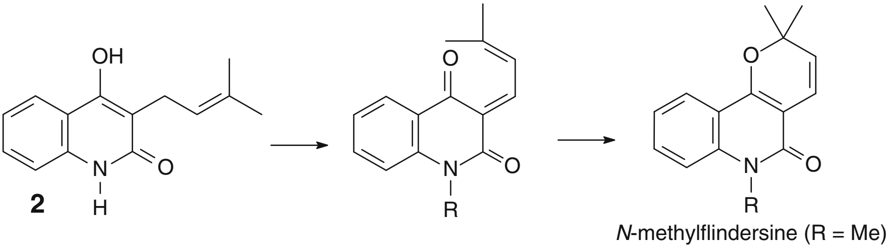
Scheme 24.1 Biosynthesis (for **4** and **7**) and biogenesis of the quinolin/one alkaloids

These data have firmly established that, in *R. graveolens* at least, *N*-methylation of anthranilate prior to CoA ligation is crucial for acridone alkaloid formation. Thus, acridones arise from condensation of the starter substrate *N*-methylanthraniloyl CoA with three units of malonyl CoA, which are modified further by prenylation and cyclization reactions to more complex furo- or pyranoacridones (Scheme 24.10). The biosynthesis of rutacridone (**34**, 1″,2″-dehydro) in cell suspension cultures of *R. graveolens* from 1,3-dihydroxy-*N*-methylacridone and hemiterpenoid pyrophosphates via glycocitrine II (**33**, 1,3-diOH, 4-$CH_2CH = CMe_2$, 10-Me) has been investigated [118, 119]. The enzyme acridone synthase catalyzes all these steps and belongs to the family of plant type III polyketide synthases (PKSs) [120]. A group of acridone alkaloids formally incorporating a geranyl group at C-2 form a 2′-(4-methyl-3-penteneyl) pyranoacridone (**41**, Scheme 24.10).

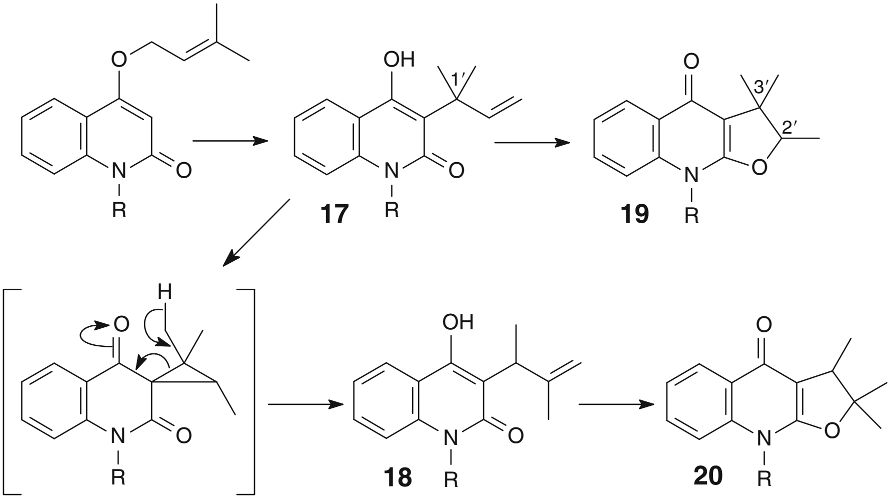
No data is available on the biosynthesis of dimeric acridone alkaloids, which have been found in Rutaceae. Acridone-coumarin/xanthone/lignin dimers have also been found in Rutaceae (Table 24.17). The bark of the New Caledonia tree *Sarcomelicope megistophylla* (Rutaceae) have yielded an intriguing range of


(+)-(R)-balfourodine
(+)-(R)-isobalfourodine
(+)-(R)-isoplatydesmine
(–)-(S)-ribalinine
OAc
OMe
Me
⁻OAc
⁻OH

Scheme 24.2 Route for the conversion of (+)-(R)-balfourodine into (+)-(R)-isobalfourodine and (+)-(R)-isoplatydesmine into (−)-(S)-ribalinine with acetic anhydride in pyridine and ulterior hydrolyze. Mechanism as proposed by Fersht and coworker [141], in which pyridine catalysis involves initial formation of an acyl pyridinium ion, which then reacts with the alcohol



Scheme 24.3 Synthesis of piranoquinolin-2-one

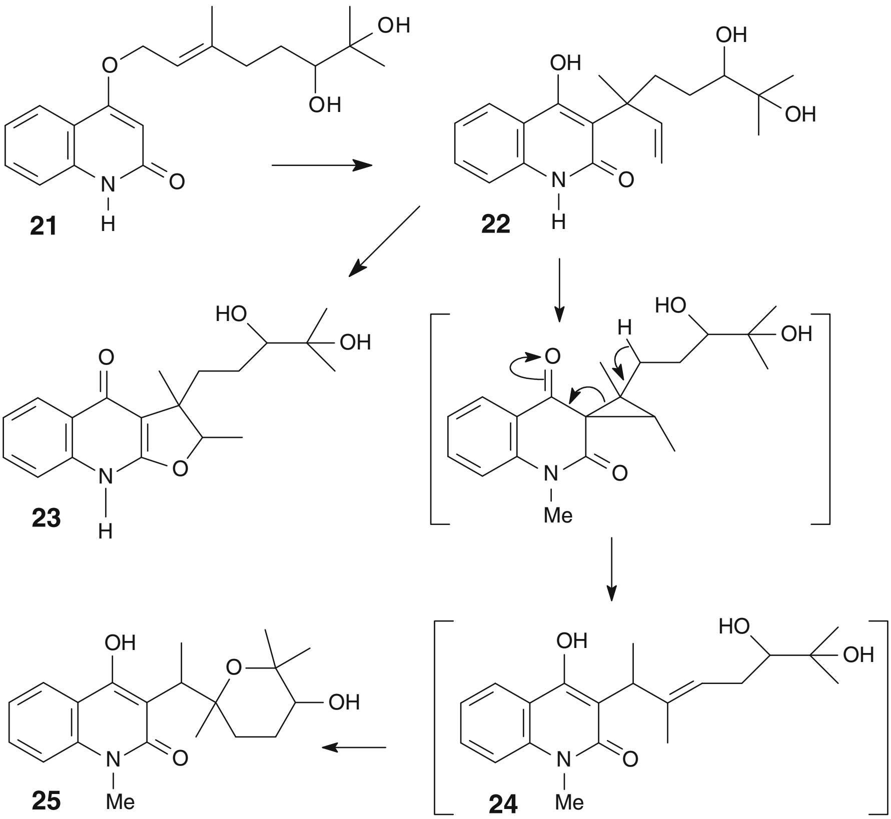


Scheme 24.4 Biogenetic pathway for the formation of quinolin-2(1*H*)-/4(1*H*)-one alkaloids by Claisen rearrangement of the prenyl ether

unprecedented acridone alkaloids, all of which are conceivably formed from highly oxygenated acridones such as melicopicine (**45**) by oxidative cleavage of ring B (Scheme 24.11). The structural similarity are clearly apparent in the (+)-sarcomejine (**47**), (−)-megistolactone (**46**), and cyclomegistine (**48**) [41]. The biosynthetic origins of megistonine I [**49**, R, R_1, R_2 = Me, R_3 = H, R_4 = 3,3-dimethylallyl] and II [**49**, R, R_1, R_2 = Me, R_3 = H, R_4 = OMe] are less obviously ascribable to acridone oxidation [41].

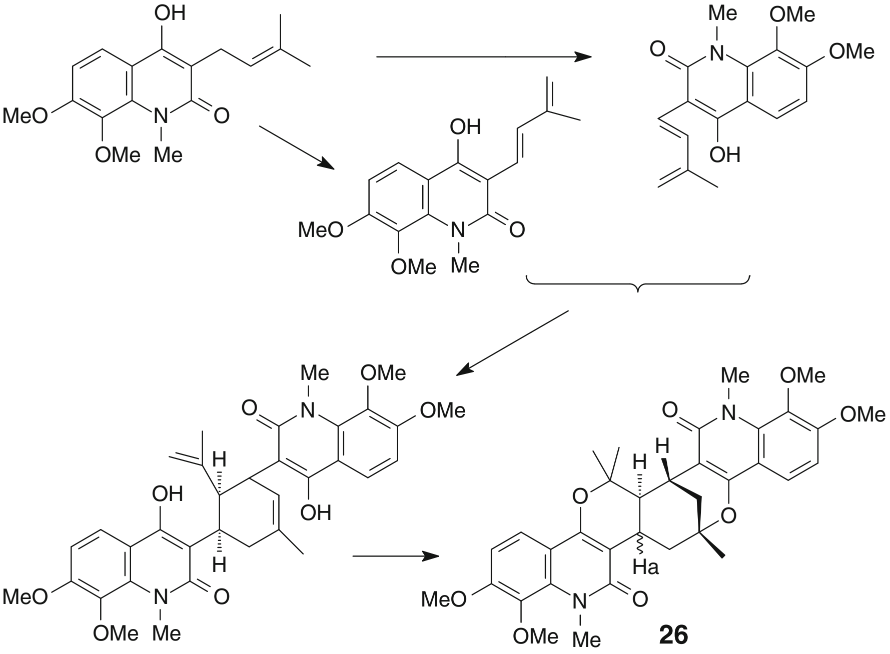
2.2 Alkyl, Aryl, Alkylarylquinoline Alkaloids

Many 2-alkylquinolin/4(1*H*)-one alkaloids bear the trivial names pseudans because of their occurrence in bacteria of the genus *Pseudomonas*. For example, *P. aeruginosa* is a ubiquitous gram-negative bacterial pathogen that causes infections not only in human hosts but also in animals and even plants.

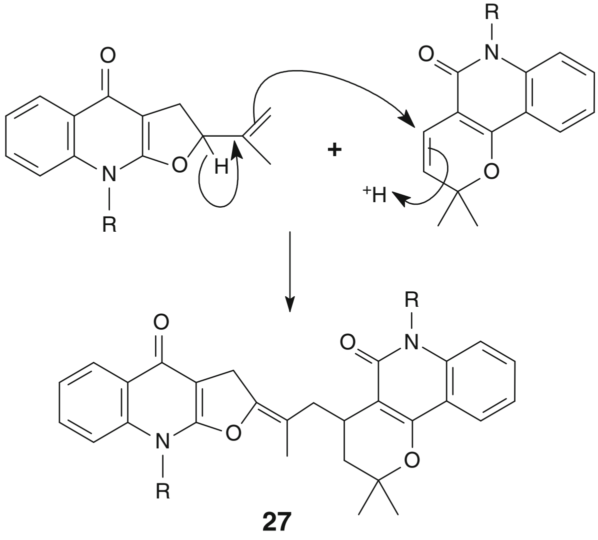


Scheme 24.5 Biogenetic pathway for the formation of quinolin-2(1*H*)-/4(1*H*)-one alkaloids by Claisen rearrangement of the geranyl ether

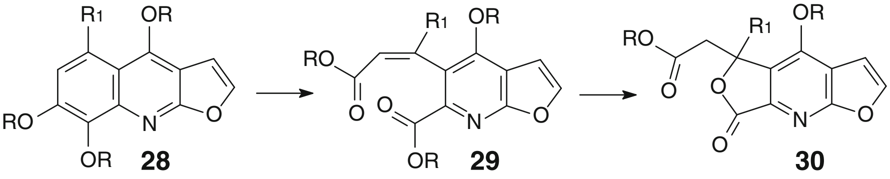
This pathogen has been identified as one of the leading causes of nosocomial infections and is responsible for fatal chronic lung infections in patients with cystic fibrosis (CF). *P. aeruginosa* coordinates its population behavior, such as biofilm formation and virulence factor production, by means of small extracellular signal molecules, so-called autoinducers, that are released into the environment under appropriate conditions. Since intercellular communication leads to cooperative and coordinated bacterial behavior in a cell density-dependent manner, it is referred to as quorum sensing. A common feature of intercellular communication is the transcriptional activation of quorum-sensing-controlled genes when the bacterial signal molecules reach a certain threshold. *P. aeruginosa* produces two major cell-to-cell signals that are members of the *N*-acyl-homoserine lactones and 4-hydroxy-2-alkylquinolines. The latter include in addition to N oxides, 3,4-hydroxy-2-heptylquinoline and 4-hydroxy-2-heptylquinoline. The latter two molecules have been shown to be involved in intracellular communication. Analysis of the underlying metabolic events of intercellular bacterial communication and elucidation of the biosynthesis of the signal molecules might contribute to the understanding and



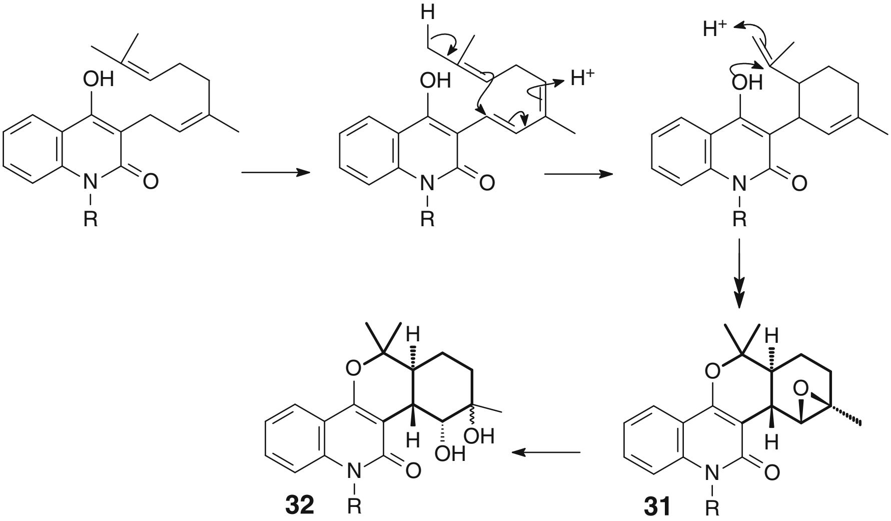
Scheme 24.6 Biogenetic pathway for the formation of dimeric quinolin-2(1*H*)-one alkaloids by Diels-Alder addition of prenyl dienes



Scheme 24.7 Biogenetic pathway for the formation of dimeric quinolin-2(1*H*)-/4(1*H*)-one alkaloids by acid-catalyzed reaction

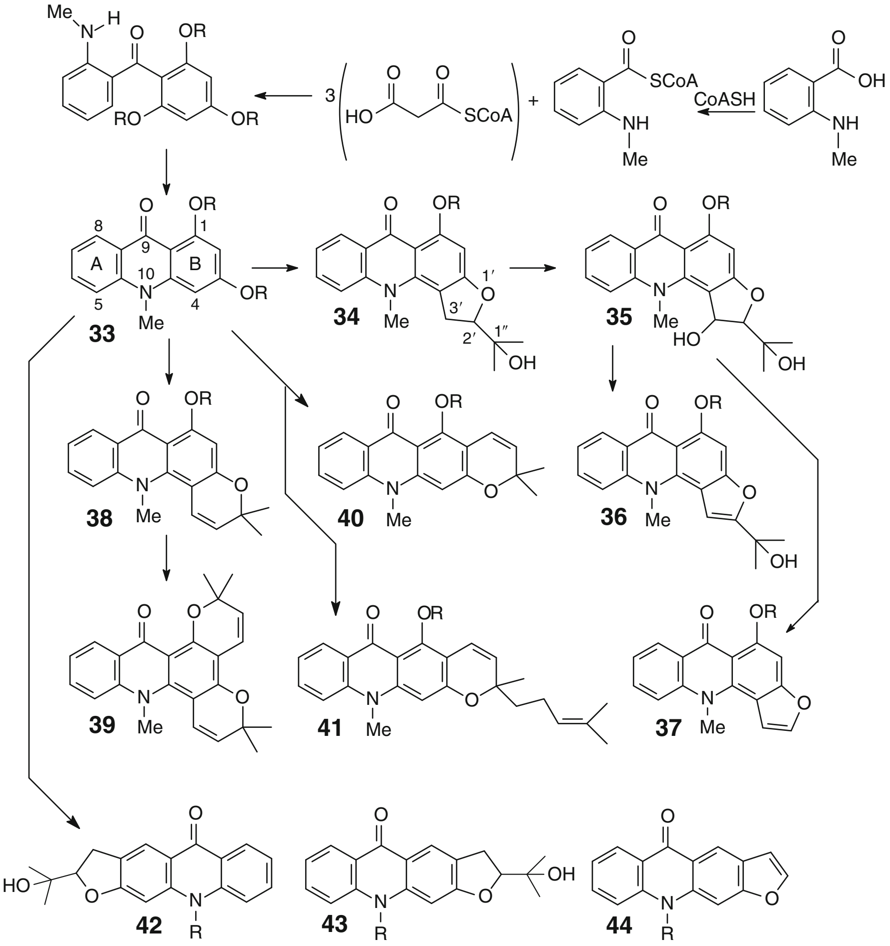


Scheme 24.8 Biogenesis of *seco*-furoquinoline alkaloids



Scheme 24.9 Biogenetic pathway for the formation of polycyclic quinolin-2(1*H*)-one alkaloids by acid-catalyzed reaction

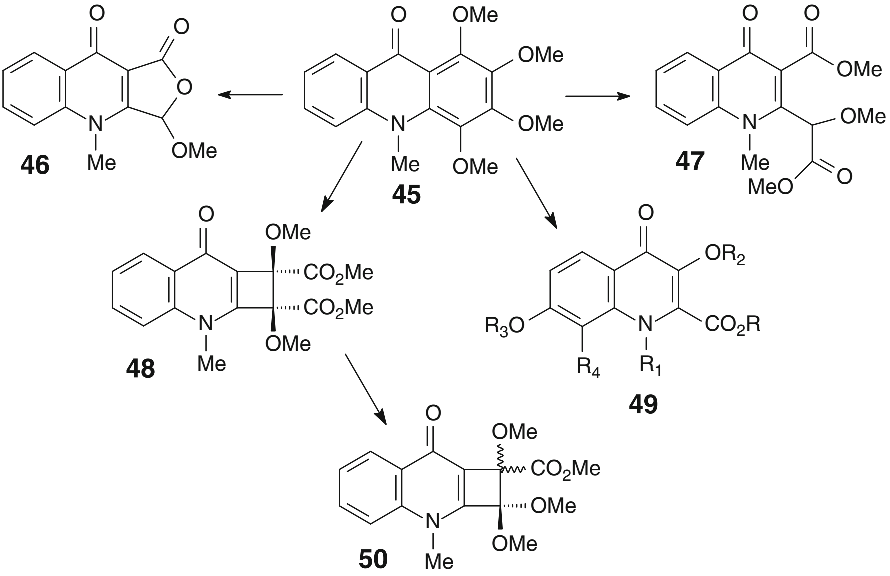
provide an opportunity to interfere with the control of virulence factor production. Moreover, a 2-heptyl-3-hydroxyquinolin-4(1*H*)-one biosynthetic gene cluster has been identified. This *pqsABCDE* operon codes for a putative coenzyme A ligase (*pqsA*), two β-keto-acyl-acyl carrier protein synthases (*pqsB*, *pqsC*), and a FabH1 homologous transacetylase (*pqsD*), whereas *pqsE* seems to encode a response effectors protein which itself is not involved in the biosynthesis of 2-heptyl-3-hydroxyquinolin-4(1*H*)-one. Although, it has clearly been demonstrated that the *pqsABCD* genes are essential for 2-heptyl-3-hydroxyquinolin-4(1*H*)-one biosynthesis, their enzymatic function remains to be elucidated [121–123]. Previous studies strongly suggested that anthranilic acid and a β-keto-acid are precursors of 2-alkylquinolin-4(1*H*)-ones [124, 125]. Further studies using feeding experiments with labeled isotopes, confirmed by gas chromatography (GC)-mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy that *P. aeruginosa* synthesizes 2-alkylquinolin-4(1*H*)-ones via a common biosynthetic pathway involving the "head-to-head" condensation of anthranilic acid and β-keto fatty acids (Scheme 24.12). Moreover, 2-heptyl-3-hydroxyquinolin-4(1*H*)-one biosynthesis



Scheme 24.10 Biosynthesis (for **33** and **34**, 1″,2″-dehydro) and biogenesis of the acridone alkaloids

seems to be dependent not only on an intact *pqsABCD* operon but also on the availability of β-keto acids. Interestingly, at least some of these acids seem to be derived from a common pool of β-hydroxy-keto acids involved in rhamnolipid biosynthesis [123].

A search of completed and uncompleted microbial genomes revealed that several species belonging to the genera *Pseudomonas* and *Burkholderia* as well as the plant pathogen *Ralstonia* contain putative homologs of the *P. aeruginosa* pqs biosynthetic genes pqsA, pqsC, and pqsD, respectively. However, apart from *P. aeruginosa*, only strains of the primary human pathogen, *B. pseudomallei* and the serologically and genetically related *B. thailandensis* were found to contain a

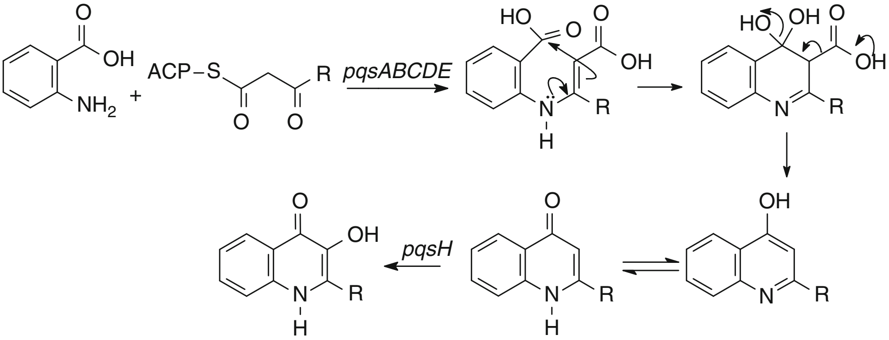


Scheme 24.11 Biogenesis of the *seco*-acridone alkaloids

complete, putative pqsABCDE operon located on chromosome 2. Interestingly, neither *B. pseudomallei* nor *B. thailandensis* possess a homolog of the *P. aeruginosa* pqsH gene. This gene facilitates the conversion of 2-heptylquinolin-4(1*H*)-one to 2-heptyl-3-hydroxyquinolin-4(1*H*)-one (Schemes 24.12 and 24.13) in *P. aeruginosa*, and its absence from *B. pseudomallei* would suggest that production of 3,4-hydroxy-2-heptylquinoline was unlikely in this organism [126].

No data is available on the biosynthesis of 2-alkylquinoline/4(1*H*)-one alkaloids from anthranilic acid in plant. However, the structures of many alkaloids isolated, especially from the Rutaceae (Tables 24.18, 24.23, and 24.24), have provided much indirect evidence for the route proposed at Scheme 24.13.

Aurachins (A-I, K, L, P) from *Stigmatella aurantiaca* strain Sg a15 are classified as A-type aurachins (A, B, F, P), which are C-3 oxygen-substituted quinolines carrying a farnesyl residue on C-4, C-type aurachins (C, D, G, H, I, K, L) are C-4 oxygen-substituted quinolines carrying a farnesyl residue on C-3, and C-type aurachin E has a [1,1a,8,d]imidazoloquinoline structure (Schemes 24.14, 24.15). Feeding of ^{13}C-labeled precursors showed that the quinoline ring is constructed from anthranilic acid and acetate, and the farnesyl residue from acetate by both the mevalonate and nonmevalonate pathways [84]. Further, feeding of labeled aurachin C indicated the A-type aurachins are derived by a novel intramolecular 3,4-migration of the farnesyl residue that is induced by a 2,3-epoxidation and terminated by a reduction step. ^{18}O-Labeling experiments indicated the new oxygen substituents originate from atomospheric oxygen [84]. On the basis of these results a biosynthetic scheme covering all aurachins is proposed (Schemes 24.14, 24.15). Insight into the loading process of anthranilate into the biosynthetic pathway for the

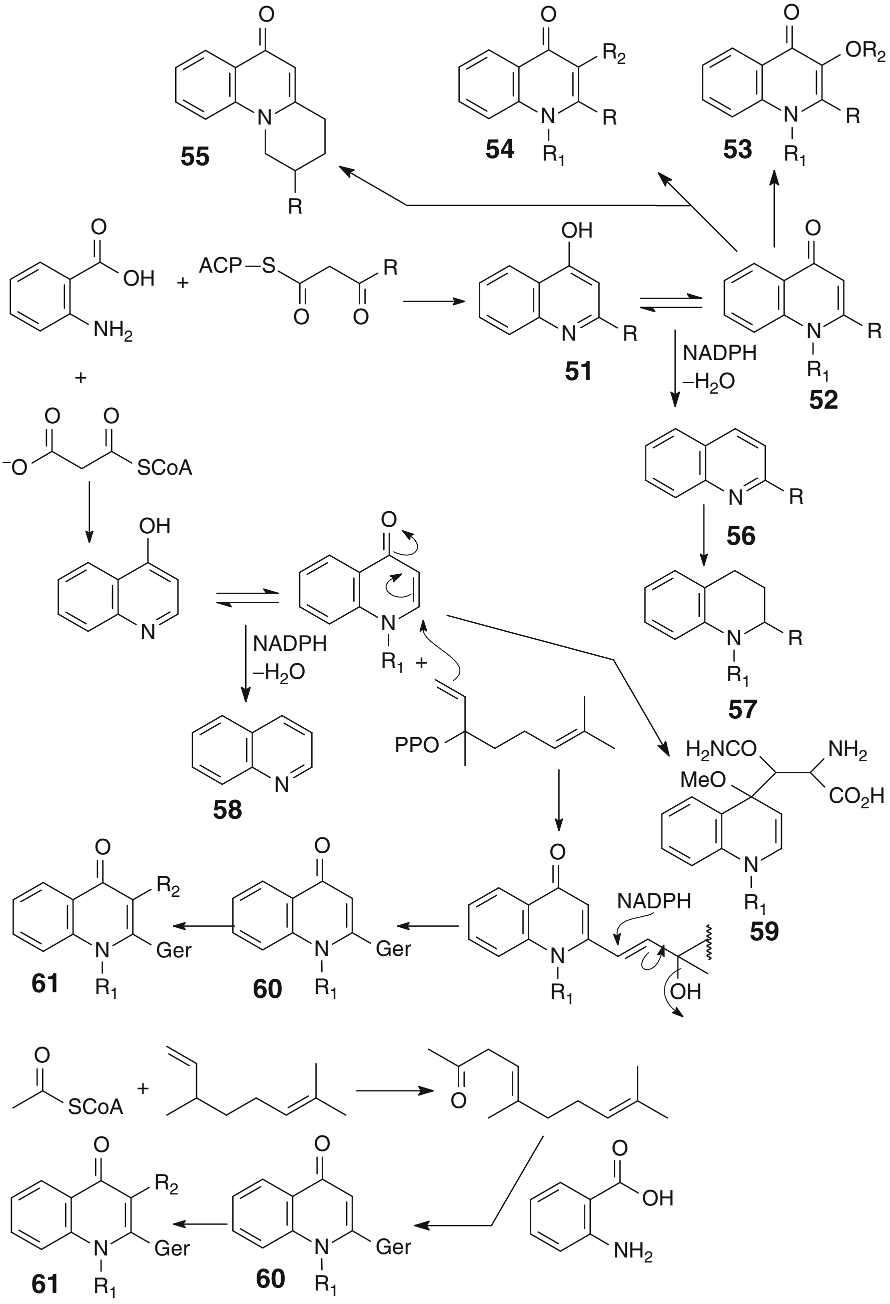


Scheme 24.12 Biosynthesis of a pseudan [41]

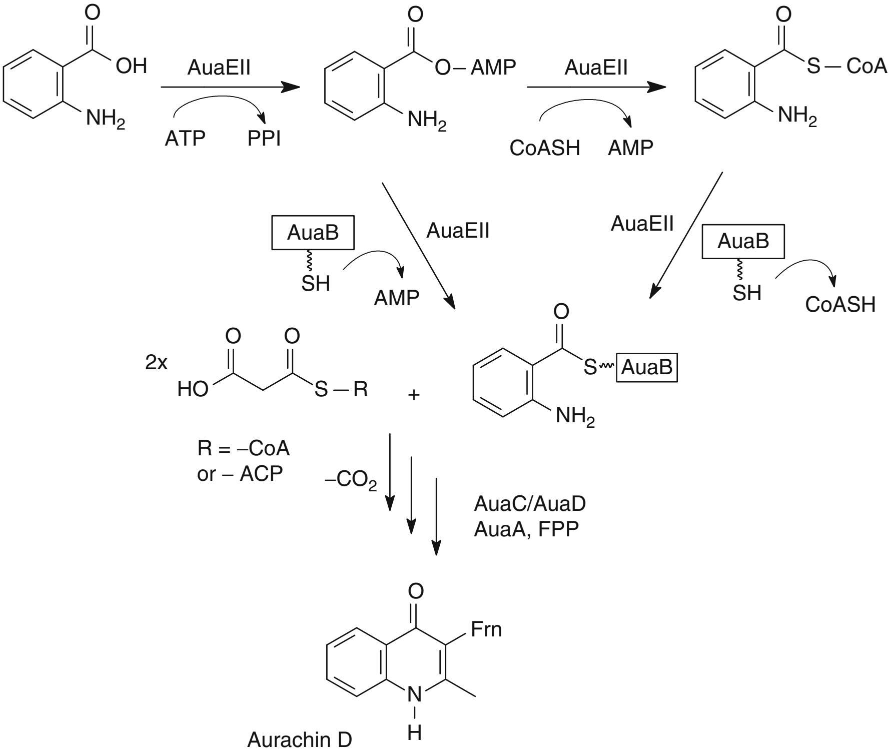
quinoline alkaloids aurachins has been provided by the sequencing of a partial biosynthetic gene cluster in the myxobacterium *Stigmatella aurantiaca*. The cluster encodes a predicted aryl: CoA ligase AuaE that was hypothesized to activate and transfer anthranilate to the acyl carrier protein AuaB [127, 128]. However, gene inactivation and in vitro experiments described by Pistorius and coworkers [129] contradicted this model. Aided by the genome sequence of *S. aurantiaca*, they identified an additional aryl:CoA ligase homologue, AuaEII, encoded in a different gene operon, which is additionally required for anthranilate priming. They report the characterization of both enzymes and the elucidation of a novel non-acetate priming strategy in thio-templated biosynthetic machineries (Scheme 24.14).

2-Geranylquinolin-4(1*H*)-ones were isolated from bacterium *Pseudonocardia sp.* (CL38489; Table 24.28) [41] and their biogenesis may involve direct reaction between a quinoline or quinolin-4(1*H*)-one and geranyl diphosphate, but chemical reactions do not justify this route, because quinoline and quinolin-4(1*H*)-one are insufficiently nucleophilic at C-2 for that reaction to occur readily. Another reasonable biogenesis for them may involve direct reaction between a quinolin-4(1*H*)-one and linalyl diphosphate (LPP) by Michael addition at C-2 to give 1-methyl-2-(3-hydroxy-3,7-dimethyl-octa-1,6-dienyl)quinolin-4(1*H*)-one of naturally occurring (**60**, **61**; Scheme 24.13, Table 24.28) [41]. Therefore, they are of uncertain biogenetic origin, it is not known whether they are formed via a biosynthetic pathway involving the condensation of anthranilic acid and β-keto intermediate with one acetate unit attached to the geranyl group (**60**, **61**; Scheme 24.13) [41]. Höfle and Kunze [84] based in Aurachins experiments proposed that 2-geranylquinolin-4(1*H*)-ones are formed by rearrangement mechanisms similar to that for A-type aurachins (Scheme 24.16).

3-Phenylquinolines (**62**), previously unknown as natural products, are surprising new metabolites of the Chinese medicinal plant *Peganum nigellastrum* (Zygophyllaceae; Table 24.21) [41]. The same plant source also yielded unusual mixed alkaloids 3-(1*H*-indol-3-yl)quinoline (**63**) and quinoline-quinazoline (**64**) (Scheme 24.17). The biogenesis of the latter was plausibly suggested to be from pegamine, a quinazoline alkaloid found in *P. nigellastrum* [41]. Oxidation


O
55
R
O
R$_2$
N
R
54
R$_1$
O
OR$_2$
N
R
53
R$_1$
O
OH
NH$_2$
+
ACP—S
R
O
O
OH
N
R
51
O
N
R
R$_1$
52
NADPH
–H$_2$O
+
O
O
$^{-}$O
SCoA
N
R
56
N
R
R$_1$
57
OH
N
O
N
R$_1$ +
NADPH
–H$_2$O
PPO
N
58
H$_2$NCO
NH$_2$
MeO
CO$_2$H
N
R$_1$
59
O
R$_2$
N
Ger
61
R$_1$
O
N
Ger
60
R$_1$
O
N
R$_1$
NADPH
OH
O
SCoA
+
O
O
R$_2$
N
Ger
61
R$_1$
O
N
Ger
60
R$_1$
O
OH
NH$_2$

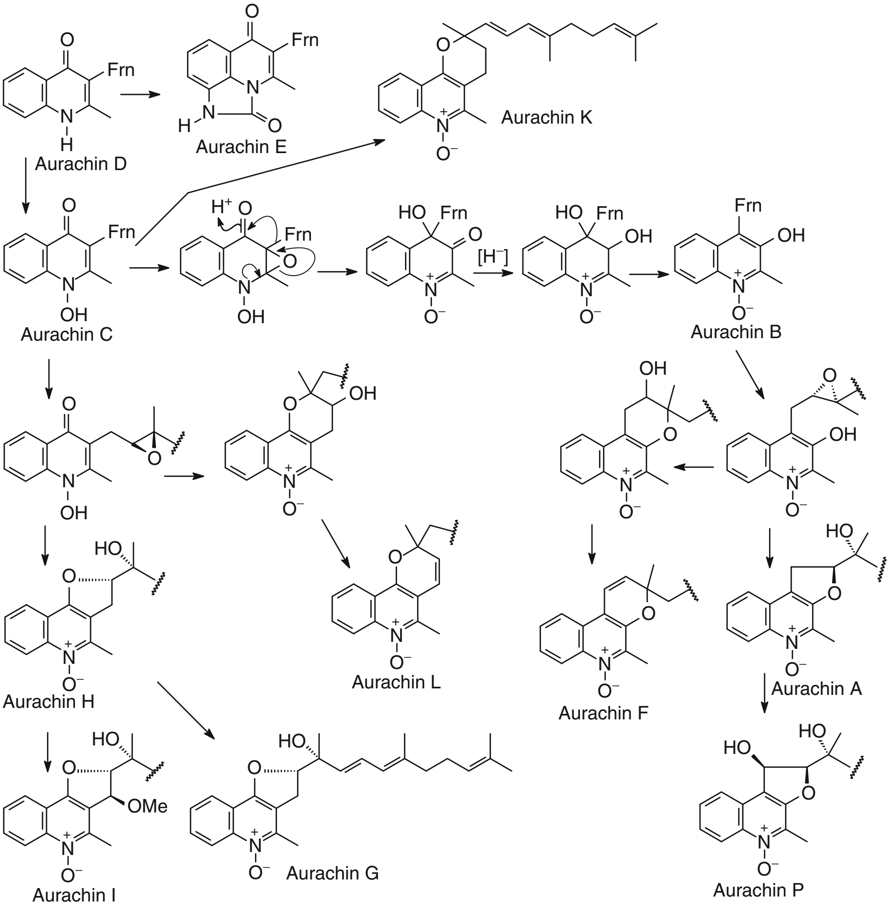
Scheme 24.13 Biogenesis of the quinolin and alkylquinolin/4(1*H*)-one alkaloids



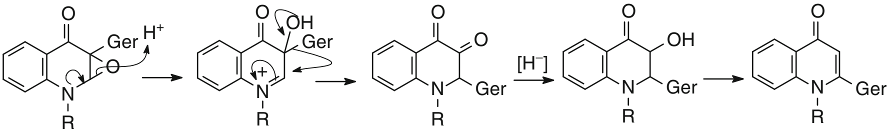
Scheme 24.14 Biosynthesis for Aurachin D alkaloid [129]

of pegamine to the corresponding aldehyde followed by imine formation with anthranilic acid was suggested to produce an intermediate imine, cyclisation and further elaboration of which leads to the luotonin F (**64**) (Scheme 24.17). Thus, it seems likely that quinoline-3-carboxamide (**65**) is a product of oxidative cleavage of luotonin F (Table 24.21). Therefore, these compounds do not have the same biogenetic origin observed in other 3-substituted quinolines. Phenylquinolines and 3-(1*H*-indol-3-yl)quinoline likely are derived from quinoline, however a mechanism for the formation of them is not clear. They may be produced by the coupling of two phenolic or phenolic and indol systems, by means of free radical reactions, as occur in lignans found in Zygophyllaceae [130].

Blaschke-Cobet and coworkers [131] have shown that the non-anthranilate portion of the 2-arylquinolin-4(1*H*)-one of graveoline of *Ruta graveolens* is derived from the decarboxylation and deamination of phenylalanine. Feeding experiments with labeled isotopes in *R. graveolens* indicated that the hydroxylation to the aromatic ring precede cyclization to the quinolin-4(1*H*)-one system. A β-keto ester and a 3-carboxyquinolin-4(1*H*)-one are probable intermediates in the biosynthesis of graveoline (**66**) (Scheme 24.18).

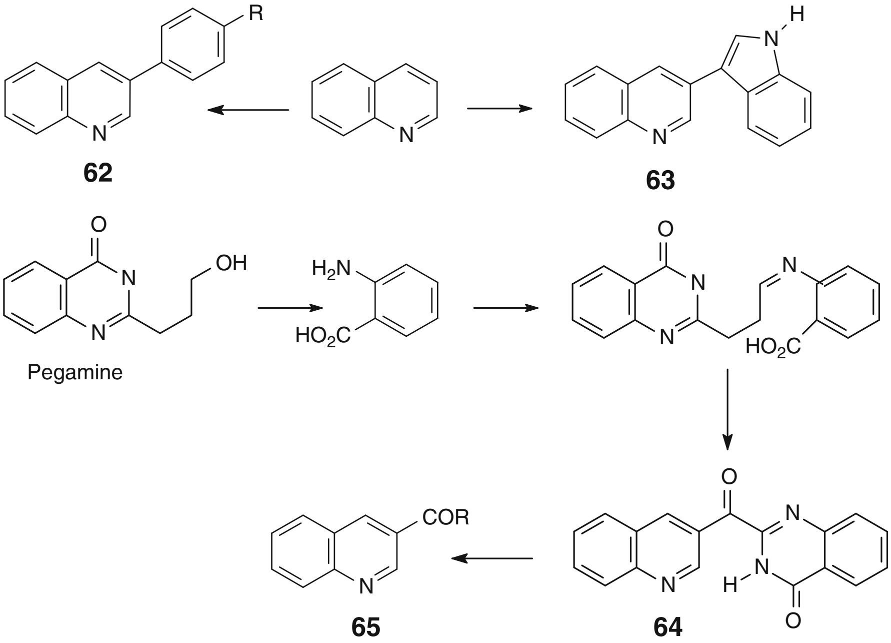

Aurachin D
Aurachin E
Aurachin K
Aurachin C
Aurachin B
[H⁻]
Aurachin L
Aurachin F
Aurachin A
Aurachin H
Aurachin I
Aurachin G
Aurachin P

Scheme 24.15 Biosynthesis (for Aurachin A–D) and biogenesis of the Aurachin alkaloids



Scheme 24.16 Biogenesis of the 2-geranylquinolin-4(1*H*)-one alkaloids [84]

No data is available on the biosynthesis of alkaloids of rutaverine type found in *R. graveolens* (Table 24.26), in which quinolin-4(1*H*)-one and phenyl ring systems are separated by a multiple carbon chain (2-alkylarylquinolin-4(1*H*)-ones). A similar pathway to that leading to graveoline (**66**), but involving condensation of β-keto intermediate with more than one acetate unit to give a polyketide chain attached to the carboxylic acyl group of the amino acid can be proposed.

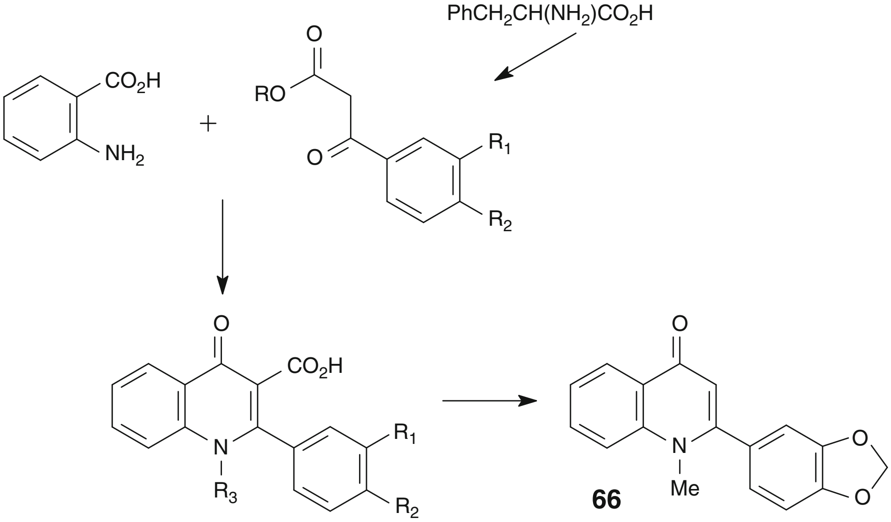


Scheme 24.17 Biogenesis of 3-substituted quinolines

It condenses with anthranilate to form the 2-alkylarylquinolin-4(1*H*)-ones. Direct supports for biogenetic ideas through the appropriate labeling studies are little from Rutaceae, but the structures of many aryl- and alkylaryquinolin/ones isolated from the rutaceous genus (Tables 24.19, 24.20, 24.26), have provided much indirect evidence for the route proposed at Schemes 24.19 and 24.20.

The coexistence of the NH-quinolin-4(1*H*)-one and 4-hydroxyquinoline isomers has been confirmed by spectrometric analysis. However, the exclusive NH-4-oxo nature of these compounds in solution phase (NMR) and solid state (IR and X-ray) have been corroborated [132]. For example, galipoline, isolated from *Angostura trifoliata* (Table 24.20), has a potential to exist in the tautomeric equilibrium (Scheme 24.21), explaining a number of reports which have appeared showing the structure of galipoline [1, 44] as 2-(3,4-dimethoxyphenylethyl)-4-hydroxyquinoline or 2-(3,4-dimethoxyphenylethyl)quinolin-4(1*H*)-one [44]. A number of reports have been confirmed the carbonyl nature by ^{13}C NMR of NH-2-alkylquinolin-4-one, indicating the predominance of the NH-oxo forms, which are widely distributed in rutaceous genus and *Pseudomonas* species (Tables 24.23, 24.28).

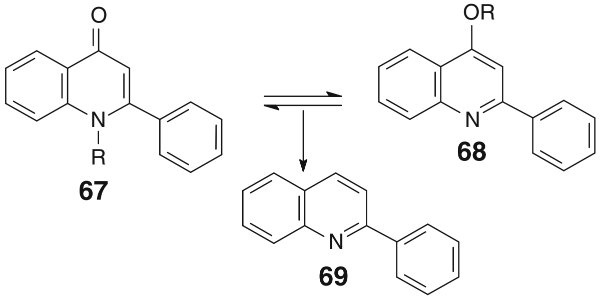
Novel quinolone antibiotics, quinolactacins A-C (Schemes 24.22 and 24.23; Table 24.28) were first discovered from the cultured broth of *Penicillium* sp. EPF-6, which was isolated from the larvae of the mulberry pyralid (*Margaronia pyloalis* Welker) [41]. More recently, the two quinolactacin A diastereomers were isolated from solid-state fermentation of *Penicillium citrinum* 90648 and named

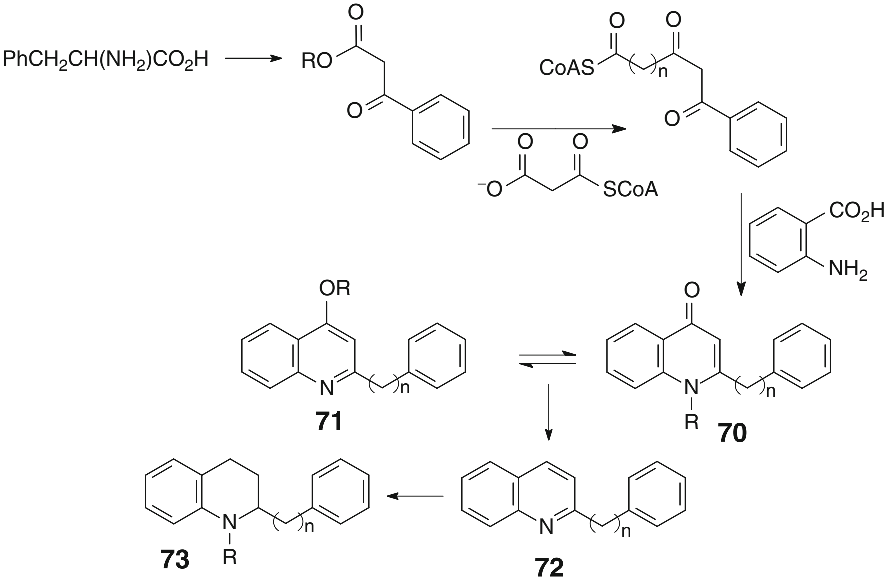


Scheme 24.18 Biosynthesis of graveoline in *Ruta graveolens* L [131]

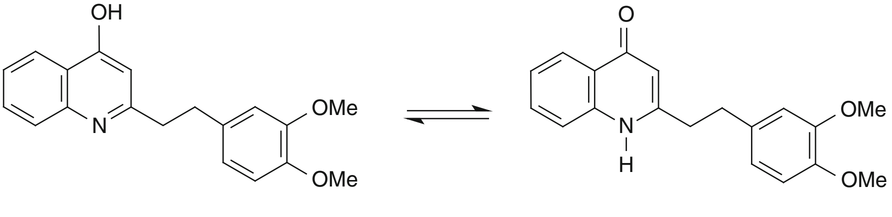
quinolactacin A1 and quinolactacin A2. The relative configuration of quinolactacin A2 is assumed to be the same as that of the originally assigned quinolactacin A from the cultured broth of *Penicillium* sp. EPF-6 on the basis of spectroscopic analysis. Quinolactacin A1 is the C-1′ diastereomer of A2 [41]. Further studies with *Penicillium citrinum* VKM FW-800 obtained from the permafrost region of Northern Russia, led to the isolation of the (+)-quinocitrinine A and (−)-quinocitrinine B, which are diastereomers at the C-1′ and C-9 stereocentres [41]. These compounds are the first pyrrolo[3,4-*b*]quinoline-types isolated from microbial metabolites. The structures are unique in that a quinolone skeleton is conjugated with a γ-lactam ring. A biomimetic total synthesis of quinolactacin B has been reported by Tatsuta's group [133]. A new enantioselective synthesis of quinolactacins A2 and B, via skeletal rearrangement of a β-carboline to the pyrrolo[3,4-*b*]quinolin-4(1*H*)-one system by an oxidative process, confirmed the absolute configuration of quinolactacin B as assigned by the literature, (S)-(−)-quinolactacin B [134]. It also established both absolute configurations of quinolactacins A1 and A2, which have not been clearly determined. Quinolactacin A2 was obtained as a single enantiomer and C-9 and C-1′ stereochemistry were established as the *S* configuration (9S, 1'S). Via the same synthetic route a pair of C-1′ diastereomers were obtained. Chiral HPLC was used for separation of these two diastereomers. From ^{1}H NMR of the diastereomeric mixture, the C-1′ diastereomer of quinolactacin A2 could be differentiated by comparison of the spectrum of A2. The spectrum was in agreement with the data of quinolactacin A1 [41, 134]. This result confirmed quinolactacin A1 is the C-1′ diastereomer of A2 (9S, 1'R) [134]. The first synthesis suggested that these compounds might be biologically synthesized from three components, amino acids L-valine and L-isoleucine, anthranilic acid, and acetic acid [133] (Schemes 24.22, 24.23).

Scheme 24.19 Biogenesis of arylquinolin/4(1*H*)-ones


O
N
R
67
OR
N
68
N
69

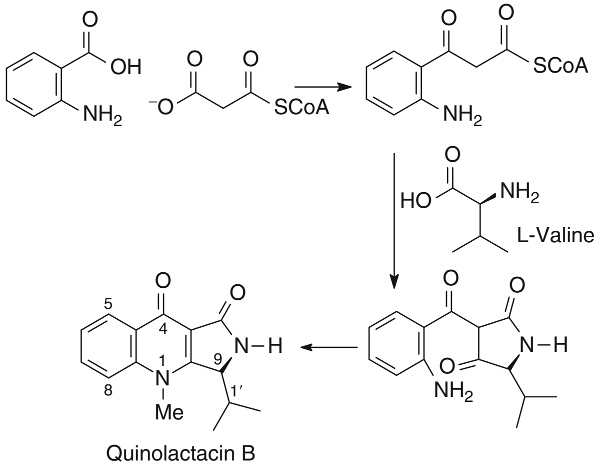

PhCH2CH(NH2)CO2H
RO
O
O
CoAS
O
O
n
O
−O
O
O
SCoA
CO2H
NH2
OR
N
n
71
O
N
R
n
70
N
n
R
73
N
n
72

Scheme 24.20 Biogenesis of alkylarylquinolin/4(1*H*)-ones


OH
N
OMe
OMe
O
N
H
OMe
OMe

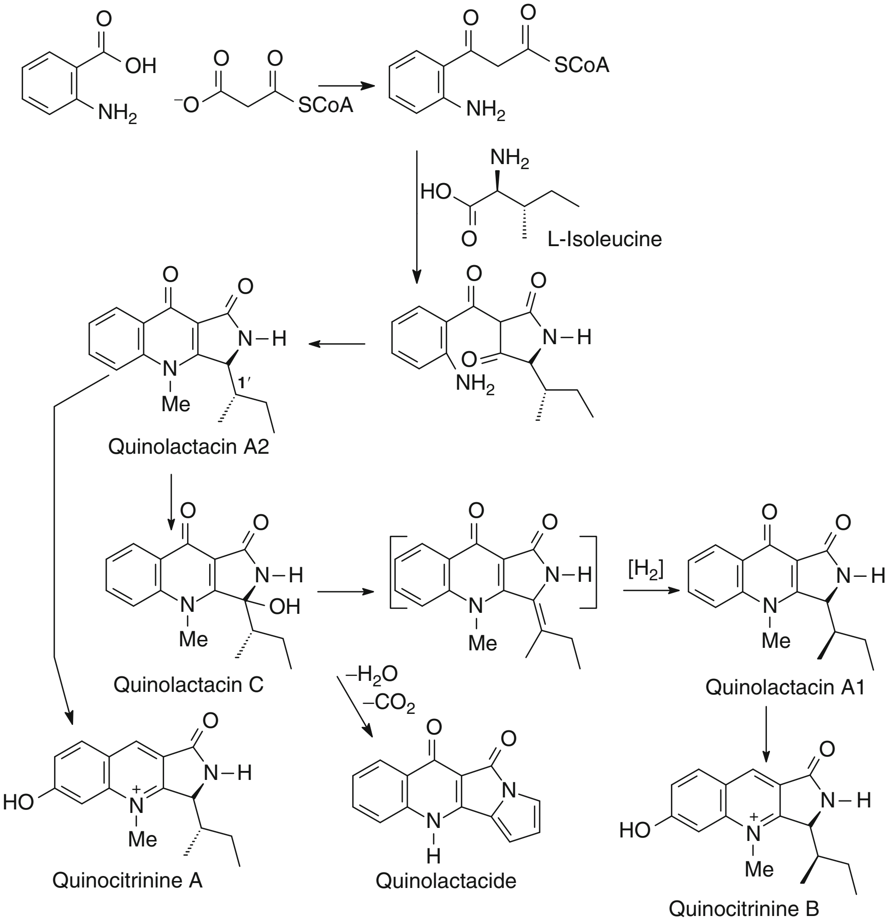
Scheme 24.21 Galipoline tautomeric equilibrium

Scheme 24.22 Biogenesis of quinolactacin B from anthranilic acid



Quinolactacin A1 does not appear to have the same biogenetic origin observed in quinolactacin A2, since other possible isoleucine stereoisomer is not generally found in living organisms. In addition, the β-carbon is insulated from the amino and carboxy groups which by electron-withdrawing effects and resonance stabilization promote epimerization at the α-position. Thus, the β-epimerization rates of the isoleucine are predicted to be extremely slow in comparison to their α-epimerization rates. Quinolactacin C was also found in *Penicilliun* genus, leading to the hypothesis that the quinolactacin A1 is not derived from β-epimerization, but perhaps from the former by elimination of H_2O and subsequent hydrogenation producing the C-1′ diastereomer of A2 (Scheme 24.23). However, the stereochemistry at the two stereogenic centers in quinolactacin C was not elucidated. A new quinolactacin with a pyrrole ring (quinolactacide) was isolated from *Penicillium citrinum* Thom F 1539 [41]. The additional ring could be formed from quinolactanin C by oxidation of methyl group at C-1′, decarboxylative elimination ($-CO_2$ and $-H_2O$) and subsequent oxidative cyclization (Scheme 24.23).

The second synthesis, which suggested pyrrolo[3,4-*b*]quinolin-4(1*H*)-one system to derive from tryptamine [134], is other alternative pathway (Scheme 24.24). This route using the keto acid derived from amino acid L-leucine (or L-valine) by transamination and tryptamine, under some stereochemical control, can also form quinolactacins stereospecifically as C-9 (*S*)-diastereomer (Scheme 24.24). Biological oxidation of indol ring by tryptophan 2,3-dioxygenase could form the 3-hydroperoxy-β-carboline, subsequent intramolecular addition of 3-hydroperoxy to iminium ion at C-2 followed by heterolytic cleavage of the peroxide bond would give the dione intermediate, which rearrange to quinolin-4(1*H*)-one. For convenience the first proposals were codified. Therefore, any proposal cannot yet be regarded as the main pathway, proofs are required.

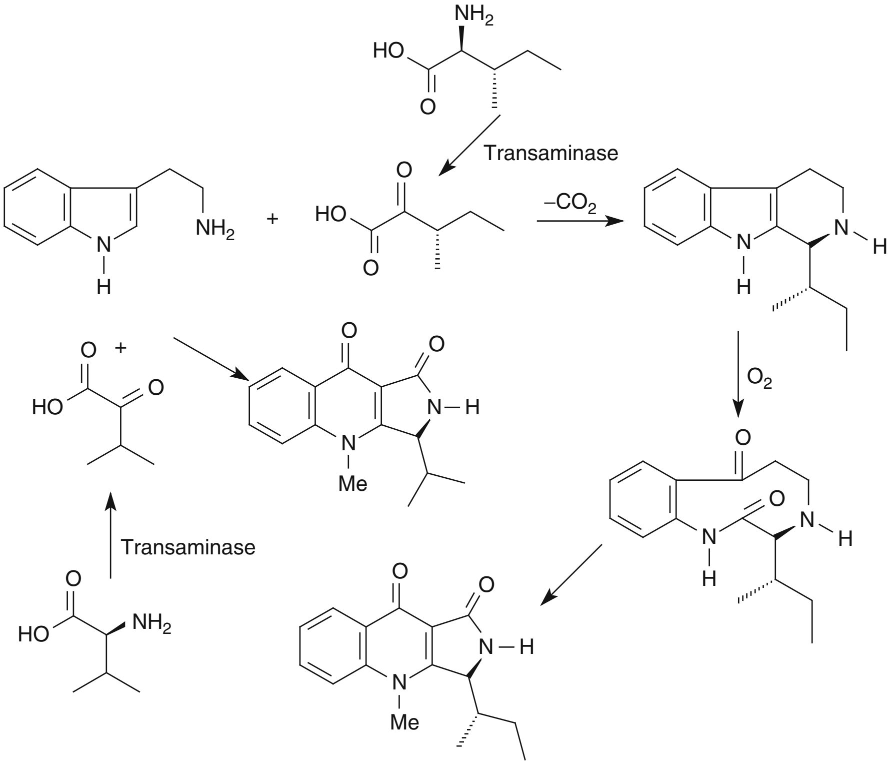


OH
NH2
⁻O
SCoA
SCoA
NH2
NH2
HO
O
L-Isoleucine
N–H
1′
N
Me
Quinolactacin A2
OH
Quinolactacin C
[H2]
Quinolactacin A1
–H2O
–CO2
HO
Quinocitrinine A
N
H
Quinolactacide
Quinocitrinine B

Scheme 24.23 Biogenesis of quinolactacins A1, A2, and C, quinocitrinines A and B, and quinolactacide from anthranilic acid

2.3 Quinazoline Alkaloids

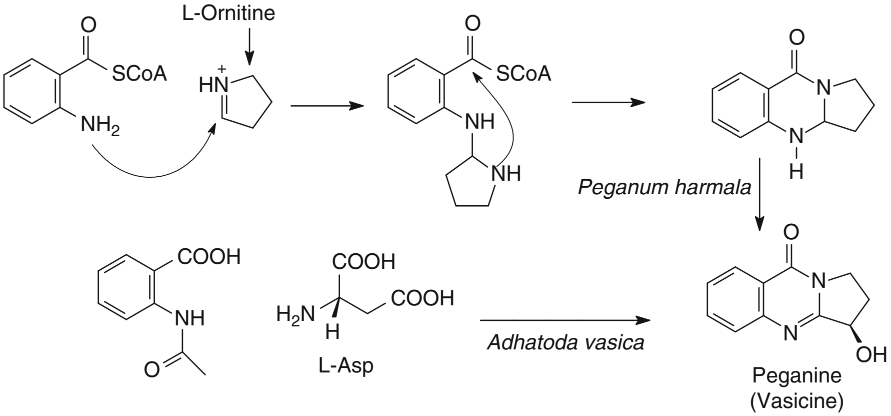
Quinazolinone is a building block for naturally occurring alkaloids isolated to date from a few of families of the plant kingdom, from animals, and from microorganisms (Tables 24.29–24.31). The first known quinazoline alkaloid was vasicine isolated from *Adhatoda vasica* (Acanthaceae). The alternative name peganine is also used for vasicine, which is found in *Peganum harmala* (Zygophyllaceae). Few data are available on the biosynthesis of these alkaloids, studies in *Peganum harmala* have clearly demonstrated peganine to be derived from anthranilic acid, the remaining part of the structure being a pyrrolidine ring supplied by ornithine (Scheme 24.25). Remarkably, this pathway is not operative in *Adhatoda vasica*



Scheme 24.24 Biogenesis of quinolactacins A2 and B from tryptamine

(*Justicia adhatoda*), and a much less predictable sequence from *N*-acetylanthranilic acid and aspartic acid is observed (Scheme 24.25) [135–140].

Aspergillus fumigatus Af293 is a known producer of quinazoline natural products, including the antitumor fumiquinazolines, of which the simplest member is fumiquinazoline F with a 6-6-6 tricyclic core derived from anthranilic acid, tryptophan, and alanine (Scheme 24.26). Fumiquinazoline F is the proposed biological precursor to fumiquinazoline A in which the pendant indole side chain has been modified via oxidative coupling of an additional molecule of alanine, yielding a fused 6-5-5 imidazoindolone. Ames and coworkers [94] identified fungal anthranilate-activating nonribosomal peptide synthetase (NRPS) domains through bioinformatics approaches. One domain previously identified is part of the trimodular NRPS Af12080, which the authors predict is responsible for fumiquinazoline F formation. They also show that two adjacent *A. fumigatus* ORFs, a monomodular NRPS Af12050 and a flavoprotein Af12060, are necessary and sufficient to convert fumiquinazoline F to fumiquinazoline A. Af12060 oxidizes the 2′,3′-double bond of the indole side chain of fumiquinazoline F, and the three-domain NRPS Af12050 activates L-Ala as the adenylate, installs it as the pantetheinyl thioester on its carrier protein domain, and acylates the oxidized indole

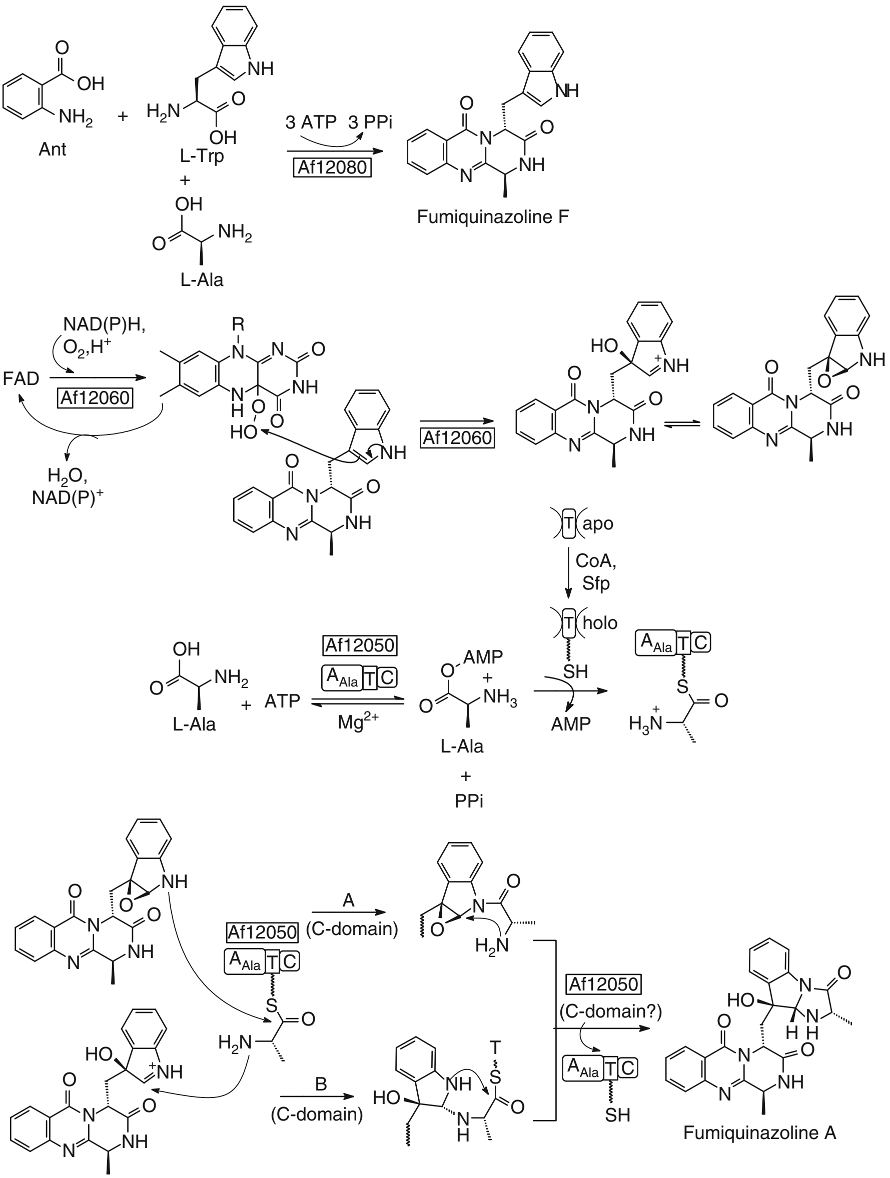


Scheme 24.25 Biosynthesis of Peganine

for subsequent intramolecular cyclization to create the 6-5-5 imidazolindolone of fumiquinazoline A (Scheme 24.26). This work provides experimental validation of the fumiquinazoline biosynthetic cluster of *A. fumigatus* Af293 and describes an oxidative annulation biosynthetic strategy likely shared among several classes of polycyclic fungal alkaloids.

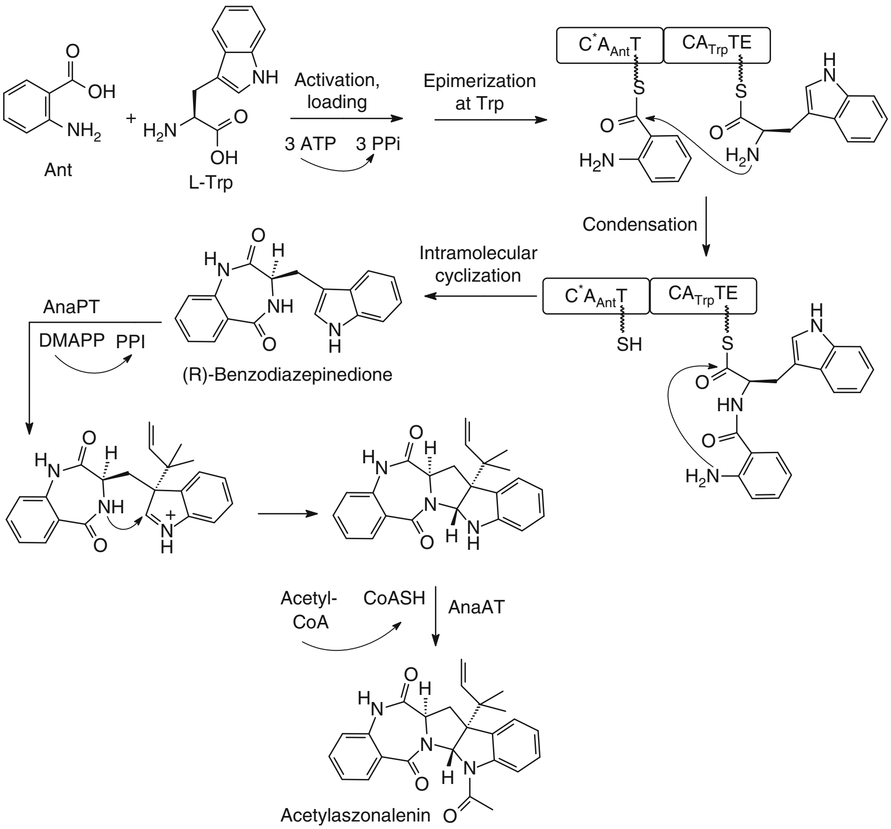
Ames and Walsh [96] validated that the first module of the acetylaszonalenin synthetase of *Neosartorya fischeri* NRRL 181 activates anthranilate to anthranilyl-AMP. With this as a starting point, they then used bioinformatic predictions about fungal adenylation domain selectivities to identify and confirm an anthranilate-activating module in the fumiquinazoline A producer *Aspergillus fumigatus* Af293 as well as a second anthranilate-activating NRPS in *N. fischeri*. This establishes an anthranilate adenylation domain code for fungal NRPS and should facilitate detection and cloning of gene clusters for benzodiazepine and quinazoline-containing polycyclic alkaloids with a wide range of biological activities (Scheme 24.27).

The biosynthesis of the bioactive compound tryptoquivaline, which are characterized by an acetylated quinazoline ring connected to a 6-5-5 imidazoindolone ring system via a five-membered spirolactone, has remained uncharacterized. Gao and coworkers [95] recently reported the identification of a gene cluster (tqa) from *Penicillium aethiopicum* that is involved in the biosynthesis of tryptoquialanine. The pathway has been confirmed to go through an intermediate common to the fumiquinazoline pathway, fumiquinazoline F, which originates from a fungal trimodular nonribosomal peptide synthetase (NRPS), as showed above. By systematically inactivating every biosynthetic gene in the cluster, followed by isolation and characterization of the intermediates, Gao and coworkers [95] were able to establish the biosynthetic sequence of the pathway. An unusual oxidative opening of the pyrazinone ring by an FAD-dependent berberine bridge enzyme-like oxidoreductase has been proposed based on genetic knockout studies. Notably, a 2-aminoisobutyric acid (AIB)-utilizing NRPS module has been identified and


Ant
L-Trp
L-Ala
3 ATP 3 PPi
Af12080
Fumiquinazoline F
NAD(P)H, O2,H+
FAD
Af12060
H2O, NAD(P)+
R
HO
Af12060
T apo
CoA, Sfp
T holo
SH
Af12050
AAla T C
ATP
Mg2+
AMP
L-Ala
PPi
A
(C-domain)
B
(C-domain)
Af12050
(C-domain?)
Fumiquinazoline A

Scheme 24.26 Biosynthesis of Fumiquinazolines F and A in *Aspergillus fumigatus* Af293 [94]

reconstituted in vitro, along with two putative enzymes of unknown functions that are involved in the synthesis of the unnatural amino acid by genetic analysis. This work provides new genetic and biochemical insights into the biosynthesis of this group of fungal alkaloids, including other related to tryptoquialanine (Scheme 24.28).


Ant
L-Trp
Activation, loading
3 ATP
3 PPi
Epimerization at Trp
C*A_{Ant}T
CA_{Trp}TE
Condensation
Intramolecular cyclization
SH
(R)-Benzodiazepinedione
AnaPT
DMAPP
PPI
Acetyl-CoA
CoASH
AnaAT
Acetylaszonalenin

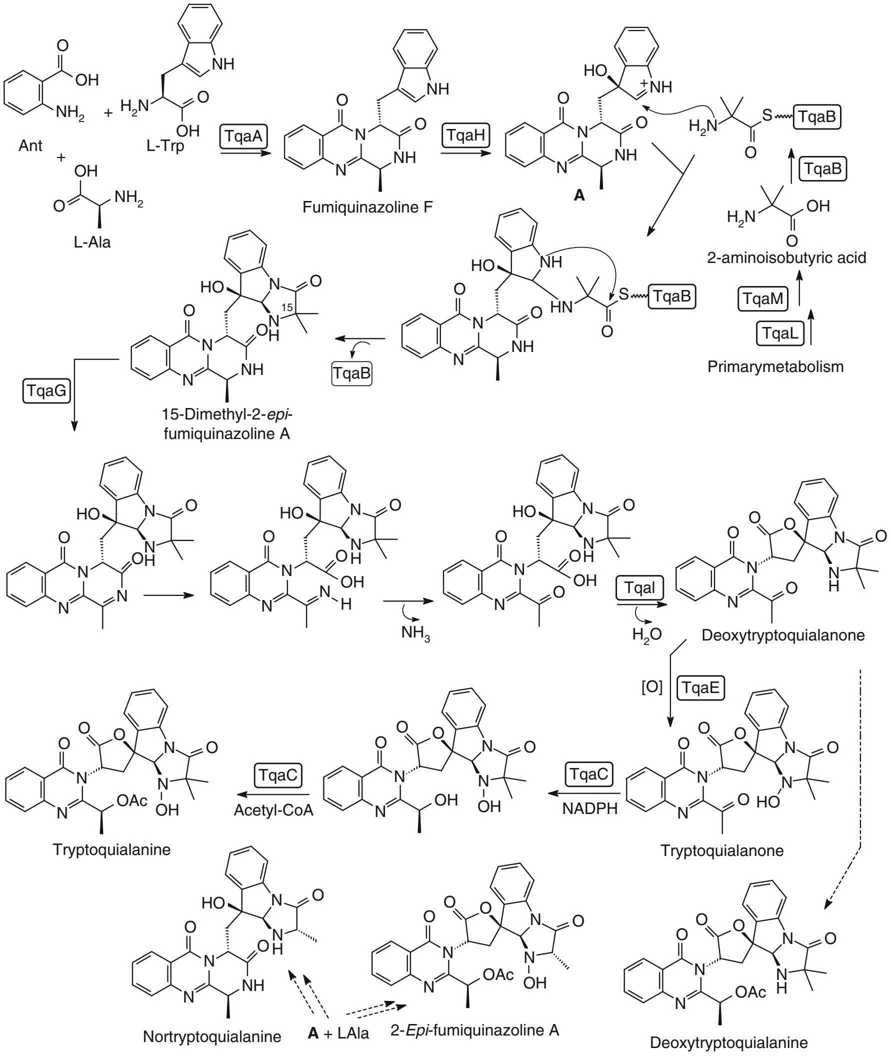
Scheme 24.27 Biosynthesis of Benzodiazepinedione and acetylaszonalenin in *Neosartorya fischeri* [96]

Similar biosynthetic logic is likely involved in the formation a number of fungal quinazoline alkaloids (Table 24.31), some examples are showed in Scheme 24.29.

The biosynthesis of the main groups of quinoline, acridone, and quinazoline alkaloids is established. However, a great deal of more detailed work remains to be done. The topics included here have been selected to stimulated further experimental biosynthetic research in the area.

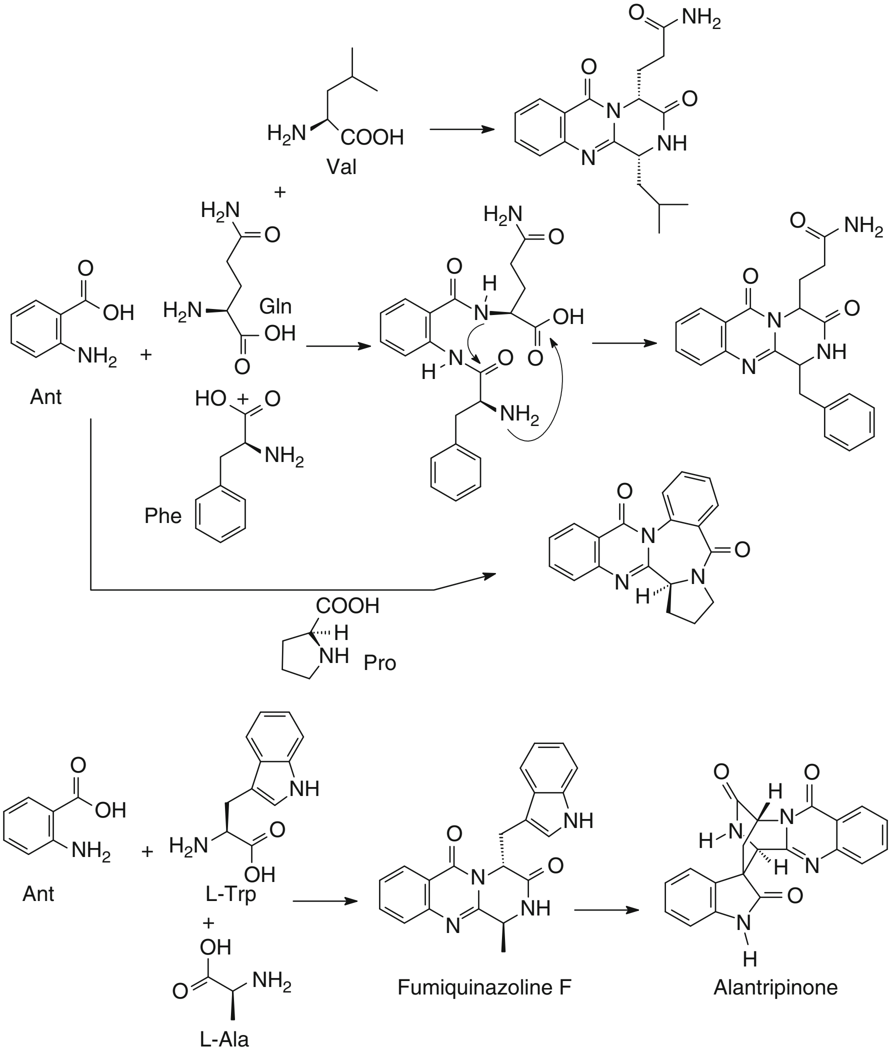
3 Biological Activity

Interest in the 2-substituted quinolin/ones, quinazolines and acridones alkaloids has accelerated rapidly with the finding that these compounds display marked biological activities, such as leishmanicidal, antimicrobial, anticancer activities.


Ant
L-Trp
L-Ala
TqaA
Fumiquinazoline F
TqaH
A
TqaB
2-aminoisobutyric acid
TqaM
TqaL
Primarymetabolism
15-Dimethyl-2-epi-fumiquinazoline A
TqaG
NH3
TqaI
H2O
Deoxytryptoquialanone
[O]
TqaE
Tryptoquialanone
NADPH
TqaC
Acetyl-CoA
Tryptoquialanine
Nortryptoquialanine
A + LAla
2-Epi-fumiquinazoline A
Deoxytryptoquialanine

Scheme 24.28 Biosynthesis of natural intermediates to Tryptoquialanine in *Penicillium aethiopicum* [95]

A wide spectrum of other biological properties for these types of alkaloids has been discovered. It is proposed in this chapter to present the latest developments in the 2-substituted quinolin/ones, quinazolines, and acridones alkaloids biological activities as well as a overview of all members of these classes of compounds.


Val
Ant
Gln
Phe
Pro
L-Trp
L-Ala
Fumiquinazoline F
Alantripinone

Scheme 24.29 Biogenesis of some fungal quinazoline alkaloids (Table 24.31)

3.1 Antiprotozoal Activity

The need for novel and more selective agents to treat protozoal diseases remains. The classic examples of these diseases are Chagas, sleeping sickness, leishmaniasis, and malaria.

3.1.1 Leishmaniasis

Leishmaniasis is a group of tropical diseases caused by a number of species of protozoan parasites belonging to the genus *Leishmania*. In humans, *Leishmania* spp. causes a variety of clinical diseases, which have been used by the World Health Organization as the basis to classify leishmaniasis in four clinical forms: visceral, mucocutaneous, cutaneous diffuse or disseminated, and cutaneous. Certain species of the parasite have been associated with the different clinical forms of the diseases; *Leishmania donovani* complex causes visceral leishmaniasis, while the *Leishmania tropical* complex is known to induce cutaneous and cutaneous diffuse leishmaniasis in several countries of Latin America. During its biological cycle, parasites of the *Leishmania* genus exist in two forms that develop in a different host, a flagellated extracellular form known as promastigote and an intracellular one designated as amastigote. The form that infects both man and other vertebrate hosts is the promastigote. The vertebrate host is infected with the promastigote form of the parasite as a result of a sting by the vector insect (*Phlebotomus* and *Lutzomyia*). After this, the promastigotes are quickly phagocytized by the macrophages of the host and inside of them the promastigotes change to the amastigote form. The clinical manifestation of the disease is a consequence of the multiplication of the amastigotes inside the macrophages. Drugs currently used to cure leishmaniasis are derivatives of pentavalent antimony, which are potentially toxic and generally administered via the parenteral route in a hospital setting for relatively long periods. In many endemic regions, the classic treatments are too expensive or unavailable to the population suffering from cutaneous leishmaniasis [143]. Most of the studies directed toward the detections of plant secondary metabolites with leishmanicidal activity have been done using the promastigote form of the parasite because it is easier to maintain under in vitro conditions. However, since the promastigote is not the infective form of the parasite in vertebrate hosts, evaluations done with theme have only an indicative value of the possible leishmanicidal activity of the metabolite tested. The study must be complemented with an evaluation using intracellular amastigotes in macrophages. Interest in the 2-substituted quinolines has accelerated rapidly with the finding of Fournet and his colleagues in the early 1990s [41, 144–146] that these compounds display marked leishmanicidal activity (Table 24.32).

3.1.2 Chagas

Chagas disease is a protozoosis caused by the flagellate protozoan *Trypanosoma cruzi*, which belongs to the Kinetoplastida order and the Trypanosomatidae family. Four main evolutionary forms can be identified during the *T. cruzi* life cycle. The trypomastigote is the infective flagellate form of the parasite found in the blood of the mammalian host (blood trypomastigote) and in the terminal part of the digestive and urinary tracts of vectors (metacyclic trypomastigote). Metacyclic trypomastigotes are more infective than blood trypomastigotes. The epimastigote is the replicative form of the parasite in the insect vector and in the acellular culture medium. The amastigote is the intracellular replicative form of the parasite in the vertebrate host. The spheromastigote is found in the stomach of the vector [147]. Besides low efficacy, the drugs currently available, nifurtimox and benznidazole have strong side effects. A number of 2-substituted quinoline and acridone alkaloids were found to display antitrypanosomal activity (Table 24.32).

3.1.3 Malaria

Human malaria is caused mainly by four species of *Plasmodium*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. falciparum*. Of these four species *P. falciparum* is responsible for the vast majority of the 300–500 million episodes of malaria worldwide and accounts for 0.7–2.7 million annual deaths. *P. vinckei* was first isolated in 1952 although it was not recognized as an independent species until 1975. Four subspecies are recognized, *P. vinckei vinckei*, *P. vinckei petteri*, *P. vinckei lentum*, and *P. vinckei brucechwatti*. The parasite is readily grown in mice and laboratory-reared thicket rats, where it shows a preference for mature red blood cells. Infections are synchronous with a periodicity of 24 h. Malaria parasites are transmitted by female *Anopheles* mosquitoes. *A. gambiae* is one of the best known, because of its predominant role in the transmission of the most dangerous *P. falciparum*. *P. vinckei* may be transmitted by *Anopheles stephensi* mosquitoes over a wide temperature range. The parasites multiply within red blood cells, causing symptoms that include anemia (light headedness, shortness of breath, tachycardia, etc.), as well as other general symptoms such as fever, chills, flu-like illness, and in severe cases, coma and death. Malaria infections are treated through the use of antimalarial drugs, such as chloroquine or pyrimthamine, although drug resistance is increasingly common [148, 149]. These factors increase the urgency of the search for novel antimalarials. Many of the reviewed alkaloids possessed antimalarial activity (Table 24.32).

3.2 Molluscicidal Activity

Schistosomiasis or bilharzia is a disease affecting many people in developing countries. The most common way of getting schistosomiasis in developing countries is by wading or swimming in lakes, ponds, and other bodies of water which are infested with the snails (usually of the *Biomphalaria*, Bulinus, or *Oncomelania* genus) that are the natural reservoir of the *Schistosoma* pathogen. *Schistosoma mansoni* is found in parts of South America and the Caribbean, Africa, and the Middle East; *S. haematobium* in Africa and the Middle East; *S. japonicum* in the Far East; and *S. mekongi* and *S. intercalatum* focally in Southeast Asia and central West Africa, respectively. Freshwater snails of the genus *Biomphalaria* are intermediate snail hosts for the transmission of the medically important *S. mansoni*; of the seven species of *Biomphalaria* transmitting schistosomiasis in the Western Hemisphere, *B. glabrata* is the most important and the best studied experimentally. It is found mostly in South America and the Greater and Lesser Antilles, where the snails occupy habitats that are often temporary due to frequent floods and droughts. They are dispersed to new habitats during times of flooding. A few countries have eradicated the disease, and many more are working toward it. Snail control by means of molluscicides is today considered an auxiliary method within integrated control of schistosomiasis [150]. Many of the reviewed alkaloids possessed antimolluscicidal activity (Table 24.32).

3.3 Antimicrobial Activity

Discovery of new antimicrobial substances is an important research objective, due to the continuing evolution of microbial resistance in medicine and agriculture (Antimicrobial activity against plant pathogens; Activity against fungus, symbiotic fungus and algicide). A number of plant 2-substituted quinolin/ones, quinazoline and acridones alkaloids have been shown potent antimicrobial activity: (a) Bacteria: *Arthrobacter aurescens*, *Bacillus cereus*, *B. circulans*, *B. licheniformis*, *B. sphaericus*, *B. subtilis*, *Brevibacterium ammoniagenes*, *Corynebacterium fascians*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Escherichia coli*, *Helicobacter pylori*, *Klebsiella pneumonia*, *Kurthia zopfii*, *Listeria monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *Micrococcus luteus*, *Mycobacterium fortuitum*, *M. phlei*, *M. tuberculosis*, *M. smegamatis*, *Proteus mirabilis*, *Pseudomanas aeruginosa*, *Salmonella typhi*, *S. setubal*, *Sarcina lutea*, *Staphylococcus aureus*, *S. epidermidis*, *Streptomyces viridochromogenes*, *Streptococcus epidermidis*, *Vibrio harveyi*, *V. anguillarum*; (b) Bacterial plant pathogens: *Agrobacterium tumefaciens*, *Corynebacterium michiganense*, *Erwinia carotovora*, *Pseudomonas solanacearum*, *Xanthomonas campestris* pv *oryzae*; (c) Fungus: *Aspergillus niger*, *A. fumigatus*, *Candida albicans*, *C. neoformans*, *Mucor hiemalis*, *M. miehei*, *Penicillim* sp., *Saccharomyces cerevisiae*, *Sporobolomyces almonicolor*, *Trichophyton interdigitale*; (d) Fungal plant pathogens: *Botrytis cinerea*, *Cochliobolus miyabeanus*, *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, *Fusarium oxysporum*, *Gaeumannomyces graminis*, *Phomopsis obscurans*, *P. viticola*, *Pyricularia oryzae*, *Pythium ultimum*, *Rhizoconia solani*, *Verticillium dahlia*; (e) Yeasts: *Debaryomyces hansenii*, *Rhodotorula acuta*, *Schizosacharomyces pombe*, *Cryptococcus* sp.; (f) Soil-borne pathogen: *Fungo*: *Phytophthora capsici*, B*acteria*: *Pseudomonas cepacia*; (g) Symbiotic fungus: *Leucoagaricus gongylophorus*; (h) Micro-algae: *Chlorella vulgaris*, *C. sorokiniana*, *Scenedesmus subspicatus* (Table 24.32).

3.4 Antitumor Activity

According with WHO (World Health Organization 2011) cancer will be the main cause of death in the world population and it will supplant the cardiovascular disease [163]. In Brazil, it is estimated that 520,000 people will develop cancer until 2013 (INCA 2011) [164].

Cancer is the main cause of death in developed country and research in this field with quinoline, quinazoline, and acridone alkalois has showed high potential use of these compounds in the cancer treatment. These compounds were bioassayed for these activities in several models (Toxicity in the brine shrimp; Inhibitory effect on tumor necrosis factor (TNF) production; Antitumour activity; Nuclear Factor of Activated T Cells – NFAT Inhibitor; Cytotoxic activity; Antimutagenic). Many of the reviewed alkaloids were found to display anticancer activity (Table 24.32).

3.5 Enzyme Inhibitors

Enzymes are frequently chosen as targets for drug design and discovery. For example, a potential strategy for the treatment of diseases caused by parasites is the design of compounds which selectively inhibit enzymes that are pivotal for survival of the parasite within the host that are part of biochemical pathways that are specific to the parasite. In recent years, a number of the reviewed alkaloids have been found to be inhibitors of enzymes which are involved in many important biological processes: (a) Inhibitory activity on CYP3A4 and CYP2D6: Cytochrome P450 enzymes are essential for the metabolism of many medications, the two most significant enzymes being CYP3A4 and CYP2D6, they can be inhibited or induced by drugs, resulting in clinically significant drug-drug interactions that can cause unanticipated adverse reactions or therapeutic failures, knowledge of the most important drugs metabolized by cytochrome P450 enzymes, as well as the most potent inhibiting and inducing drugs, can help minimize the possibility of adverse drug reactions and interactions; (b) Cytochromes *bo* and *bd* – two terminal respiratory oxidases found in *E. coli* and many other bacteria; (c) Lipoxygenase; (d) NADH oxidation; (e) Angiotensin II receptor binding; (f) Diacylglycerol acyltransferase; (g) Type B monoamine oxidase – potential value in the treatment of neurological disorders such as Parkinson’s and Alzheimer’s diseases and Huntington’s chorea; (h) Screening of acetylcholinesterase and butyrylcholinesterase inhibition – Alzheimer’s diseases; (i) Leukotrienes such as LTB_4 are highly potent mediators of inflammation and allergic reactions, they are formed in human neutrophils by 5-lipoxigenase (5-LOX) which catalyzes the first step in the conversion of arachidonic acid – leukotriene biosynthesis inhibitory activity; (j) Cathepsin V enzyme has been considered as a potential diagnostic marker for colon tumors (Table 24.32).

3.6 Antiplatelet Activity

Platelets are a type of blood cell. They play a key role in normal blood clotting. During the clotting process, platelets clump together to plug small holes in damaged blood vessels. The purpose of clotting is to stop bleeding. The number of platelets in blood can be affected by many diseases. Platelets may be counted to monitor or diagnose diseases or identify the cause of excess bleeding. If the number is higher than normal (thrombocytosis), this may be associated with: Polycythemia vera, post-splenectomy syndrome, primary thrombocytosis, certain malignancies, early chronic myelogenous leukemia (CML), and anemia. Theoretically, antiplatelet agents can be developed that target each step in the platelet activation or inhibition mechanisms. For example, the antiplatelet activity of aspirin was discovered while studying cyclooxygenase inhibitors. Numerous new antiplatelet agents were developed based on their inhibitory effects on platelet activation. Nonetheless, the number of antiplatelet agents ready for clinical is still insufficient, and deleterious side effects are also associated with most of the existing agents [151]. Therefore, the search for an ideal antiplatelet agent is still an important goal for the medical and pharmaceutical industries. Antiplatelet activity has been found in a number of the reviewed alkaloids (Table 24.32).

3.7 Insecticide Activity

Insects compete with the men by feed and control them with natural products is a method that can avoid contaminating the environments. The quinoline, quinazoline, and acridone alkaloids were found to display insecticide activity (Table 24.32).

3.8 Miscellaneous Biological Activities

Quinoline, quinazoline, and acridone alkaloids were found to display several other activities such as:

- Inhibitory effect on germination
- Anti-HIV activity
- Muscle Relaxant
- Estrogenic activity
- Anti-inflammatory activity
- Neurodegenerative diseases
- Phtotoxicity (anti-psoriasis activity)
- Potassium channel blockers
- Antiarrythmic activity
- Vasorelaxing activity
- Pollen-growth inhibitors
- Anti-inflammatory and antihypertensive
- Endotoxic to limulus amoebocyte lysate test
- Tremor and paralysis effects
- Pharmacokinetic
- Raising body temperature
- Anticoccidial
- Oxytocin effect and stimulation of muscular contraction
- Antispasmodic activity
- Antioxidant activity
- Antiviral activity
- Antiallergic activity
- Phagocyte oxidative burst (Table 24.32)

4 Conclusions

Alkaloids derived from anthranilic acid undoubtedly occur in greatest abundance in plants from the Rutaceae family. Particularly well represented are alkaloids based on quinoline (633 derivatives) and acridone skeletons (256 derivatives). The furoquinoline alkaloids of the dictamnine group (117 compounds) are the most widespread in the Rutaceae. The simplest representative is dictamnine and the most common is skimmianine (Table 24.5). Traditionally, the Rutaceae has been subdivided into six subfamilies of which three, the Rutoideae, Toddalioideae, and Aurantioideae, are large [41]. The furoquinolines occur widely in genera of Rutoideae, Toddalioideae, and Flindersioideae, and only to a limited extent among the Aurantioideae. They appear to be absent in the smaller subfamily Dictyolomatoideae and Spathelioideae. Of the 633 quinoline derivatives 28 % are dihydro- and furoquinolines, followed by quinolin-2(1*H*)-ones (22 %), alkyl-, aryl-, and alkylarylquinolin-4(1*H*)-ones (18 %), dihydro- and pyranoquinolines (12 %), simple quinolones (11 %), quinolin-4(1*H*)-ones (5 %), and dimeric quinolin-2(1*H*)-one/4(1*H*)-ones (4 %).

Acridones have been recorded in genera of the three large subfamilies Rutoideae, Toddalioideae, and Aurantioideae. Simple, dimeric, pyrano- (including dihydro) and furoacridones (including dihydro) are also known in order decreasing of their derivatives 53 %, 18 %, 17 %, and 12 %, respectively. The literature registers the occurrence of two simple acridones in Simaroubaceae and one in Asteraceae, one furoacridone in Piperaceae, and one pyranoacridone in Apocynaceae. The first case is not surprising since Simaroubaceae also belong to the order Sapindales. The other cases are more interesting since the families Asteraceae, Piperaceae, and Apocynaceae do not even belong to the Sapindales. It can of course be argued that the plant specimens were wrongly identified. Indeed the sporadic occurrence of particular micromolecular types in unrelated taxa is a general phenomenon. A few quinoline derivatives (43 compounds) occur sporadically in other families of Sapindales (Simaroubaceae, Meliaceae, Zygophylaceae) and in other unrelated families (Acanthaceae, Annonaceae, Apocynaceae, Araliaceae, Asteraceae, Brassicaceae, Elaeocarpaceae, Euphorbiaceae, Fabaceae, Fagaceae, Loganiaceae, Menispermaceae, Nitrareaceae, Poaceae, Periplocaceae, Ranunculaceae, Rubiaceae, Santalaceae, Sterculiaceae, Zingiberaceae).

The 2-alkylquinolin/4(1*H*)-one alkaloids isolated from some bacteria are typical of the Rutaceae. From the systematic standpoint the co-occurrence of 2-*n*-nonylquinolin-4(1*H*)-one in *Ruta graveolens*, *Raulinoa echinata*, *Boronia bowmanii*, 2-*n*-undecylquinolin-4(1*H*)-one in the former, *Ptelea trifoliate* (Rutaceae), and both in *Pseudomonas* (Proteobacteria) is astonishing. In addition, it is noteworthy that many of these alkaloids in bacteria were firmly established as derived from anthranilic acid, while the Rutaceae were less biosynthetically investigated. The analysis of distribution of alkylquinolin/ones among five genera of Proteobacteria division showed that *Pseudomonas* (Pseudomonadaceae) is the major sources of 2-alkylquinolin/4(1*H*)-ones. There is a much less proliferation of this alkaloid type in *Alteromonas* (Alteromonadaceae). Both produce these alkaloids with variation only in the aliphatic chains at C-2. These two families are included in the orders Pseudomonadales and Alteromonadales, respectively, and in the same class Gammaproteobacteria [41]. *Stigmatella*, which form part of Cystobacteraceae family (Myxococcales order and Deltaproteobacteria class), explore 2-alkylquinolin/4(1*H*)-ones chemistry along several different biosynthetic pathways, which leads to compounds containing farnesyl as substituent (Scheme 24.15). Of the four genera from Actinobacteria division examined, notably *Pseudonocardia* yield 2-alkylquinolin-4(1*H*)-ones with an uncommon substitution pattern, containing a geranyl group at C-2 (Scheme 24.13) instead of an aliphatic chain derived from β-keto fatty acids (Scheme 24.13). These genera are included in the Actinomycetales order and the quinolin/ones are of sporadic occurrence in the three remaining genera. *Arthrobacter* (Micrococcaceae) contain 2-alkylquinolin-4(1*H*)-one with a methyl group at C-3 (Scheme 24.13, Table 24.28), while in *Janibacter* (Intransporangiaceae) alkylation in a quinoline skeleton appear in position C-8 as oxidized methyl group (Table 24.22).

The quinolin/4(1*H*)-one alkaloids with a linear aliphatic side chain at C-2 appear to be absent in fungi. Fungus *Penicillium* (Ascomycota phylum, Ascomycetes class, and Eurotiales order) has yielded a novel class of quinolin/ones [41]. They are based on the combination of amino acids L-valine and L-isoleucine, anthranilic acid, and acetic acid [41] (Schemes 24.22, 24.23), or these amino acids and tryptamine [41] (Scheme 24.24). They constitute two small groups which can be considered as 2-substituted quinolin-4(1*H*)-ones: quinolactacins A1, A2, B, and C, quinolactacide; and 2-substitued quinolines: quinocitrinines A and B. Both are alkaloid types at present unknown from any other source. Thus, clearly fungi from Ascomycetes class deserve more attention in order to find new quinolactacins, since they showed interesting biological effect on tumor necrosis factor (TNF) [41].

Quinazoline derivatives are less common (11 compounds) in Rutaceae. Interestingly, these alkaloids (50 derivatives) occur in the following taxonomically unrelated plant families: Acanthaceae, Apocynaceae, Araliaceae, Asteraceae, Brassicaceae, Fabaceae, Hydrangeaceae, Malvaceae, Orchidaceae, Polygonaceae, Ranunculaceae, Rubiaceae, Saxifragaceae, Scrophulariaceae, and Zygophylaceae. A variety of quinazoline (62 derivatives) are also produced by bacteria, fungi, and marine animals.

The alkaloids reported in the Rutaceae can help to confirm the affinity between subfamilies because there is greater chemical diversity. On the other hand, the bacteria and fungi are needed for more substantial chemical studies. When more data become available, it is likely that useful systematic correlations will emerge. More detailed studies regarding the biosynthetic pathways of quinolines and quinazolines in the Rutaceae are needed. Such studies would clarify the differences in the pathways based on their derivation from anthranilic acid in bacteria and in rutaceous plants.

Finally, this survey indicates that the Rutaceae, and various bacterial and fungal species offer considerable potential for the discovery of new or known alkaloids with significant and possibly valuable biological activities.

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Alkaloids Derived from Histidine: Imidazole (Pilocarpine, Pilosine) 25

Ana Paula Santos and Paulo Roberto H. Moreno

Contents

1 Introduction 862
2 Histidine-Derived Alkaloids 863
2.1 Alkaloids Formed from Histamine and Derivatives 864
2.2 *Pilocarpus* Alkaloids 868
3 Imidazole Alkaloids Biosynthesis 871
3.1 *Pilocarpus* Alkaloids Biosynthesis 872
3.2 Dolichotheline Biosynthesis 873
4 Biotechnological Approaches 874
4.1 Biotechnology in Pilocarpine Production 874
5 Biological Activity 876
6 Conclusions 878
References 878

Abstract

This chapter will cover the alkaloids containing the imidazole nucleus which is the smallest group of alkaloids in terms of number of compounds known. This class of alkaloids is synthesized by precursors derived directly from the amino acid, L-histidine. The known structures can be divided in those derived from histamine as a precursor and the other ones called *Pilocarpus* alkaloids that

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contained a distinct precursor. The state of the art on the biosynthesis of these alkaloids, biotechnological approaches for their production, and their biological activity are also discussed.

Keywords

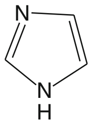
Biological activities • Biosynthesis • Cell culture • Histidine derivatives • Imidazole alkaloids • *Pilocarpus* alkaloids • Plant sources

Abbreviations

^{13}C NMR	^{13}C Nuclear magnetic resonance
^{1}H NMR	1H Nuclear magnetic resonance
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
CNS	Central nervous system
ESI-MS	Electron spray ionization – mass spectrometry
GA_3	Gibberellin A3
HDC	Histidine decarboxylase
HPLC	High performance liquid chromatography
HPLC–ESI–MS/MS	High performance liquid chromatography with electrospray Ionization tandem mass spectrometry
HT	Histidine aminotransferase
IUPAC	International Union of Pure and Applied Chemistry
MS medium	Medium Murashige and Skoog
PEG	Polyethylene glycol

1 Introduction

Alkaloids form a large group of secondary metabolites with a structural diversity comparable to that of terpenoids, representing ca. 20% of all known natural substances [1]. In spite of this abundance, proportionally few alkaloids contain the imidazole group (**1**).



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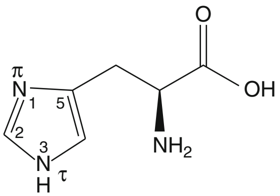


2

In general, the nitrogen atom in alkaloids, normally forms a heterocyclic ring, is derived from amino acids. L-Histidine (**2**) is the only amino acid that contains an imidazole ring (glyoxaline) (**1**), an aromatic heterocyclic group with one nitrogen atom hybridized as in pyridine and the other one hybridized as in pyrrole. These two nitrogen atoms confer a relatively high basicity to the imidazole ring because the protonated species can be stabilized by resonance. This amino acid is considered the precursor of all alkaloids containing this ring system because of their close structural relationship [2].

About the nomenclature of histidine and its derivatives, there has been some confusion for a considerable time, mainly concerning the numbering of atoms in the imidazole ring. Biochemists generally numbered the nitrogen atom adjacent to the side chain as 1 and organic chemists designating it as 3. In this chapter, we followed the IUPAC recommendation for numbering the nitrogen atoms in the imidazole ring of histidine. The nitrogen nearest the alanine side chain is called *pros* ("near," abbreviated π) and the farthest one, *tele* ("far," abbreviated τ). This means that the pros-nitrogen atom (N^{π}) has position 1 and the *tele*-nitrogen (N^{τ}), position 3. Consequently, the carbon atom between the two-ring nitrogen atoms is numbered 2 (as in imidazole), and the carbon atom next to the τ nitrogen is numbered 5. The carbon atoms of the aliphatic chain are designated α and β. This numbering is also used for the decarboxylation product histamine and for substituted histidine derivatives [3].

Battersby and Openshaw [4] wrote one of the first reviews on imidazole alkaloids. At that time, this group was even smaller, and the discussion dealt mainly with pilocarpine and related alkaloids. Since the 1980s, the number of alkaloids containing the imidazole ring has increased mainly from marine organisms such as mussels, sea urchins, and sponges and in smaller number from plants [5]. These marine alkaloids will be discussed in a specific chapter in this handbook (▶ Chap. 9, "Marine Pyrroloiminoquinone Alkaloids, Makaluvamines and Discorhabdins, and Marine Pyrrole-Imidazole Alkaloids").

On the other hand, their occurrence in plants is restricted to some specific genera or even species from different families without a distribution pattern. The only exception is *Pilocarpus* Vahl (Rutaceae) in which all the species are known to accumulate imidazole alkaloids. No new sources from these alkaloids from higher plant have been reported since 1990.

In this chapter, we will only discuss about nonmarine alkaloids which are originated directly from histidine and are not simple peptide alkaloids. The alkaloids here discussed were classified, accordingly to their biosynthetic origin, in simple histamine derivatives and the so-called *Pilocarpus* alkaloids which are originated directly from histidine but from different precursors.

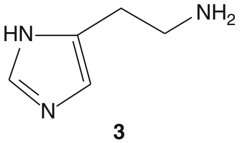
2 Histidine-Derived Alkaloids

Few imidazole alkaloids can undoubtedly be associated to L-histidine as biogenetic precursor. Nevertheless, some of these are closely related to this amino acid despite lack of biosynthetic experimental data. In this chapter, imidazole alkaloids were divided according to their chemical features as histamine and derivatives, simple histamine amides, and *Pilocarpus* alkaloids. Plant species, isolation, and detection are highlighted in this topic.

2.1 Alkaloids Formed from Histamine and Derivatives

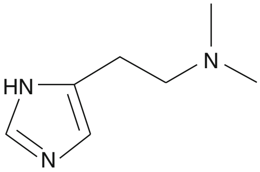
2.1.1 Histamine and Simple Amines

Histamine is a biogenic amine formed by L-histidine decarboxylation mediated by HDC (histidine decarboxylase) (EC EC 4.1.1.22) and naturally occurs in some fungi, marine, and plant species [6, 7]. It was isolated from aerial parts of *Capsella bursa-pastoris* (L.) Medik. (Brassicaceae), *Lolium perenne* L. (Poaceae) [8], and *Spinacea oleracea* L. (Chenopodiaceae) [7, 9]. This amine may act as an intermediate in many imidazole derivatives biosynthesis such as amides.



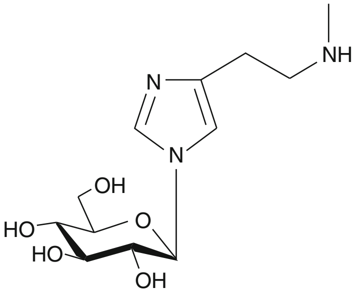
3

S. oleracea [7], *Echinocereus blanckii* Palm. and *E. triglochidiatus* Engelm. var. *paucispinus Engelm.* ex W.T. Marshall (Cactaceae) [10] provide N^{α},N^{α}-dimethylhistamine (4) in addition to other alkaloids. Extraction and isolation procedures were performed accordingly to classical phytochemical techniques, using partition, column, and thin layer chromatography. *E. triglochidiatus* var. *neomexicanus* yielded 0.11% of this alkaloid with the following major mass fragment: 139 (8%) M^+, 95 (10%), and 58 (100%).



4

This imidazole alkaloid is also present in *Casimiroa edulis* Llave et Lex (Rutaceae), which also produces a glycosylated derivative of N^{α}-methylhistamine, casimidine (**5**), and the unusual alkaloid containing a sulfur atom, zapotidine (**6**) [8, 11–13].



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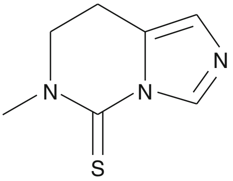
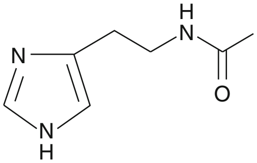


6

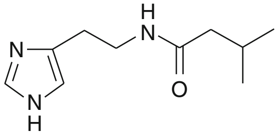
2.1.2 Simple Histamine Amides

The simplest histamine derivative from this group, N^{α}-acetylhistamine (**7**), was firstly identified by Appel and Werle in *S. oleracea* [7] crude alkaloid extract.



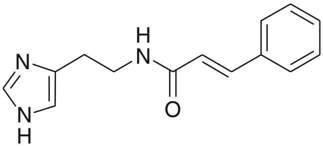
7

Also in this group, dolichotheline (*N*-isovalerylhistamine) (**8**) was isolated, in 1969, from a small cactus named *Dolichothele sphaerica* (Dietrich) Br. and R. [14, 15]. The alkaloid was obtained from crystallization from nonphenolic fraction with a benzene-acetone mixture. Identification was carried out by thin layer chromatography using chromogenic Pauly's reagent (for histidine and tyrosine metabolites) and spectroscopy methods. Structural identity of dolichotheline was provided by means of mass spectral data with molecular ion at *m*/*z* 195 and an *m*/*z* 85 and *m*/*z* 111 fragments due to the loss of the isovaleryl and histamine moieties, respectively [14]. This was the first imidazole alkaloid reported to a Cactaceae plant and out of jaborandi species.



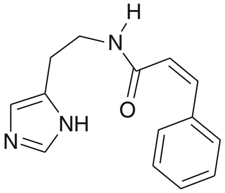
8

N^{α}-cinnamoylhistamine (**9**) was isolated from several plant species such as *Argyrodendron peralatum* (Bailey) Edlin ex J.H. Boas (Sterculiaceae), *Acacia* spp. (Fabaceae), and *Glochidion philippicum* (Cav.) C.B.Rob. (Euphorbiaceae) [8, 16]. Using current analytical methods, this alkaloid was isolated as colorless crystals (m.p. 178–179 °C) and characterized as the *trans* isomer due to high coupling constant for the olefinic protons signs [16].

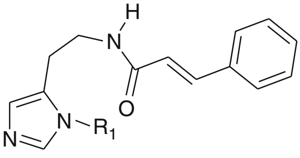


9

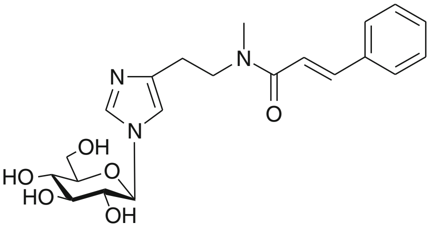
The *cis* isomeric form (**9a**) was not previously reported in former species, but it was isolated from aerial parts of *Lycium cestroides* Schltdl. (Solanaceae) [17, 18]. By means of ^{1}H NMR spectroscopy the *cis*-*trans* isomers of N^{α}-cinnamoylhistamine were characterized as two quasi-bicyclic forms with a hydrogen bond between the carbonyl amidic group and the N^{π}-H imidazolic ring [17]. According to the authors, these isomers spontaneously undergo to lineal form especially the *trans* isomer (**9b**). Methyl derivatives were also isolated from crude ethanolic extract (**10a** and **10b**). The Rutaceae species *C. edulis* affords casimiroedine (**11**) [11, 12], a glycosylated derivative of N^{α}-cinnamoyl-N_{π}-methylhistamine from *L. cestroides*.



9a R_1= H
10a R_1= $-CH_3$

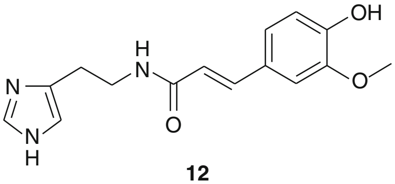


9b R_1= H
10b R_1= $-CH_3$



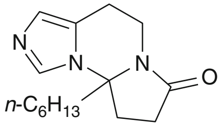
11

Ephedra spp. roots are known as major source of ephedrine and derivatives alkaloids, but this plant species also contains an imidazole derivative, feruloylhistamine (**12**), which was identified in methanolic extract of the drug in 1983 [19, 20]. After column chromatography and crystallization procedures, feruloylhistamine was characterized by ^{1}H and ^{13}C NMR and mass spectroscopy. A molecular ion peak was observed at *m*/*z* 287 consistent to a $C_{15}H_{17}N_3O_3$ fragment, and the ^{13}C NMR spectrum revealed signals for aliphatic and aromatic carbons and also one carbonyl group. The synthetic derivative was also obtained and afforded the same physical data as the natural compound [19].



12

The isomers glochidine (**13**) and glochidicine (**14**) (both with a lactam ring) and *N*-(4-oxodecanoyl) histamine (**15**) were isolated from *G. philippicum*. Structure elucidation was performed by elementary analysis, ^{1}H and ^{13}C NMR and mass spectroscopy. The *m*/*z* 176 fragment, which corresponds to *n*-hexyl loss from C-12, was present in both isomeric forms [21].



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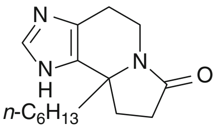

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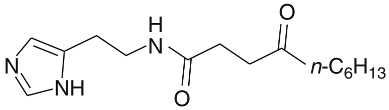
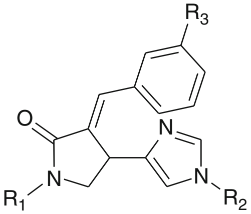
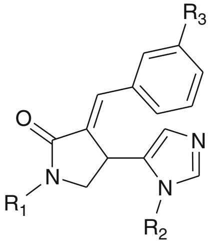


15

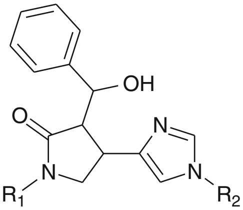
Other examples of histamine amides that also contain a lactam portion are the alkaloids from *Cynometra* spp. (Fabaceae). Structurally, they are very similar to that one found in *Pilocarpus* species. *C. hankei* Harms, *C. ananta* Hutch. & Dalziel, and *C. lujae* De Wild. and are indigenous to west tropical Africa, which contain lactams derivatives, in which occur isomerization at imidazole ring level, such as anantine(**16**)/isoanantine (**19**) and cynodine (**18**)/isocynodine (**19**) [22]. As these alkaloids are only found in *Cynometra* species, possibly, they have a chemotaxonomic importance to this genus.



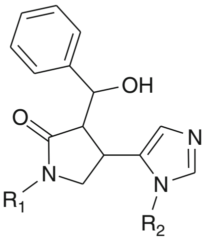
16 $R_1 = H \quad R_2 = -CH_3 \quad R_3 = H$
17 $R_1 = R_2 = R_3 = H$
18 $R_1 = H \quad R_2 = -CH_3 \quad R_3 = OH$



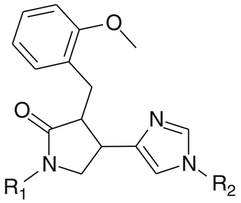
19 $R_1 = H \quad R_2 = -CH_3 \quad R_3 = H$



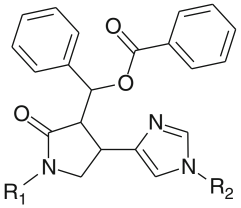
20 $R_1 = R_2 = -CH_3$
21 $R_1 = -CH_3 \quad R_2 = H$



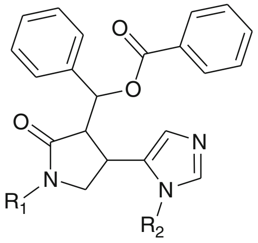
22 $R_1 = R_2 = -CH_3$



23 $R_1 = H \quad R_2 = -CH_3$



24 $R_1 = R_2 = -CH_3$
25 $R_1 = -CH_3 \quad R_2 = H$



26 $R_1 = R_2 = -CH_3$

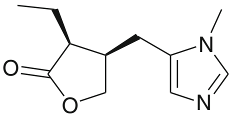
2.2 *Pilocarpus* Alkaloids

This is undoubtedly the most important class of L-histidine derivatives only because of pilocarpine's (**27**) therapeutic uses [8, 23, 24]. Since Byasson's first report of jaborandi alkaloids isolation from the leaves of *Pilocarpus* spp. (Rutaceae) [8], in 1875, several other imidazole alkaloids have been reported in this genus. The identification of these was facilitated due to recent advances in hyphenated chromatographic techniques such as HPLC–ESI–MS/MS (high performance liquid chromatography with electrospray ionization tandem mass spectrometry).

Chemically, the majority of *Pilocarpus* alkaloids hold simultaneously an imidazole and a γ-lactone ring. Epimerization at C2 and C3 carbons of the lactone moiety is commonly observed, and this change in absolute configuration gives rise to some compounds that can naturally occur in *Pilocarpus* species or are generated during extraction procedures [23, 25].

Pilocarpine (**27**) was first isolated, in 1875, by Hardy and by Gerrard, in distinct works, as colorless crystalline salts from the leaves of *P. jaborandi* Holmes [8, 23]. This alkaloid is also found in several other species such as *P. microphyllus* Stapf., *P. pennatifolius* Lemmaire, *P. racemosus* Vahl, *P. trachyllophus* Holmes, and *P. carajaensis* Skorupa, where total pilocarpine content ranges from *ca.* 3.0% to

70% of total alkaloids. Isopilocarpine (**28**) is the epimerization product of pilocarpine produced by enolization pathway, accordingly to Döpke and d'Heureuse [26].



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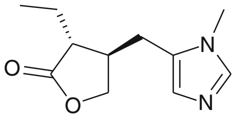
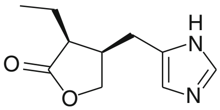


28

Initially, pilocarpine structure was incorrectly attributed as a betaine derivative of a pyridine-lactic acid compound. Further research, based on chemical degradation, synthesis, and X-ray analysis, provided pilocarpine structure as (2*S*,3*R*)-2-ethyl-3-[(1-methylimidazol-5-yl)methyl]-4-butanolide, as extensively described in previous reviews [8].

Pilocarpidine (**29**) and isopilocarpine (**30**) are the non-N^{π} derivatives of this series of compounds [8, 23, 24]. They were isolated in 1900, by means of classical phytochemical procedures, from *P. jaborandi*, which was confirmed as the only source of these alkaloids by HPLC–ESI–MS/MS [27–29].



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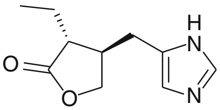
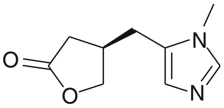


30

Pilosinine (**31**) is a naturally occurring alkaloid in *P. microphyllus*, structurally, it resembles pilocarpine (**27**) since it lacks the ethyl substituent at C2 [8]. This substance plays an important role in pilocarpine synthesis as a key intermediate as indicated by recent works [30, 31]. Tandem mass spectroscopy studies indicated that *P. carajaensis*, *P. spicatus* A. St. Hil., *P. trachyllophus*, *P. pennatifolius*, *P. jaborandi*, and *P. racemosus* accumulated 2,3-dehydropilosinine (**32**). Confirmatory fragmentation pattern was made by comparison with that described to the synthetic derivative.



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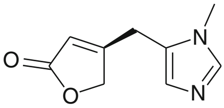
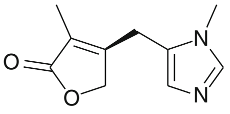


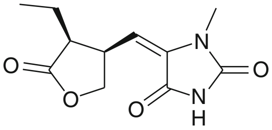
32

The alkaloid 13-*nor*-7(11)-dehydro-pilocarpine (**33**) was firstly isolated from *P. trachyllophus* [32] as a nitrate salt. Recently afforded, as mentioned before, by chromatographic techniques improvements, this substance was identified in five other species, namely, *P. microphyllus*, *P. carajaensis*, *P. spicatus*, *P. racemosus*, and *P. pennatifolius* [29]. As it was found in this genus and could possibly participate in different biosynthetic routes.



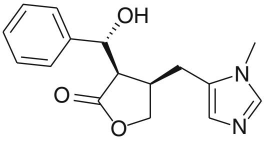
33

The imidazole alkaloid 4,6-dehydro-1,2,4,5-tetrahydro-2,5-dioxopilocarpine (**34**) was isolated from *P. grandiflorus* Engl. stems, as a yellowish oil. Its structure was determined by high-resolution mass spectroscopy (*m*/*z* 238) and ^{1}H and ^{13}C NMR that indicated a change in the structure of pilocarpine due to the signals at 177.7, 162.4, 152.4, 132.2, and 112.3 ppm, associated to two additional carbonyl groups and a double bond [33].



34

Seven isomers of molecular formula $C_{16}H_{18}N_2O_3$ are reported to *Pilocarpus* species, six out of them have proposed structures: pilosine (**35**), isopilosine (**36**), epipilosine (**37**), epiisopilosine (**38**), and the N^{τ}-methyl derivatives piloturine (**39**) and epiisopiloturine (**40**) [29, 34, 35]. All they have is an α-hydroxylbenzyl group instead of the ethyl substituent at C-2 in the lactone ring. Except for epipilosine (**34**), all these alkaloids were identified in *P. microphyllus*, *P. jaborandi*, and *P. carajaensis* species [29].



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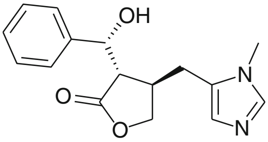

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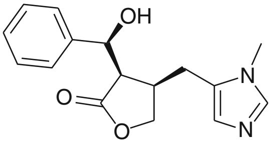

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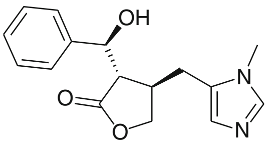

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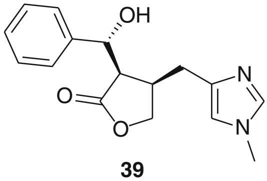

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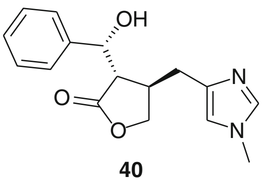
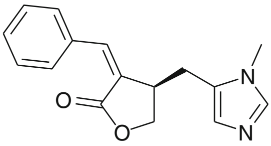


40

High-resolution mass spectrometry of the total alkaloids of *Pilocarpus* species (*P. microphyllus*, *P. spicatus*, and *P. carajaensis*) indicated the presence of three isomers of m/z 269. The fragmentation pattern was consistent of that from anhydropilosine (**41**) [28, 29].



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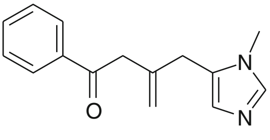
As previously mentioned, HPLC advances improved *Pilocarpus* alkaloids' identification, and several new derivatives of this class were characterized. Three protonated molecules (**42**) (*m*/*z* 241), (**43**) (*m*/*z* 259), and (**44**) (*m*/*z* 285) were identified in *P. microphyllus* ESI-MS fingerprints, whose structures are shown below. These new compounds display a common and characteristic fragment of the benzoyl ion, $C_6H_5CO^+$ (ion of *m*/*z* 105). Screening studies in other *Pilocaprus* species showed (**42**) (and an isomer) were also present in *P. carajaensis*, *P spicatus*, *P. trachyllophus*, and *P. racemosus* [27–29].



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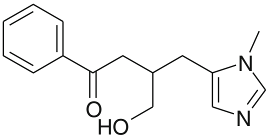

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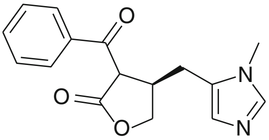

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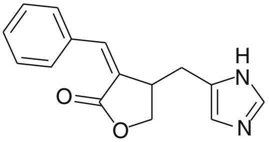
Some of these new *Pilocarpus* derivatives were found specifically in *P. pennatifolius* and *P. microphyllus* samples. Following compounds occur as isomeric forms in which absolute configuration was not elucidated [29].



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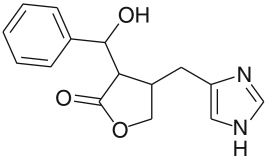

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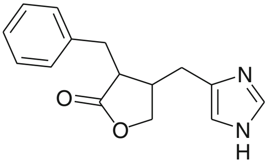

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3 Imidazole Alkaloids Biosynthesis

Alkaloids are important secondary metabolites with marked physiological effects and some of them with unmeasured pharmacological interest [23, 36]. It is a general belief that, in plants, alkaloids may be associated with protection against predators or as nitrogen storage/transport and even be involved in physiological balance of the plant [23]. The biosynthesis of these nitrogen derivatives depends on the presence of amino acid precursors (such as ornithine, nicotinic acid, tyrosine, anthranilic acid, and histidine), carbon units, and also key enzymes that conjugate them [23, 36, 37].

Biosynthetic studies have experienced great development since initial works on imidazole alkaloids biogenesis [36]. The use of isotopic labeled precursors, in the 1950s, is the first boundary in secondary metabolite biosynthesis. Cell cultures were also extensively used as tool to track active enzymes involved in alkaloid production. Advances in molecular biology have led biosynthetic researches to gene level, successfully used in genetic engineering studies guiding alkaloids or other secondary metabolites accumulation in plants [36–38]. Despite these recent improvements in biosynthetic approaches, biogenesis of imidazole alkaloids remains obscure.

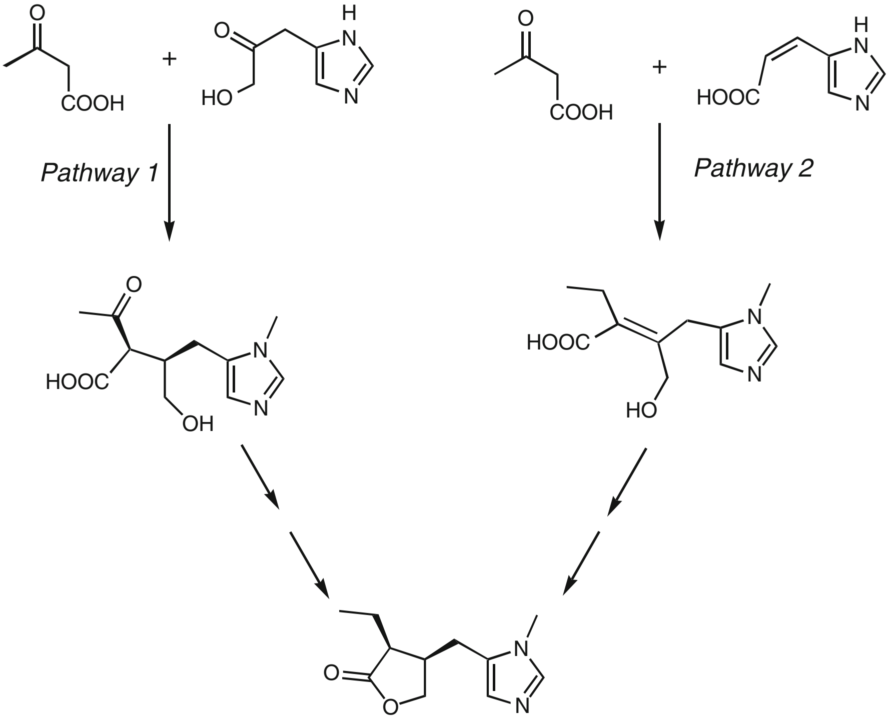
Only the biosynthesis of *Pilocarpus* alkaloids and dolichotheline, from *Dolichothele sphaerica*, were reported in literature as described below.

3.1 *Pilocarpus* Alkaloids Biosynthesis

The amino acid L-histidine is the assumed building block in the biosynthesis of pilocarpine (**27**) and other imidazolic alkaloids due to the presence of glyoxaline ring [8, 23]. First attempts to clarify this pathway were proposed by Boit and Leete that considered the phosphate derivative of 2-oxo-3-(5-imidazolyl)-propanol, also known as imidazole pyruvic acid, as imidazole ring precursor (Scheme 25.1- Pathway 1) [8]. This initial biosynthesis proposal was improved detailing the lactone ring formation by aldol condensation. Another biosynthetic approach suggested condensation of 2-oxobutyric acid (lactone moiety) with urocanic acid (imidazole moiety) (Scheme 25.1- Pathway 2) [8, 39].

Isotopic studies with labeled potential precursors such as sodium acetate-^{14}C, threonine-^{14}C, histidine-^{14}C, histidinol-^{14}C, and L-methionine-(*S*-methyl-^{14}C) were carried out with *P. pennatifolius* in an attempt to prove the proposed pathways [8, 39]. Only the methylation of pilocarpidine, last step in both mechanisms, was attested by significant incorporation of radioactivity in the methyl group attached to the imidazole nucleus. This data confirmed an important assumption, the one that considers methionine the biological source of the *N*-methyl group of pilocarpine. Also, one should consider that these radiolabeled studies were carried out with stems, no other parts of the plant were analyzed, and thus some other site involved in biosynthesis was not considered in this study.

The presence of the enzyme histidine aminotransferase, (HT) (EC 2.6.1.38) was reported in *P. pennatifolius* roots [40], which could link L-histidine to imidazole alkaloids biosynthesis and reinforce the hypothesis of a specific site for pilocarpine production. The enzyme activity was estimated in 46.09 nKat.mg^{-1} of protein and optimal reaction at pH 8–9. HPLC data from enzymatic reaction indicated the formation of a product with the same retention time of the imidazole pyruvic acid standard obtained by synthesis. Such an enzyme activity could not be detected in *P. pennatifolius* leaves, reinforcing these organs could be involved only in the later steps of the biosynthesis, such as *N*-methylation, or could play a role as accumulation site and not in the production.



Scheme 25.1 Hypothetical pathways for the pilocarpine biosynthesis

A cell suspension study with *P. microphyllus* recorded after several subcultures a stable cell line in pilocarpine and other imidazole alkaloids production and the same alkaloid profile found in leaves [41]. This study also characterized three new alkaloids with imidazole ring in this plant species for the first time, by means of ESI-MS. Posterior studies using the same technique in leaves of several samples of *Pilocarpus* suggested three different pathways may occur in imidazole alkaloids biosynthesis [27, 41].

Identification of the genes involved in imidazole alkaloids biosynthesis could be the next and final boundary in pilocarpine production in plants. Nevertheless, isolation from natural sources is still a feasible way to obtain this alkaloid.

3.2 Dolichotheline Biosynthesis

Dolichotheline is an amide derivative from histamine of *Dolichothele sphaerica* (Cactaceae) whose structure suggests the condensation of the imidazole unit with isovaleric acid [14, 15]. Its biosynthesis was evaluated by means of radiolabeled tracers such as DL-[2-^{14}C]histidine, DL-[2-^{14}C]leucine, DL-[2-^{14}C]mevalonic acid lactone, and sodium [1-^{14}C]isovalerate [42]. The extension of radioactivity incorporation indicated that histidine was involved in dolichotheline biosynthesis and also that leucine or mevalonic acid could be the carbon unit donor, since both of them can be reputed as isovaleric acid precursor [43].

The enzymatic system that promotes the amide linkage between L-histamine and isovaleric acid in *D. sphaerica* is not specific, so aberrant or unnatural alkaloids (derivatives produced in the absence of natural precursors) can be produced. Different aberrant alkaloids were produced in labeled studies; one could infer that this system is suitable to prepare biologically active natural products that are difficult to synthesize [44–46].

4 Biotechnological Approaches

Plant tissue culture was initially used for growing isolated plant cells, tissues, and organs under controlled conditions aiming regeneration or propagation of entire plants. This technique relies on cell totipotency, which means that single cells undergo differentiation and thus regenerate an entire plant [47].

Large-scale production of secondary metabolites by plant cell culture seems to be feasible and attractive to industrial production. It has two major advantages over traditional monoculture methods [48]: (1) controlled production of fine natural chemicals independent of climatic, edaphic, and political conditions and (2) higher quality and yield of the final product in well-defined systems. In recent years, metabolic engineering has opened a new promising perspective for improved secondary metabolites production. This approach can be used to improve production not only in cell culture but also in the plant itself or in other plant species or even other organisms.

Biotechnological approaches in imidazole alkaloids production are not so developed as other classes of secondary metabolites, and the studies deal exclusively with pilocarpine production. Nevertheless, the reported results do not lead to a profitable accumulation of this alkaloid but are interesting approaches considering that *Pilocarpus* species are threatened plants.

4.1 Biotechnology in Pilocarpine Production

Despite pilocarpine pharmaceutical importance and structural simplicity, synthetic approaches available for large-scale production are time consuming and do not achieve high yields. Even under ideal storage conditions, *Pilocarpus* spp. leaves can lose at least half of their alkaloid content after 1 year via regular degradation routes [25, 49]. Alternative methodologies aiming increased production of this secondary metabolite are thus a major objective to fine chemicals industries.

First attempts to obtain pilocarpine in cell cultures were performed during the 1990s. Seedlings and cell suspension cultures obtained from *P. microphyllus* species accumulate pilocarpine in a content of 1/5th to 1/20th of the amount of naturally growing plant [50]. Hairy roots, obtained from genetic transformation by *Agrobacterium rhizogenes*, exhibit about the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants. Thus, a hairy root culture of *P. microphyllus* was established in a Murashige and Skoog medium supplemented with 2,4-D and kinetin as growth regulators. Pilocarpine was accumulated in cells and also excreted to the medium in which the amount of alkaloid varied from 300 to 500 μg/g and were equivalent of the original plant [51].

Micropropagation of *P. microphyllus* seedlings were carried out in absence of GA_3 resulted in a higher percentage of germination and a lower rate of contamination [52]. The apical segment was the best explant for shoot formation grown on medium with different combinations and concentrations of BAP, zeatin, and kinetin.

P. microphyllus seedlings were subjugated to different stressing factors and elicitors, such as hypoxia, salt stress, wounding, nutrient (nitrogen/potassium) omission, salicylic acid, and methyl jasmonate [53]. A time of exposure/concentration relationship of salicylic acid and methyl jasmonate treatments increased pilocarpine accumulation over control in a fourfold factor. Further conditions on the other hand reduced pilocarpine production.

In *P. microphyllus* model, callus were also subjected to conditions and elicitors in an MS-liquid medium supplemented with 2,4-D as growth regulator [54]. Different nutrient concentration, pH value, type/concentration of elicitors (histidine, threonine and methyl jasmonic acid), and osmotic and salt stress (PEG and NaCl) were evaluated. Light incidence influence was also analyzed. Pilocarpine was released in the liquid medium from callus kept in the dark. Detection was carried out using HPLC-MS/MS techniques, and pilocarpine quantification was performed by HPLC. Elicitors induced highest accumulation of the alkaloid in a time/concentration relationship. Light incidence and methyl jasmonic acid inhibited pilocarpine release to the medium.

This model was also used in the evaluation of stable cell lineages with the same alkaloid profile than that of *Pilocarpus* leaves [55]. After 12 subcultures, imidazole alkaloids' composition of the cell cultures was similar to that of the leaves; thus, one can infer that imidazole alkaloid metabolism in *P. microphyllus* cells is similar to that found in leaves. After 24 subcultures, the overall alkaloid distribution remained the same indicating that cell cultures can be cultivated for long periods, and identification of three new imidazole derivatives suggested that this is a suitable model to biosynthesis studies. The effect of pH variations (from 4.8 to 9.8) on stable cell lineages changed pilocarpine/pilosine distribution between the cell and the medium, and highest pilocarpine accumulation was reached at pH 8.8–9.8 [56].

Callus and cell suspension culture of *P. pennatifolius* was established using young leaves on an MS medium (pH 5.7) supplemented with 2,4-D and kinetin as growth regulators [49]. Light-green- to yellowish-colored friable callus was obtained after six subcultures in solid media, and cell suspension culture was obtained (same growth regulators composition) and afforded 35 g/L of biomass after 20 days of maintenance. The callus cultures and suspended cells accumulated, respectively, 1.01 and 0.045 μg/g dry weight of pilocarpine.

5 Biological Activity

Some authors do not consider histamine, (2-[5-imidazolyl] ethylamine) as an alkaloid because it is a universal compound, mostly it is regarded as a biogenic amine. However, histamine is the simplest derivative from histidine presenting a remarkable biological activity. Histamine is formed in living cells by the 1 rate-limiting step exothermic decarboxylation of L-histidine, catalyzed by HDC [6, 7, 57]. This compound was discovered in 1910 by Dale and Laidlaw, as an isolate from the mold ergot capable of not only stimulating smooth muscle contraction but also inducing a shock-like syndrome when injected into mammals [58].

Nowadays, histamine is classified as a natural body constituent and a mediator on anaphylactic reactions, and it is implicated in several physiological functions, being one of the most extensively studied chemical compounds playing a central role in inflammation processes, gastric acid secretion, and neurotransmission. The research with histamine receptors allowed the development of drug therapies specifically targeted for allergies, gastric ulcers, asthma, and anaphylaxis. The control of histamine receptors has also some applications in sepsis control, hemorrhagic shock, anesthesia, surgery, cardiovascular disease, cancer, CNS disorders, and immune-mediated diseases [59–61].

Some histamine amides that have been isolated from some cactus species also showed pharmacological properties. In general, cactus alkaloids belong to β-phenethylamines or to the related tetrahydroisoquinolines that are potentially psychoactive compounds. The studies with *Dolichothele sphaerica* started due to its ethnobotanical relationship with the hallucinogenic *Lophophora williamsii* (Lem.) Coult., commonly known as peyote [62]. The chemical investigation of this cactus resulted in the isolation of the unusual imidazole alkaloid dolichotheline (*N*-isovalerylhistamine) [14], but no pharmacological studies were carried out with the isolated alkaloid.

Feruloylhistamine has also been isolated from the underground part of *Ephedra* plants, which was considered the hypotensive principle of the crude drug “maō-kon” [19]. Based on this finding, some feruloylhistamine analogues were prepared and their hypotensive and some other potential pharmacological properties were examined. The feruloyl group in the amide was changed by a caffeoyl, cinnamoyl, *p*-coumaroyl, and synapoyl substituent maintaining some hypotensive activity but much lower than that of histamine itself. Among the analogues, *p*-coumaroylhistamine presented a remarkable histidine decarboxylase inhibitory activity which was comparable with that of α-fluoromethylhistidine, a reference inhibitor for this enzyme. Consequently, it was expected that this analogue might decrease the gastric acid release, leading to new antiulcer drugs. However, *p*-coumaroylhistamine exerted no antiulcer activity either in the antistress ulcer formation assay or in the antiacid secretion test. All the feruloylhistamine analogues also exhibited some antihepatotoxic activity. Caffeoylhistamine was the analogue that presented the most significant antihepatotoxic activity in the two model assays [20].

The most important pharmaceutical alkaloids in this group are the so-called *Pilocarpus* alkaloids, mainly pilocarpine, which is used as an ophthalmic cholinergic drug. Pilocarpine and other related alkaloids were isolated from several *Pilocarpus* species which are popularly known by the name of "jaborandi" which was derived from the Tupi-Guarani language, *ya-mbor-endi* (the one who causes slobbering). The first record in Europe on the use of these species was made in 1570 by Gabriel Soares de Souza, a Portuguese explorer who traveled through Bahia, describing that the indigenous population used these plants to treat mouth ulcers. Adolf Weber was responsible for introducing pilocarpine as a medical treatment for glaucoma in 1877, soon after its isolation by Hardy and Gerrard in 1875 [63].

Pilocarpine is a parasympathomimetic agent that binds selectively to muscarinic receptors and exerts a broad spectrum of pharmacological effects, which include stimulation of bladder, tear ducts, and sudoriferous and salivary glands. These effects make this alkaloid the drug of choice in glaucoma treatment, because it increases the intraocular aqueous outflow through the trabecular meshwork by contracting the ciliary muscle and a mechanical pull on the trabecular meshwork [64]. Pilocarpine is also used as a cholinergic sialogogue to treat xerostomia, a subjective feeling of a dry mouth, which is relatively common in patients on chronic hemodialysis and after radiotherapy treatment of head and neck cancers. The beneficial effect of pilocarpine on xerostomia probably can be attributed to the stimulation of the salivary glands function [65, 66].

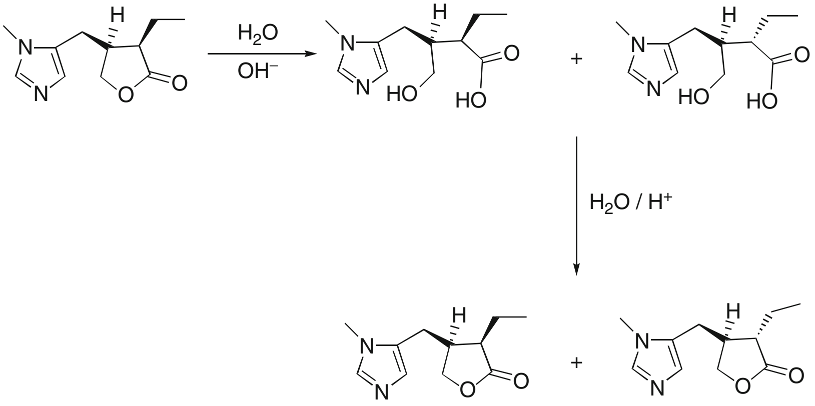
Sjögren's syndrome is a systematic autoimmune disease characterized by dysfunction of the lacrimal and salivary glands, its therapy remains largely empirical and symptomatic targeting, mainly the alleviation of the dryness symptoms. The stimulation of muscarinic M3 receptors to increase aqueous secretions by pilocarpine is the cornerstone of the current therapy [67, 68].

Systemic or intracerebral administration of pilocarpine hydrochloride in high doses induces seizures in rodents. These seizures are characterized by a sequential development of behavioral patterns and electrographic activity [69]. The pilocarpine seizure induces *status epilepticus* like in humans mimicking the pathogenesis and progression of mesial temporal lobe epilepsy [70]. It can be useful to exploit these model properties to design new therapeutic approaches for treating refractory epilepsies.

Isopilocarpine coexists with pilocarpine in nature; in aqueous solutions, pilocarpine can hydrolyze to both pilocarpinic and isopilocarpinic acids. The latter, in acidic pH, can recyclize to isopilocarpine (Scheme 25.2). The contrary does not happen, because the isopilocarpine hydrolysis yields only isopilocarpinic acid [25]. This change in the spatial structure decreases the binding affinity of isopilocarpine to approximately one-tenth to that of pilocarpine in bovine muscarinic cholinergic receptors [71].

Epiisopiloturine (**40**) was found in the leaves of *P. microphyllus* and presented an effect against *Schistosoma mansoni*, causing death on schistosomes from several ages at doses from 150 to 300 μg/mL and was also able to inhibit in 100% worm eggs laying at 100 μg/mL with no cytotoxicity to mammalian cells [72].

Although more than a dozen alkaloids have been isolated from *Pilocarpus* spp., the majority has not been screened for biological activities. Besides their structural resemblance, several are epimers, only pilocarpine possesses a relevant CNS activity.



Scheme 25.2 Epimerization of pilocarpine into isopilocarpine

6 Conclusions

Imidazole alkaloids are one of the smallest groups within alkaloids. This group does not have a defined distribution pattern among the plant species as observed for other groups, the only genus where all species accumulate imidazole alkaloids is *Pilocarpus*. Although the medicinal value of pilocarpine is widely recognized and the fact that the control of histamine receptors might have an important role in several therapies, the majority of the imidazole alkaloids have not yet received a thorough investigation.

The biosynthetic route leading to imidazole alkaloids is a puzzle to be solved. These alkaloids are most likely formed from L-histidine, either by its decarboxylation or deamination. The biosynthesis of those alkaloids derived from histamine, decarboxylation route, is already well established. On the other hand, the biosynthesis of *Pilocarpus* alkaloids is not yet completely elucidated. These alkaloids are thought to be formed by the deamination route having either imidazole pyruvic acid or urocanic acid as precursors. The hypothesis that the former acid is in fact the initial precursor in pilocarpine biosynthesis was supported when only HAT activity was detected in *P. pennatifolius* roots. However, the identification of new alkaloids in *P. microphyllus* cultures suggested that other biosynthetic pathways might be involved, making this an interesting field to be explored.

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Alkaloids Derived by Amination Reaction: Acetate-Derived (Coniine) 26

Rumen Binev

Contents

1 Introduction 884
1.1 Habitat 885
1.2 Botanical Characterization 885
1.3 Alkaloids 886
2 Phytochemistry and Isolation of Some Hemlock Plant Alkaloids 888
2.1 Isolation of the Alkaloids 888
2.2 Phytochemistry of the Alkaloids 889
3 Biosynthesis of γ-Coniceine and Coniine 893
4 Biosynthesis of *C. maculatum* Alkaloids 893
5 Biological Activities 896
5.1 Mechanism of Action 897
5.2 Species-Related Sensitivity 898
5.3 Acute Poisoning 898
5.4 Chronic (Teratogenic) Toxicity 900
5.5 Medicinal Uses 903
6 Conclusion 903
References 903

Abstract

This chapter is intended to be a presentation of alkaloids derived by amination reaction (acetate derived) from chemical, biological, and ecological points of view. The phytochemistry, isolation, biosynthesis, and biological activities of

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these alkaloids are described. The acetate-derived alkaloids (coniine) contained mainly in the plant poison hemlock (*Conium maculatum*); therefore, some of the botanical characteristics of this plant are mentioned.

It is, however, pertinent to mention at this juncture that the enormous volume of authentic information accumulated so far with regard to the isolation of alkaloids from a variety of plant species and their subsequent characterization by the help of latest analytical techniques they may be classified as alkaloids derived from amination reactions. These include:

(a) Acetate-derived alkaloids
(b) Phenylalanine-derived alkaloids
(c) Terpenoid alkaloids
(d) Steroidal alkaloids

Keywords
Acetate-derived alkaloids • biological activities • biosynthesis • coniine • phytochemistry

1 Introduction

It has been duly established that the larger section of alkaloids is virtually derived from amino acid precursors with the help of certain specific processes that essentially introduce into the final structure not only an N-atom but also an amino acid carbon skeleton or a major part of it. However, a good number of alkaloids do not essentially conform with this analogy. They are usually synthesized primarily from non-amino acid precursors having the N-atom inserted into the structure at a comparatively latter stage. Interestingly, such structures are predominantly based on both steroidal and terpenoid skeletons. Besides, a few comparatively simpler alkaloids also appear to be derived exclusively with the help of similar late amination processes. In extensive and intensive studies on certain alkaloids, it has been observed that the N-atom is specifically donated from an amino acid source through a transamination reaction using an appropriate ketone or aldehyde.

The taste of the hemlock potion offered to Socrates to induce his “suicide” should have been very bitter, but no comments about this are reported by Plato in the last chapter of *Fedone*, describing Socrates death, where he is reported to drink the poison “peacefully,” maybe thanks to his interior consciousness. If the bitter taste is indeed a poorly relevant feature of poisons used for suicide, it is a very negative and undesirable characteristic for poisons used in homicides. Thus, the poison present in it is really too dangerous for herbal administration by the uninitiated.

The coniine is the most important alkaloid of the hemlock plants. Coniine, one of the major toxic alkaloids present in poison hemlock (*Conium maculatum*), occurs in two optically active forms [36, 45].

1.1 Habitat

The poison hemlock (*Conium maculatum*) is a very common and worldwide plant species. It is one of the most toxic plants known [12, 18, 34, 38, 70, 85]. Poison hemlock is native to Europe and western Asia and has been brought in America and Oceania as an ornamental plant [12–14, 18, 43, 51, 53], although in other countries the plant has been introduced through the transport of seeds [14].

1.2 Botanical Characterization

Conium maculatum L., a member of *Apiaceae* (formerly *Umbelliferae*) of rhizomal plants, the carrot family [52], is an annual, biennial, or in favorable conditions perennial plant, usually 120–180 cm high. During the first year of growth, *Conium maculatum* reaches 45 cm height forming dense stands around the parent plants. The second year new plants grow from rosettes, with larger leaves which are dark green, bisected, triangular, and glabrous [12, 35, 85]. The root is long, forked, tuberous, pale yellow and reminds about a carrot. The stem is mottled with irregular purple spots, is erect, bright green and slightly ridged, much branched above and hollow. The leaves are fern-like [38], numerous, alternate, long-stalked, and tripinnate (which means that they are divided along the midrib into opposite pairs of leaflets which in turn are divided and subdivided) [85]. Its flowers are white, grouped in umbels, which are small and numerous and have a terminal position, with 12–16 rays per umbel. It produces a large number of green fruits, 2–3 mm long and about 2 mm wide [69, 70], grayish at maturity, and formed by two closed mericarps [51, 52]. The fruit is a broadly ovoid and is composed of two grayish-brown seeds with five wavy, longitudinal ridges. The petals of the small flowers are white, and the stamens of the flowers are longer than the petals and have white anthers. The inflorescence is produced mainly from June to September. At germination, the cotyledons are narrow and lanceolate; the first true leaves have two or more leaflets along an axis and are hairless. The plant has a bitter taste, and the odor reminds about a mouse. Where plants are numerous, the odor can be very pervasive. The seeds or fruits do not have a very marked odor, but if crushed or mixed with an alkali as potassium hydroxide solution, the same characteristic, odor of mouse urine is produced [38, 52, 70, 85].

The poison hemlock grows in ground, hedgerows, roadsides and woodland, pastures, banks of streams and rivers, meadows and waste grounds modified soils, along fences, roadsides and ditches, around windmills, abandoned constructions, and around woods, under which it can vegetate during the winter [38]. The poison hemlock is a nitrophile plant which means that it prefers moist soils with high nitrogen level. Since it belongs to a group of very common, wide-spreaded weed species, its germination is an important target of investigations. Poison hemlock can be a persistent weed species particularly in moist habitats. A single plant may produce 35,000–40,000 seeds, which usually fall near the parent plant, but can be spread by water and animals [35, 85].

1.3 Alkaloids

The *Conium maculatum* produces alkaloids, linear furanocoumarins have been isolated from the plant, and synthesis and accumulation sites have not yet been clearly identified [19]. All tissues of *Conium maculatum* were very rich in alkaloids [1, 3, 4, 12, 14, 20, 28, 31, 84]. The alkaloid synthesis in *Conium maculatum* took place more readily in tissues of the shoot than the root [4, 20].

There are eight known piperidine alkaloids in poison hemlock [3, 4, 12, 20, 30, 35, 44, 46, 54, 67–70, 85, 88] (Fig. 26.1).

Conium maculatum plants have typically a mousy odor when bruised due to the presence of piperidine alkaloids. One of the chief components was identified long ago as coniine, 2-propyl-piperidine, although this is sometimes replaced by γ-coniceine, accompanied by six minor ones [36].

Two of them, coniine and γ-coniceine, are frequently found in largest amounts. Coniine is reported to be eight times more toxic than γ-coniceine [3, 4, 20, 30, 67, 85].

A large width in the outcome regarding the alkaloid content, both qualitatively and quantitatively, has been found, depending on that different researchers have not used exactly the same method, which stage one study and which organ [1, 20, 51, 52]. Other parameters which had an effect on the alkaloid concentration were rain (gives an increase of γ-coniceine level in fruits and flowers) and temperature [19, 81]; even the diurnal concentration varied in the alkaloids [20]. The quantity of alkaloid was twice during the sunny seasons compared to cloudy seasons. As the fruits ripen, the alkaloid content increase, and this also depends upon the soil moisture and its exposure to the sun. Since green fruits contain more alkaloids than mature fruits and seeds, the level of alkaloids in fruits reaches its peak while ripening but reduces near maturity. The concentrations of coniine and coniceine are found in similar degrees in cloudy summers, while coniine is most prevalent during dry summers [1, 4]. A significant increase is detected in the alkaloid concentration of *Conium maculatum* on nitrogen-fertilized soils [36, 70, 85]. Coniine (2-propylpiperidine) and *N*-methylconiine progressively increase in flowers and fruits, while γ-coniceine decreases during plant development. Highest concentration of alkaloids is in seeds which can contaminate poultry and swine cereal grains. The coniine is derived from acetic acid [30, 33, 48, 55, 70, 78, 88], formed from an eight-carbon poly-β-keto acid. Obviously, γ-coniceine is a precursor of coniine and the other hemlock substances, but the knowledge of the eight-carbon compound which is converted to the former alkaloid is still like a black box [13, 19, 35, 51–53, 78].

At least eight piperidine alkaloids have been found in various parts of the plant [13, 14, 52]. The two predominant toxic alkaloids are coniine (mature plant and seeds) and γ-coniceine (young growing plant) [20, 30]. The leaves and stems prior to the development of seed heads are the most toxic part of the plant. The seeds themselves are highly toxic and can be a source of poisoning when they contaminate cereal grains fed to livestock. Alkaloid content varies with stage of

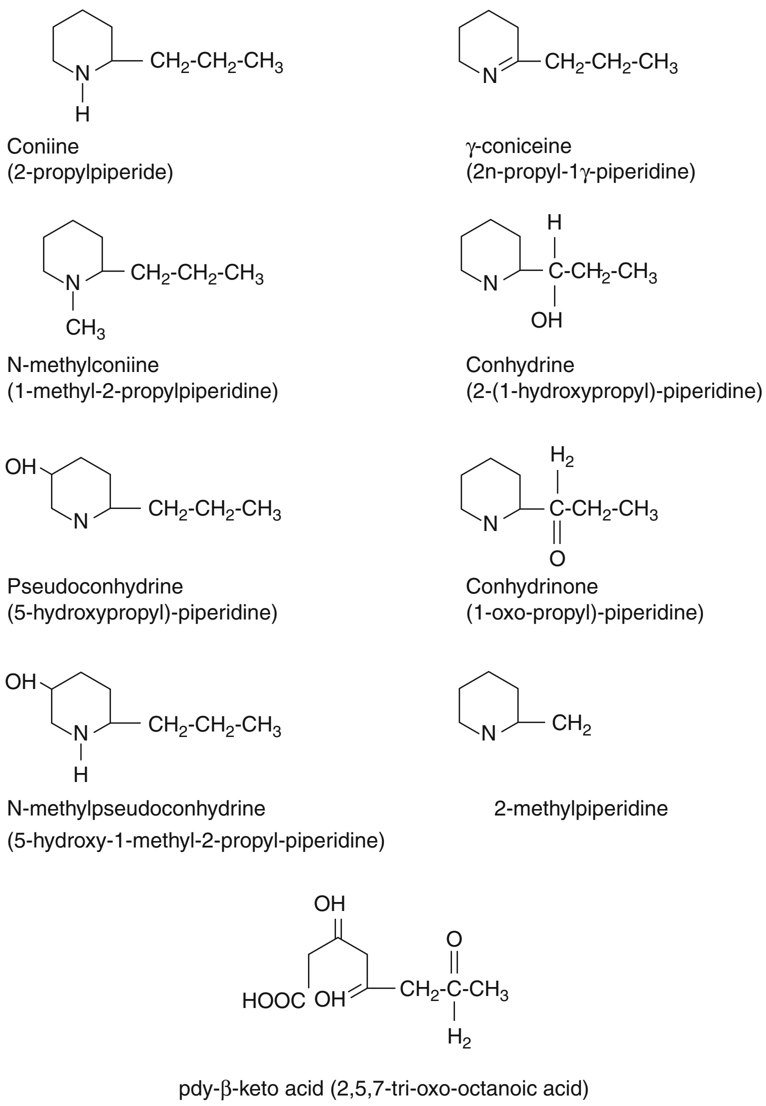

CH2-CH2-CH3
N
H
Coniine
(2-propylpiperide)
CH2-CH2-CH3
N
γ-coniceine
(2n-propyl-1γ-piperidine)
CH2-CH2-CH3
N
CH3
N-methylconiine
(1-methyl-2-propylpiperidine)
H
C-CH2-CH3
OH
N
Conhydrine
(2-(1-hydroxypropyl)-piperidine)
OH
CH2-CH2-CH3
N
Pseudoconhydrine
(5-hydroxypropyl)-piperidine)
H2
C-CH2-CH3
O
N
Conhydrinone
(1-oxo-propyl)-piperidine)
OH
CH2-CH2-CH3
N
H
N-methylpseudoconhydrine
(5-hydroxy-1-methyl-2-propyl-piperidine)
CH2
N
2-methylpiperidine
OH
O
HOOC
OH
CH2-C-CH3
H2
pdy-β-keto acid (2,5,7-tri-oxo-octanoic acid)

Fig. 26.1 Piperidine alkaloids in poison hemlock

development and stage of reproduction of plant. Young plants in the first year of growth are less toxic than mature plants, and those growing in the warmer southern states appear to be more toxic than those in the northern areas. Plants in the second year have alkaloid contents of approximately 1 % in all plant parts [4–6, 14, 18, 79] (Table 26.1).

Table 26.1 Individual and total alkaloid contents of *Conium maculatum*

	Coniceine (%DM)	Coniine (%DM)	Others (%DM)	Total alkaloid
Roots	–	–	–	<0.01
	ND	0.026	0.074	0.5
Dry stems	–	–	–	0.009
Leaves (vegetative growth)	–	–	–	1.49
	0.29	ND	0.03[b]	0.32
	0.86	0.06	0.02[a]	0.94
Flowers	0.75	0.2	0.09[a]	1.04
Green fruits	–	–	–	1.62
	0.36	0.42	0.25	1.03
	–	–	–	3
	0.23	ND	–	–
Ripe fruits	0.01	0.42	0.85[a]	1.07
	–	–	–	0.217
Shoots (vegetative growth)	0.15	ND	0.02[a]	0.17
	–	–	–	0.075
	–	–	–	0.023
	–	–	–	0.029
	–	–	–	0.02
	–	–	–	0.761
	0.18	0.01	–	–
Seeds	–	–	–	0.75
	–	–	–	0.92
	–	–	–	–
	–	–	–	0.019

ND not detected, *%DM* percentage in dry matter
[a]N-methylconiine
[b]Conhydrine

2 Phytochemistry and Isolation of Some Hemlock Plant Alkaloids

2.1 Isolation of the Alkaloids

Of the total alkaloids of hemlock isolated by the method of Chemnitius and fractionally distilled, the portion boiling up to 190 C contains most of the coniine, γ-coniceine, and *N*-methylconiine, while conhydrine and ψ-conhydrine remain in the higher boiling residues. For the separation of coniine from coniceine, recommends conversion into hydrochlorides. These are dried and extracted with acetone, which dissolves coniceine hydrochloride, leaving the coniine salt, from which the base may then be regenerated. For final purification, the coniine is converted into the ᴅ-hydrogen tartrate. It is sometimes necessary to start crystallization by adding a crystal of the desired salt. The crude-mixed alkaloids distill until the temperature rises to 190 C, benzoylate the distillate, extract the tertiary bases by shaking an ethereal solution with dilute acid, pour the concentrated ethereal solution into light petroleum to precipitate most of the benzoyl-δ-aminobutyl propyl ketone formed by the action of benzoyl chloride on coniceine, distill the solvent from the filtrate, and collect from the residue the fraction boiling at 200–210 °C/16 mmHg, which is nearly pure benzoylconiine (bp. 203–4 C/16 mmHg). From this a mixture of D- and L-coniines is obtained by hydrolysis, the former predominating.

2.2 Phytochemistry of the Alkaloids

These piperidine alkaloids have the simplest structures for alkaloids so far described. The hemlock plants are comprised of several potent alkaloids, such as *coniine, γ-coniceine, conhydrine, N-methylconiine, and pseudoconhydrine*. These alkaloids shall now be discussed as follows.

2.2.1 Coniine

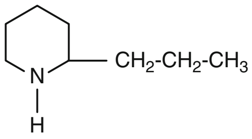
Coniine (2-propylpiperidine) can occur in two optically active forms according to configuration at C2, the (*S*) and (*R*) forms of which the former is the naturally occurring one. It is liquid at room temperature (melting point, −2 °C) and boils at 166 °C. Because of its volatility, it is best isolated as the hydrochloride (melting point, 220 °C), which crystallizes easily. Many synthetic routes have been-described in the literature, the earliest ones being a condensation between α-picoline and acetaldehyde or reduction of 2-allylpyridine by sodium in ethanol and the latest using modern, sophisticated reactions [55, 69, 70].

Synonyms: Cicutine, Conicine.

Biological sources: It is obtained from the unripe, fully grown dried fruits of *Conium maculatum* L. (*Umbelliferae*).

It also occurs in the plant *Aethusa cynapium L.* (*Apiaceae*) (Fool's Parsley), *Cicuta maculata* L. (*Apiaceae*) (water hemlock).

Chemical structure:



Coniine
(S)-2-Propylpiperidine.

It occurs naturally as the (S)-(+)- isomer.

Isolation. Coniine may be isolated by adopting the various following steps, namely:

(a) The powdered unripe, fully grown dried fruits of hemlock are mixed with a dilute solution of KOH and then subjected to stream distillation. The distillate is collected and neutralized carefully with dilute HCl and evaporated to dryness preferably under vacuum.

(b) The residue obtained as stated in (a) above is extracted with alcohol, filtered, and the alcohol evaporated to dryness under vacuum. The alcohol helps in extracting the alkaloidal salts that are dissolved in water; it is then rendered alkaline either with diluted KOH solution or with dilute NH_4OH and finally extracted with ether successively.

(c) The ether from the combined ethereal layer is evaporated completely, when an oily liquid consisting of the free bases remains in the residue.

(d) Finally, the residue is subjected to fractional distillation in a current of H_2 gas when the alkaloids could be broadly separated, and a mixture containing *coniine* and *γ-coniceine* shall pars over as the first fraction at 171–172 °C. These two alkaloids are consequently made to their corresponding hydrochloride salts, evaporated to dryness, and extracted with acetone. Thus, coniine hydrochloride would be separated as an insoluble product, while the *γ-coniceine* may be recovered by evaporating acetone under vacuum.

Coniine enjoys the unique distinction of being the first alkaloid produced synthetically.

Characteristic features:

- It is a colorless alkaline liquid.
- It darkens and polymerizes on being exposed to air and light.
- It has a mousy odor.
- Its physical parameters are as follows: bp 166–166.5 °C; $d\frac{20}{4}$ 0844–0848; $n\frac{23}{D}$ 1.4505.
- It is steam volatile.

Solubility: 1 ml dissolves in 90 ml of water, less soluble in hot water. The base dissolves in about 25 % water at room temperature. It is found to be soluble in alcohol, ether, acetone, benzene, amyl alcohol, and slightly soluble in chloroform.

Identification tests:

(a) It readily forms the corresponding hydrobromide ($C_8H_{17}N.HBr$), obtained as prisms, mp 211 °C, 1 g dissolves in 2 ml water, 3 ml alcohol, and is soluble freely in ether and chloroform.

(b) Its hydrochloride ($C_8H_{17}N.HCl$) forms rhomboids, mp 221 °C, freely soluble in water, alcohol and chloroform.

(c) It gives a red coloration with sodium nitroprusside slowly, which on addition of acetaldehyde changes to violet or blue.

Caution. It exhibits potential symptoms of overexposure as weakness, drowsiness, paresthesias, ataxia, nausea, excessive salivation, and bradycardia followed by tachycardia.

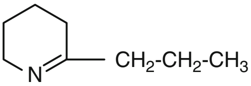
Uses. Externally, the coniine salts are used as ointments and infrequently employed for their local analgesic action in the symptomatic relief of pruritus, hemorrhoids, and fissures [45, 48, 74].

2.2.2 γ-Coniceine

γ-Coniceine (2,3,4,5-tetrahydro-6-propylpyridine) often accompanies coniine, sometimes occurring in greater quantities and is more toxic. As the free base is liquid (boiling point, 171 °C) at room temperature, it was better isolated as the hydrochloride (melting point 143 °C). The alkaloid was first synthesized by alkylation of phthalimide [70].

Biological source: It is obtained from the seeds of *Conium maculatum* L. (*Umbelliferae*).

Chemical structure:



γ-Coniceine
2, 3, 4, 5-Tetrahydro-6-propylpyridine.

Characteristic features:

- It is a colorless liquid alkaloid.
- It possesses a distinct mousy odor.
- It is steam volatile.
- Its physical parameters are bp 171 °C; d_{4}^{15} 0.8753; n_{D}^{16} 1.4661.
- It is slightly soluble in water, but freely soluble in ethanol, chloroform, and ether.

Identification test:

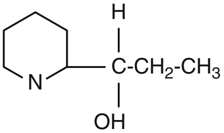
- γ-Coniceine when subjected to reduction, it gives rise to a racemic mixture of dl-coniine.
- It forms γ-coniceine hydrochloride ($C_8H_{15}N.HCl$) which gives hygroscopic crystals from ether mp 143 °C [21, 45, 46, 50, 70, 72, 75, 77].

2.2.3 Conhydrine

Conhydrine (2-(1-hydroxypropyl)piperidine) was recognized from early days as a minor, poisonous component of hemlock, where it occurs in the (+) form and could be separated by fractional distillation or crystallization. It is more soluble in water than coniine and crystallizes readily from ether (melting point, 121 °C). It was separated on a large scale from coniine [70].

Biological source: It is obtained from the seeds of *Conium maculatum* L. (*Umbelliferae*).

Chemical structure:



Conhydrine
[R-(R, S*)-a-Ethyl-2-piperidine methanol.*

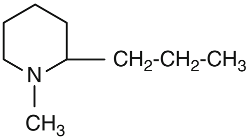
Characteristic features:

- The crystals obtained from ether have mp 121 °C, bp 226 °C, and $[\acute{\alpha}]_D$ + 10 °C.
- It is slightly soluble in water, but easily soluble in ethanol, ether, and chloroform [3, 45].

2.2.4 *N*-Methylconiine

Biological source: It is same as for conhydrine above.

Chemical structure:



N-Methylconiine

1-Methyl-2-propylpiperidine.

Isolation. The d-form is stated to occur in hemlock in small quantities, while thel-form may be isolated from residues left in the preparation of coniine by crystallization of the hydrobromides.

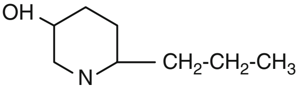
Characteristic features: The physical characteristic features of d-, l-, and dl-forms are given below (Table 26.2).

2.2.5 Pseudoconhydrine

Pseudoconhydrine (ψ-conhydrine, 6-propyl-3-piperidinol) is an isomer of conhydrine with the hydroxyl group on C5 (melting point, 105 °C), present in *Conium* usually as a minor component but reported unexpectedly as a major component in a certain American strain, growing in a greenhouse in early autumn, where it is formed from γ-coniceine [70].

Biological source: Its biological source is same as for coniine, through *N*-methylconiine above.

Chemical structure:



Pseudoconhydrine

(3S-trans)-6-Propyl-3-piperidinol.

Characteristic features:

(a) It gives hygroscopic needles from absolute ether.

(b) Its mp stands at 106 °C, whereas its monohydrate, scales, gives mp 60 °C from moist ether.

(c) Its physical parameters are bp 236 °C; $[\acute{\alpha}]^{20}_{D}$ +11° (c = 10 in alcohol); pK (18 °C): 3.70.

(d) It is soluble in water.

Table 26.2 The physical characteristic features of d-, l-, and dl-forms of the *N*-methylconiine [45]

Form	Nature	bp (°C)	d^{24}	$[\acute{\alpha}]_{D}^{24}$	n_{D}^{13}	Solubility
d-	Oily liquid	173–174	0.8318	+81°	1.4538	Slightly in water, soluble in organic solvents
l-	–	–	0.8318	–84°	1.4538	Slightly in water, soluble in organic solvents
dl-	–	10.5–56.6	–	–	–	–

Identification tests: It readily forms the hydrochloride salt ($C_8H_{17}NO.HCl$) as the crystals from ethanol having mp 213 °C [45].

Leaves and stems of a South African *Conium* species growing at high altitudes were found to contain *N*-methylpseudoconhydrine as a major component along with conhydrine. It occurred as only a minor alkaloid in plants growing at low altitudes. The free base is a liquid and is best prepared as its hydrochloride (melting point 157 °C).

Oxidation of the secondary alcoholic group of conhydrine yields conhydrinone, isolated from the plant as the crystalline hydrochloride (melting point, 249 °C) [3, 50, 70].

3 Biosynthesis of γ-Coniceine and Coniine

The γ-coniceine and coniine are generally the most abundant, and they account for most of the plant's acute and chronic toxicity. These alkaloids are synthesized by the plant from eight acetate units from the metabolic pool, forming a polyketoacid which cyclizes through an aminotransferase and forms gamma-coniceine as the parent alkaloid via reduction by an NADPH-dependent reductase.

A fatty acid precursor octanoic acid (capric acid) is employed, which is subsequently transformed into the ketoaldehyde through successive oxidation and reduction steps (Fig. 26.2). The resulting ketoaldehyde acts as a substrate for a transamination reaction; the amino moiety is derived from L-alanine [1, 8, 21, 30, 33, 45, 47, 49, 50, 55, 68, 70, 72, 74, 75]. The ultimate transformation leads to the formation of imine, giving the heterocyclic ring present in γ-coniceine, and then reduction of the coniine, as shown in Fig. 26.2.

4 Biosynthesis of *C. maculatum* Alkaloids

For a long time, secondary plant metabolites were considered as waste substances, accumulating during the plant life. Actually, they have a dynamic interaction with the primary metabolism and are involved in turnover and catabolic processes, fully participating in the whole scheme of the plant metabolism [3, 5, 44, 51, 52]. *C. maculatum* alkaloids are no exception. Their concentrations can undergo

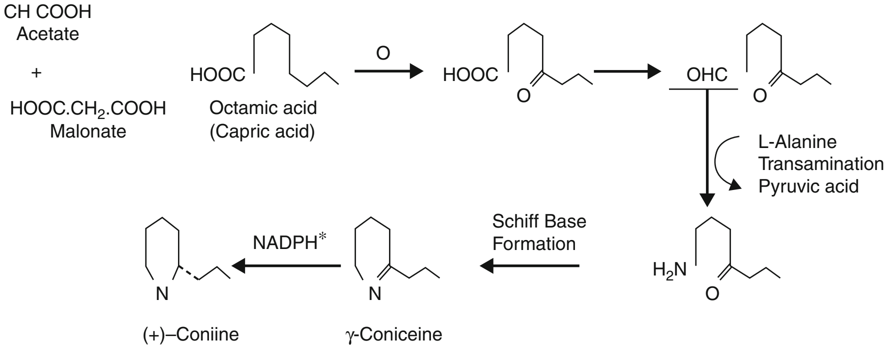


Fig. 26.2 Biosynthesis of γ-Coniceine and Coniine

frequent changes during the plant growth. It is remarkable that seeds present a high alkaloid content, while during the early stages of growth, a much lower alkaloid content is found in the foliage. Rapid changes in the alkaloid concentration are observed in different circumstances: wet or dry weather, different seasons, and degree of herbivory.

In early experiments related to changes of the concentrations of the two major alkaloids found in *C. maculatum* (coniine and γ-coniceine, the latter in much lower concentrations), it was noticed that coniine reached a maximum of about 6 μg/fruit at 4–5 weeks of growth depending on the year of collection, while γ-coniceine simultaneously, although not stoichiometrically, decreased to a minimum. Diurnal, large changes were also noticed in the concentrations of both alkaloids, the increase in one corresponding to a decrease in the other. According to these authors, minor quantities of other alkaloids were noticed: when coniine was at its maximum, *N*-methylconiine cooccurred, while conhydrine occurred in small amounts when coniceine predominated, but in this case *N*-methylconiine was absent [33, 52].

Biosynthesis of these compounds proceeds through a completely different route from that of the other piperidine alkaloids (Fig. 26.3). Early work pointed to γ-coniceine as the first alkaloid to be formed, followed by transformation to coniine, conhydrine, and *N*-methylconiine. Levels of these compounds varied greatly within the plant and under different environmental conditions and between varieties. Variations were even found between 2- or 4-h periods of a day and between days. This can be surprising, when, for instance, Leete and coworkers usually found coniine and conhydrine in equal amounts in their plants but on a subsequent occasion when the plants were grown in a greenhouse in the early autumn, coniine and pseudoconhydrine were the major components [44, 50–52, 68, 88].

It was proposed that the piperidine ring of *C. maculatum* alkaloids is produced by cyclization of lysine. However, attempts to demonstrate that lysine is a precursor of the piperidine ring in coniine by feeding D,L-lysine-2-^{14}C to the stems of *C. maculatum* plants, failed in producing labeled coniine.

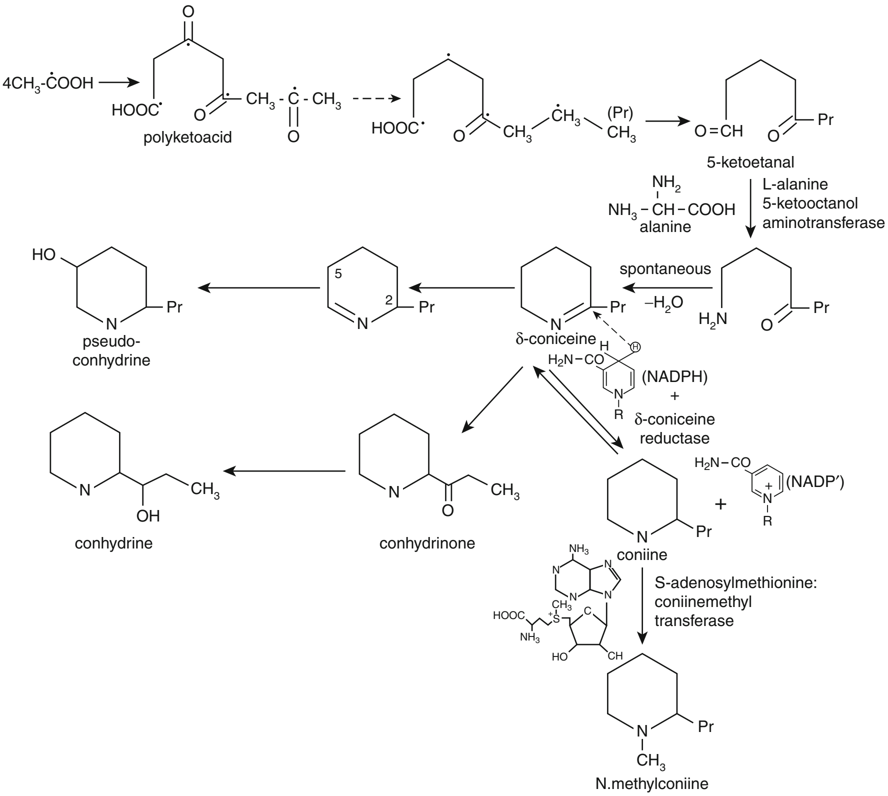

4CH3-ĊOOH
HOOC•
polyketoacid
CH3 - Ċ - CH3
O
HOOC•
CH3
ĊH3
(Pr)
CH3
O=CH
Pr
5-ketoetanal
NH2
NH3 - CH - COOH
alanine
L-alanine
5-ketooctanol
aminotransferase
spontaneous
−H2O
H2N
Pr
δ-coniceine
H2N-CO
(NADPH)
+
δ-coniceine
reductase
R
H2N-CO
(NADP′)
+
R
coniine
S-adenosylmethionine:
coniinemethyl
transferase
NH3
HOOC
CH3
HO
CH
N.methylconiine
CH3
Pr
HO
pseudo-
conhydrine
5
2
conhydrine
OH
CH3
conhydrinone
CH3

Fig. 26.3 Biosynthesis of *C. maculatum* Alkaloids

Uniformly labeled L-lysine-^{14}C administered to hemlock plants afforded radioactive coniine, but no degradative studies were produced by these authors to prove that labeling was present in the piperidine ring. Instead, feeding sodium acetate-1-^{14}C to 2-year-old *C. maculatum* plants indicated that alkaloids obtained from the plant (coniine, γ-coniceine, and conhydrine) presented labeling. Chemical degradation of the obtained coniine showed that activity was mostly and evenly distributed in even-numbered C-atoms, which allowed to speculate that hemlock alkaloids are derived from an 8-C aliphatic polyketo chain produced by the linear attachment of four acetate units probably through the condensation of 4 molecules of acetyl-CoA. The results of may be due to metabolization of lysine-^{14}C to radioactive acetate units, then incorporated to coniine.

Feeding γ-coniceine-1′-^{14}C to *C. maculatum* plants showed that all the activity was transferred to the C-1′ of the propyl side chains of coniine and pseudoconhydrine indicating that γ-coniceine can be the precursor of the former. It is interesting to note that similar feeding experiments conducted in a greenhouse and outdoors produced conhydrine and pseudoconhydrine, respectively, suggesting that changes in the environment can induce changes in the alkaloid composition of *C. maculatum.*

Later experimental work provided evidence that the 8-carbon polyketoacid intermediate in the synthesis of γ-coniceine is derived from octanoic acid since this acid was shown to be readily incorporated into coniine. Further work indicated that 5-keto-octanoic acid and 5-keto-octanal were produced during the biosynthesis of γ-coniceine. A transaminase (L-alanine: 5-keto-octanal aminotransferase) was obtained from *C. maculatum*. This transaminase catalyzes the reaction between 5-keto-octanal with L-alanine as the amino group donor to form the piperidine ring and the propyl side chain. Another *C. maculatum* alkaloid, *N*-methylconiine, was shown to be produced by another enzyme from the plant: a coniine methyltransferase which acts as a transmethylator utilizing *S*-adenosyl-L-methionine as a methyl group donor.

The production of coniine is catalyzed by an NADPH-dependent γ-coniceine reductase in a reversible reaction, since earlier experiments showed a fast interconversion between both substances, even in an hourly basis.

Glutamate-oxaloacetate aminotransferase (GOT) and GPT, possible aliphatic aminotransferases in the amination of 5-keto-octanal instead of L-alanine:5-keto-octanal aminotransferase, were excluded from that function by further work which separated the activities of the former from that of the latter, in turn shown to be composed of two isozymes, although no individual function was described.

γ-Coniceine has also been proposed as the source of pseudoconhydrine. Tautomerization of the double bond between N and C2 of the piperidine ring could produce the shift of the double bond to N and C6, yielding an allylic C5 which in turn could be oxidized to produce a hydroxy C5. It has been proposed that conhydrine is produced by oxidation of γ-coniceine to conhydrinone and its subsequent reduction to conhydrine [1, 3, 4, 8, 21, 30, 33, 44–50, 55, 68–70, 72–78, 88].

The d-stereoisomer of the *N*-methylconiine was separated from coniine, from which it was distinguished by its rotary power, in a plant extract provided by the Merck factory, and the l-stereoisomer separated as the hydrobromide, again from coniine, in a plant extract also from the Merck factory [15].

Five unidentified polar compounds from Conium fruits and leaves were recognized chromatographically and distinguished by their differing solubilities in aqueous ethanol. On hydrolysis, they yielded coniine or related alkaloids. It is postulated that the alkaloid moiety may be part of a molecule resembling NAD, replacing the nicotinamide portion. Furthermore, other activities for NAD and NADP have been described, especially those involving signaling [8], and, who knows, maybe coniine is involved in these.

5 Biological Activities

Despite its poisonous nature, *Conium* is included in several herbals as Succus conii, described as a narcotic, sedative, analgesic, spasmolytic, antiaphrodisiac, and anticancer agent. It was even listed at one time (up to 1934) in the British

Pharmacopoeia and the British Pharmaceutical Codex as a sedative and antispasmodic, the latter property leading it to be recommended as an antidote to strychnine [32]. More recently, it was speculated that by modifying the structure to remove some of the toxic properties, notably the teratogenic effects [52], a substitute for curare could be obtained for surgical purposes. The coniine alkaloids are neurotoxins, and death results from respiratory failure. Problems arise with livestock eating the plants which may grow in fields adjoining watercourses, or dried material inadvertently becoming included in hay.

The biological activities of the poison hemlock (*Conium maculatum*) are due to the content of piperidine alkaloids – coniine (2-propylpiperidine), γ-coniceine (2n-propyl-1γ-piperidine), conhydrine (2-(1- hydroxypropyl)-piperidine), *N*-methylconiine (1-methyl-2- propylpiperidine), pseudoconhydrine ((5-hydroxypropyl)-piperidine), conhydrinone ((1-oxo-propyl)-piperidine), *N*-methylpseudoconhydrine (5-hydroxy-1-methyl-2-propyl-piperidine), and 2-methylpiperidine. The poisoning effect of hemlock supplements by the presence of poly-β-keto acid (2,5,7-tri-oxo-octanoic acid), quercetin, kaempferol, and glycoside dioxymid [4, 12, 13, 20, 52, 85, 87].

5.1 Mechanism of Action

The mechanism of action of the conium alkaloids is complex because they have a profound effect in blocking spinal cord reflexes. After an initial stimulatory effect, the autonomic nervous system ganglia become depressed [6, 26, 56, 87]. Large doses of alkaloid cause skeletal muscle stimulation followed by neuromuscular blockade and paralysis similar to that caused by nicotine on the central and peripheral nervous systems [52]. In small quantities, the alkaloids cause skeletal defects in the fetal calf if poison hemlock is grazed by pregnant cows. Hemlock alkaloids have been found to have an action on spinal cord reflexes and depress autonomic activity and in large quantities cause neuromuscular blockade [6, 7, 13, 28, 56, 57, 61, 62, 87]. This action may lead to respiratory depression [9] and anoxic brain injury, with eventual death following within 24 h of ingestion [17]. Despite rhabdomyolysis and associated acute renal failure have been recorded regarding hemlock poisoning, no direct toxic effects have been described concerning the liver or kidneys [29, 71, 79].

The action of poison hemlock of the central nervous system seems to play a minor role, as the senses seemingly remain intact in humans and in animals. Since stimulation of the sensory cells does not result in muscle contraction, there is an increased resistance in the sensory cells [7, 13, 26, 56, 87]. The influence the hemlock alkaloids have on the heart is of minor importance. After preliminary stimulation the sympathetic and parasympathetic ganglia become paralyzed, the same reaction takes place with the respiratory medullary center, which results in a respiratory arrest and asphyctic agonal convulsions [9, 17].

5.2 Species-Related Sensitivity

In the literature, spontaneous cases and experimentally induced intoxications with poison hemlock (*Conium maculatum*) in cattle [10, 11, 37, 56–58, 61, 65, 66, 85, 87], pigs [16, 23, 24, 37, 59, 60, 85, 86], sheep [10, 62], goats [10, 63, 64], horses [10, 52], donkeys [10], elk [39], rats [2, 25, 26], rabbits [25], birds [27], and humans [9, 17, 22, 71, 79, 85, 87] are reported.

These investigations show that in animals, poison hemlock intoxication occurs in two clinical forms – acute and chronic [52, 85]. For the acute form, the sensitivity of animal species in descending order is as follows: cattle, sheep, goats, and swine whereas for the chronic form – cattle, swine, and sheep [52]. Our data [10, 11] for the sensitivity to acute intoxication showed that cattle were the most sensitive, followed by horses, sheep, and goats. Cattle have been fatally poisoned by eating as little 0.5% of their body weight of green hemlock [37, 56, 61].

Experimental hemlock poisoning in cattle, pigs, and sheep has been produced by a wide range of doses, suggesting that there is considerable variation in the toxic alkaloid content of the plant [37]. Cattle dosed with 1 mg/kg developed clinical signs, while 5.3 mg/kg of green plant was lethal [66]. In sheep, repeated poison hemlock doses of 10 mg/kg body weight were lethal [62]. Cattle were most susceptible to pure coniine administered by stomach tube, requiring 3.3 mg/kg body weight to induce severe poisoning. Mares required 15.5 mg/kg and sheep 44.0 mg/kg of the alkaloid to induce severe poisoning [66]. The lethal dose following ingestion of poison hemlock seeds in swine is 1 g/kg [59–61], and in men – 2 g/kg [29, 38, 52, 85, 87]. People are usually poisoned when they mistakenly eat hemlock for plants [70, 85]. A tea made from poison hemlock was reportedly used to kill Socrates [70].

Toxic incidents in men, after consumption of milk from cows having ingested poison hemlock in minimal toxic doses, have been reported [38]. Lethal doses of fresh *C. maculatum* are given below (Table 26.3).

There is information concerning the acute toxicity of pure coniine, γ-coniceine, and *N*-methylconiine. The oral LD50s in mice are 12 mg/kg for coniceine, 100 mg/kg for coniine, and 204.5 mg/kg for *N*-methylconiine [15, 83]. The single oral doses of pure coniine inducing acute toxic effects in some domestic animals are indicated in Table 26.4.

Livestock seldom eat hemlock because of its strong odor, but they will do so if no other forage is available or if it is incorporated in hay or silage [52].

5.3 Acute Poisoning

The most important and toxic of these is coniine, which has a chemical structure similar to nicotine [13, 14, 80]. Coniine is a neurotoxin, which disrupts the workings of the central nervous system and is toxic to humans and all classes of livestock [85]. Ingestion in any quantity can result in respiratory collapse and death.

Table 26.3 Acute lethal oral doses of fresh *Conium maculatum* in some domestic animals [52]

Animal species	Lethal dose (g/kg LW)	Alcaloid content (%)	γ-Coniceine (%)	γ-Coniceine doses (g/kg LW)
Sows	8.0	0.023	98	1.8
Cows	5.3	0.40	98	21
Sheep	10.0	0.40	98	39

Table 26.4 Acute effects of single oral doses of coniine in different domestic animals [52]

Species	Dose (mg/kg)	Degree of symptoms
Cows[a]	3.3	Severe
Mares[a]	15.3	Severe
Ewes[a]	44.0	Moderate
Quails[b]	25.0	Severe
Chicks[b]	50.0	Severe
Turkey chicks[b]	100.0	Severe

[a] Adult animals (maximum nonlethal doses)
[b] Three- to six-week-old animals (lethal to some individuals)

Coniine causes death by blocking the neuromuscular junction in a manner similar to curare; this results in an ascending muscular paralysis with eventual paralysis of the respiratory muscles which results in death due to lack of oxygen to the heart and brain [80]. Death can be prevented by artificial ventilation until the effects have worn off 48–72 h later. For an adult, the ingestion of more than 100 mg of coniine (approximately 6–8 fresh leaves, or a smaller dose of the seeds or root) may be fatal [70, 85].

When this group of piperidine alkaloid-containing plants is involved, the symptoms of poisoning are similar in all livestock production. The initial symptoms include nervousness, depression, grinding of the teeth, frothing around the mouth, relaxation of the nictitating membrane of the eye, frequent urination and defecation, and lethargy. Then eventually follows muscular weakness, tremors and fasciculations, ataxia, collapse, respiratory failure, and death [17, 38, 85]. Signs of toxicosis may appear as early as 1 h after ingestion and get worse the next 24–48 h even if further ingestion does not occur [9, 38, 70].

The poison hemlock intoxications in men are accompanied with ataxia, vibration, convulsions, nausea, vomiting, general indisposition, muscle weakness, slow movements and speech, loss of skin sensitivity, arrhythmia, and mydriasis. Prior to the death, paralysis of limbs, eyelid collapse, and mental sluggishness [22, 43, 67, 79, 87] occur.

Our studies [10, 11] upon the acute form of poison hemlock intoxications in cattle showed that the body temperature was elevated but in the course of the intoxication, prior to the lethal issue, it falls under the reference values. These data could be used for determination of the prognosis and the outcome of this type of intoxication in cattle. The heart rate is accelerated and the pulse – weak, soft, and arrhythmic. The respiration is accelerated, labored, and accompanied with stridors. In cattle, a high-grade frothy bloat with alkaline pH of rumen liquid is observed. Apart these alterations, refusal of feed, lack of defecation and urination, hypersalivation, lack of thirst, diffusely reddened conjunctivae and mydriasis, eyelid collapse, gasping and moaning, teeth grinding, disturbances in locomotion, muscle cramps, ataxia, hyporeflexia, dermal anesthesia, and convulsions were also observed.

In goats and sheep, the bloat is remitting and transient. In sheep having ingested poison hemlock when grazing, a profuse, bad-odored greenish diarrhea was observed by us. In donkeys, a bilateral abdominal distention, arexia, lack of defecation, weak and brief episodes of colic alternating with long painless pauses have been observed. During rectal examination, intestinal meteorism is determined. In horses, the episodes of colic are stronger and prolonged, as a result of acute ventricular dilatation [10].

5.4 Chronic (Teratogenic) Toxicity

Since the piperidines behave in a specific way and are teratogenic, they also fulfill specific criteria for teratogenesis [23, 24, 38, 70, 85]. The structural characteristics of these piperidines need to be determined and their main differences outlined to be able to find out their mechanism of action, as fetal movement and malpositioning. The birth defects caused by *Conium maculatum* are the same, and their biological activities occur by a similar mechanism of action [16, 52, 65, 66, 70]. As usual with biological active compounds one use to characterize the toxicity into acute and chronic forms. The peripheral actions of coniine are similar to those of nicotine, but it produces more pronounced paralysis of the central nervous system and of the skeletal muscle nerve [65, 70].

The molecular structure determines the teratogenicity (chronic toxicity) of *C. maculatum* alkaloids (no information has been found relating the acute toxicity and the structure of these alkaloids). The side chain must be a propyl group or larger. For instance, 2-ethylpiperidine has been shown to be nonteratogenic. Partial unsaturation seems to increase toxicity since coniceine is more toxic than coniine, while 2-propylpyridine is nonteratogenic, i.e., aromatization of the ring suppresses toxicity.

The related malformations caused by poison hemlock have been described in calves, piglets, kits, and lambs: at birth they show arthrogryposis, scoliosis, torticollis, cleft palate, and excessive flexure of the carpal joints (Fig. 26.4).

These injuries were reproduced experimentally to cows during the 55–75 days and sows during the 30–45 days of pregnancy [38, 40–42, 70, 85]. The piperidine toxins from poison hemlock induce in livestock reduction of fetal movement and fetal malpositioning [24, 40, 41, 59, 60, 62–66, 70, 82] and produce a similar sedative and anesthetic effect on the fetus as on the dam [16]. The fetal mandibular movements (particularly, the downward movement of the lower jaw) and tongue retraction are required for palatal shelves to become closer, allowing a normal closure of the

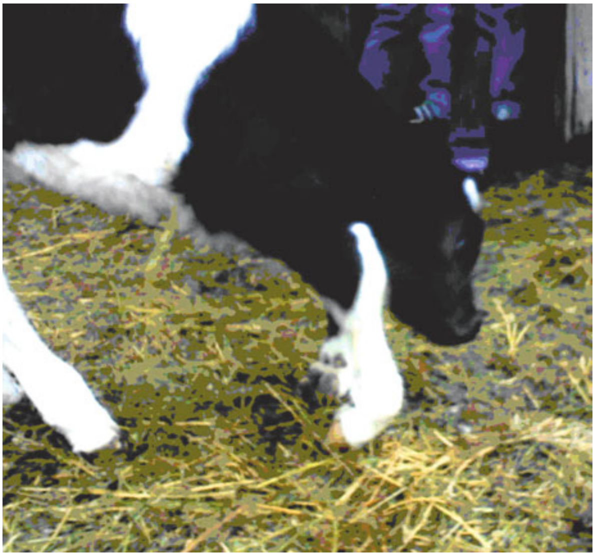


Fig. 26.4 Malformations caused by Poison Hemlock in calves: At birth they show arthrogryposis, scoliosis, torticollis, cleft palate and excessive flexure of the carpal joints

upper jaw. The absence of these movements can produce palatoschisis, since the tongue occupies the place where the palatal shelves must fuse.

Arthrogryposis has been questioned as an accurate name for the malformation, and it has been proposed to be replaced by multiple congenital contracture (MCC) [82]. These authors found that MCC is caused by loss of muscle mass with imbalance of muscle power at the joints which provokes a collagenic response consisting of partial replacement of muscle volume and collagenous thickening of the joint capsules, the latter leading to joint fixation.

It is possible that the known anesthetic effect of the alkaloids of *C. maculatum* inhibits the mentioned fetal movements. In consequence, the tongue obstructs the place where the shelves fuse together. Furthermore, the closure of the palatal shelves also depends on the separation of the fetal inferior jaw from the breast bone, which in turn depends on the fetal movements. If these movements are impaired, the lower jaw compresses the tongue against the upper part of the oral cavity, which impedes the mutual approaching of both palatal shelves [24].

Seeds and fresh *C. maculatum* administered orally to goats twice daily during 30–60 days of gestation produced malformations in the offspring (palatoschisis and arthrogryposis). Ultrasonic control of fetal movement was performed during 12 h at 45, 51, 55, and 60 days of pregnancy. *C. maculatum* seeds produced almost total fetal immobility, while the fresh plant produced the same effect, but lasting only 5h. Goat kids born to dams dosed with seeds showed palatoschisis and arthrogryposis, while those born to dams fed with the fresh plant showed minor flexures of forelimbs, which receded a few weeks after birth [63, 64]. This lack of fetal movement caused by *C. maculatum* had been also observed in pigs [23, 24, 59, 60, 82] and in sheep [62]. In the latter, only slight front limb abnormalities were found, which

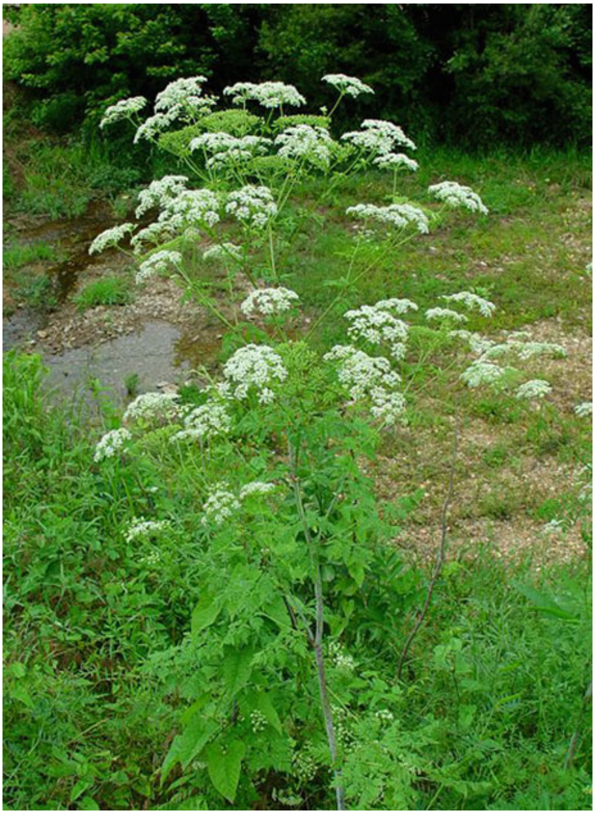


Fig. 26.5 Poison Hemlock (*Conium maculatum*)

receded in about 8 weeks, this being consistent with the already mentioned lower chronic toxicity produced by *C. maculatum* in sheep.

The four main alkaloids are coniine, γ-coniceine, *N*-methylconiine, and conhydrine. Conhydrine occurs in the smallest proportions and has the weakest pharmacological action [3, 4, 19, 20, 31, 67, 85, 87]. The most distinctive action of the three other hemlock alkaloids plus nicotine is their ability, provided the dose is small, to inhibit the crossed extensor reflex and the so-called knee-jerk by an action potential in the spinal cord. Since neurons in the spinal cord may both be inhibited and activated by the action of hemlock, the mechanisms involved are a bit complicated. If the alkaloids initially stimulate inhibitory neurons rather than blocking excitatory ones, the antagonistic relationship of the two substances may be explained. There is evidence that its central synapse is controlled by polysynaptic inhibitory pathways, although the patellar reflex is monosynaptic [3, 19, 67]. It is possible that the *C. maculatum* piperidine alkaloids may act upon the mechanism that regulates the amniotic liquid, adding to the production of malformations. Finally, lack of fetal movement, whatever its origin, can also cause limb malformations.

5.5 Medicinal Uses

Poison hemlock has been used as a sedative and for its antispasmodic properties. It was also used by Greek and Persian physicians for a variety of problems, such as arthritis. It was not always effective, however, as the difference between a therapeutic and a toxic amount (the therapeutic index) is very slight. Overdoses can produce paralysis and loss of speech, followed by depression of the respiratory function, and then death.

6 Conclusion

The study and use of alkaloids over the past 200 years is a success story. The acetate-derived alkaloids contained mainly in the plant poison hemlock (*Conium maculatum*) which is a very common and worldwide plant species, and it is one of the most toxic plants known. Levels of the various alkaloids are fluid with formation and interconversions being strongly influenced by plant variety, plant part, age, season, and growing conditions. Toxicity is due to a group of alkaloids of which the representative members are coniine and γ-coniceine. It has been suggested that the compounds are involved in an oxidation–reduction system of metabolic significance in the plant. The acetate-derived alkaloids derive biosynthetically from acetate units via the polyketide pathway in contrast to other piperidine alkaloids which derive from lysine. Coniine stimulates and then depresses autonomic ganglia and motor end plates in skeletal muscle. Large doses can cause neuromuscular block. Both acute and chronic symptoms have been described. The compounds are neurotoxins, and death results from respiratory failure. Chronic nonlethal ingestion by pregnant livestock leads to fetal malformation.

My purpose was to compose a beneficial text for an academic and professional audience that could also serve as a source of knowledge for anyone who is interested in the fascinating subject of alkaloids.

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27 Ephedra Alkaloids-Alkaloids Derived by Amination Reaction: Phenylalanine Derived

Ali Parsaeimehr and Elmira Sargsyan

Contents

1 Introduction 910
2 *Ephedra* 910
2.1 Ephedrine, its Derivatives, and their Pharmacological Uses 912
2.2 The Biosynthesis Pathway and Expression of Ephedrine in *Ephedra* 913
2.3 Methods of Analysis for Ephedrine and its Derivatives 915
3 Percentage of Ephedrine in Different *Ephedra* Species 916
4 Biotechnological Methods for Ephedrine Production 917
4.1 In Vitro Culture of *Ephedra* and Ephedrine Production 917
4.2 Biotransformation Techniques for Ephedrine Production 919
5 Perspective 920
References 920

Abstract

Ephedrine is an alkaloid derived from various plants in the genus *Ephedra* (family Ephedraceae). The alkaloid is comparable in actions to adrenaline. It has many medicinal and pharmaceutical applications; it is a bronchodilator to relieve shortness of breath, chest tightness, wheezing, and cough associated with bronchial asthma. Amino acids such as phenylalanine and tyrosine serve as the precursors for a large number of alkaloids, including ephedrine, capsaicin, and colchicines. Although ephedrine is produced by chemical synthesis or enzymatic methods, it is also obtained from *Ephedra* species. This chapter provides general information about *Ephedra*, ephedrine, and its derivatives, discusses methods to analyze them, and finally presents achievements in this area of science.

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Biotransformation • *ephedra* • ephedrine • pseudoephedrine • tissue culture

Abbreviations

2 4-D	2, 4-dichlorophenoxyacetic acid
BA	Benzyl adenine
BAP	6-Benzylamino purine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kin	Kinetin
MS	Murashige and Skoog’s medium
NAA	1-naphthaleneacetic

1 Introduction

In today’s world, some plant-derived secondary metabolites play a great role in pharmaceutical production, among them nitrogen-containing natural compounds called alkaloids. Some alkaloids are produced by synthesis or enzymatic methods and others are obtained from plants. Unfortunately, overexploitation of these plants has made them endangered species. Therefore, appropriate technologies for sustainable use and sufficient focus on the biosynthetic routes and methods of isolation of these secondary metabolites derived from the plants are required.

Ephedrine, an alkaloid with an amphetamine-like structure, is currently used in pharmaceuticals, with potentials to affect nerves, muscle, blood pressure, and heart rate. In 2004, the US Food and Drug Administration (FDA) banned ephedrine alkaloids marketed for reasons other than asthma, colds, allergies, other disease, or traditional Asian use. Ephedrine and its derivatives (stereoisomers and diastereomers) are naturally found in varied proportions in different plant species such as *Catha edulis* [12], *Roemeria refracta* [48], *Taxus baccata* [20], *Sida cordifolia* [7], *Pinellia ternata* [24], *Aconitum napellus* [8], and *Ephedra* species [22, 29, 32, 37].

This chapter describes *Ephedra* alkaloids, their biosynthetic pathway, methods to analyze them, and biotechnological methods for their production.

2 *Ephedra*

Ephedra is a leafless evergreen plant, the only genus in the family of Ephedraceae and order of Gnetales, and a genus of gymnosperm that is introduced as a medicinal plant. This plant grows in dry climates in Central Asia, North America, the southern part of Europe, and the northern part of Africa. It reaches to about 60–100 cm high [23]. The Chinese have used *Ephedra sinica* (*má huáng*) for thousands of years to treat allergies, bronchial asthma, chills, colds, coughs, edema, fever, flu, headaches, and nasal congestion. There are more than forty species recognized in the literature,

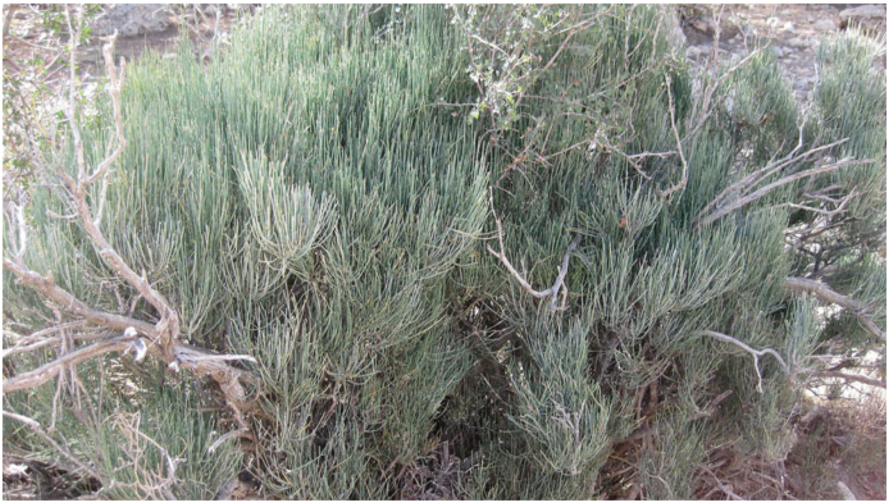

Fig. 27.1 Habitat of *Ephedra strobilacea*

however, the primary species of *Ephedra* are *E. sinica* Stapf, *E. major* Host, *E. gerardiana* Wall, *E. equisetina* Bunge, and *E. intermedia* Schrenk & Meyer [9]. Generally, the plant is a small woody bush, as shown in Fig. 27.1.

Ephedra is a strong stimulant and acrid-tasting herb. According to legends, the bodyguards of Genghis Khan, who were threatened with beheading if they fell asleep on sentry duty, used to drink a tea containing *Ephedra* to stay alert [3]. *Ephedra* has been a natural source of alkaloids such as ephedrine, pseudoephedrine, norpseudoephedrine, and other related compounds that are highly correlated with stimulatory actions. The total alkaloid content of *Ephedra* is approximately 1–2.5 %, and ephedrine and pseudoephedrine are more than 80 % of the alkaloid content of dried plant. Generally, extraction of ephedrine-type alkaloids from crude plant material involves an acid/base extraction procedure [4, 27, 35, 40].

There have been significant developments in the isolation of the other pharmacological compounds from different species of *Ephedra* besides ephedrine and its derivatives, such as kynurenic acid derivatives, methanoproline amino acids, flavones, tannins, carboxylic acids, volatile terpenes, and other compounds such as *cis*-3, 4-methanoproline, and a cyclopropyl analogue of proline or quinaldic acid known as an antimicroorganism agent [10, 19, 21, 44]. In this regard, researchers have reported the antimicrobial activity of *Ephedra* extracts against such microorganisms as *Vibrio parahaemolyticus*, *Clostridium perfringens*, *Bacillus subtilis*, and *Staphylococcus aureus*. Furthermore, there are significant reports on the presence of antioxidants in *Ephedra* species. Using a FRAP method (ferric reducing antioxidant power) on *Ephedra strobilacea*, an amount of 1.61 ± 0.08 mmol eq quercetin g^{-1} extract was recorded [32]. The antioxidant activity of *Ephedra* extract is perhaps correlated with the existence of reported phenolic compounds such as benzoic, p-hydroxybenzoic, cinnamic, p-coumaric, vanillic, and protocatechuic [10].

Another outstanding aspect of *Ephedra* lays behind its antimutagenic activity. The extracts of in vitro culture of *Ephedra* were tested against benzo[a]pyrene, 1, 6-dinitropyrene and 3, 9-dinitrofluoranthene and showed a remarkable inhibition against benzo[a]pyrene, whereas that inhibition against 1, 6-diNP and 3, 9-diNF was varied from 20 % to 86 %. Researchers concluded that the differences in inhibition might be due to inactivation of metabolic enzymes [12].

Recently, many dietary supplements containing *Ephedra* powder have received increasing attention for their use in aiding weight control and boosting performance and energy. However, there is also evidence that dietary supplements containing ephedrine alkaloids pose a risk of serious adverse events including heart attack, stroke, and death. Due to these results, the FDA has prohibited the sale of some dietary supplements containing *Ephedra* alkaloid, excluding its use in decongestants and medicines for colds and the treatment asthma [4, 25].

2.1 Ephedrine, its Derivatives, and their Pharmacological Uses

Ephedrine is an alkaloid, a sympathomimetic amine with molecular formula C_{10} H_{15} NO_1, a molecular mass of 165.2, and the structural name (1*R*, 2*S*)-2-methylamino-1-phenylpropan-1-ol. This bitter colorless or white solid-crystal is completely soluble in water, alcohol, chloroform, ether, and glycerol. Ephedrine is also produced by chemical synthesis and there is significant documentation of commercial ephedrine production using microbial biotransformation techniques [42]. Ephedrine has a structure close to methamphetamines, and its stimulant actions are comparable to epinephrine (adrenaline), a hormone produces by the adrenal glands that enhances heart rate and constriction of blood vessels in high-stress situations. Medicinal use of ephedrine began around 3000 B.C with the Chinese from *má huáng*, but its isolation was first reported in 1855 and its pharmaceutical application started in 1930 [22]. Studies on ephedrine's molecular structure show that two asymmetric carbon atoms are involved in ephedrine's molecular skeleton; therefore, four optically active stereoisomers forms naturally occur as follows: (1*R*, 2*S*)-(−)-ephedrine, (1*S*, 2*R*)-(+)-ephedrine, (1*R*, 2*R*)-(−)-pseudoephedrine, (1*S*, 2*S*)-(+)-pseudoephedrine (Fig. 27.2).

Notably, pseudoephedrine is a diastereomer of ephedrine and considerably racemic ephedrine (dl-ephedrine) has not been found naturally, however, it is prepared synthetically and is inactive for commercial purposes. Ephedrine and pseudoephedrine are completely stable compounds under changing temperature conditions, but they are quite unstable when exposed to sunlight or in the presence of oxygen pressure [2]. The unique molecular structure of ephedrine causes its different stereoisomers to be valuable for pharmaceutical applications such as nasal decongestant, pupil dilator, bronchodilator, and central nervous system stimulant. Ephedrine is a sympathomimetic substance and the principle mechanism of ephedrine activity is its influence, by enhancing the activity of noradrenalin, on post-synaptic α- and β-receptors in the nervous system. Stimulation of α 1-adrenergic receptors produces contraction of vascular smooth muscle,

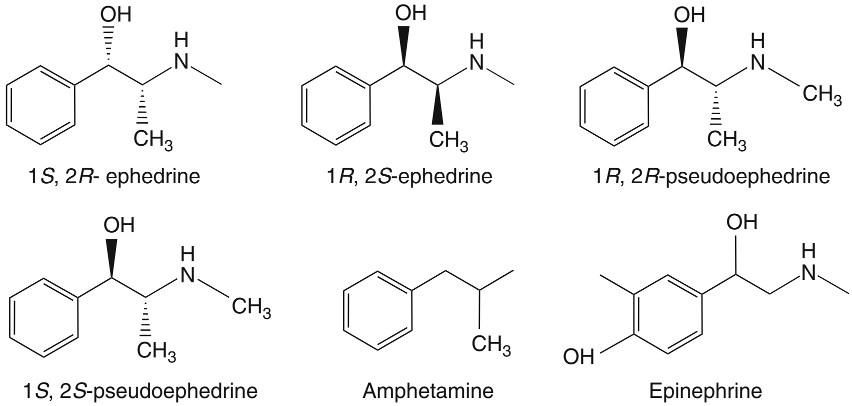

Fig. 27.2 Comparison of ephedrine and its derivatives with Amphetamine and Epinephrine

increased contractile force of the heart and arrhythmias, glycogenolysis, gluconeogenesis, hyperpolarization, and relaxation of intestinal smooth muscle. Stimulation of β_2-adrenergic receptors decreases insulin secretion, platelet aggregation, and the release of norepinephrine from the nerve terminals, and causes contraction of vascular smooth muscle [9]. It is also reported that ephedrine has the ability to metabolize to norephedrine (phenylpropanolamine), which may be responsible for the central nervous system stimulant effects that lead to weight lose; it seems that the mechanism for loss appears to be by increasing the metabolic rate of adipose tissue [6, 26]. Unfortunately, ephedrine has been correlated with toxic psychosis and based on this result the demand is increasing for d-pseudoephedrine, the ephedrine diastereomer that has fewer side effects compared with ephedrine; it also has a weaker, longer-lasting influence on the central nervous and cardiac system [43, 47].

2.2 The Biosynthesis Pathway and Expression of Ephedrine in *Ephedra*

About 5,000 different alkaloids are known and most of them are derived from amino acids. Typically, amino acids such as phenylalanine, tyrosine, and their corresponding decarboxylation products serve as the precursor for a large number of alkaloids, including ephedrine, capsaicin, colchicines, and even opium alkaloids. The class of phenylalkylamines in *Ephedra*, such as ephedrine, is originally derived from phenylalanine [15, 16, 41]. Evidently, the production of ephedrine and its derivatives in *Ephedra* starts via the biosynthetic pathway of shikimic acid by transformation of phenylalanine in to benzaldehyde (C_6H_5CHO) that consequently leads to a reaction to synthesize cathinone (benzoylethanamine), a monoamine alkaloid molecule from which ephedrine and pseudoephedrine are

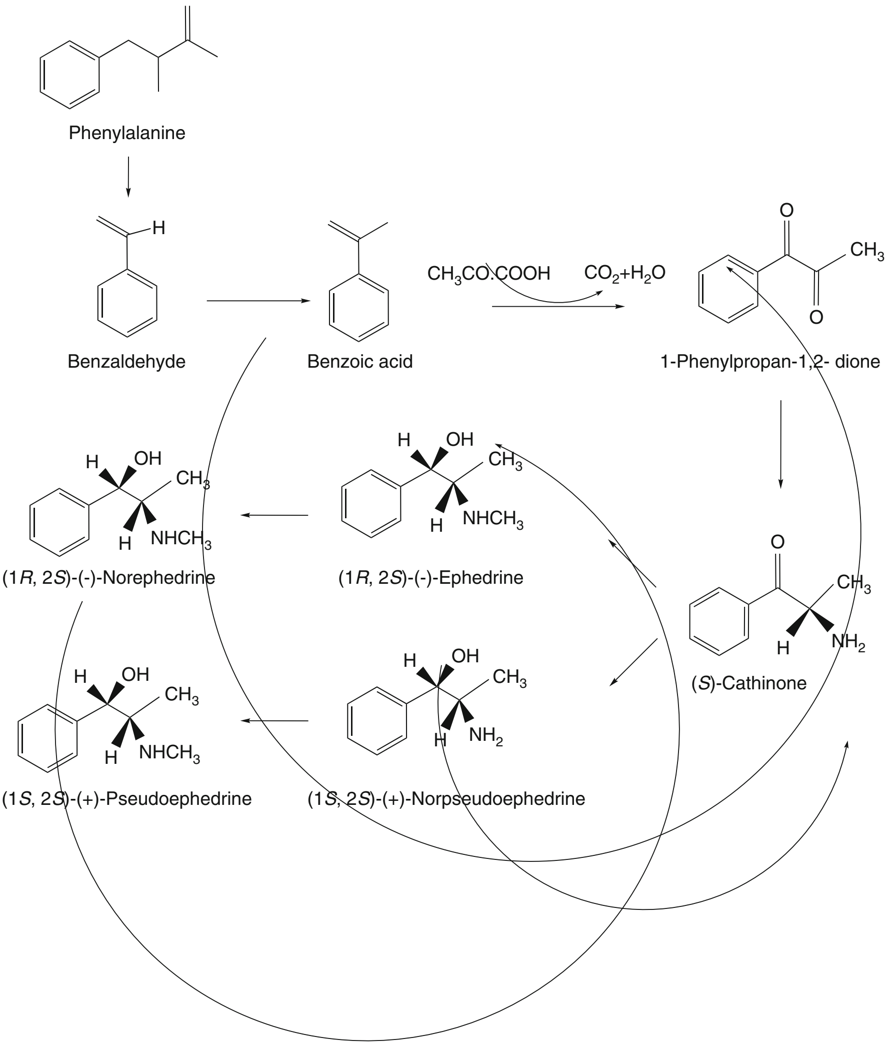
Phenylalanine
H
Benzaldehyde
Benzoic acid
$CH_3CO.COOH$
CO_2+H_2O
O
CH_3
O
1-Phenylpropan-1,2- dione
H
OH
CH_3
H
$NHCH_3$
(1*R*, 2*S*)-(-)-Norephedrine
H
OH
CH_3
H
$NHCH_3$
(1*R*, 2*S*)-(-)-Ephedrine
O
CH_3
H
NH_2
(*S*)-Cathinone
H
OH
CH_3
H
$NHCH_3$
(1*S*, 2*S*)-(+)-Pseudoephedrine
H
OH
CH_3
H
NH_2
(1*S*, 2*S*)-(+)-Norpseudoephedrine

Fig. 27.3 The biosynthesis pathway of ephedrine in Ephedra

derived (Fig. 27.3). Using ^{13}C nuclear magnetic resonance spectroscopy, researchers reported that the labeled C_2 fragment of [2, 3-$^{13}C_2$] pyruvic acid is transferred intact into the *C*-methyl group of the adjacent carbon atom of the *Ephedra* alkaloids. This observation lead to identification of pyruvate as the elusive precursor of the aliphatic C_2 terminus of the skeleton of ephedrine. In other words, the carbon skeleton of ephedrine is generated from pyruvate and either benzaldehyde or benzoic acid by a reaction analogous to the formation of acetoin or diacetyl from pyruvate and acetaldehyde or acetic acid, respectively [11].

2.3 Methods of Analysis for Ephedrine and its Derivatives

Secondary metabolites include a wide variety of compounds with different structures and chemical properties. Typically, isolation and estimation of secondary metabolites are carried out by extraction followed by quantification and separation using thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC) and mass spectrometry (MS), nuclear magnetic resonance (NMR), or absorption spectroscopy.

Choosing the right solvent system and extraction method leads to reliable and reproducible results. Sonication and microwave extraction methods are frequently used to optimize the yield of the target compound. Currently, different methods are reported by different groups to extract ephedrine and its derivatives, and solvents such as dichloroethane, benzene, chloroform, toluene, chlorobenzene, amyl-, ethyl- and isopropyl- alcohol, and aqueous solutions have been widely tested [10]. The most frequently used method is the extraction by methanol-water (5:5) under the influence of sonication for 15–25 min, followed by centrifugation at 5,400–7,000 rpm for 7–10 min, and subsequently the supernatants of the samples are quantitatively and qualitatively explored by TLC, HPLC, or GC/MS devices [31, 33, 34, 45, 46].

To determine the presence of ephedrine and its derivatives using TLC, a solution of butanol, glacial acetic acid, and water (4:1:1) as the mobile phase and ninhydrin (2,2-Dihydroxyindane-1,3-dione) for localizations has typically been used [28, 29, 34]. O'Dowd and colleagues reported that ethanol extract of in vivo or in vitro grown plants often contains compounds that show a positive test for ephedrine and pseudoephedrine with ninhydrin in TLC, but such compounds were absent in HPLC analysis. Therefore, TLC cannot be used as a sufficient and reliable method and, in parallel, a more confirmative method must be conducted [28].

A simple method developed for the quantitative determination of ephedrine in *Ephedra* callus was reported using a HPLC device. In this method, a Tris–NaOH–H_3PO_4 ($pH = 3.2$) run buffer was used; the detector was adjusted to 185 nm. In order to increase the solubility of hydrophobic analyses and improve the separation efficiency, 15 % acetonitrile was used in the buffer as a modifier [22]. Sheu and Huang have presented two methods for ephedrine and its derivatives determinations. As a Cosmosil 5C18-MS column was used in their experiment with a gradient solvent system consisting of a phosphate buffer and acetonitrile, the detector was adjusted at 210 nm with a retention time of 50 min. Alternatively, an isocratic solvent system of a sodium dodecylsulfate-acetonitrile solution was also tried with a retention time of 35 min [45].

Quantitative analysis of *Ephedra* alkaloids using a GC or GC/MS devices is difficult considering their retention time and the task of distinguishing them from diastereomers. Cui et al. analyzed 12 species of Chinese *Ephedra* by trimethylsilyl (TMS) derivatization of *Ephedra* alkaloids using a GC/MS device equipped a nitrogen phosphorous detector and helium as the carrier gas with a flow rate of 0.9 mL min^{-1}; the smallest quantity detected by this method was less than 2 ng [5].

Another reliable quantitative analysis of ephedrine alkaloids using an H-NMR was also described by Kim et al. A certain amount of sample was mixed with 0.5 M H_2SO_4 solution and sonicated for 1 h, then centrifuged for 20 min at 3,000 rpm. Subsequently, the supernatant layer was transferred to a tube containing 5 M KOH solution, NaCl and diethyl ether. Samples were centrifuged again and the diethyl ether layers were combined into a flask and evaporated after addition of an internal standard. Finally, dried samples were dissolved in $CDCl_3$ (deuterated chloroform) and used for H-NMR measurement. The peak of each *Ephedra* alkaloid was quite well separated from the others. The H-NMR method was also found to be highly accurate with low standard deviation (5–10 %). This result was similar to that of HPLC and all four alkaloids can be detected without any derivatization [17].

3 Percentage of Ephedrine in Different *Ephedra* Species

A wide variation exists in alkaloid content among different species of *Ephedra* in different parts of the world, with the highest total alkaloid content of 2.5 % in *Ephedra equisetina*. In fact, the yield of ephedrine in the extract of different species of *Ephedra* varies from 55 % to 83 % [10, 13]. Cui and colleagues reported that the highest amount of ephedrine among different species analyzed was in *Ephedra monosperma* Gmel. Ex Mey (1.401 %), while the highest amount of pseudoephedrine was in *Ephedra intermedia* Schrenk ex (0.912 %) [5] (Table 27.1).

Parsaeimehr et al., using a HPLC gradient method, reported the presence of ephedrine and pseudoephedrine in three species of *Ephedra* and observed that the content of ephedrine and pseudoephedrine varied from species to species. The highest amount of ephedrine was recorded in *E. strobilacea* (2.7 mg g^{-1} dry weight), while the highest amount of pseudoephedrine was recorded in *Ephedra procera* (3.7 mg g^{-1} dry weight) [33] (Table 27.2).

Ephedra alkaloids content were determined by O'Dowd and colleagues, who reported the highest amount of ephedrine and pseudoephedrine in *E. equisetina*, while the lowest amount was recorded in *Ephedra andina* [28] (Table 27.3).

Using an H-NMR device, Kim and colleagues reported that the content of the four analyzed ephedrine alkaloids in their samples was quite different, varying from 1 % to 2 % of dry weight. Among two species, *E. intermedia* had a lower amount of total alkaloids (0.94 %) and ephedrine content compared with pseudoephedrine than *E. sinica* (Table 27.4) [17].

Molecular analysis combined with chemical assessments on collected *s*pecies of *Ephedra* from Mongolia revealed that the *Ephedra* plants were divided into nine genotypes based on the nucleotide sequences of 18S ribosomal RNA (rRNA) gene and *trn*K gene. The chemical analysis showed that the amount of ephedrine and its derivatives vary from genotype to genotype or even in similar genotypes. The *E. sinica* genotype Si-II showed the highest ephedrine accumulation; the lowest accumulation ephedrine and its derivatives was recorded in *Ephedra przewalskii* irrespective of its different genotypes [18].

Table 27.1 Alkaloid content (% dry weight) of different species of *Ephedra* (After Ref. [5])

Species	NPE	NE	E	PE	ME	MPE
E. equisetina	0.155	0.198	1.250	0.580	0.025	ND
E. intermedia	0.111	0.075	0.550	0.912	0.026	ND
E. likiangensis	0.170	0.051	0.630	0.606	0.027	Trace
E. monosperma	0.330	0.180	1.401	0.860	0.054	0.005
E. gerardiana	0.074	0.078	0.765	0.101	0.040	Trace
E. lomatolepis	0.320	0.037	0.167	0.830	0.005	Trace
E. przewalskii	0.005	0.003	0.029	0.006	0.003	ND

NPE norpseudoephedrine, *NE* norephedrine, *E* ephedrine, *PE* pseudoephedrine, *ME* methylephedrine, *MPE* methylpseudoephedrine, *ND* Not detected

Table 27.2 Alkaloid content (µg/g dry weigh) of different species of *Ephedra* (After Ref. [33])

Alkaloids	*E. procera*	*E. pachyclada*	*E. strobilacea*
Ephedrine	884.42 ± 59.85	821.01 ± 81.42	2670.68 ± 10.15
Pseudoepehdrine	3679 ± 69.75	948 ± 75.40	1575.17 ± 62.09

Table 27.3 Alkaloid content (% dry weight) of different species of *Ephedra* (After Refs. [28, 29])

Alkaloids	*E. andina*	*E. equisetina*	*E. intermedia*	*E. major*	*E. minima*
Ephedrine	Trace	0.80	0.06	0.27	0.50
Pseudoepehdrine	0.13	0.50	0.15	0.75	1.04

Table 27.4 Alkaloid content (mg/g dry weight) of different species of *Ephedra* using H-NMR (After Ref. [17])

Species	E	PE	ME	MPE	Total alkaloids (%)
E. sinica	8.98 ± 1.23	3.92 ± 0.44	1.30 ±0.18	0.75 ±0.10	1.49
E. intermedia	2.15 ± 0.31	6.29 ± 0.87	0.22 ± 0.04	0.76 ± 0.14	0.94

Age of the plant, altitude, season of collection, and the sample tissue (primary or secondary shoots) on all have a significant influence on alkaloid content of *Ephedra*. However, the highest amount of ephedrine accumulates in older green stems at the mid-internodes [1, 14, 28, 29] (Table 27.5).

4 Biotechnological Methods for Ephedrine Production

4.1 In Vitro Culture of *Ephedra* and Ephedrine Production

Plant cell and tissue cultures are excellent systems for the production of secondary metabolites. O'Dowd and colleagues examined the influence of different types of plant growth regulators on alkaloid content of callus derived from different species

Table 27.5 Alkaloid content (% dry weight) of different species of *Ephedra* grown in Mongolia (After Ref. [18])

Species	Genotype	E	PE	NE	NEP	ME
E. sinica	Si-II	2.14	0.22	Trace	0.07	0.32
E. sinica	Si-I	0.99	0.84	0.15	0.29	Trace
E. equisetina	E-I	0.14	4.45	0.12	0.12	0.07
E. equisetina	E-I	0.15	3.66	Trace	0.07	0.10
E. przewalskii	Pr-I	–	Trace	–	–	–
E. przewalskii	Pr-II	–	Trace	–	–	–
E. regeliana	Rg-I	0.59	2.25	0.13	0.14	0.08
E. regeliana	Rg-II	0.41	1.44	Trace	Trace	0.10

of *Ephedra*. All *Ephedra* species were grown on MS medium supplement with 0.25 μM kinetin and 5.0 μM 2,4 dichlorophenoxy acetic or 1-naphtaleneacetic acid as callus cultures, and trace amounts of ephedrine and 0.01 % of pseudoephedrine on a dry weight basis were recorded. In comparison, 0.8 % and 0.5 % of ephedrine and pseudoephedrine, respectively, on a dry weight basis were recorded in the stem of *Ephedra equistina* [28, 29].

Many factors influence callus growth and secondary metabolite content, but the major ones are genotype, physiological status of the plant, tissue source, chemical composition and physical state of the culture medium, and the culture conditions of callus growth. Callus cultures of *Ephedra foliata* and *Ephedra gerardiana* were grown on MS medium supplemented with 2.3 μM Kin and 54 μM NAA. *E. foliata* callus contained no alkaloids whereas that of *E. gerardiana* contained 0.17 % ephedrine after 8 weeks growth. The authors recorded one tenth the ephedrine content in the callus of *E. gerardiana* compared with the stem. 2, 4-D reduced the yield of ephedrine content to 0.13 %, but by replacing NAA with IBA, the maximum level of ephedrine (0.3 % of dry weight) was recorded [37–39].

Different plant growth regulators had a significant impact on growth of in vitro cultures of *E. monosperma* and accumulation of alkaloids in callus [36]. In our laboratory, we recorded a significant influence of NAA 2.0 mgl^{-1} and Kin 1.0 mgl^{-1} incorporated in the medium on ephedrine and pseudoephedrine production. They also recorded superiority of kinetin over BAP for ephedrine production in callus cultures (Table 27.6) [34].

O'Dowd and Richardson reported for the first time the transformation in *Ephedra* by *Agrobacterium rhizogenes* producing in vivo and in vitro roots and tumors. Two-year-old in vivo tumor tissues of *Ephedra fragilis* and *Ephedra minima* contained up to 0.3 % ephedrine on a dry weight basis, and slow-growing in vitro cultured tumor tissues of *E. fragilis* contained up to 0.01 % ephedrine; alkaloid was not detected in faster-growing isolates [30].

When L-phenylalanine was used as precursor for ephedrine and pseudoephedrine production in *Ephedra* suspension culture, the maximum production of

Table 27.6 Effect of plant growth regulators on fresh weight and alkaloids content in *E. procera* (After Ref. [34])

Auxin (mg l^{-1})	Cytokinin (mg l^{-1})	FW (g/explant)	Ephedrine (μg g^{-1} DW)	Pseudoephedrine (μg g^{-1} DW)
NAA	BAP			
2.0	0.5	1.970 ± 0.20	37.4 ± 0.1	274.2 ± 1.9
1.0	1.0	0.970 ± 0.13	45.9 ± 0.5	320.8 ± 0.4
1.5	1.0	1.517 ± 0.16	64.5 ± 1.5	355.4 ± 0.3
2.0	1.0	2.421 ± 0.25	83.9 ± 1.3	466.2 ± 0.5
NAA	Kin			
2.0	0.5	2.670 ± 0.19	47.4 ± 0.9	322.2 ± 0.1
1.0	1.0	2.487 ± 0.27	62.4 ± 1.4	356.3 ± 0.6
1.5	1.0	3.766 ± 0.27	65.1 ± 0.5	356.9 ± 0.6
2.0	1.0	6.657 ± 0.26	108.1 ± 0.6	730.3 ± 1

Nd not detectable, *Ns* callus didn't survive, *Nr* not recorded, ——— No callus induction observed in the medium, *RGR* relative growing rate (g g^{-1} day^{-1}). The following formula was used to record RGR

RGR = $3(\mathrm{Wf}^{1/3}–\mathrm{Wi}^{1/3})/\mathrm{tf}–\mathrm{ti}$, where: *Wi* callus initial mass (at ti), *Wf* final callus mass (at tf), t = time, *tf*–ti = 28 days of subculture period

ephedrine and pseudoephedrine was recorded with 7.5 mM L-phenylalanine after the 12th day: 1.1 mg g^{-1} dry weight and 5.9 mg g^{-1} dry weight for ephedrine and pseudo-ephedrine, respectively [31]. Suspension cultures are excellent systems for isolating the suitable cell lines to be used in bioreactors through medium optimization by selection of correct precursors, elicitors, and growth regulators, appropriate design of a bioreactor, and selection of a suitable matrix for cell immobilization.

4.2 Biotransformation Techniques for Ephedrine Production

Significant progress has been made in the field of microbial biotransformation, and valuable compounds such as antibiotics, pigments, glycosides, terpenoids, and alkaloids have been successfully produced by biotransformation. Yeasts such as *Candida utilis*, *Saccharomyces cerevisiae*, and *Hansenula* spp. have been used for commercial production of ephedrine. The microbial biotransformation process starts with condensation of an active benzaldehyde (from pyruvic acid). This benzaldehyde (by enzyme pyruvate decarboxylase) leads to production of L-phenylacetylcarbinol, a precursor for the biosynthesis of ephedrine and its derivatives (Fig. 27.4). Other strategies using yeasts for the production of ephedrine and its derivatives include fed-batch culture, continuous culture, biotransformation methods using yeasts' immobilized cells, and biotransformation methods using purified pyruvate decarboxylase free enzyme [42].

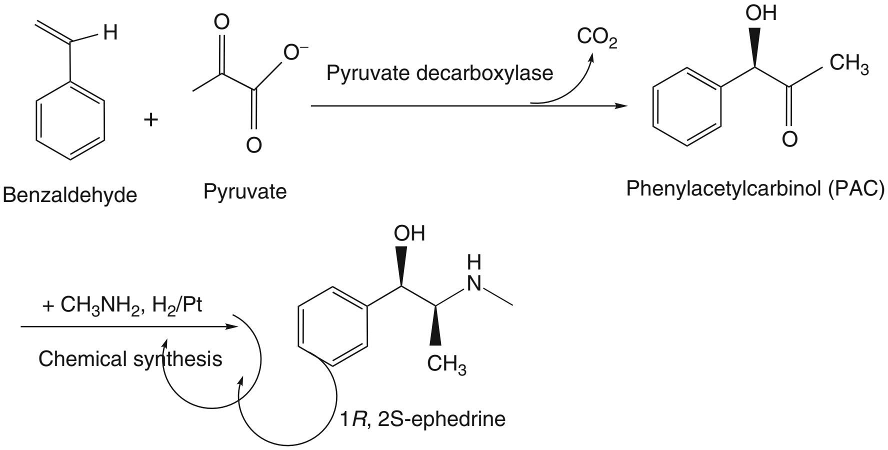

Fig. 27.4 The biotransformation process for ephedrine production

5 Perspective

Traditional uses of *Ephedra* have been known for thousands of years, however, the plant still needs proper phytochemical evaluation for antimutagen, anticarcinogen, antioxidant, antimicrobial, and anti-inflammatory agents. Proper evaluation of plant extract of this age-old drug may provide new clues. The mystery of somras (a drink of the gods in Hindu mythology) has yet to be revealed, though Ephedra is considered a potent candidate for its preparation. Biotechnological methods such as tissue culture, biotransformation, and gene manipulation techniques can produce secondary metabolites like ephedrine and its derivatives.

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Terpenoid Alkaloids Derived by Amination Reaction 28

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Contents

1 Introduction 924
2 Occurrence 925
3 Phytochemistry 929
3.1 Monoterpenoid Alkaloids 929
3.2 Diterpenoid Alkaloids 931
4 Chemotaxonomic Studies 940
5 Biosynthesis 941
5.1 Biosynthesis of Monoterpenoid Alkaloids 941
5.2 Biosynthesis of Diterpenoid Alkaloids 942
6 Biological Activities 944
6.1 Cardiovascular Activity 944
6.2 Cytotoxic Activity 945
6.3 Antinociceptive Activity 945
6.4 Toxicity 946
6.5 Mode of Action 946
6.6 Other Activities 947
7 Conclusion 947
References 947

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Abstract

Terpenoid alkaloids are a class of *pseudoalkaloids*, in which a nitrogen atom is inserted in the skeleton on a late biosynthetic stage. This class of compounds, sharing structural diversity, interesting chemistry, and promising pharmacological properties, has for a long time stimulated the interest of the scientific community and is covered in this chapter. The occurrence, phytochemistry with classification, and biological activities of monoterpenoid alkaloids reported between 2000 and Feb. 2012 and that of diterpenoid alkaloids reported between 2009 and Feb. 2012 are presented together with the biosynthetic approaches.

Key Words

Aconitum • amination reaction • *Delphinium* • diterpenoid alkaloids • incarvillea • monoterpenoid alkaloids • Ranunculaceae • Terpenoid alkaloids

Abbreviations

6T-CEM	Human acute T lymphocyte leukemia cell line
AChBP	Acetylcholine-binding protein
ED_{50}	Effective dose fifty percent
ent-CPP	Ent-copalyl diphosphate
GA–MLRs	Genetic algorithm–multiple linear regressions
GA–PLS	Genetic algorithm–partial least squares
GGPP	Geranylgeranyl pyrophosphate
hERG	Human ether-*á-go-go*-related gene
HR-ESI-MS	High-resolution electrospray ionization mass spectrum
IR	Infrared
LoVo	Human colon carcinoma cell line
MCF-7	Michigan Cancer Foundation-7
MDA-MB-435	Human breast carcinoma cell
MLA	Methyllycaconitine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nAChR	Nicotinic acetylcholine receptor
NMR	Nuclear magnetic resonance
nor-BNI	Norbinaltorphimine
NTI	Naltrindole
QSAR	Quantitative structure–activity relationship
SRB	Sulforhodamine B
β-FNA	*β*-funaltrexamine

1 Introduction

Terpenoid alkaloids are an ever-growing class of plant natural products possessing various chemical structures and interesting biological activities.

Based upon biogenesis, the alkaloids are broadly classified as the *true alkaloids* and the *pseudoalkaloids*. The majority of alkaloids are *true alkaloids* which are derived from α-amino acid precursors. Other alkaloids such as terpenes and steroids are named *pseudoalkaloids* because a relatively late amination process occurs in a transamination reaction by donating a nitrogen atom of an amino acid source [1]. A large number of alkaloids based on mono- and diterpenoid skeleton produced in an amination reaction have been isolated from natural sources and characterized. Several book chapters and review papers have been published to describe the mono- and diterpenoid class of alkaloids, with an intriguing chemistry and numerous varied bioactivities which constitute the largest and the most complicated group of terpenoid alkaloids [2–7].

Terpenoid alkaloid-producing plants have been utilized for centuries as traditional medicines in China, Japan, Mongolia, Russia, India, Pakistan, and Nepal [5, 8]. For instance, the plants of genus *Aconitum* have for a long time been used in Asia, Alaska, and Europe [9] in traditional medicine for the treatment of traumatic injury [10], as febrifuge and bitter tonic [11], and as ingredients in intoxicating liquor [12]. The most popular use of this species is described in Chinese traditional medicine as an analgesic and cardiotonic herbal medicine [13] and for the treatment of some inflammations like gastritis, hepatitis, nephritis, etc. [14, pp. 186–187]. These plants have also been used as poisons for hunting since ancient times and, later, in homicides [7, 15]. Similarly, the species of genus *Delphinium* have been used as a traditional medicine in China since ancient time for the treatment of ringworm, scabies, other skin diseases, and inflammation [14, p. 365], whereas in Nepal, it is used for the treatment of jaundice and fever [8]. In general, the terpenoid alkaloids exhibit different actions varying from poisonous (e.g., aconitine) to therapeutic (e.g., lappaconitine), even though they share similar molecular skeletons [16].

2 Occurrence

Terpenoid alkaloids are nonuniformly distributed in the plant kingdom. Monoterpenoid alkaloids are mostly found in the plant family Gentianaceae, whereas diterpenoid alkaloids are mainly located in the family Ranunculaceae. Gentianaceae, a rich source of monoterpenoid alkaloids, is a family of flowering plants comprising approximately 70–80 genera and 900–1,200 species [17]. Most of the monoterpenoid alkaloids have been isolated from the genus *Incarvillea* of Gentianaceae. Similarly, the majority of the diterpenoid alkaloids have been isolated from the species of *Aconitum* and *Delphinium* of Ranunculaceae family that consists of more than 300 and 400 species, respectively, distributed throughout the Northern hemisphere including Asia, Europe, and North America. Out of those, more than 113 species of *Delphinium* [18] and more than 200 species of *Aconitum* [19] are endemic to China.

Table 28.1 Monoterpenoid alkaloids (compound **1–9**: actinidine-type and **10–18**: skytanthine-type monoterpenoid alkaloids)

S.N.	Compound name (no.)	Mol. for.	Mol. wt.	Opt. rot^{n}.	Plant source	References
1	Delavayine B (**1**)	$C_{21}H_{25}NO_4$	355	−6.5 (Pyr.)	*Incarvillea delavayi*	[20]
2	Delavayine C (**2**)	$C_{16}H_{24}NO_4$	294	−72.3 (MeOH)	*I. delavayi*	[20]
3	Incargutosine C (**3**)	$C_{13}H_{17}NO_2$	219	–	*Incarvillea arguta*	[21]
4	Incargutosine D (**4**)	$C_{13}H_{17}NO_2$	219	–	*I. arguta*	[21]
5	10-Acetoxy-actinidine (**5**)	$C_{12}H_{15}NO_2$	205	–	*Argylia radiata*	[22]
6	Argutane A (**6**)	$C_{20}H_{22}N_2O_2$	322	+25.4 (MeOH)	*I. arguta*	[23]
7	Argutane B (**7**)	$C_{20}H_{22}N_2O_2$	322	+21.0 (MeOH)	*I. arguta*	[23]
8	Argutine A (**8**)	$C_{13}H_{15}NO$	201	–	*I. arguta*	[24]
9	Argutine B (**9**)	$C_{10}H_{12}N_2O$	176	–	*I. arguta*	[24]
10	4-Nor-7,8-dehydro-10-hydroxy-skytanthine (**10**)	$C_{10}H_{17}NO$	167	–	*A. radiata*	[22]
11	Delavayine A (**11**)	$C_{19}H_{28}NO_2$	302	−5.1 (Pyr.)	*I. delavayi*	[25]
12	Incarvillateine E (**12**)	$C_{53}H_{81}N_3O_8$	887	−6.4 ($CHCl_3$)	*Incarvillea sinensis*	[26]
13	Incarvine E (**13**)	$C_{21}H_{35}NO_3$	349	−6.3 ($CHCl_3$)	*I. sinensis*	[27]
14	Incarvine F (**14**)	$C_{29}H_{47}NO_6$	505	−6.6 ($CHCl_3$)	*I. sinensis*	[27]
15	Isoincarvilline (**15**)	$C_{11}H_{21}NO$	183	+2.0 ($CHCl_3$)	*Incarvillea mairei*	[28]
16	Mairine A (**16**)	$C_{11}H_{21}NO$	183	−12.0 (MeOH)	*I. mairei*	[29]
17	Mairine B (**17**)	$C_{11}H_{21}NO$	183	−5.0 (MeOH)	*I. mairei*	[29]
18	Mairine C (**18**)	$C_{11}H_{19}NO_2$	197	+21.0 (MeOH)	*I. mairei*	[29]

The naturally occurring monoterpenoid alkaloids reported between 2000 and Feb. 2012 and the diterpenoid alkaloids reported between 2009 and Feb. 2012 are listed in Tables 28.1 and 28.2, respectively, including their name and compound number, molecular formula, molecular weight, optical rotation, plant source, and reference number. The chemical structure of those compounds together with their classification details is given in Sect. 3.

Table 28.2 Diterpenoid alkaloids (compound **1–2**: ranaconitine-type C_{18}-diterpenoid alkaloids, **3–13**: aconitine-type C_{19}-diterpenoid alkaloids, **14–24**: lycoctonine-type C_{19}-diterpenoid alkaloids, **25**: C_{19}-rearranged-type C_{19}-diterpenoid alkaloid, **26–27**: atisine-type C_{20}-diterpenoid alkaloids, **28**: hetidine-type C_{20}-diterpenoid alkaloid, **29–36**: hetidine-type C_{20}-diterpenoid alkaloids, **37–41**: denudatine-type C_{20}-diterpenoid alkaloids, **42–51**: vakognavine-type C_{20}-diterpenoid alkaloids, **52**:atisine–denudatine-type C_{20}-diterpenoid alkaloid)

S.N.	Compound name (no.)	Mol. for.	Mol. wt.	Opt. rot^n.	Plant source	References
1	Anthriscifolcine F (**19**)	$C_{25}H_{37}NO_8$	479	−27.8 ($CHCl_3$)	*Delphinium anthriscifolium*	[30]
2	Anthriscifolcine G (**20**)	$C_{25}H_{37}NO_7$	463	−56.7 ($CHCl_3$)	*D. anthriscifolium*	[30]
3	Ouvrardianine B (**21**)	$C_{33}H_{43}NO_{10}$	613	−28.1 ($CHCl_3$)	*Aconitum ouvrardianum*	[31]
4	10-Hydroxy-8-*O*-methyltalatizamine (**22**)	$C_{25}H_{41}NO_6$	451	−7 ($CHCl_3$)	*Aconitum anthora*	[32]
5	1,15-Dimethoxy-3-hydroxy-14-benzoyl-16-ketoneoline (**23**)	$C_{32}H_{43}NO_9$	585	–	*Aconitum kusnezoffii*	[13]
6	Aconitramine D (**24**)	$C_{32}H_{43}NO_6$	537	−0.17 (MeOH)	*Aconitum transsectum*	[33]
7	Aconitramine E (**25**)	$C_{33}H_{47}NO_8$	585	+5.6 (MeOH)	*A. transsectum*	[33]
8	*N*-Deethylaconine (**26**)	$C_{23}H_{37}NO_9$	471	+25.4 (MeOH)	*Aconitum carmichaelii*	[34]
9	Beiwutinine (**27**)	$C_{24}H_{39}NO_{10}$	501	+22.0 (MeOH)	*A. carmichaelii*	[34]
10	Macrorhynine C (**28**)	$C_{33}H_{43}NO_{11}$	629	−57.5 ($CHCl_3$)	*Aconitum macrorhynchum*	[35]
11	Ouvrardianine A (**29**)	$C_{32}H_{41}NO_9$	583	+50.7 (MeOH)	*A. ouvrardianum*	[31]
12	Liangshantine (**30**)	$C_{26}H_{37}NO_7$	475	+118.5 ($CHCl_3$)	*Aconitum liangshanicum*	[36]
13	Aconitorientaline (**31**)	$C_{25}H_{39}NO_7$	465	± 0.0 ($CHCl_3$)	*Aconitum orientale*	[37]
14	Umbrosumine A (**32**)	$C_{38}H_{53}N_3O_{11}$	727	+21.7 ($CHCl_3$)	*Delphinium umbrosum*	[38]
15	Umbrosumine B (**33**)	$C_{38}H_{53}N_3O_{11}$	727	+48.6 ($CHCl_3$)	*D. umbrosum*	[38]
16	Umbrosumine C (**34**)	$C_{38}H_{52}N_2O_{12}$	728	+30.6 ($CHCl_3$)	*D. umbrosum*	[38]
17	Majusine A (**35**)	$C_{32}H_{44}N_2O_9$	600	+41.3 ($CHCl_3$)	*Delphinium majus*	[16]
18	Anthriscifoldine A (**36**)	$C_{25}H_{37}NO_7$	463	+66.7 ($CHCl_3$)	*D. anthriscifolium*	[30]
19	Delphidenine (**37**)	$C_{25}H_{37}NO_7$	463	+68.0 ($CHCl_3$)	*Delphinium densiflorum*	[39]

(*continued*)

Table 28.2 (continued)

S.N.	Compound name (no.)	Mol. for.	Mol. wt.	Opt. rot^{n}.	Plant source	References
20	Majusine C (**38**)	$C_{26}H_{37}NO_8$	491	+27.9 ($CHCl_3$)	*D. majus*	[16]
21	Anthriscifoldine B (**39**)	$C_{25}H_{39}NO_7$	465	−22.5 ($CHCl_3$)	*D. anthriscifolium*	[30]
22	Anthriscifoldine C (**40**)	$C_{27}H_{41}NO_7$	491	−15.4 (Acetone)	*D. anthriscifolium*	[30]
23	Majusine B (**41**)	$C_{24}H_{37}NO_6$	435	+2.1 ($CHCl_3$)	*D. majus*	[16]
24	Sharwuphinine A (**42**)	$C_{23}H_{35}NO_8$	435	+25.0 (MeOH)	*Delphinium sharwurense*	[40]
25	Yunnanenseine A (**43**)	$C_{45}H_{47}NO_{15}$	665	+27.1 ($CHCl_3$)	*Delphinium yunnanense*	[41]
26	Delphatisine C (**44**)	$C_{24}H_{31}NO_5$	413	+32.2 ($CHCl_3$)	*Delphinium chrysotrichum*	[42]
27	Honatisine (**45**)	$C_{33}H_{49}NO_6$	555	−18.1 ($CHCl_3$)	*Delphinium honanense*	[43]
28	Anthriscifolmine I (**46**)	$C_{30}H_{41}NO_7$	527	−83.3 ($CHCl_3$)	*D. anthriscifolium*	[44]
29	Anthriscifolmine C (**47**)	$C_{29}H_{31}NO_7$	505	−33.3 (MeOH)	*D. anthriscifolium*	[45]
30	Yunnanenseine B (**48**)	$C_{28}H_{37}NO_7$	499	−24.7 ($CHCl_3$)	*D. yunnanense*	[41]
31	Yunnanenseine C (**49**)	$C_{25}H_{35}NO_6$	457	−15.7 ($CHCl_3$)	*D. yunnanense*	[41]
32	Tatsienenseine B (**50**)	$C_{24}H_{31}NO_4$	397	+21.9 ($CHCl_3$)	*Delphinium tatsienense*	[46]
33	Tatsienenseine C (**51**)	$C_{24}H_{31}NO_3$	381	+9.9 ($CHCl_3$)	*D. tatsienense*	[46]
34	Anthriscifolmine J (**52**)	$C_{34}H_{40}NO_{10}$	621	−35.0 ($CHCl_3$)	*D. anthriscifolium*	[44]
35	Majusidine A (**53**)	$C_{22}H_{29}NO_5$	387	−38.0 ($CHCl_3$)	*D. majus*	[16]
36	Majusidine B (**54**)	$C_{25}H_{33}NO_4$	411	+27.9 ($CHCl_3$)	*D. majus*	[16]
37	Anthriscifolmine A (**55**)	$C_{25}H_{37}NO_5$	431	−38.4 (MeOH)	*D. anthriscifolium*	[45]
38	Anthriscifolmine B (**56**)	$C_{25}H_{37}NO_6$	447	−34.0 (MeOH)	*D. anthriscifolium*	[45]
39	Liangshantine A (**57**)	$C_{22}H_{29}NO_3$	355	+23.6 ($CHCl_3$)	*A. liangshanicum*	[36]
40	Liangshantine B (**58**)	$C_{21}H_{33}NO_4$	363	−79.0 (MeOH)	*A. liangshanicum*	[36]
41	Liangshantine C (**59**)	$C_{23}H_{33}NO_4$	387	−73.7 ($CHCl_3$)	*A. liangshanicum*	[36]

(*continued*)

Table 28.2 (continued)

S.N.	Compound name (no.)	Mol. for.	Mol. wt.	Opt. rot^n.	Plant source	References
42	Anthriscifolmine D (**60**)	$C_{33}H_{41}NO_9$	595	+13.0 (MeOH)	*D. anthriscifolium*	[47]
43	Anthriscifolmine E (**61**)	$C_{40}H_{49}NO_{13}$	751	−24.9 ($CHCl_3$)	*D. anthriscifolium*	[47]
44	Anthriscifolmine F (**62**)	$C_{40}H_{49}NO_{12}$	735	−25.0 ($CHCl_3$)	*D. anthriscifolium*	[47]
45	Anthriscifolmine G (**63**)	$C_{37}H_{43}NO_{13}$	709	+12.1 ($CHCl_3$)	*D. anthriscifolium*	[47]
46	Anthriscifolmine H (**64**)	$C_{37}H_{43}NO_{12}$	693	+8.4 ($CHCl_3$)	*D. anthriscifolium*	[47]
47	Tatsienenseine A (**65**)	$C_{43}H_{45}NO_{13}$	783	+27.1 ($CHCl_3$)	*D. tatsienense*	[46]
48	Majusimine A (**66**)	$C_{45}H_{47}NO_{15}$	841	−67.2 ($CHCl_3$)	*D. majus*	[16]
49	Majusimine B (**67**)	$C_{43}H_{45}NO_{14}$	799	−54.0 ($CHCl_3$)	*D. majus*	[16]
50	Majusimine C (**68**)	$C_{41}H_{43}NO_{13}$	757	−44.4 ($CHCl_3$)	*D. majus*	[16]
51	Majusimine D (**69**)	$C_{34}H_{37}NO_{12}$	651	−24.7 ($CHCl_3$)	*D. majus*	[16]
52	Piepunine (**70**)	$C_{44}H_{64}N_2O_4$	684	−51.0 ($CHCl_3$)	*Aconitum piepunense*	[48]

3 Phytochemistry

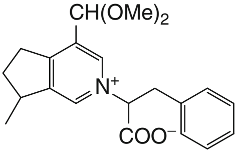
Terpenoid alkaloids comprise a large number of compounds biosynthetically derived from pathways that lead to the various groups of terpenes such as monoterpenoids and diterpenoids. Due to structural diversity, these alkaloids are of great interest of study with respect to the phytochemistry, synthesis, and pharmacological activities.

3.1 Monoterpenoid Alkaloids

A number of important alkaloids are derived from monoterpenes. Monoterpenoid alkaloids are comparably simple compounds formed by addition of nitrogen atom to an existing monoterpene, mainly of the secologanin type. Several monoterpenoid alkaloids have been isolated and characterized and classified based upon their structural characteristics. The more common and pharmaceutically important types such as actinidine, skytanthine, and gentianine are discussed herein.

3.1.1 Actinidine-Type Alkaloids

Actinidine, one of the rare types of monoterpenoid alkaloid [49], is a steam-volatile methylcyclopentane pyridine derivative found mainly in the essential oils of the species of *Actinidia* and *Incarvillea* [20]. Nine new actinidine-type monoterpenoid alkaloids (**1**–**9**) have been isolated in the period of 2000 to Feb. 2012 [20–24]. Two novel actinidine-type monoterpene alkaloids, delavayine B (**1**) and delavayine C (**2**), were isolated from aerial part of *I. delavayi* [20]. Phytochemical investigation on *I. arguta* yielded incargutosine C (**3**) and incargutosine D (**4**). The absolute configurations of both **3** and **4** were determined by a synthetic method [21]. Compound **5** obtained from *A. radiate* contains a 10-acetoxy moiety [22]. Two novel monoterpenoid alkaloid dimers named argutane A (**6**) and argutane B (**7**) were obtained from the roots of *I. arguta* [23]. Similarly, two new compounds argutine A (**8**) and argutine B (**9**) having an *α*,*β*-unsaturated carbonyl moiety and an amide moiety, respectively, were reported from *I. arguta* [24].



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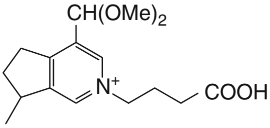

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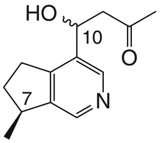
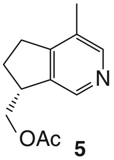


3 *7R*,10*R*
4 *7R*,10*S*



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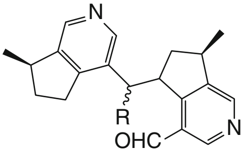
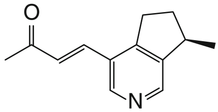


6 R= *β*-OH
7 R= *α*-OH



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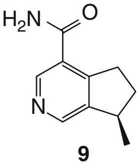

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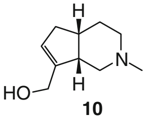
3.1.2 Skytanthine-Type Alkaloids

A 4-nor-7,8-dehydro-10-hydroxy-skytanthine (**10**) has been obtained from *A. radiata* [22]. Compound **10** and actinidine-type compound **5** are the alkaloid compounds that have been isolated from this plant species for the first time, and it is a reasonable hypothesis that the monoterpenoid structures of **10** and **5** should be derived from the previously isolated iridoids in this plant, which are known to be the biogenetic precursors of this type of alkaloids [50]. A novel skytanthine-type alkaloid named delavayine A (**11**) that comprises a benzoate ester has been isolated from *I. delavayi* [25]. Similarly, another novel skytanthine-type alkaloid, incarvillateine E (**12**), possessing three incarvilline moieties has been isolated and characterized from the aerial parts of *I. sinensis* [26]. Incarvine E (**13**) and incarvine F (**14**) [27] isolated from *I. sinensis* and isoincarvilline (**15**) [28] and mairines A–C (**16**–**18**) [29] from *I. mairei* are other new skytanthine-type monoterpenoid alkaloids. Compounds **16** and **18** contain a rare *δ*-skytanthine *N*-oxide in the structure [29].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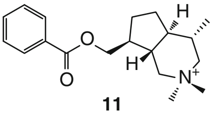
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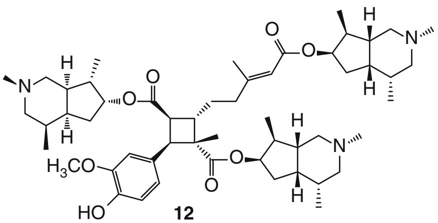
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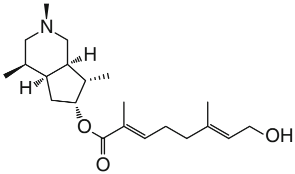
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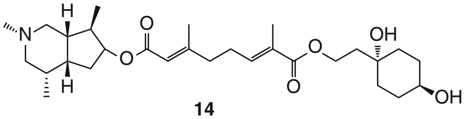
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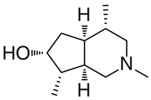
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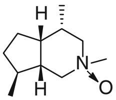
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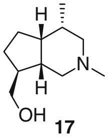
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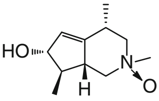
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3.1.3 Gentianine-Type Alkaloids

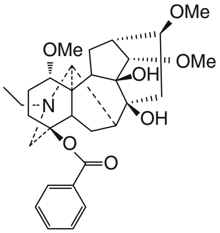
Gentianine-type monoterpenoid alkaloids are widely distributed in plants of the family Gentianaceae. Several studies were carried out on this type in the late 1950s. Gentianine is a bitter, crystalline monoterpenoid alkaloid possessing varying pharmacological activities [17]. This compound has been isolated from a number of plant genus including *Anthocleista* [51], *Gentiana* [52], *Fagraea* [53], and *Swertia* [54]. There is no report of isolation of new gentianine-type monoterpenoid alkaloid in the period of 2000 to Feb. 2012.

3.2 Diterpenoid Alkaloids

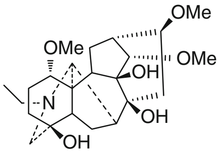
Plant species of the genera *Aconitum*, *Delphinium*, and *Consolida* of Ranunculaceae family are rich sources of diterpenoid alkaloids of pharmacological and economic importance [4]. Several review papers and chapter in books have been published in this class of alkaloids mainly by the research group of Wang from China and Atta-ur-Rahman from Pakistan [3–7]. The diterpenoid alkaloids are broadly classified into three categories: the C_{18}-, C_{19}-, and C_{20}-diterpenoid alkaloids. All these classes of alkaloids have been reviewed until the end of 2008 by Wang and coworkers [3]. The diterpenoid alkaloids reported from 2009 to Feb. 2012 will be discussed here. The chemical structures displayed in this chapter are predominantly those of new alkaloids. However, some of the known structures are also presented whenever necessary. During this period, the new diterpenoid alkaloids were isolated only from the genera *Aconitum* and *Delphinium* of Ranunculaceae family.

3.2.1 C_{18}-Diterpenoid Alkaloids

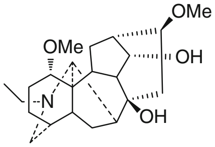
The C_{18}-diterpenoid alkaloids are a member of small groups within the diterpenoid alkaloids. The first C_{18}-diterpenoid alkaloid named lappaconitine was isolated from the plant *Aconitum septentrionale* [55], and its structure was confirmed after several years by extensive chemical studies [56]. In the beginning of the 1970s, three more C_{18}-diterpenoid alkaloids named lappaconidine [57], aconosine [58], and excelsine [59] were isolated and characterized which advanced this class of diterpenoid alkaloids. Until today, over 100 compounds of this class have been isolated from around 40 plant species mainly from *Aconitum* followed by *Delphinium*.



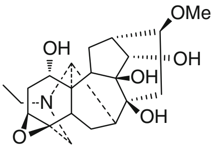
Lappaconitine



Lappaconidine



Aconosine

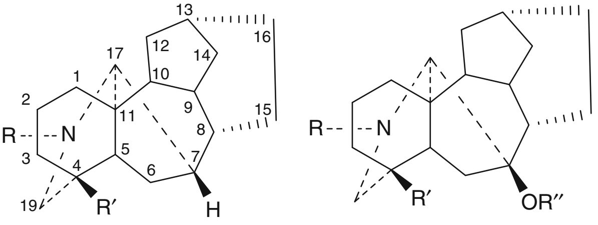


Excelsine

For a long time, lappaconitine and other C_{18}-diterpenoid alkaloids were classified as belonging to the C_{19}-diterpenoid class. Wang and Fang suggested a new class "C_{18}-diterpenoid alkaloids" in 1983 [60]. This was further classified as norditerpenoid alkaloids [61] or bisnorditerpenoid alkaloids [62]. However, the class "C_{18}-diterpenoid alkaloids" is common, and recent literature papers classified the compounds accordingly. Ichinohe et al. in 2004 divided the C_{18}-diterpenoid alkaloids into two categories as the lappaconitine type (A-1) and the ranaconitine type (A-2) (Fig. 28.1) based on presence or absence of an oxygen functionality attached at C-7 [63].

Lappaconitine-Type Alkaloids

The lappaconitine-type C_{18}-diterpenoid alkaloids are structurally classified by lack of an oxygen functionality and presence of a methine unit at C-7 [6]. This is a fairly



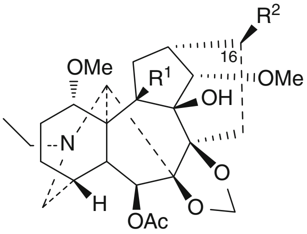
Lappaconitines (A–1)

Ranaconitines (A–2)

Fig. 28.1 C_{18} types of diterpenoid alkaloid: R and R″ can be hydrogen, acyl, or alkyl groups, and R′ can be hydrogen, hydroxyl, alkoxy, or acyloxy groups small class of compounds so far isolated. Only 16 compounds were reported in the period from 1998 to 2008 [3], and there is no report of isolation of this class of compounds since then.

Ranaconitine-Type Alkaloids

The ranaconitine-type C_{18}-diterpenoid alkaloids possess an oxygen functionality at C-7. This class also contains a small number of diterpenoid alkaloids. Only two new compounds anthriscifolcines F (**19**) and G (**20**) isolated from *D. anthriscifolium* between 2009 and Feb. 2012 are added in this series [30].



19 $R^1 = R^2 = OH$ **19a** $R^1 = OH$ $R^2 = OAc$

20 $R^1 = H$ $R^2 = OH$ **20a** $R^1 = H$ $R^2 = OAc$

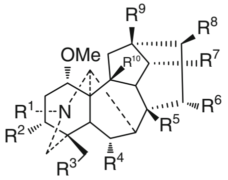
Compounds **19** and **20** represent ranaconitine-type C_{18}-diterpenoid alkaloids having hydroxyl group at C-16 position. Initially, the orientation of the 16-OH group in **19** and **20** was ambiguous since no evident NOE correlation could be found when H-16 was irradiated. To accomplish this, the acetylated derivatives **19a** and **20a** were prepared where NOE correlations could be observed between 16-OAc and 14-OCH_3 and H-16 and H-17 in both **19a** and **20a**. The configuration of carbon atom 16 in **19** and **20** therefore was unambiguously established as *β*. The conformation of the D-ring in **19a** and **20a** was suggested as a boat, which would enable the 16-OAc substituent to remain in an equatorial position [30].

3.2.2 C_{19}-Diterpenoid Alkaloids

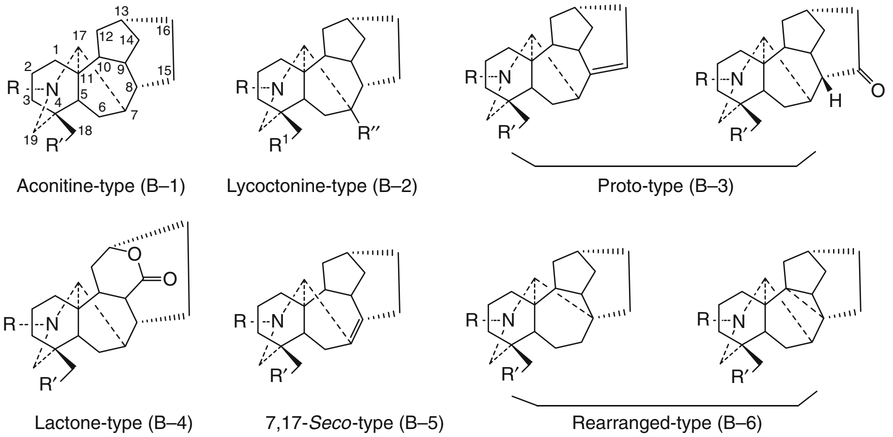
The C_{19}-diterpenoid alkaloids are the largest class of naturally occurring diterpenoid alkaloids with more than 700 compounds so far isolated and characterized from around 315 species of plants mainly from the genera *Aconitum* and *Delphinium*. This class of alkaloids is divided into six types: aconitine type (B-1), lycoctonine type (B-2), pyro type (B-3), lactone type (B-4), 7,17-*seco* type (B-5), and rearranged type (B-6) (Fig. 28.2). The order of types from B-1 to B-6 is based upon the higher to lower number of alkaloids obtained so far. In between 2009 and Feb. 2012, only aconitine, lycoctonine, and rearranged-type C_{19}-diterpenoid alkaloids have been isolated and are discussed herein.

Aconitine-Type Alkaloids

The main feature of aconitine-type C_{19}-diterpenoid alkaloid is the lack of an oxygen-containing functionality at C-7. The characteristic functionalities in this type include an amine, an imine, an amide, and the *N*,*O*-mixed acetal. A majority of the compounds bear oxygen functionalities at C-1, C-6, C-8, C-14, and C-16. In a very small number, alkaloids of this class including ouvrardianine B (**21**) possess the formyl moiety that is attached to the nitrogen [31]. 1,15-Dimethoxy-3-hydroxy-14-benzoyl-16-ketoneoline (**23**), isolated from *A. ouvrardianum*, is the first member of amine group that contains carbonyl group at C-16 [13]. *N*-Deethylaconine (**26**) is a rare aconitine with a secondary amine, while in most cases, a tertiary amine with ethyl or methyl groups is found [34]. Interestingly, beiwutinine (**27**) is the first aconitine-type C_{19}-diterpenoid alkaloid that has seven hydroxyl groups attached to the skeleton [34].



	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9	R^{10}
21	CHO	H	OMe	OMe	OAc	H	OBz	OMe	OH	H
22	C_2H_5	H	OMe	H	OMe	H	OH	OMe	H	OH
23	C_2H_5	OH	OMe	OMe	OH	H	OBz	=O	H	H
24	C_2H_5	H	H	H	OBz	H	OAc	OMe	H	H
25	C_2H_5	H	OMe	OMe	OAs	H	OH	OMe	H	H
26	H	OH	OMe	OMe	OH	OH	OH	OMe	OH	H
27	CH_3	OH	OMe	OMe	OH	OH	OH	OH	OH	OH

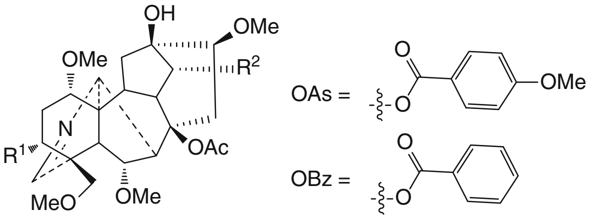


Aconitine-type (B–1) Lycoctonine-type (B–2) Proto-type (B–3)

Lactone-type (B–4) 7,17-*Seco*-type (B–5) Rearranged-type (B–6)

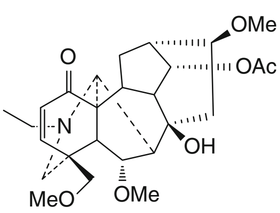
Fig. 28.2 C_{19}-diterpenoid alkaloids; R can be hydrogen or alkyl groups; R′ can be hydrogen, hydroxyl, alkoxy, or acyloxy groups; R″ can be hydroxyl, alkoxy, or acyloxy groups

The aconitine-type C_{19}-diterpenoid alkaloids, namely, macrorhynine (**28**) and ouvrardianine A (**29**), with a rare C-19 imino unit, were isolated from *A. macrorhynchum* and *A. ouvrardianum*, respectively. The group at C-19 in both compounds **28** and **29** was determined on the basis of its 1D and 2D NMR and other spectroscopic data analysis [31, 35].

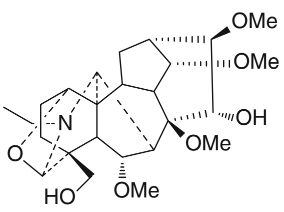


	R^1	R^2
28	OH	OAs
29	H	OBz

Liangshantine (**30**) features an *α*,*β*-unsaturated carbonyl group at ring A [36]. A novel aconitine-type C_{19}-diterpenoid alkaloid named aconitorientaline (**31**) was isolated from the roots of *A. orientale* [37]. This compound contains an epoxy group between C-1 and C-19.



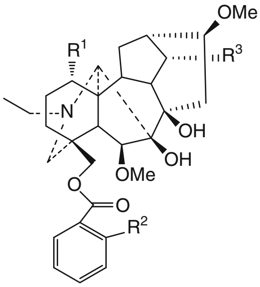
30



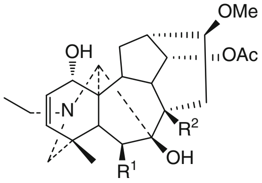
31

Lycoctonine-Type Alkaloids

The characteristic feature of this type of C_{19}-diterpenoid alkaloids is the presence of an oxygenated functionality at C-7. Like that of aconitine type, these alkaloids are also subdivided into amines, imines, amides, and *N*,*O*-mixed acetals based upon the substitution pattern of the nitrogen. A total of 11 lycoctonine-type C_{19}-diterpenoid alkaloids (**32**–**42**) have been obtained between 2009 and Feb. 2012. Compounds **32**–**35** possess a benzoyl ester moiety at C-18 with a variety of amide groups in the *ortho* position. Anthriscifoldine A (**36**) [30] and delphidenine (**37**) [39] are identical compounds reported by different research groups almost at the same time from *D. anthriscifolium* and *D. densiflorum*, respectively.

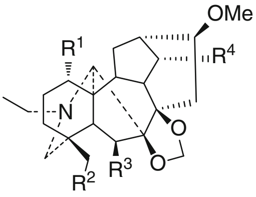


	R1	R2	R3
32	OMe	$NHCOCH_2CH(CH_3)CONH_2$	OAc
33	OMe	$NHCOCH(CH_3)CH_2CONH_2$	OAc
34	OMe	$NHCOCH_2CH(CH_3)COOH$	OAc
35	OH	$NHCOCH_3$	OH

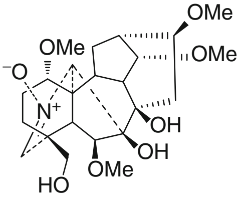


	R1	R2
36	OH	OMe
37	OH	OMe
38	OAc	OH

Compound **39–41** bears a common methylenedioxy group at C-7 and C-8 [16, 30], whereas compound **42** is the first natural diterpene alkaloid with a nitrone functionality [40]. Biogenetically, compound **42** might be derived from lycoctonine by formation of *N*-oxide followed by *N*-deethylation followed by conversion into a hydroxylamine derivative.



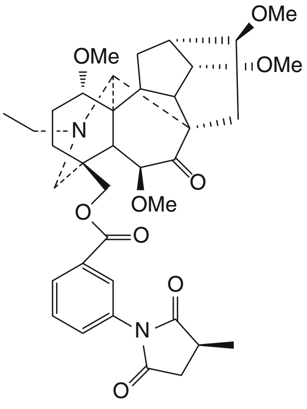
	R1	R2	R3	R4	R5
39	OMe	OMe	H	OH	OH
40	OMe	OMe	H	OAc	H
41	OH	H	OMe	OH	H



42

C_{19}-Rearranged-Type Alkaloids

Some of the C_{19}-diterpenoid alkaloids have a rearranged skeleton in which either the bridge between C-8 and C-17 is formed instead of common bond between C-7 and C-17 or an additional bond formed between C-8 and C-10. Only one compound named yunnanenseine A (**43**) of this type having a bridge between C-8 and C-17 [41] was isolated between 2009 and Feb. 2012.


OMe
OMe
OMe
N
O
OMe
O
O
O
N
O

43

3.2.3 C_{20}-Diterpenoid Alkaloids

The C_{20}-diterpenoid alkaloids are the second largest group of diterpenoid alkaloids after C_{19}-diterpenoid alkaloids and are mainly obtained from the genera of *Aconitum* and *Delphinium*. The classification of C_{20}-diterpenoid alkaloids has not been very clear. Different group suggested different classifications [5]. Wang et al. [3] classified it as atisines, denudatines, hetidines, hetisines, vakognavines, napellines, kusnezolines and omeielines, racemulosines, arcutines, and tricalysiamides. During the period of 2009–Feb. 2012, compounds only from the types atisine (C-1), hetidine (C-2), hitisine (C-3), denudatine (C-4), and vakognavine (C-5) have been isolated (Fig. 28.3). Additionally, one more new class of C_{20}-diterpenoid alkaloid, atisine–denudatine type, has been isolated recently from the roots of *A. piepunense* [48].

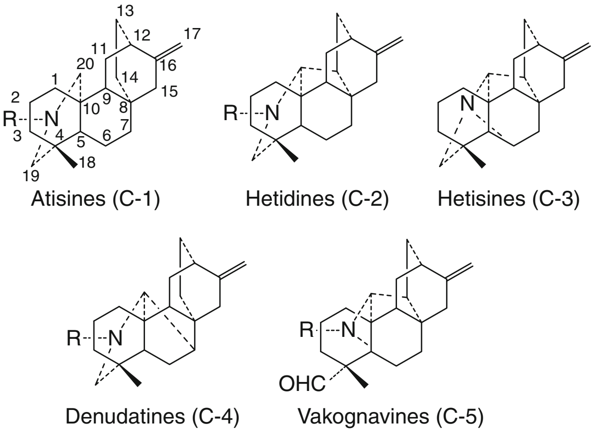
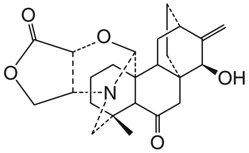

13
12
17
11
20
16
14
1
15
2
9
R
N
10
8
7
3
4
6
5
18
19
Atisines (C-1)
R
N
Hetidines (C-2)
N
Hetisines (C-3)
R
N
Denudatines (C-4)
R
N
OHC
Vakognavines (C-5)

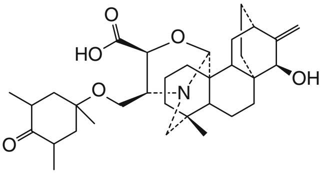
Fig. 28.3 Isolated compounds from the types atisine (C-1), hetidine (C-2), hitisine (C-3), denudatine (C-4), and vakognavine (C-5)

Atisine-Type Alkaloids

This type of comparatively simpler C_{20}-diterpenoid alkaloids bears a pentacyclic core in the structure. Only two new compounds named delphatisine C (**44**) from *D. chrysotrichum* [42] and honatisine (**45**) from *D. honanense* [43] have been isolated between the periods of 2009 and Feb. 2012. Compound **44** contains a five-membered lactone ring connecting to the oxazolidine ring. Extensive NOESY experiments were required to elucidate the relative configuration. Compound **45** is a novel C_{20}-diterpenoid alkaloid containing an unique 1′,3′,5′-trimethyl-4′-oxocyclohexyloxy unit attached to C-24.



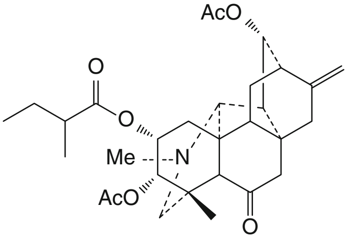
44



45

Hetidine-Type Alkaloids

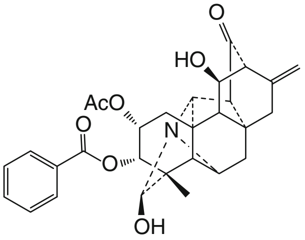
This type of alkaloids has a hexacyclic core with an additional bond at C-20–C-14. They are more complex than the atisine alkaloids. One new hetidine-type C_{20}-diterpenoid alkaloid named anthriscifolmine I (**46**) was reported between 2009 and Feb. 2012 from *D. anthriscifolium* [44].



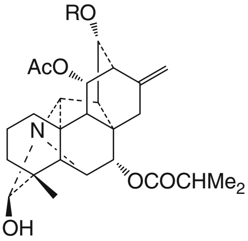
46

Hetisine-Type Alkaloids

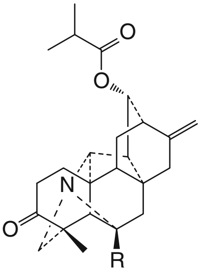
This type is one of the most complex groups derived from the atisine skeleton having a heptacyclic system with an additional N-C-6 bond. A total of eight hetisine-type C_{20}-diterpenoid alkaloids (**47**–**54**) were isolated in the period of 2009 to Feb. 2012 [16, 41, 44–46]. Besides the complexity of the skeleton, acetyl, benzoyl, tertiary methyl, hydroxyl, *O*-formyl groups as well as an exocyclic double bond and a ketone make this skeleton a unique structure.


O
HO
AcO
O
O
N
OH

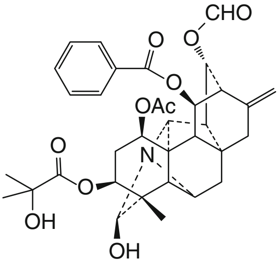
47


RO
AcO
N
OCOCHMe$_2$
OH

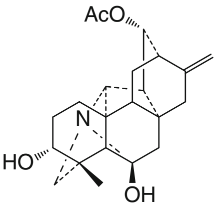
48 R = Ac
49 R = H



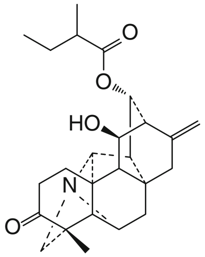
50 R = OH
51 R = H


CHO
O
O
O
OAc
O
N
O
OH
OH

52



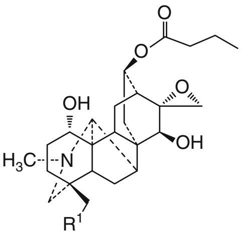
53



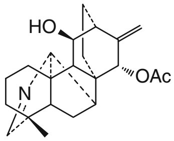
54

Denudatine-Type Alkaloids

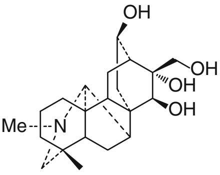
This type of alkaloids is similar to the atisine type except of an additional bond between C-20 and C-7. Five new compounds, two of them, namely, anthriscifolmines A (**55**) and B (**56**) from *D. anthriscifolium* [41] and liangshantines A–C (**57**–**59**) from *A. liangshanicum* [36], have been isolated from 2009 to Feb. 2012. Compound **56** is a rare C_{20}-diterpenoid alkaloid bearing a CH_2OH-18 moiety attached to C-4.


O
O
O
OH
OH
H$_3$C
N
R^1

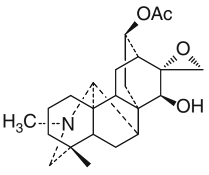
55 R^1= H
56 R^1= OH



57


OH
OH
OH
OH
Me
N

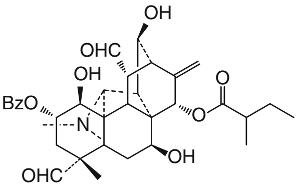
58


OAc
O
OH
H$_3$C
N

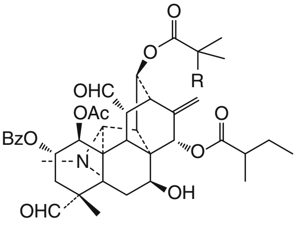
59

Vakognavine-Type Alkaloids

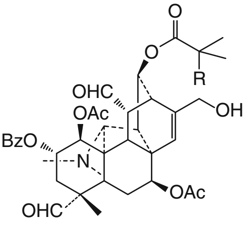
This type of alkaloid have an *N*,19-*seco* hetisine skeleton in addition to a C-4 aldehyde moiety. This type is named after the first compound vakognavine [64, 65]. Ten new vakognavine C_{20}-diterpenoid alkaloids have been isolated between 2009 and Feb. 2012. Five new alkaloids of this type, anthriscifolmines D–H (**60–64**), were isolated from *D. anthriscifolium* [47]. Anthriscifolmines G (**63**) and H (**64**) are exceptional cases of this type having double bond in C-15 and C-16 [47]. Other compounds of this type are tatsienenseine A (**65**) isolated from *D. tatsienense* [46] and majusimines A–D (**66–69**) from *D. majus* [16].


OH
OHC
OH
BzO
N
O
O
OH
OHC

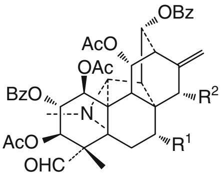
60


O
O
R
OHC
OAc
BzO
N
O
O
OH
OHC

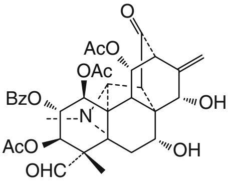
61 R = OH
62 R = H


O
O
R
OHC
OAc
OH
BzO
N
OAc
OHC

63 R = OH
64 R = H


OBz
AcO
OAc
BzO
N
R2
AcO
OHC
R1

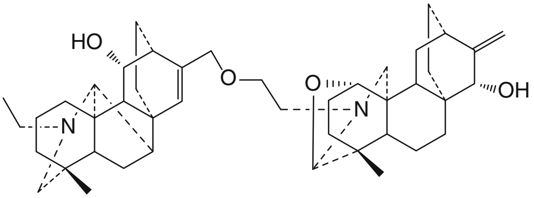
	R^1	R^2
65	H	OAc
66	OAc	OAc
67	OH	OAc
68	OH	OH


O
AcO
OAc
BzO
N
OH
AcO
OH
OHC

69

Atisine–Denudatine-Type Alkaloids

In the year 2010, a novel C_{20}-diterpenoid alkaloid named piepunine (**70**) has been isolated from *A. piepunense* containing both atisine and denudatine skeletons [48]. Compound **70** represents the first example of this type.



70

4 Chemotaxonomic Studies

During the review period of monoterpenoid and diterpenoid alkaloids, only one paper on the chemotaxonomic studies of diterpenoid alkaloids was published [18].

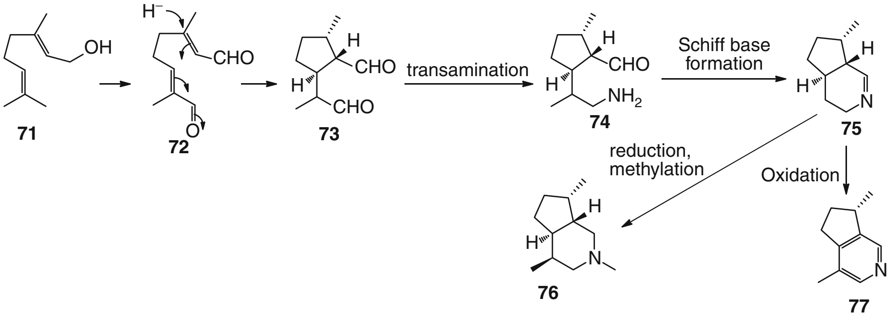
This paper described the chemotaxonomy characteristics of two C_{20}-diterpenoid alkaloids and five C_{19}-diterpenoid alkaloids from *Delphinium albocoeruleum* Maxim of Chinese origin. The study indicated that the compound delelatine may be considered as a chemotaxonomic marker for the *Delphinium* genus as it occurs in *Delphinium elatum*, *Delphinium barbeyi*, *D. tatsinenense* [66], and *Delphinium giraldii* [67]. Delphinine, a major compound, may possibly be utilized for identification of *D. albocoeruleum* Maxim.

5 Biosynthesis

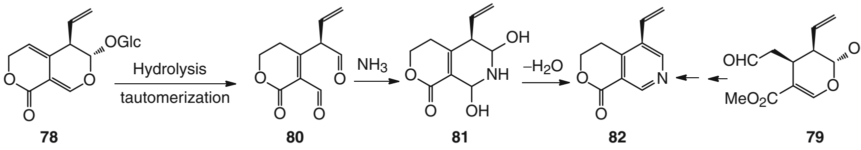
The majority of the alkaloids are derived from amino acid precursors through enzymatic processes. In most cases, the nitrogen atom is donated from an amino acid source through enzymatic transamination. Terpenoid alkaloids are most simplistically described as aminated terpenes [68]. The outcome of this unique biosynthesis is the formation of natural products containing unprecedented structures. A variety of mono- or diterpenoid alkaloid skeletons has been isolated and characterized, but the exact information about their biosynthesis in nature is still somewhat sparse [1]. It is considered that like in terpene biosynthesis, prenyl units are first linked together to form geranyl-, farnesyl-, or geranylgeranyl pyrophosphates, which then undergo enzyme-mediated cyclizations and Wagner–Meerwein rearrangements followed by oxidation and in some cases further rearrangement. Nitrogen incorporation can occur either before, during, or after the cyclase phase and thus convert terpene to terpenoid alkaloid.

5.1 Biosynthesis of Monoterpenoid Alkaloids

Monoterpenoid alkaloids are structurally related to iridoid compounds, the oxygen heterocycle being replaced by a nitrogen-containing ring [1]. A possible biosynthesis of actinidine and *β*-skytanthine starting from geraniol is proposed in Scheme 28.1.



Scheme 28.1 Possible biosynthesis of *β*-skytanthine (**76**) and actinidine (**77**) alkaloids from geraniol (**71**)



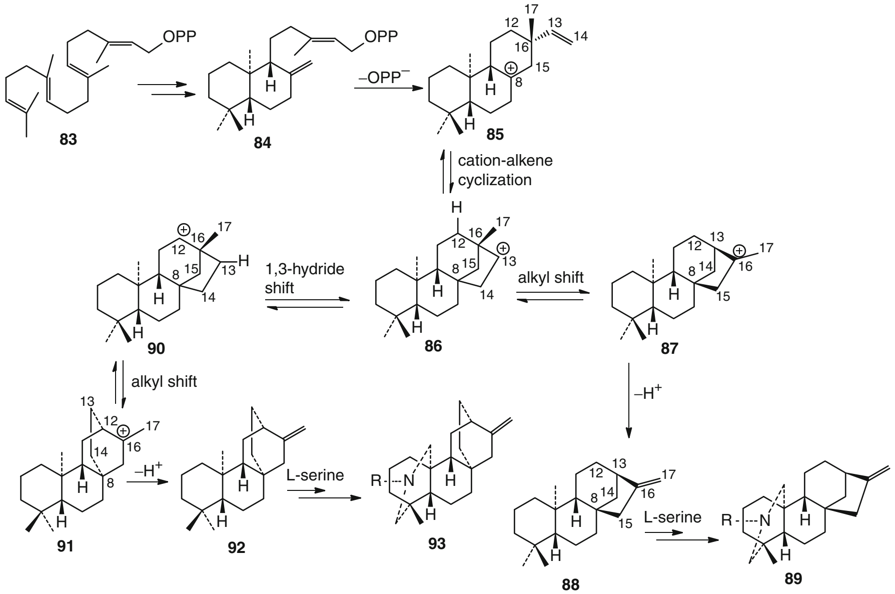
Scheme 28.2 Possible biosynthetic pathways of gentianine (**82**) from gentiopicroside (**78**) and secologanin (**79**)

The amination via an amino acid of an iridodial diastereomer (**73**), which was obtained from geraniol (**71**) after forming a dialdehyde intermediate (**72**), yielded compound **74** and its cyclization product **75**. Compound **75** upon reduction and methylation yielded β-skytanthine, (**76**) whereas oxidation produces actinidine (**77**).

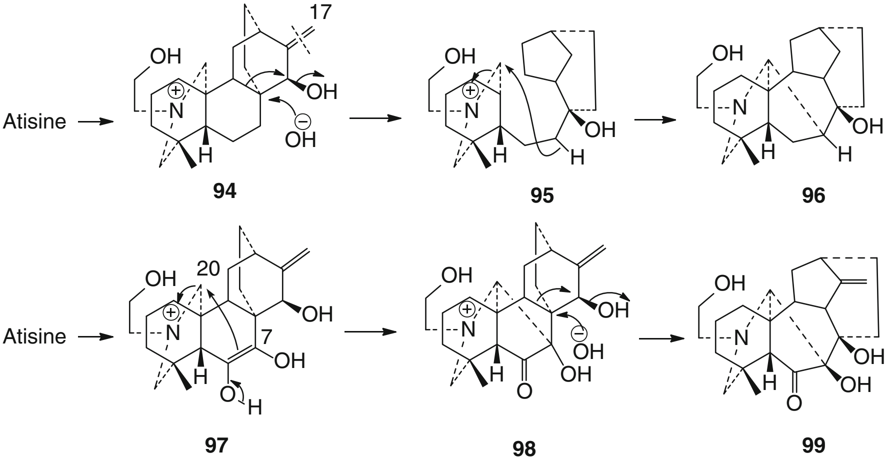
Gentianine, one of the most common monoterpenoid alkaloids, might be formed from iridoid compounds such as gentiopicroside (**78**) or secologanin (**79**) [1] (Scheme 28.2). Compound **78** undergoes enzymatic hydrolysis of the β-glucoside followed by tautomerization to produce **80**, which upon hemiaminal formation and subsequent dehydration gives gentianine (**82**). Likewise, compound **79** reacts with ammonia followed by cyclization to give **82**.

5.2 Biosynthesis of Diterpenoid Alkaloids

Investigations on the biosynthesis of diterpenoid alkaloids have been scarcely reported [1, 5, 7, 68]. Specific details of the biosynthetic processes are yet unclear. Recent biosynthetic studies showed that the diterpenoid alkaloids are derived from *ent*-kaurene and *ent*-atisane diterpenoid alkaloid families via enzyme-mediated carbocationic rearrangements [69]. Geranylgeranyl pyrophosphate (GGPP, **83**) is cyclized enzymatically to give *ent*-CPP (**84**), which after loss of pyrophosphate further cyclizes to give the pimarenyl carbocation (**85**) (Scheme 28.3). The pimarenyl carbocation (**85**) is generally proposed to first undergo cation–alkene cyclization to form a secondary carbocation, the beyeranyl cation (**86**). Beyeranyl cation (**86**) is a key branch point in the reaction pathways leading to *ent*-kaurenes (**88**) and *ent*-atisanes (**92**). An alkyl shift (C12 migrating from C16 to C13) in beyeranyl cation (**86**) leads to the kauranyl cation (**87**), the precursor to *ent*-kaurenes (**88**). A 1,3-hydride shift in beyeranyl cation (**86**) leads to another secondary cation (**90**), which can form *ent*-atisanes (**92**) via a subsequent alkyl shift (C13 migrating from C16 to C12) to form cation (**91**) followed by deprotonation. The veatchine-type (**89**) and atisane-type (**93**) C_{20}-diterpenoid alkaloids are derived from the amination of the *ent*-kaurane-type (**88**) and *ent*-atisane-type (**92**) diterpenoids, respectively [70]. Zhao et al. [71] confirmed that the aminoethanol moiety in C_{20}-diterpenoid alkaloids was derived biosynthetically from L-serine, which was consistent with the biogenetic hypothesis of Benn [72]. Subsequently, further oxidations can occur, and more complex C_{20}-diterpenoid alkaloids skeleton can form. Similarly, loss of carbon atom(s) from C_{20}-diterpenoid alkaloids forms C_{19}- and C_{18}-diterpenoid alkaloids [68].


OPP
83
OPP
84
–OPP⁻
85
cation-alkene
cyclization
1,3-hydride
shift
alkyl shift
90
86
87
alkyl shift
–H⁺
–H⁺
91
92
L-serine
R
N
93
88
L-serine
R
N
89

Scheme 28.3 Proposed biosynthetic pathway of veatchine-type (**89**) and atisane-type (**93**) diterpenoid alkaloids


Atisine
17
OH
N
OH
OH
94
95
96
Atisine
20
7
OH
OH
O
H
97
98
99

Scheme 28.4 Possible biosynthetic pathways of aconitine-type (**94**) and lycoctonine-type (**97**) C_{19}-diterpenoid alkaloids

The aconitine-type alkaloid (**96**) is most probably formed from the atisine skeleton through different modifications as indicated in Scheme 28.4. The intermediate **94** after a Wagner–Meerwein rearrangement process converts two fused six-membered rings into a (7 + 5)-membered bicyclic system in which C-17 exocyclic double bond is lost in the presence of a specific enzyme to produce **95**. Compound **95** undergoes internal rearrangement to give aconitine-type C_{19}-diterpenoid alkaloid **96**. Similarly, formation of lycoctonine-type C_{19}-diterpenoid alkaloid **99** from atisine alkaloids takes place via formation of the (7 + 5)-membered bicyclic intermediates **97** and **98** [7].

6 Biological Activities

Alkaloid-containing plants have been used by humans since ancient times for therapeutic and recreational purposes. The terpenoid alkaloids possess a diverse array of pharmacological properties, which has been studied for a century. Cordell reviewed the pharmacology of monoterpene alkaloids of the family Gentianaceae with focus on gentianine and skytanthine in 1977 [73]. Gentianine was found to exhibit central muscle-weakening action, inhibition of provoked aggression, and analgesic potentiating effects, whereas skytanthine exhibits no curare-like action but does induce tremor. More study is so far focused on biological activities of diterpenoid alkaloids obtained from Ranunculaceae [3].

6.1 Cardiovascular Activity

Liu and coworkers performed bioassay-guided fractionation of an *n*-BuOH extract of the lateral roots of *A. carmichaeli* that yielded five cardioactive C_{19}-diterpenoid alkaloids [34]. Among them, mesaconine, hypaconitine, and beiwutinine showed the strongest cardiac actions on the isolated perfused bullfrog heart. Also, the compound mesaconine is found to have protective effects, including improved inotropic effect and left ventricular diastolic function, on myocardial ischemia–reperfusion injury in rat at a dose of nM concentration and has almost no effect on heart rate.

Allapinine (lappaconitine hydrobromide) has passed clinical trials and was commercially available as a drug since 1987. Cardiologists believe that allapinine is the first-line drug for the treatment of certain types of cardiac arrhythmia [74]. The investigation of the structure–activity relationship of more than 200 diterpenoid alkaloids and their derivatives revealed that the structural elements are responsible for the arrhythmogenic and antiarrhythmic properties [75]. It is also noted that diterpenoid alkaloids containing the same skeletal and similar functionalities exhibited both arrhythmogenic and antiarrhythmic properties.

Mesaconitine, an aconite-type C_{19}-diterpenoid alkaloid, inhibited phenylephrine-induced contraction in the endothelium-intact but not endothelium-denuded, aortic rings [76]. Compounds named sharwuphinine A, lycoctonine, and deoxymethylenelycoctonine were tested when phenylephrine (PE) 3×10^{-7} M was applied to thoracic aortic rings with endothelium after achieving a maximal response. Only sharwuphinine A showed a moderate vasorelaxant activity (40% relaxation at 10^{-4} M) and that vasodilation may be influenced by the hydrophobicity of a nitrogen atom [40].

Forgo and coworkers studied 24 C_{19}- and C_{20}-diterpene alkaloids representing the structural diversity of *Aconitum* alkaloids for the human ether-à-go-go-related gene (hERG) channel activity by the whole-cell patch clamp technique, using the QPatch-16 automated patch clamp system [32]. At 10 μM, aconitine, 14-benzoylaconine-8-*O*-palmitate, songoramine, gigactonine, and neolinine demonstrated significant hERG K^+ channel inhibition; all other compounds exerted only low (6–21%) inhibitory activity. The study revealed that some structurally and functionally unrelated C_{19}- and C_{20}-diterpene alkaloids may block the hERG K^+ channel and may therefore act in cardiac action potential repolarization, with prolongation of the QT interval and increase of the risk of potentially fatal ventricular arrhythmias.

6.2 Cytotoxic Activity

A large number of diterpenoid alkaloids isolated from *Delphinium* and *Aconitum* exhibited significant cytotoxic activities [3]. The four compounds, delphatisine, delpheline, delbrunine, and delectinine, isolated from *Delphinium chrysotrichum* were determined by SRB assay for their cytotoxicity in which delphatisine showed significant cytotoxic activities (IC_{50} = 2.36 μM) against the A549 cell line [42]. A novel diterpenoid alkaloid named honatisine together with six other compounds of same series obtained from *D. honanense* were evaluated by a SRB assay for their cytotoxicity in which honatisine showed a significant cytotoxic activity (IC_{50} = 3.16 μM) against the MCF-7 cell line [43]. Similarly, six diterpenoid alkaloids isolated from a Tibetan medicinal plant *Aconitum richardsonianum* were also evaluated for their cytotoxicity activity in which a compound named delelatine showed cytotoxic activities ($IC_{50} = 4.36\ \mu$M) against the human cell line P388 [77]. An actinidine-type monoterpenoid named argutine A isolated from *I. arguta* was evaluated for cytotoxicity using four tumor cell lines, A549, LoVo, 6T-CEM, and MDA-MB-435 (MDA), in the MTT assay found to show modest cytotoxicity against these four cell lines with IC_{50} values in the range of 55.4–289.4 μM [21].

6.3 Antinociceptive Activity

Nakamura and coworker reported that the extract of *I. delavayi* moderately decreased the number of writhings and stretchings in the model of acetic acid-induced writhing test in mice in a dose-dependent manner [25]. While testing the pure compounds, orally administered 8-epodeoxyloganic acid (100 mg/kg) showed a weak antinociceptive activity. Similarly, subcutaneous injection of delavayine A (50 mg/kg), a novel monoterpene alkaloid, showed a more significant level of antinociceptive activity. The same group further studied the structure-antinociceptive activity of incarvillateine, a monoterpenoid alkaloid from *I. sinensis*, found to show significant antinociceptive activity in a formalin-induced pain model in mice [78]. Chie et al. in 2005 found that incarvillateine produced graded inhibition of both the neurogenic (early phase) and inflammatory (late phase) phases in a dose-related manner [79]. Comparing the antinociceptive effects of different doses of incarvillateine and morphine, the ED_{50} values of incarvillateine were *ca.* 1.06 (early phase) and 1.33 (late phase) times lower than those of morphine. The same research group further determined the antinociceptive mechanism of incarvillateine, which was antagonized by nor-BNI (κ-receptor antagonist) and β-FNA (μ-receptor antagonist), while NTI (δ-receptor antagonist) did not influence the effect. Furthermore, the antinociceptive effect of incarvillateine was blocked by theophylline, an adenosine-receptor antagonist. These results suggested that the antinociceptive effect arose from the activation of μ- and κ-opioid receptors and adenosine receptor [79].

6.4 Toxicity

Plants of the genus *Aconitum* are highly toxic and have been used for centuries as poisons for spears and arrows and later in homicides. Many C_{19}-diterpenoid alkaloids can cause arrhythmia [75]. The diterpenoid alkaloid aconitine is used by pharmacologists for the development of a cardiac arrhythmia model (the so-called aconitine model). It is one of the strongest plant toxins with potency comparable to the cobra venom [80]. Aconitine poisoning mainly occurs either by confusion with edible plants or accidental ingestion by children [81]. Similarly, larkspurs (*Delphinium* spp.) are responsible for more than 15% of cattle death in North America where larkspurs are abundant. The lycoctonine and the 7,8-methylenedioxylycoctonine-type of C_{19}-diterpenoid alkaloids are the major constituents of poisonous in larkspurs [3]. The highest levels of cardio- and neurotoxicity are expressed by the C_{19}-diterpenoid alkaloids and particularly those which contain two ester groups such as aconitine, mesaconitine, and hypaconitine [82].

6.5 Mode of Action

Ameri summarized the biological activities and mechanism of action of several diterpenoid alkaloids [83], and Wang and coworkers reviewed the mode of action of a number of alkaloids of this class [3]. Turabekova et al. studied 19 diterpenoid alkaloids with curare-like activity obtained from *Aconitum* and *Delphinium* and perform QSAR analysis and molecular docking into AChBP [84]. In this study, GA–MLRA and GA–PLS approaches have been used to build QSAR models to predict *N*-cholinolytic activity measured in vivo (blockade of neuromuscular conductivity, BNMC, and third eyelid relaxing activity, TYRA) and in vitro (suppression of frog's abdominal straight muscles on acetylcholine, SAM) for all 19 diterpenoid alkaloids. The H-bond interactions are revealed to be the prevalent factors modulating *N*-cholinolytic activity by both QSAR analysis and docking studies with additional contribution of steric and reactivity features as identified for TYRA and SAM data, respectively. The alkaloid–receptor complexes were further analyzed by means of the docking software using the binding site of MLA (an α7-type nAChR subtype selective antagonist) complexed with AChBP (homolog of the ligand-binding domain of nAChRs) as template. All compounds fitted in the binding pocket of native MLA. An additional docking on lappaconitine and aconite alkaloids revealed that they are known to bind to voltage-gated Na^+ channel but not to nAChRs.

6.6 Other Activities

A brine shrimp bioassay of the crude extract and the pure compounds gentianine and gentianadine obtained from *Gentiana olivieri* was found to be nontoxic [49].

7 Conclusion

Terpenoid alkaloids derived from a late-stage amination reaction, the so-called *pseudoalkaloids*, have for a long time been in the focus of investigation because of their intriguing structure, interesting chemistry, and ethnomedicinal and pharmaceutical importance. The majority of monoterpenoid alkaloids including some novel structure have been isolated from the genus *Incarvillea* of Gentianaceae family, while the majority of the diterpenoid alkaloids have been obtained from the genus *Aconitum* and *Delphinium* of Ranunculaceae family. The skeletal arrangement of monoterpenoid alkaloids is simple compared to the diterpenoid alkaloids. However, the structural peculiarities equally attract scientists to investigate monoterpenoid alkaloids. The diterpenoid alkaloids, on the other hand, are characterized by complicated but interesting structural features with a large number of stereocenters. Because of the structural diversity, diterpenoid alkaloids are classified into C_{18}-, C_{19}-, and C_{20}-diterpenoid alkaloids with many types.

The terpenoid alkaloids are considered to be derived from prenyl units that undergo enzymatic cyclization, while the nitrogen atom is inserted in the skeleton on a late stage in the biosynthesis. Some of the terpenoid alkaloids exhibit potent cardiovascular, cytotoxic, and antinociceptive activity, while others show extreme toxicity. The mode of action of some diterpenoid alkaloids including curare-like activity has also been studied. More investigations are needed on this type of alkaloids to provide a detailed understanding of the interdependence between structure, activity, and mode of action in order to develop future drug candidates.

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Purine Alkaloids, Cytokinins, and Purine-Like Neurotoxin Alkaloids

29

Hiroshi Ashihara, Takao Yokota, and Alan Crozier

Contents

1 Introduction 954
2 Occurrence 955
  2.1 Caffeine 955
  2.2 Theobromine and Other Methylxanthines 956
  2.3 Methyluric Acids 956
  2.4 Cytokinins 956
  2.5 Saxitoxin and Tetrodotoxin 957
3 Phytochemistry 957
  3.1 Purine Alkaloids 957
  3.2 Cytokinins 958
  3.3 Saxitoxin and Tetrodotoxin 958
4 Biosynthesis 959
  4.1 Theobromine and Caffeine 959
  4.2 1-Methylxanthine, 3-Methylxanthine, and Theophylline 961
  4.3 Methyluric Acids 962
  4.4 Cytokinins 963
  4.5 Saxitoxin 963
  4.6 Tetrodotoxin 963
5 Biological Activities 965
  5.1 The Role of Purine Alkaloids *in Planta* 965
  5.2 Biological Activity of Purine Alkaloids in Humans 965

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5.3 Toxicity of Purine Alkaloids in Animals 969
5.4 The Role of Cytokinins in Planta 969
5.5 Biological Activity of Saxitoxin and Tetrodotoxin 970
6 Biotechnology Approaches 970
7 Conclusion/Prospects 971
References 972

Abstract

Purine alkaloids, such as caffeine, theobromine, theophylline, 7-methylxanthosine, and theacrine, are produced in several plant species including tea, coffee, cacao, and maté. The major route of caffeine biosynthesis is the xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway. Two hypotheses concerning the ecological roles of caffeine in plants, chemical defense and allelopathic function theories, have been proposed. Genetically modified plants of agriculturally important plant species have been obtained. One is construction of coffee plants in which caffeine production is suppressed, and the others are caffeine-producing tobacco and chrysanthemum plants which resist infection by fungal and insect pests. The impact of caffeine on human health is discussed, and, in addition to purine alkaloids, cytokinins, adenine-derived phytohormones, and the neurotoxin alkaloids, saxitoxin and tetrodotoxin are also described briefly.

Keywords

Caffeine • theobromine • methylxanthine • methyluric acid • *trans*-zeatin • phytohormone • neurotoxin • tea • coffee • cacao

1 Introduction

In a narrow sense, purine alkaloids comprise methylxanthines and methyluric acids [1]. This chapter, however, also includes information on cytokinins, which are purine ring-containing phytohormones, and the purine-like neurotoxin alkaloids, saxitoxin and tetrodotoxin.

The most well-known purine alkaloid is caffeine (1,3,7-trimethylxanthine) **1** which, along with theobromine (3,7-dimethylxanthine) **2**, accumulates in leaves of tea and maté and beans of coffee and cacao, which are popular components of nonalcoholic beverages and/or chocolate products [2]. The pharmacological effects of purine alkaloids in animals, such as stimulation of the central nervous system, have been investigated extensively [3]. Caffeine is also often utilized in cytological studies to induce the formation of binucleate cells and is, therefore, used to measure the duration of the mitotic cycle [4].

Caffeine was first isolated from coffee in 1820 by the German chemist Friedlieb Ferdinand Runge and, independently, in 1821 by French chemists, Pierre Jean Robiquet, Pierre Joseph Pelletier, and Joseph Bienaimé Caventou. The biosynthesis and catabolism of caffeine were not fully elucidated until the 1990s [1]. Highly purified caffeine synthase, a key enzyme in the caffeine biosynthetic pathway, was obtained from tea leaves in 1999 [5], and the first isolation of a caffeine synthase gene from tea leaves was reported in 2000 [6]. Other methylxanthines, such as theophylline (1,3-dimethylxanthine), paraxanthine (1,7-dimethylxanthine), 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, and 7-methylxanthosine (**3–8**), are intermediates in the caffeine biosynthetic pathway as well as being catabolites of caffeine. Methyluric acids include structures **9–12**. Cytokinins, such as N^6-(Δ^2-isopentenyl)adenine (iP) **13**, *trans*-zeatin **14**, and *cis*-zeatin **15**, are hormones that promote cell division in plant tissues. Saxitoxin **16** and tetrodotoxin **17** are purine-like alkaloids responsible for shellfish and puffer fish poisoning, and these neurotoxins are also discussed briefly in this chapter.

2 Occurrence

2.1 Caffeine

Methylxanthines such as caffeine and theobromine have been found in nearly 100 species in 13 orders of the plant kingdom [7, 8]. Compared with other plant alkaloids, such as nicotine, morphine, and strychnine, purine alkaloids are distributed widely in plant species. The phylogenetic relationship between caffeine-synthesizing plant orders was shown in a review by Ashihara and Crozier [8] using Takhtajan's phylogenetic tree for angiosperms [9].

The accumulation of high concentrations of methylxanthines is restricted to a limited number of species belonging to the genera *Camellia*, *Coffea*, *Cola*, *Paullinia*, *Ilex*, and *Theobroma* [8]. The caffeine content of young leaves of first flush shoots of the teas *Camellia sinensis*, *Camellia assamica*, and *Camellia taliensis* is 2–3 % of dry weight, while the level in *Camellia kissi* is less than 0.02 %. The caffeine content of seeds of different *Coffea* species varies from 0.4 % to 2.4 % dry weight [10]. Green coffee beans (as opposed to roasted beans, which are used to prepare the beverage) of commercially cultivated species contain substantial quantities of caffeine; arabica coffee (*Coffea arabica*) beans typically contain 1.2–1.4 % caffeine [11], while robusta coffee (*Coffea canephora*) contains 1.2–3.3 % [10, 11]. There are also several wild coffee species where the green beans contain either no caffeine or extremely low levels. Such coffees include *Coffea eugenioides* and 30 species in the six subsections of *Mascarocoffea* [10, 12–14].

Young leaves of maté (*Ilex paraguariensis*) are a further source of caffeine (0.8–0.9 %) [15] as are seeds of guarana where caffeine is located mainly in the cotyledons (4.3 %) and testa (1.6 %) [16]. Citrus flowers can accumulate up to 0.17 % methylxanthines on a fresh weight basis with caffeine being the main component. Trace quantities of caffeine have also been found in the nectar of citrus flowers [17].

2.2 Theobromine and Other Methylxanthines

Theobromine **2** is an intermediate of caffeine biosynthesis and is often found as a minor methylxanthine in tissues that are actively synthesizing caffeine, such as young flush tea shoots and immature coffee seeds. However, it is the major methylxanthine in mature seeds of *Theobroma cacao* (cacao). The cotyledons of cacao beans contain 2.2–2.7 % theobromine on a dry weight basis and 0.6–0.8 % caffeine [18]. Analysis of several cacao genotypes representing the three horticultural races Criollo, Forastero, and Trinitario revealed considerable variations in the purine alkaloid content of the seed, with slightly higher levels found within the Criollo types [19]. Theobromine is also found as a predominant purine alkaloid (5.0–6.8 %) in young leaves of *Camellia ptilophylla* (cocoa tea) [20] and *Camellia irrawadiensis* (~0.8 %) [21]. Theophylline is a minor purine alkaloid in tea, coffee, maté, and guaraná [8].

2.3 Methyluric Acids

Methyluric acids are found in a very limited number of plant species. Theacrine (1,3,7,9-tetramethyluric acid) **10**, accompanied by caffeine, is a major purine alkaloid in the leaves of a Chinese tea called "kucha" (*Camellia assamica* var. kucha) [22]. The endogenous levels of theacrine and caffeine in expanding buds and young leaves of kucha can be up to 2.8 % of dry weight [23]. Mature leaves of wild coffee species, *Coffea liberica*, *Coffea dewevrei*, and *Coffea abeokutae*, contain different types of methyluric acids, namely theacrine along with liberine [*O*(2),1,9-trimethyluric acid] **11**, and methylliberine [*O*(2),1,7,9-tetramethyluric acid] **12** [24, 25]. Theacrine is the principal purine alkaloid in seeds of 11 species of *Theobroma* and nine species of *Herrania* [19]. *Theobroma grandiflorum* (cupu) contains 0.25 % liberine in cotyledons and 0.08 % in the nutshells [26].

2.4 Cytokinins

Naturally occurring cytokinins are adenine-type compounds synthesized in plant tissues. Major sites of cytokinin biosynthesis are roots, cambium, and other actively dividing tissues. Transport of *trans*-zeatin-type cytokinins **14** is via the xylem, while that of N^6-(Δ^2-isopentenyl)adenine-type cytokinins **13** occurs in the phloem. In addition to acting as local signals, cytokinins communicate acropetal and systemic long-distance signals, and structural side-chain variations mediate different biological messages [27].

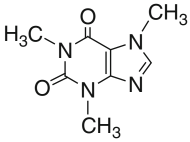
2.5 Saxitoxin and Tetrodotoxin

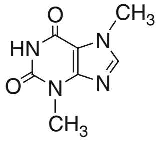
Saxitoxin **16** is produced by certain marine dinoflagellates and cyanobacteria [28]. Ingestion of saxitoxin, usually through shellfish contaminated by toxic algal blooms, is responsible for the human illness known as paralytic shellfish poisoning. The term saxitoxin originates from the name of the butter clam (*Saxidomus giganteus*) [29]. Like saxitoxin, tetrodotoxin **17** is produced by symbiotic bacteria, such as species of *Pseudomonas* and *Vibrio*, which reside within globefish, and other aquatic animals. The name of tetrodotoxin derives from *Tetraodontiformes*, the order that includes the globefish and related species which carry this toxin [30].

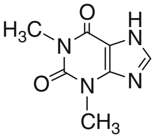
3 Phytochemistry

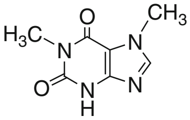
3.1 Purine Alkaloids

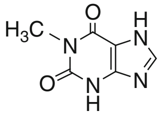
Purine is a heterocyclic aromatic organic compound, consisting of a pyrimidine ring fused to an imidazole ring. Purines, including substituted purines and their tautomers, are the most widely distributed nitrogen-containing heterocycle in nature [31]. Methylxanthines and methyluric acids **1–12** are classified as purine alkaloids, and their respective structures are based on xanthine and uric acid skeletons. Methyl groups originating from *S*-adenosyl-L-methionine are attached to nitrogen atoms at positions 1, 3, 7, and 9 or an oxygen at position 2.

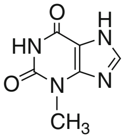

Caffeine **1**

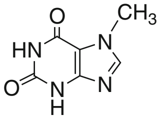

Theobromine **2**

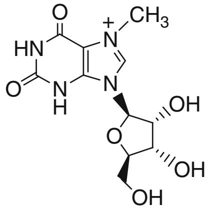

Theophylline **3**

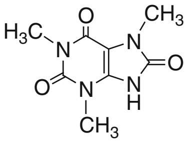

Paraxanthine **4**

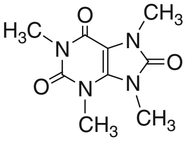

1-Methylxanthine **5**

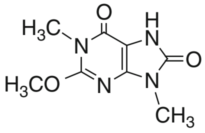

3-Methylxanthine **6**

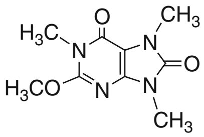

7-Methylxanthine **7**


7-Methylxanthosine **8**


1,3,7-Trimethyluric acid **9**

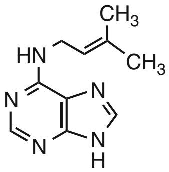

Theacrine **10**

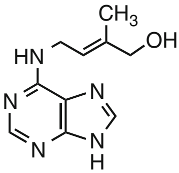

Liberine **11**

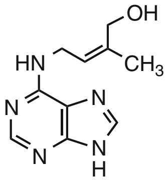

Methylliberine **12**

3.2 Cytokinins

Structurally, cytokinins are classified into two groups, namely, adenine-type cytokinins represented by zeatin, kinetin, and 6-benzylaminopurine, and phenylurea-type cytokinins which include diphenylurea and thidiazuron. The naturally occurring cytokinins N^6-(Δ^2-isopentenyl)adenine **13**, *trans*-zeatin **14**, and *cis*-zeatin **15** differ in the stereoisomeric hydroxylation at the isoprenoid side chain. *trans*-Zeatin and N^6-(Δ^2-isopentenyl)adenine are physiologically the most important cytokinins in plants [27].

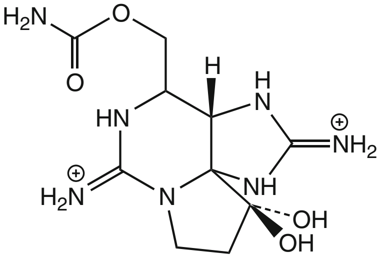

N^6-(Δ^2-Isopentenyl)adenine **13**

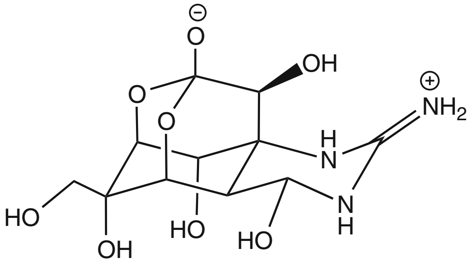

trans-Zeatin **14**


cis-Zeatin **15**

3.3 Saxitoxin and Tetrodotoxin

Chemical formula of saxitoxin **16** is $C_{10}H_{17}N_7O_4$ and that of tetrodotoxin **17** is $C_{11}H_{17}N_3O_8$. Both compounds are water soluble because of their ionic structures. There are many saxitoxin-related compounds which contain additional sulfate ester and/or sulfocarbamoyl moieties. In the case of tetrodotoxin, numerous analogues have been isolated from a variety of aquatic animals including puffer fish, newts, frog, and octopus [32].


Saxitoxin **16**


Tetrodotoxin **17**

4 Biosynthesis

4.1 Theobromine and Caffeine

Methylxanthines are formed from purine nucleotides in plants. Results from studies on in situ metabolism of ^{14}C- and ^{15}N-labeled precursors and from identification of enzymes and genes have established that the main caffeine biosynthetic pathway is a four-step sequence consisting of three methylations and one nucleosidase reaction with xanthosine acting as the initial substrate (Fig. 29.1). Although the information has been obtained mainly from coffee (*Coffea arabica* and *Coffea canephora*) and tea (*Camellia sinensis*), the available evidence suggests that the pathway is essentially the same in other methylxanthine-forming plants [8, 33, 34]. The formation of monomethylxanthine in the main caffeine biosynthetic pathway is initiated by the conversion of xanthosine to 7-methylxanthosine. This reaction is catalyzed by 7-methylxanthosine synthase (EC 2.1.1.158). The second step involves a nucleosidase which catalyzes the hydrolysis of 7-methylxanthosine. It was considered that *N*-methyl nucleosidase (EC 3.2.2.25), which occurs in tea leaves, participates in this reaction [35], but recent structural studies on coffee 7-methylxanthosine synthase suggested that both the methyl transfer and nucleoside cleavage are coupled and catalyzed by a single enzyme [36]. The third step in the caffeine biosynthesis is also catalyzed by a SAM-dependent *N*-methyltransferase.

Highly purified caffeine synthase (EC 2.1.1.160) obtained from young tea leaves has a broad substrate specificity and catalyzes the two-step conversion of 7-methylxanthine to caffeine via theobromine [5]. This enzyme is distinct from the *N*-methyltransferase that catalyzes the first methylation step in the caffeine pathway. Plural genes encoding *N*-methyltransferases have been cloned (Table 29.1). The recombinant enzymes obtained from these genes show different substrate specificities. The recombinant coffee caffeine synthase (EC 2.1.1.160) can utilize paraxanthine, theobromine, and 7-methylxanthine as substrates. The activity of the recombinant theobromine synthase (EC 2.1.1.159) is specific for the conversion of 7-methylxanthine to theobromine. Theobromine synthase, but not the dual-functional caffeine synthase, appears to participate principally in theobromine synthesis in theobromine-accumulating plants, such as *Theobroma cacao*, *Camellia ptilophylla*, and *Camellia irrawadiensis* [37]. Conversion of theobromine to caffeine is performed by the dual-functional caffeine synthase (EC 2.1.1.160). The methylation of *N*1 of 7-methylxanthine by caffeine synthase is much slower than that of *N*3, and, as a consequence, there is only transient accumulation of theobromine in caffeine-synthesizing tissues (Fig. 29.1). In addition to the main caffeine biosynthetic pathway, various minor routes may also operate (Fig. 29.1). Presence of these alternative pathways appears to be a consequence of the broad specificities of the *N*-methyltransferases. For example, caffeine synthase catalyzes *N*3- and *N*1-methylations; thus, 3-methylxanthine and paraxanthine are synthesized, respectively, from xanthine and 7-methylxanthine [5]. However, only restricted accumulation of these

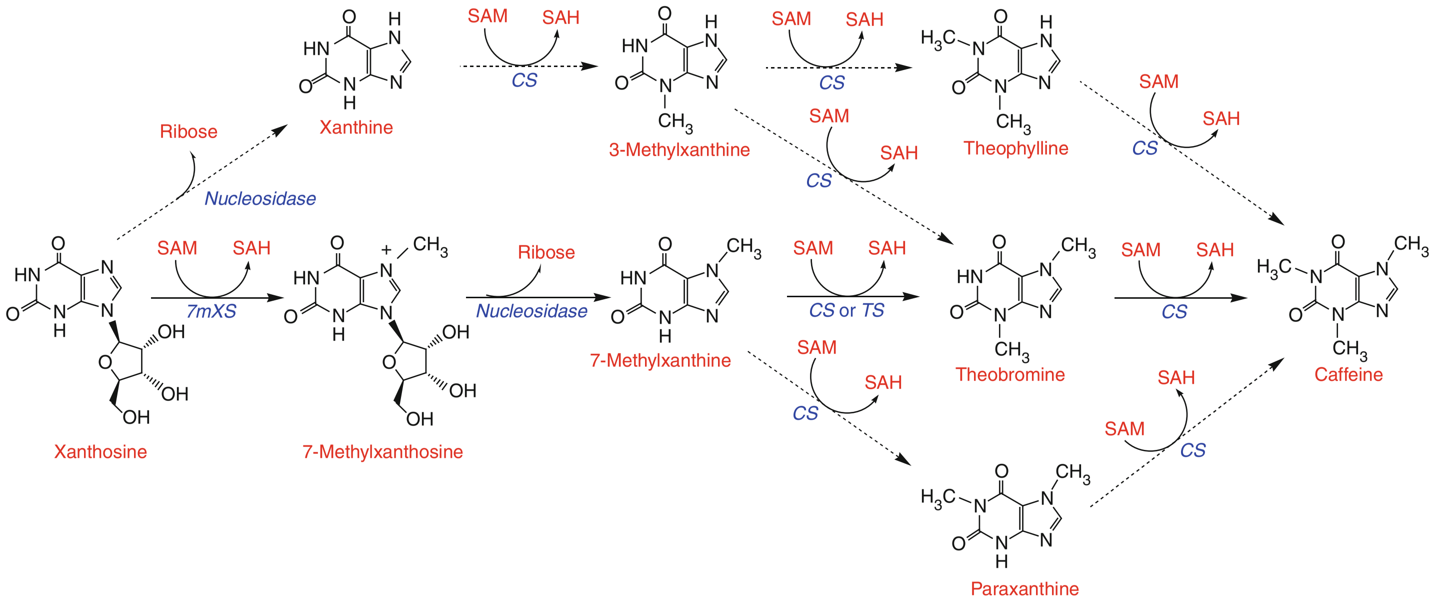

Xanthosine
Ribose
Nucleosidase
Xanthine
SAM
SAH
7mXS
7-Methylxanthosine
Ribose
Nucleosidase
7-Methylxanthine
SAM
SAH
CS
3-Methylxanthine
SAM
SAH
CS
Theophylline
SAM
SAH
CS
SAM
SAH
CS or TS
Theobromine
SAM
SAH
CS
Paraxanthine
SAM
SAH
CS
SAM
SAH
CS
SAM
SAH
CS
Caffeine

Fig. 29.1 The biosynthetic pathways of caffeine from xanthosine. The major pathway that consists of four steps is shown with *solid arrows*. Conversion of 7-methylxanthosine to 7-methylxanthine is catalyzed by nucleosidase activity involved in 7-methylxanthosine synthase or a specific *N*-methyl nucleosidase. Minor pathways, shown with *dotted arrows*, are theoretically possible. They may occur because of the broad substrate specificity of caffeine synthase or other *N*-methyltransferases. Enzymes: *7mXS* 7-methylxanthosine synthase, *CS* caffeine synthase, *TS* theobromine synthase, *SAM S*-adenosyl-L-methionine, *SAH S*-adenosyl-L-homocysteine

Table 29.1 Genes of *N*-methyltransferases involved in caffeine biosynthesis isolated from coffee (*Coffea arabica*) plants

Enzyme name	EC number	Gene accession number
7-Methylxanthosine synthase[a]	2.1.1.158	AB034699, AB048793
S-Adenosyl-L-methionine:xanthosine N^7-Methyltransferase[b]		
Xanthosine methyltransferase[c]		
Theobromine synthase[a]	2.1.1.159	AB034700, AB054841 AB048794
S-Adenosyl-L-methionine:7-methylxanthine N^3-Methyltransferase[b]		
7-Methylxanthine methyltransferase[c]		
Caffeine synthase[a]	2.1.1.160	AB086414, AB984125
S-Adenosyl-L-methionine:3,7-dimethylxanthine N^1-Methyltransferase[b]		
3,7-Methylxanthine methyltransferase[c]		

[a] Accepted name
[b] Systematic name
[c] Other name by the IUBMB Enzyme Nomenclature are shown

compounds occurs: 3-methylxanthine can be catabolized to xanthine, and paraxanthine appears to be immediately converted to caffeine. Paraxanthine is the most active substrate of caffeine synthase [5], but only limited amounts of the dimethylxanthine appear to be produced in plant tissues because *N*1-methylation of 7-methylxanthine is very slow [1].

Xanthosine, the initial substrate of purine alkaloid synthesis, is supplied by at least four different pathways: *de novo* purine biosynthesis (*de novo* route), degradation of adenine nucleotides (AMP route), the SAM cycle (SAM route), and guanine nucleotides (GMP route) [1, 38]. The pathways leading to xanthosine formation are illustrated in Fig. 29.2.

4.2 1-Methylxanthine, 3-Methylxanthine, and Theophylline

Production of 1-methylxanthine **5**, 3-methylxanthine **6**, and theophylline **3** is carried out by minor branches of the caffeine biosynthetic pathway as described in Sect. 4.1; however, they are also involved in the degradation of caffeine [1, 34]. In contrast to theobromine, which is the immediate precursor of caffeine, theophylline, which is also a dimethylxanthine, is the first intermediate of caffeine catabolism. Caffeine accumulates in mature plant tissues, principally as a consequence of being degraded very slowly with the sequential removal of the methyl groups, resulting in the conversion of theophylline to xanthine, with 1- and 3-methylxanthine serving as intermediates. Xanthine is further degraded by the conventional purine catabolic pathway in higher plants [39–41] (Fig. 29.3).

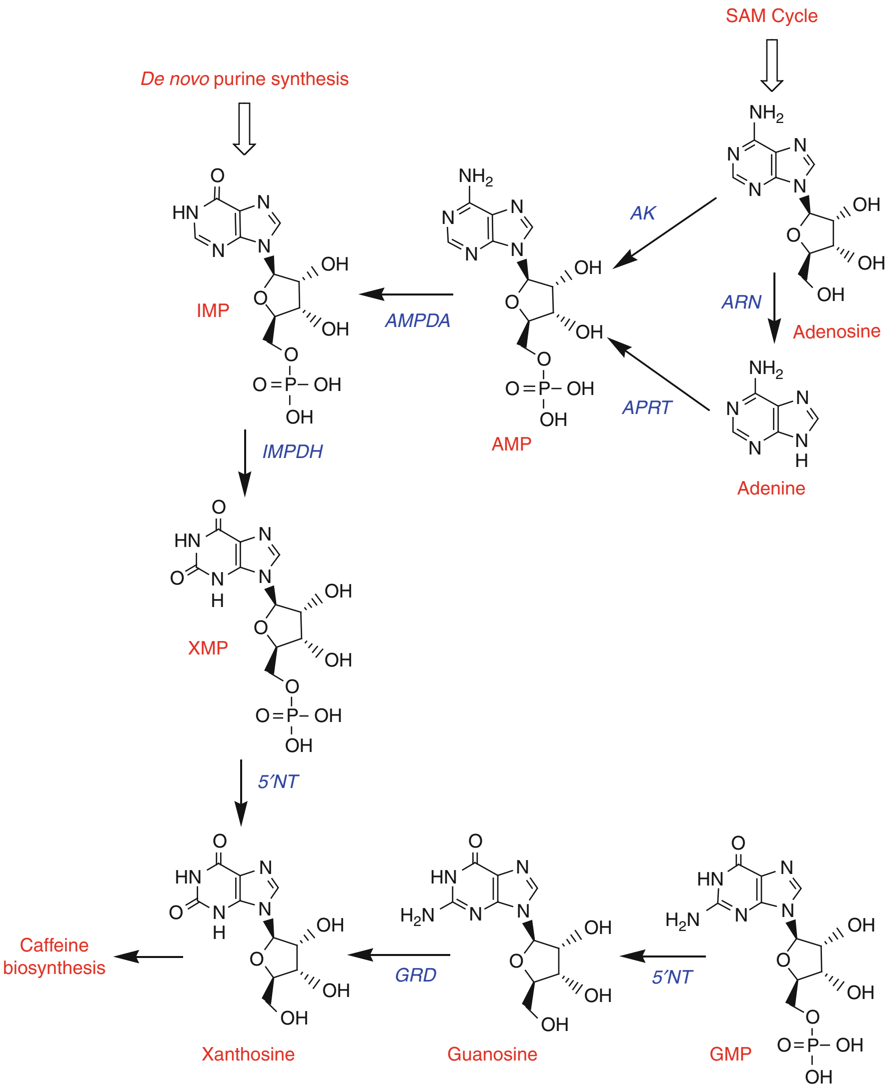

SAM Cycle
De novo purine synthesis
IMP
AMPDA
AMP
AK
ARN
Adenosine
APRT
Adenine
IMPDH
XMP
5′NT
Caffeine biosynthesis
Xanthosine
GRD
Guanosine
5′NT
GMP

Fig. 29.2 Caffeine is produced from xanthosine derived from four routes: (1) inosine-5′-monophosphate (IMP) originating from *de novo* purine synthesis (*de novo* route), (2) adenosine released from the *S*-adenosylmethionine (SAM) cycle, (3) the cellular adenosine nucleotide pool (AMP route), and (4) the guanine nucleotide pool (GMP route). Enzymes: *AMPDA* AMP deaminase, *APRT* adenine phosphoribosyltransferase, *AK* adenosine kinase, *ARN* adenosine nucleosidase, *GRD* guanine deaminase, *IMPDH* IMP dehydrogenase, *5′NT* 5′-nucleotidase

4.3 Methyluric Acids

Little is known on the biosynthetic pathways of methyluric acids. Radiolabeled feeding experiments indicate that theacrine **10** is synthesized from caffeine.

Conversion of caffeine to theacrine in kucha probably occurs by successive oxidation and methylation steps with 1,3,7-trimethyluric acid **9** acting as the intermediate [23]. Leaves of *Coffea dewevrei*, *Coffea liberica*, and *Coffea abeokutae* converted caffeine **1** to liberine **11** probably via theacrine **10** and methylliberine **12** [25] (Fig. 29.4).

4.4 Cytokinins

Biosynthetic pathways of naturally occurring cytokinins are illustrated in Fig. 29.5. The first step of cytokinin biosynthesis is the formation of N^6-(Δ^2-isopentenyl) adenine nucleotides catalyzed by adenylate isopentenyltransferase (EC 2.5.1.27). In higher plants, N^6-(Δ^2-isopentenyl)adenine riboside 5′-triphosphate or N^6-(Δ^2-isopentenyl)adenine riboside 5′-diphosphate are formed preferentially. In Arabidopsis, N^6-(Δ^2-isopentenyl)adenine nucleotides are converted into *trans*-zeatin nucleotides by cytochrome P450 monooxygenases. Bioactive cytokinins are base forms. Cytokinin nucleotides are converted to nucleobases by 5′-nucleotidase and nucleosidase as shown in the conventional purine nucleotide catabolism pathway. However, a novel enzyme, cytokinin nucleoside 5′-monophosphate phosphoribohydrolase, named LOG, has recently been identified. Therefore, it is likely that at least two pathways convert inactive nucleotide forms of cytokinin to the active freebase forms that occur in plants [27, 42]. The reverse reactions, the conversion of the active to inactive structures, seem to be catalyzed by adenine phosphoribosyltransferase [43] and/or adenosine kinase [44]. In addition, biosynthesis of *cis*-zeatin from tRNAs in plants has been demonstrated using Arabidopsis mutants with defective tRNA isopentenyltransferases [45].

4.5 Saxitoxin

The biosynthetic pathway of saxitoxin **16** was originally proposed by Shimizu et al. [46]. Recently, Kellmann et al. [47] described a candidate paralytic shellfish poisoning toxin biosynthetic gene cluster (*sxt*) from *Cylindrospermopsis raciborskii* T3. The saxitoxin biosynthetic pathway is encoded by a more than 35-kb gene cluster, and comparative sequence analysis has assigned 30 catalytic functions to 26 encoded proteins. Saxitoxin biosynthesis is initiated by Claisen condensation-based acylation of arginine by propionyl-ACP produced from *S*-adenosylmethionine and acetyl-ACP by a new type of polyketide synthase. Further steps are catalyzed by enzymes responsible for heterocyclizations and various tailoring reactions that produce saxitoxin **16** (Fig. 29.6) and numerous isoforms of saxitoxin.

4.6 Tetrodotoxin

Based on the structures of tetrodotoxin **17** and its analogues, Yasumoto et al. [48] proposed that the biosynthetic pathway proceeds via condensation of arginine

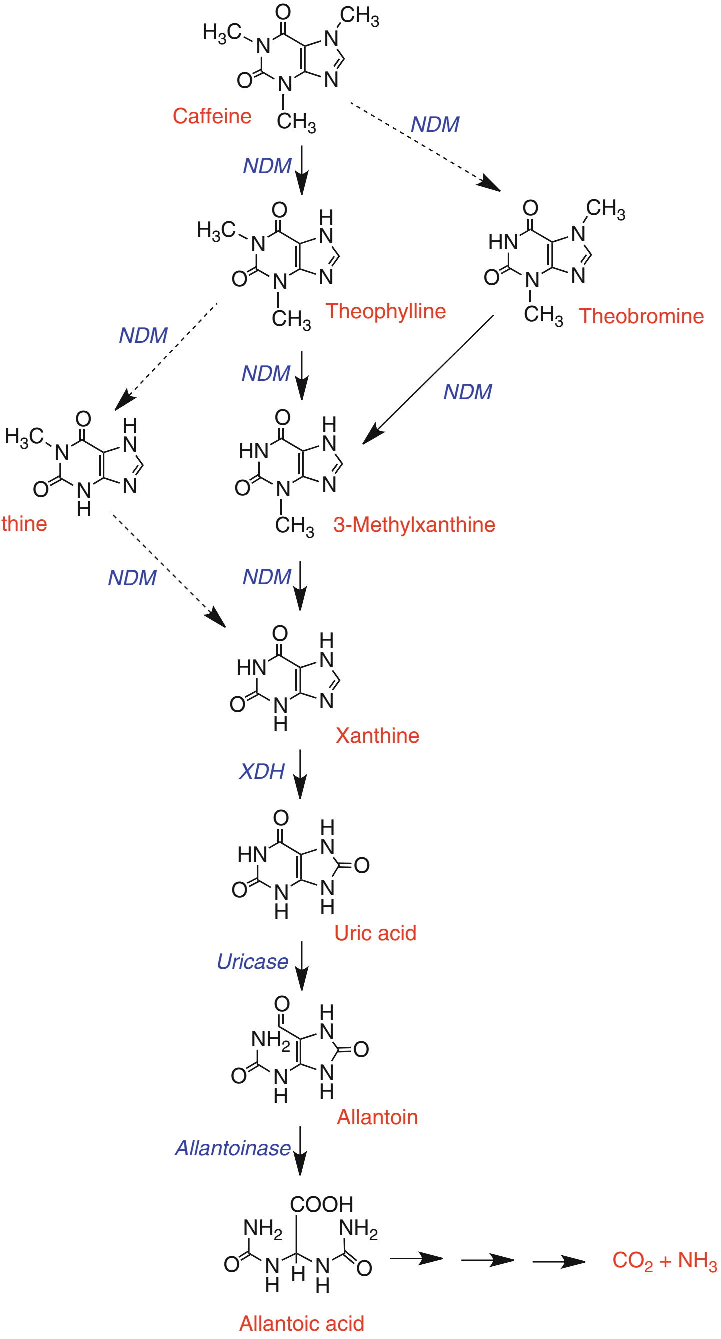

Caffeine
NDM
NDM
Theophylline
Theobromine
NDM
NDM
NDM
1-Methylxanthine
3-Methylxanthine
NDM
NDM
Xanthine
XDH
Uric acid
Uricase
Allantoin
Allantoinase
Allantoic acid
$CO_2 + NH_3$

Fig. 29.3 (continued)

and isoprene. Later, Yotsu-Yamashita et al. [49] suggested another possible pathway via condensation of guanidine, a pentose sugar and isoprene. The pathways are illustrated in Fig. 29.7, but as yet there are no firm data to support these proposals. Due to its unique structure, several plausible routes and many possible enzymes could potentially be involved in tetrodotoxin biosynthesis. Possible reactions involved in the putative pathways were discussed by Chau et al. [50].

5 Biological Activities

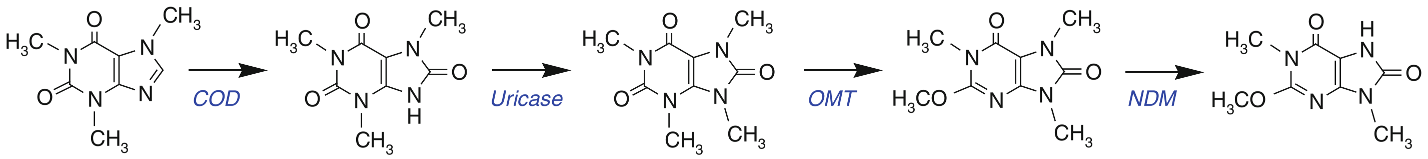
5.1 The Role of Purine Alkaloids *in Planta*

The physiological role of purine alkaloids, and in particular caffeine, *in planta* is largely unknown. They appear not to act as a nitrogen reserve since they are not transported to young developing organs and considerable amounts remain in senescing tissues. There are two hypotheses concerning the ecological roles of caffeine **1** in plants. The chemical defense theory proposes that the high concentrations of caffeine in young leaves, fruits, and flower buds of species such as tea and coffee act as a defense to protect young, delicate tissues from pathogens and herbivores [51]. The allelopathic or autotoxic function theory proposes that caffeine in seed coats and falling leaves is released into the soil to inhibit germination of seeds around the parent plants [52]. Inhibition of germination and growth of seeds by caffeine was recognized as long ago as 1912 by Ransom [53]. In caffeine-synthesizing plant species, caffeine is probably sequestered in special organelles or organs, such as vacuoles and seed coats, and as a result, no inhibition occurs. However, even in caffeine-producing plants, exogenously supplied caffeine inhibits the growth [54]. It is unclear to what extent caffeine is involved in allelopathy in natural ecosystems, especially as soil bacteria such as *Pseudomonas putida* can degrade methylxanthines [40].

5.2 Biological Activity of Purine Alkaloids in Humans

There are many publications on caffeine and human health [3, 55, 56]. The main effects of moderate consumption of caffeine by humans are an increase in attention,

Fig. 29.3 3-Methylxanthine, 1-methylxanthine, and theophylline are synthesized from caffeine as part of the caffeine degradation pathway that produces xanthine which is further degraded to CO_2 and NH_3 by the conventional oxidative purine catabolism pathway. The conversion of caffeine to theophylline is the rate-limiting step in caffeine-accumulating species such as *Coffea arabica* and *Camellia sinensis*. *Solid arrows* indicate major routes and *dotted arrow* minor conversions. The pathway was elucidated from data obtained in feeding experiments. *N*-Demethylase enzymes involved in caffeine degradation have not yet been characterized in plants. Enzymes: *NDM N*-demethylase, *XDH* xanthine dehydrogenase



Caffeine **1**

1,3,7-Trimethyluric acid **9**

Theacrine **10**

Methylliberine **12**

Liberine **11**

Fig. 29.4 Proposed biosynthesis of liberine from caffeine [26]. Enzymes: *COD* caffeine oxidase, *OMT O*-methyltransferase, *NDM N*-demethylase

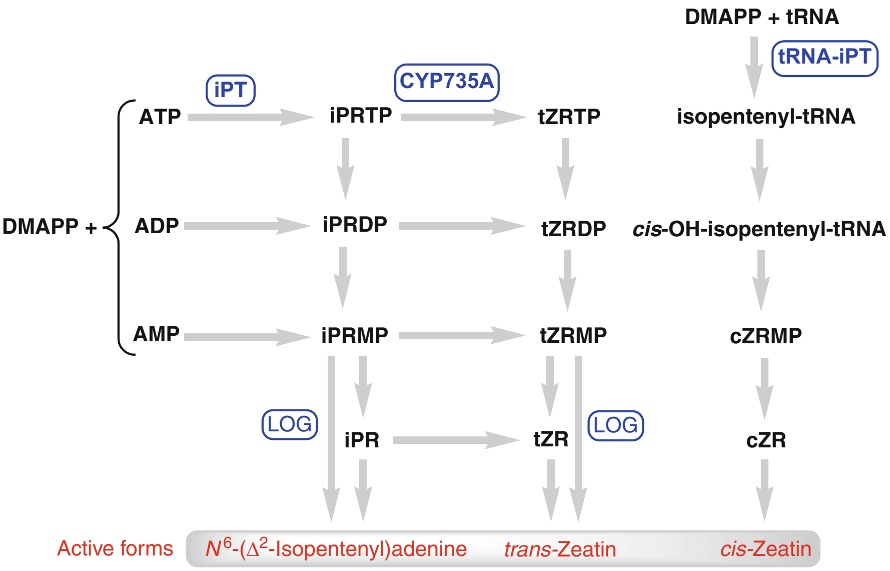


Fig. 29.5 The two cytokinin biosynthesis and activation pathways. *DMAPP* dimethylallyl diphosphate, *iP* N^6-(Δ^2-isopentenyl)adenine, *iPRMP* iP riboside 5′-monophosphate, *tZ* *trans*-zeatin, *tZRTP* tZ riboside 5′-triphosphate, *tZRDP* tZ riboside 5′-diphosphate, *tZRMP* tZ riboside 5′-monophosphate, *cZ* *cis*-zeatin, *cZRMP* cZ riboside 5′-monophosphate, *cZR* cZ riboside. Enzymes: *iPT* isopentenyltransferase, *CYP735A* cytochrome P450 CYP735A, *tRNA-iPT* transfer RNA isopentenyltransferase, *LOG* lonely guy, cytokinin nucleoside 5′-monophosphate phosphoribohydrolase (After Hirose et al. [26])

memory performance, physical performance, muscle recovery and intraocular pressure, and a possible decrease in risk of heart disease.

The attraction of tea and coffee to many consumers results from the ability of caffeine to increase alertness and a capacity to remain alert for longer periods without sleep. The biochemical mechanism underlying this effect is a synergistic interaction with catecholamines (adrenaline and noradrenaline – the major signals in the sympathetic nervous system) and antagonism of the actions of adenosine, which elevates another neurotransmitter in the brain, dopamine. Elevation of dopamine concentration in the brain is responsible for many of the central nervous system stimulants and indeed addictive properties of caffeine. Dopamine also enhances the action of serotonin on raising mood. Caffeine increases sympathetic activity and nor/adrenaline levels, and if catecholamines are elevated for any reason, such as stress, then the biological actions of caffeine are exaggerated. An important catecholamine action augmented by caffeine is inhibition of the enzyme cyclic AMP-phosphodiesterase, which results in the accumulation of cyclic AMP in cells, and promotes glucose synthesis. It also leads to excessive gastric acid secretion, which can cause symptoms of heartburn and indigestion [57]. This is one of a range of adverse effects of caffeine which includes anxiety, tremors, and other gastrointestinal problems to which some people are all but immune while

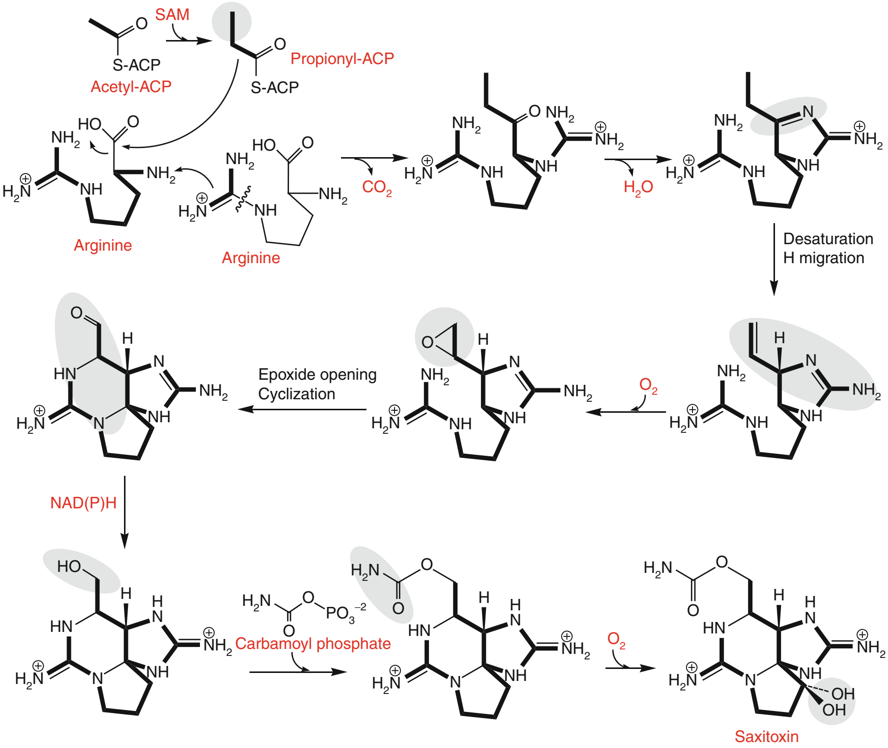


Fig. 29.6 Proposed biosynthesis of saxitoxin [47]. Pathways were envisaged by the analysis of a saxitoxin biosynthesis gene cluster in cyanobacteria. Moieties resulting from reactions are highlighted by *gray shading*

others are extremely sensitive and experience such effects after drinking of one cup of coffee [3].

After consuming tea or coffee, caffeine is rapidly absorbed in the upper gastrointestinal tract of humans and can be detected in the bloodstream within 5 min, reaching peak concentrations after ca. 30 min. It readily crosses the blood–brain barrier and enters all body fluids including serum, saliva, milk, and semen. Caffeine is metabolized in the liver by microsomal enzymes and xanthine oxidase, which together mediate demethylations and oxidations yielding products that include di- and monomethylxanthines, methylated uric acids, and uracil derivatives [3]. The half-life of caffeine is typically 2.5–4.5 h, and the elimination rate can be increased by exercise. Excretion is slower in the grossly obese and those suffering from liver disease. Oral contraceptives delay clearance by one third. Smoking, in contrast, enhances clearance by inducing the caffeine-metabolizing enzymes in the liver. Caffeine metabolism is slower, and the half-life extended up to 10 h, in pregnancy and is much longer in the developing fetus. Newborn children, especially premature infants, can have a greatly extended half-life for caffeine (>100 h) because of incomplete development of the hepatic

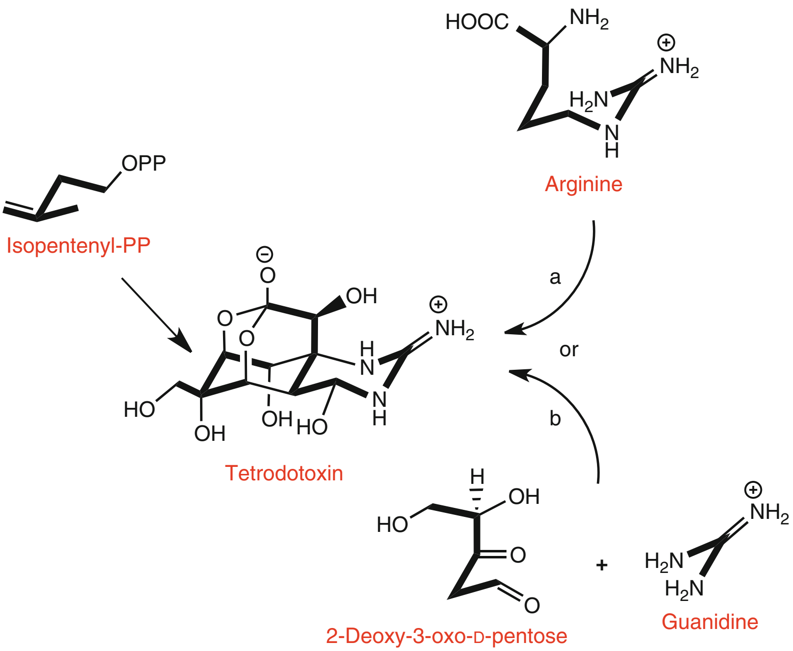

HOOC
NH2
⊕
NH2
H2N
N
H
Arginine
OPP
Isopentenyl-PP
a
or
b
⊖
O
O
O
OH
⊕
NH2
H
N
N
H
HO
OH
OH
HO
Tetrodotoxin
H
OH
HO
O
O
2-Deoxy-3-oxo-D-pentose
+
⊕
NH2
H2N
H2N
Guanidine

Fig. 29.7 Proposed biosynthesis of tetrodotoxin [48, 49]. The pathways illustrated have not yet been supported experimentally. Isopentenyl-PP, isopentenyl diphosphate

metabolizing enzymes. The occurrence of unwanted and unpleasant side effect of caffeine is, therefore, more likely in pregnancy, in young children, and those with liver disease, and for this reason, intake of caffeine-containing beverages by these groups should be limited [3].

5.3 Toxicity of Purine Alkaloids in Animals

Several species, including dogs, foxes, badgers, horses, and the New Zealand kea (an alpine parrot), have a very limited capacity to metabolize theobromine **2** which tends to accumulate and become toxic with potentially fatal consequences [58, 59]. Depending upon the size of the dog, as little as 100–200 mg of theobromine, which can be the content of ~100 g of milk chocolate or ~10 g of dark chocolate [60], can be fatal. Caffeine consumption by horses leads to theophylline accumulation and hyperactivity [61].

5.4 The Role of Cytokinins in Planta

Cytokinins are a group of mobile phytohormones that play a critical role in plant growth and development by regulating leaf senescence, apical dominance, root proliferation, phyllotaxis, reproductive competence, and nutritional signaling [27]. Cytokinins also participate in the maintenance of meristem function [42] and in the modulation of metabolism and morphogenesis in response to environmental stimuli [62].

5.5 Biological Activity of Saxitoxin and Tetrodotoxin

The ingestion of saxitoxin **16** causes paralytic shellfish poisoning. The medical importance of saxitoxin originates from its production by certain dinoflagellates which are abundant in algal blooms known as red tides. The toxin is released into the surrounding water and ingested by bivalve filter feeders such as oysters, clams, and mussels which sequester saxitoxin in their tissues. Saxitoxin is water soluble and heat resistant so is not destroyed by cooking and passes on readily into humans who consume contaminated seafood. The blocking of neuronal sodium channels which occurs in paralytic shellfish poisoning produces a flaccid paralysis that leaves its victim calm and conscious as the symptoms develop with death typically occurring from respiratory failure.

Tetrodotoxin **17**, produced by a number of marine organisms, including puffer fish and other members of Tetraodontiformes, is a potent neurotoxin that is approximately ten times more poisonous than potassium cyanide. It too blocks the action potential in nerves by binding to the voltage-gated, fast sodium channels in nerve cell membranes thereby preventing the nerve cells from firing.

The flesh of puffer fish (fugu) is considered a delicacy in Japan. The fish is prepared by specially trained chefs who are certified by the government to prepare the flesh free of the toxic, liver, skin, and gonads. Despite these precautions, many cases of tetrodotoxin poisoning are reported every year after eating fugu. The toxic dose, caused by paralysis of the diaphragm and subsequent respiratory failure, is unclear because different puffer fish contain varying levels of tetrodotoxin. A dose of 1–2 mg of purified tetrodotoxin can be lethal, and reported cases have documented poisoning after ingestion of as little as ~40 g of puffer fish [63, 64].

6 Biotechnology Approaches

Since genes of enzymes of caffeine biosynthesis have been cloned, genetically modified (GM) plants of several plant species have been obtained; one is construction of GM coffee plants in which caffeine production is suppressed, and the other is introduction of caffeine biosynthesis into what are normally non-caffeine-producing species [34]. The first approach involves introducing antisense or the double-stranded RNA interference (RNAi) constructs for enzymes of caffeine biosynthesis into *Coffea canephora* and *Coffea arabica* [65, 66]. Although transgenic coffee beans have not yet obtained, decaffeinated coffee beans are likely to be produced in the near future. The use of such genetic engineering to make full-flavored caffeine-deficient coffee and tea will be of interest to the increasing numbers of consumers who are concerned about the potentially adverse effects of caffeine consumption on their health.

The second approach was to construct caffeine-producing transgenic tobacco (*Nicotiana tobacum*) plants, which naturally do not synthesize the purine alkaloid. This idea was based on a report that exogenously applied caffeine markedly increased the resistance of plants against several pests, and thereby enhanced their growth and survival [67]. The study has been achieved using a multigene transfer system, involving cDNAs for all three *N*-methyltransferases, and the resulting transgenic tobacco plants successfully accumulated caffeine in leaves in amounts that proved unpalatable to tobacco cutworms [34, 68, 69]. Recently, Sano and coworkers reported the simultaneous activation of salicylate production and fungal resistance in transgenic caffeine-producing chrysanthemum plants [70]. The transgenic chrysanthemum, which produced an equivalent level of caffeine as the tobacco plants at ~3 μg per g fresh weight, exhibited resistance against gray mold fungal attack. Transcripts of *PR-2* gene, a marker for pathogen response, were constitutively accumulated in mature leaves without pathogen attack. The levels of salicylic acid and its glucoside conjugate in mature leaves of the transgenic lines were found to be 2.5-fold higher than in the wild-type control. These findings suggest that stimulation of endogenous caffeine production and/or deposition of salicylates may activate a series of defense reactions even under nonstressed conditions. The caffeine-producing transgenic chrysanthemum plants have also been shown to have resistance against herbivores, lepidoptera caterpillars, and aphids [71].

7 Conclusion/Prospects

It is over decade since the first report on the cloning of *N*-methyltransferases involved in caffeine biosynthesis from tea and coffee plants [1]. Much of the widespread interest in this research has been fuelled by the possibilities of using genetic engineering to obtain transgenic, low-caffeine coffee and tea that could be used to produce “natural” decaffeinated beverages. However, as yet, there are no reports on the caffeine content of coffee beans produced by such plants. The recent report of Kim and Sano [72] that tobacco plants genetically engineered to produce caffeine repel insect pests and exhibit resistance to viral and bacterial infections is potentially of major importance and likewise the resistance to fungal infection of transgenic chrysanthemum plants that accumulate caffeine and salicylic acid [70]. Almost 40 % of world agricultural production is lost because of herbivores and diseases, and a practical and economic means of pest control is one of the most urgent measures required to obtain a reliable and increasing supply of foodstuffs for the world’s expanding population. The production of low-level caffeine by agriculturally important crops may have the potential to offer protection against both insect pests and pathogens, and from an environmental prospective, it would certainly be cheaper and more environmentally friendly than treatment of crops with pesticides and fungicides.

Information on cellular metabolic organization of caffeine biosynthesis and catabolism links to purine nucleotide metabolism, intercellular translocation, and accumulation mechanisms at specific cellular sites, such as chloroplasts and vacuoles, have yet to be fully revealed. Cell-, tissue-, and organ-specific synthesis and possibly catabolism of purine alkaloids may be regulated by unique and unknown developmental- and environmental-specific control mechanisms. A great deal of fascinating purine alkaloid biology in plants still remained to be discovered.

Compared with purine alkaloids, an extensive number of studies have investigated cytokinins, and many reviews have been published. Readers interested in this topic are directed to a number of recent, excellent reviews [27, 62, 73]. The studies on paralytic shellfish toxin metabolism in the cyanobacteria suggested that saxitoxin and derivatives are converted into xanthine, urea, and further to CO_2 and NH_4^+ or recycled via primary metabolism through the purine degradation pathway [74]. Details on the current status of saxitoxin **16** and tetrodotoxin **17** can be found in recent reviews [28, 46, 47, 50, 75].

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Aziridine Alkaloids: Origin, Chemistry and Activity

30

Valery M. Dembitsky, Alexander O. Terent’ev, and Dmitri O. Levitsky

Contents

1 Introduction 978
2 Natural Aziridine Alkaloids 978
3 Selected Semisynthetic and Synthetic Aziridine Alkaloids as Analogues of Natural Products 990
4 Conclusion 1002
References 1002

Abstract

This chapter describes research on natural aziridine alkaloids isolated from both terrestrial and marine species, as well as their lipophilic semisynthetic and/or synthetic analogues. Over 100 biologically active aziridine-containing compounds demonstrate confirmed pharmacological activity including antitumor, antimicrobial, and antibacterial effects. The structures, origin, and biological activities of aziridine alkaloids are reviewed. Consequently, this

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review emphasizes the role of aziridine alkaloids as an important source of drug prototypes and leads for drug discovery.

Keywords
Alkaloids • antibacterial • anticancer • aziridine • bioactive • plant

1 Introduction

Several groups of rare natural alkaloidal metabolites incorporating the cyclobutane [1], aziridine [2, 3], and azetidine moieties [4] and/or their synthetic counterparts possess a broad spectrum of biological activities.

Aziridine alkaloids also belong to a rare and somewhat neglected group of natural products which are known to play a seminal role in the secondary metabolism of some microorganisms, plants, and various marine organisms [5]. The aziridine-containing compounds have been of interest as both immunomodulatory and anticancer agents since the late 1950s [6]. Aziridines are inherently strained making them attractive for study in terms of reactivity and pharmacodynamic action. Ethylenimine (or aziridine, **1**) and some of its simple derivatives are commercial products in different fields of applied chemistry [7]. Observations of the toxic action of aziridines have prompted extensive investigations involving their synthesis and pharmacological activity, allowing selection and advancement of suitable substances as putative cancer chemotherapeutic agents. Notably, a few are enjoying regular clinical use [8]. Bayer strain encourages ring-opening reactions of aziridines in the presence of nucleophiles, imparting useful alkylating properties, despite their powerful mutagenic and toxic activities [9].

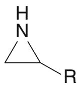
Aziridines are highly valuable heterocyclic compounds and are widely used during the synthesis of numerous drugs and biologically active natural products (and their derivatives) [10–15]. Many aziridine alkaloids have anticancer, antibacterial, and/or antimicrobial activity against selected cancer cell lines, pathogenic bacteria, and/or microorganisms strongly indicating that the presence of the aziridine ring in natural as well as synthetic compounds is essential for such activities [16–19].

This chapter reviews natural aziridine alkaloids, with high antitumor, antimicrobial, and antibacterial activities, and also highlights those semisynthetic derivatives and analogues which possess therapeutic promise.

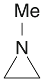
2 Natural Aziridine Alkaloids

The simple alkaloid, ethylenimine (aziridine, azacyclo-propane, or aziran, **1**) was detected in various foodstuffs including baker's yeast (*Saccharomyces cerevisiae*) autolyzate [20], in the volatile flavoring constituents of cooked chicken, beef and pork [21], and beef flavor [22]. Two metabolites (**1**) and aziridine-2-carboxylic acid (**2**) were isolated from mushrooms *Agaricus silvaticus* (class Basidiomycetes), both of which have been synthesized [23]. Aziridine-2-carboxylic acid (**2**) as well as aziridine-containing peptides are vital intermediates in the synthesis of various amino acid and peptide derivatives [24]. Furthermore, (**2**) and related compounds represent interesting substrates for clarifying enzyme mechanisms but also as the warhead of novel irreversible protease inhibitors with a number of potential therapeutic applications [25, 26].

More complex aziridines are found in various plant sources. For instance, 1-methyl-aziridine (**3**) was detected using GC-MS within onion bulbs (*Allium cepa*, class Liliopsida, order Asparagales, family Alliaceae) [27]. Flue-cured tobacco (*Nicotiana tabacum*, family Solanaceae) contains 4-(1-aziridinyl)-3-buten-2-one (**4**) [28]. Natural aziridine alkaloids (**2**,**5**–**11**) were detected and isolated from distillate, and residue in extractions of dried matter of *Petasites japonicus* (family Asteraceae, Japanese name Fuki) [29] is also known as bog rhubarb or giant butterbur. It is native to Japan, where the spring growth is relished as a vegetable. Consequently, its pharmacological properties are of considerable importance.



1 R = H
2 R = COOH



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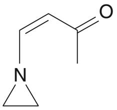

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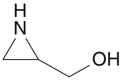
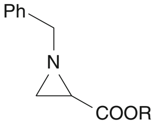
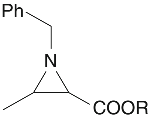


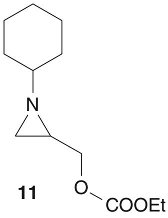
5 2-Aziridinemethanol



6 R = H
7 R = Me



8 R = Me
9 R = Et
10 R = i-Pr



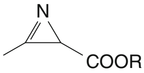
11

Since the 1950s, polymerization products of ethylenimine, their polymerizable homologs, as well as substitution products were considered useful for disinfecting and preserving textiles, leather, skins, meat, glands, blood, glue, casein (and other albuminous substances), starch, size, dressings, fruits, and vegetables. Their utility in disinfecting floors, walls, stock and portable water vessels, and medical instruments has improved health and safety [30–32].

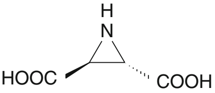
The azirinomycin (**12**), 3-methyl-2H-azirine-2-carboxylic acid, was isolated from a strain of *Streptomyces aureus*. Its methyl ester (**13**) exhibited broad spectrum antibiotic activity in vitro against both Gram-positive and Gram-negative bacteria [33, 34]. The carboxylic acid (**12**) is most active against *Staphylococcus aureus* followed by *Proteus vulgaris*, *Bacillus subtilis*, and *Streptococcus faecalis*. In contrast, the methyl ester shows its lowest activities against one of the *Staphylococcus aureus* cultures and *Streptococcus faecalis*.

(2*S*,3*S*)-Aziridine-2,3-dicarboxylic acid (also known as *S*,*S*-2,3-dicarboxyaziridine, **14**), which demonstrates antibacterial activity toward *Aeromonas salmonecida*, was isolated from the cultured broth of a *Streptomycetes* MD 398-Al (FERM-P 3217) [35]. The compound (**14**) was effective against *Pellicularia sasaki* and *Pythium debaryanum* [36]. It is a potent competitive inhibitor of various enzymes including fumarase isolated from pig heart ($K_i = 0.08\ \mu M$) [37] and aspartase of *Escherichia coli* ($K_i = 55\ \mu M$). It also shows antibacterial activity against *Aeromonas salmonicida* [38]. Ethyl esters of aziridine-2,3-dicarboxylic acid inhibited the cysteine proteinase papain [39], whereas peptides containing the aziridine-2,3-dicarboxylic acid building block are inhibitors of several cysteine proteases such as the papain-like mammalian proteases [40].

The alkaloidal antibiotic, U-47,929 (also known as ficellomycin, **15**), was isolated from *Streptomyces ficellus* [41]. Interestingly, it inhibited the growth of Gram-positive bacteria in vitro and is effective in the treatment of experimental *Staphylococcus aureus* infections in mice [42]. Structural elucidation of (**15**) [43] was eventually achieved by a combination of NMR, mass spectrometry, and formation of derivatives. The 1-azabicyclo[3.1.0]hexane moiety in (**15**) represents an unusual ring system making ficellomycin a unique natural product [43].



12 R = H
13 R = Me



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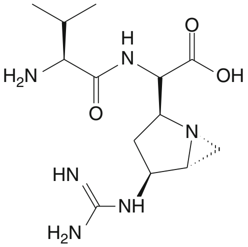
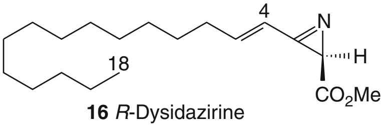


15 Ficellomycin

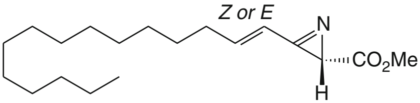
The unique cytotoxic azacyclopropene, *R*-dysidazirine (**16**), was isolated from the marine sponge *Dysidea fragilis* (Fiji) just over 20 years ago [44]. More recently, both the (*Z*) and (*E*) geometrical isomers of *S*-dysidazirine (**17a**) and (**17b**) were isolated and were also found to possess cytotoxicity. The dibrominated analogues, *S*-antazirine (**18a**) and (**18b**), were also detected within the same marine sponge *D. fragilis* collected in Pohnpei, Micronesia [45]. Three new ω-halogenated long-chain 2*H*-azirines (**19a,b** and **20**) have recently been isolated from the marine sponge *Dysidea fragilis*, two of them containing a terminal (*Z*)-1-bromo-1-chlorovinyl group, the first such example from a marine invertebrate [46]. Cytotoxic activity of (**17b** and **18b**) and new compounds (**19a**,**b**, and **20**) is shown in Table 30.1.

Table 30.1 In vitro cytotoxicity of aziridine-containing fatty acids against HCT-116 [46]

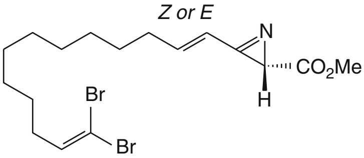
Compound	IC_{50} (μg/mL)	IC_{50} (μM)
17b	7.9	18.2
18b	8.5	19.6
19a	5.3	13.6
19b	5.9	15.2
20	8.6	24.8



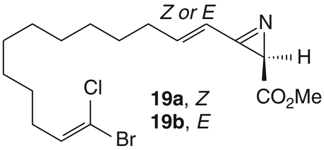
16 *R*-Dysidazirine



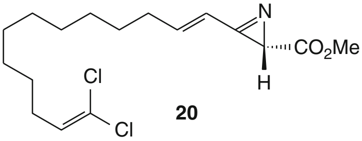
17a *S*-Dysidazirine, *Z*
17b *S*-Dysidazirine, *E*



18a *S*-Antazirine, *Z*
18b *S*-Antazirine, *E*



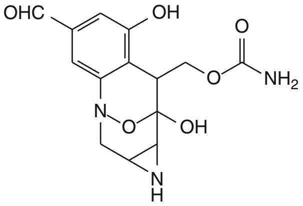
19a, *Z*
19b, *E*



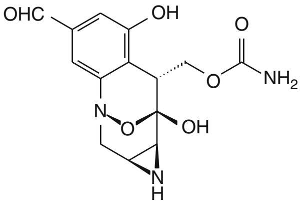
20

The antitumor antibiotic FR-900482 (**21**) was isolated from *Streptomyces sandaensis* 6897 as a mixture of the two hydroxylamine hemiketal isomers (**22**) and (**23**) [47]. FR-900482 exhibits potent cytotoxic activity against various tumor cells in vitro. Furthermore, it possessed a weak antimicrobial activity against some Gram-positive and Gram-negative bacteria [48]. Activity against human LX-1, MX-1, SC-6, and LC-6 tumor cells has been identified [49]. Quite a number of FR-900482 derivatives were synthesized, and some of them showed antileukemic activity [50].

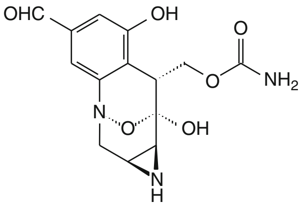
Additionally, FR-900482 inhibited DNA, RNA, and protein synthesis in cell culture of murine L1210 leukemia cells. Whereas FR 900482 did not induce DNA single-strand breaks either in the leukemia cells or in plasmid pBR322, it promoted interstrand DNA-DNA cross-links in leukemia cells. An activation of FR 900482 was required prior to induction of interstrand DNA-DNA cross-linking required for cytotoxic action [51]. FK317 (**24**), an analogue of FR-900482, had stronger cytotoxic effects against in vitro cultured B16, P388, HeLa S3, and KB tumor cell lines. In vivo experiments revealed an equivalent antitumor activity of FK317 against P388, M5076, and MX-1 and a more potent antitumor activity against L1210, Colon 38, and LX-1 cell lines as compared with FK973 (**26**) [52]. Both FR900482 (**21**) and FR66979 (**27**) are structurally novel natural products isolated by Fujisawa Pharmaceutical Co. (Japan) in 1987 and have been shown to be highly potent antitumor antibiotics structurally related to the mitomycins [5]. The N-O substructure is bioisosteric with peroxides, and the activity of natural products containing this functional group may generate free radicals, especially upon reductive activation. Not surprisingly, studies on the mode of action have established that these new agents form covalent DNA interstrand cross-links both in vitro and in vivo as a result of the reactive mitosene intermediate generated upon bioreductive activation [for details, see Refs. 53–55].



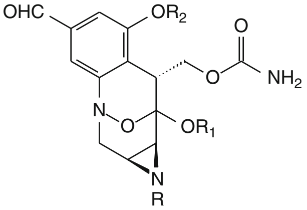
21 FR 900482



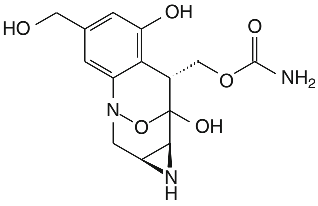
22 FR 900482
major isomer



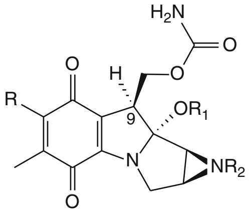
23 FR 900482
minor isomer



24 FR 073317
R = R_1 = Ac, R_2 = Me
25 FR 70496
R = Ac, R_1 = H, R_2 = Me
26 FK 973
R = R_1 = R_2 =Ac

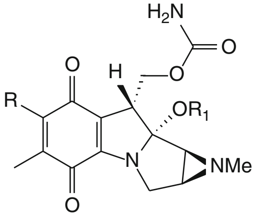


27 FR 66979

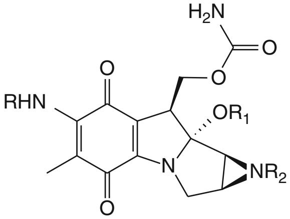


28 Mitomycin A, R = OMe, R_1,R_2 = H
29 Mitomycin F, R = OMe, R_1 = R_2 = Me
30 Mitomycin C, R = NH_2, R_1 = Me, R_2 = H
31 Porfiromycin, R = NH_2, R_1,R_2 = Me
32 9a-Demethylmitomycin A, R = OMe,
R_1 = R_2 =H
33 9-epi mitomycin B, R = OMe, R_1 = H, R_2 = Me

Semisynthetic analogues, such as FK317 (**24**) and FK973 (**26**), have been shown to be a potent cytotoxic compound; to date, no direct evidence of DNA interstrand cross-link sequence specificity has been reported. In one study, DNA interstrand cross-links were generated by treatment of a synthetic duplex DNA substrate with FK317 and its deacetylated metabolites FR70496 (**25**) and FR157471 [56]. FK973 and all its deacetylated metabolites showed strong cytotoxicity on in vitro cultured murine L1210 leukemia cells; however, FK973 remained the most potent cytotoxic agent of this series [57]. Synthesis and other biological activities of FR900482 and its analogues have been reviewed [58–60].



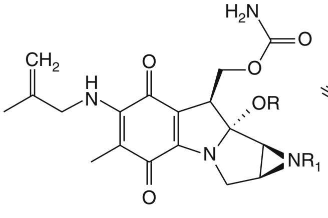
34 Mitomycin B, R = OMe, R_1 = H
35 Mitomycin J, R = OMe, R1 = Me
36 Mitomycin D, R = NH_2, R_1 = H
37 Mitomycin E, R = NH_2, R_1 = Me



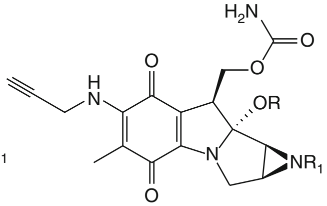
38 R = R_2 = Me, R_1 = H
39 R = R_1 = Me, R_2 = H
40 R = Et, R_1 = H, R_2 = Me
41 R = Et, R_1 = Me, R_2 = H
42 R = n-Pr, R_1 = H, R_2 = Me
43 R = n-Pr, R_1 = Me, R_2 = H

The mitomycins are potent antibiotics that belong to the family of antitumor quinones. In 1956, mitomycin A (**28**) and B (**34**) were isolated from *Streptomyces caespitosus*, and shortly thereafter, mitomycin C (**30**) was discovered within the same strain [61, 62]. The *N*-methyl derivative of (**31**), porfiromycin, was isolated in 1960 from *Streptomyces ardus*, which was followed by the discovery of mitiromycin from *Streptomyces verticillatus* [63, 64]. Among all these different mitomycins, (**31**) enjoyed early widespread clinical use as a consequence of its uniquely superior activity against solid tumors. Secondly, it possessed reduced toxicity when compared to the natural counterparts (**28**) and (**34**). Mitomycin A, B, and C and porfiromycin also were produced by a *Micromonospora* species KY 11084 [65]. Mitomycins A and C showed antimicrobial activity against *Bacillus subtilis* and *Klebsiella pneumoniae* [66].

Effects of mitomycin A (1–10 μg/mL), mitomycin B (1–50 μg/mL), mitomycin C (10–30 μg/mL), *N*-methyl-mitomycin (1–40 μg/mL), and porfiromycin (1–60 μg/mL) on the *Euglena gracilis* chloroplast system were reported. However, only *N*-methyl-mitomycin (20–40 μg/mL), porfiromycin (40–60 μg/mL), and mitomycin B (40–50 μg/mL) were effective bleaching agents.

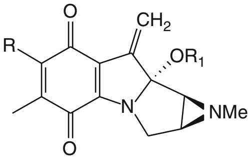


44 R = Me, R$_1$ = H
45 R = H, R$_1$ = Me



46 R = Me, R$_1$ = H
47 R = H, R$_1$ = Me

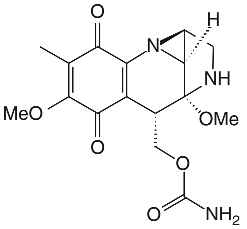
Thus, only mitomycin derivatives containing an alkyl group on the aziridine nitrogen are effective bleaching agents. The sensitivity of the *Euglena* chloroplast to small structural differences in the active centers of antibiotics demonstrates the usefulness of this organism in finding a relationship between biological activity and chemical structure [67]. Mitomycin A and C were manufactured by fermentation with mitomycin-producing *Streptomyces* and *Micromonospora* or by catalytic isomerization of mitomycin A and mitomycin C, respectively. Both isomers showed antibiotic activities against various bacteria, including *Streptococcus, Staphylococcus, Bacillus, Proteus*, and *Salmonella* [68].



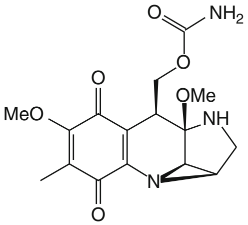
48 Mitomycin H, R = OMe, R$_1$ = H
49 Mitomycin G, R = OMe, R$_1$ = Me
50 Mitomycin K, R = NH$_2$, R$_1$ = Me
51 Mitomycin Z, R = NH$_2$, R$_1$ = H

Molecular genetic manipulation of the mitomycin pathway can elucidate the sequence of reactions involved in mitomycin biosynthesis, as well as provide access to novel mitomycin natural products. Thus, 9α-demethyl mitomycin A (**32**), 9-epi-mitomycin B (**33**), and *N*-methylmitomycin A (mitomycin F, **29**) have been obtained using mitomycin B as starting material [69]. Mitomycin J (**35**) and mitomycin D (**36**) were isolated as minor antibiotics from *Streptomyces fradiae* SCF5 [70], and mitomycin E (**37**) was obtained from *S. lavendulae* [71]. Mitomycin C, A, and F showed anthelmintic activity against gastrointestinal parasites *Hymenolepis microstoma* and *H. nana* developing in *Tribolium confusum* (Coleoptera, Tenebrionidae) [72].

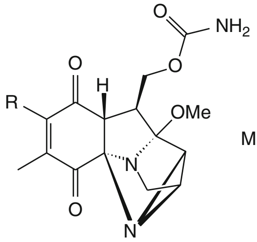
Several neoplasm inhibitor analogues (**38–47**) of mitomycin B and C were produced by *Streptomyces caespitosus*. Upon supplementation of the normal fermentation medium for the production of mitomycin C with *S. caespitosus* with a number of primary amines, two new types of mitomycin analogues, described as Type I and Type II, were produced. Type I analogues were related to mitomycin C with the amine substitution at position C7 on the mitosane ring. Type II analogues also contain the same substitutions at C7, but the conformation of the mitosane ring was related to mitomycin B, by possessing an OH at positions C9a and a Me-substituted aziridine [73]. In all cases, the Type I analogues are more active in a prophage induction test and against L1210 lymphatic leukemia in mice [73].



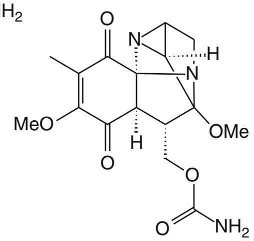
52 Isomitomycin A



53 AX-2



54 Albomitomycin A (AX-1), R = OMe
55 CX-1, R = NH_2



56 Albomitomycin C

Mitomycins H (**48**), G (**49**), and K (**50**) were produced by culturing a strain of *S. caespitosus* ATCC 29422 [74]. Mitomycins H, G, K, and Z (**51**) were also prepared from mitomycin B by cultivating *S. caespitosus* ATCC 27422 [72]. Four isolated antibiotics (**48–51**) possessed antibacterial activity [75]. Anticancer activity of some mitomycines against Sarcoma 180 cell line is shown in Table 30.2.

The neoplasm inhibitors, isomitomycin A (**52**) and albomitomycin A (**54**) and (**56**), were isolated, together with mitomycin A from *S. caespitosus* culture broth. Both antibiotics were obtained by intramolecular rearrangement of mitomycin A [76]. Anticancer antibiotics AX-2 (**53**) and CX-1 (**55**) were isolated from the culture broth of *S. caespitosus* and obtained from mitomycin C [77]. Other biological activities of different mitomycines, their mechanisms of action, and therapeutic utility have been described in various reviews [5, 9, 77–83].

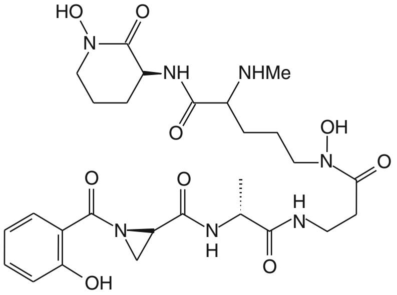
Table 30.2 Anticancer activity of some mitomycines against sarcoma 180 cell line[a]

No.	Δ 9	LD_{50}	ED_{50}
28	β	2.1	1.1
29	β	5.0	1.3
30	β	8.4	4.4
31	β	57.0	22.0
32	β	7.5	4.9
34	α	4.5	2.5
35	α	9.0	10.0
48	γ	12.0	6.8
49	γ	130.0	100.0
50	γ	22.0	35.0
51	γ	210.0	82.0

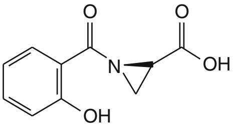
[a] Substituent at 9 position, a (carbamoyloxy) methyl group with α and β configurations and a vinyl group were taken into account. LD_{50} and ED_{50} were used as measures of biological activity. LD_{50} values of administration of an i.p. route were measured in male ddY mice by probit analysis. ED_{60} doses that gave 50% inhibition of tumor growth were calculated from the dose-response curve. Sarcoma 180 cells (5 × 106/mouse) were inoculated s.c. into ddY mice on day 0, and drugs were injected i.p. on day 1. Tumor volume was measured on day 7

A few naturally occurring peptides containing an aziridine ring have been discovered in living organisms. For instance, peptide madurastatin A1 (**57**) and madurastatin B1 (**58**), consisting of Ser and salicylic acid moieties, were isolated from the culture broth of a pathogenic *Actinomadura madurae* IFM 0745 strain. Both metabolites showed antibacterial activity against *Micrococcus luteus*, indicating that the presence of the aziridine ring is essential for such activity. Since (**57**) has a strong affinity with ferric ion attributed to the presence of two hydroxamic acids and a salicylic acid, this low molecular weight chelator is considered a siderophore [84].

Miraziridine A (**59**) isolated from the marine sponge *Theonella* aff. *mirabilis* unifies within one molecule three structurally privileged elements: (a) (2*R*,3*R*)-aziridine-2,3-dicarboxylic acid, (b) (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (statine), and (c) (E)-(*S*)-4-amino-7-guanidino-hept-2-enoic acid (vinylogous arginine).

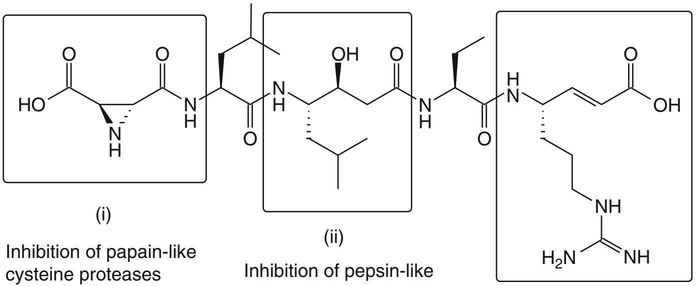


57 Madurastatin A1



58 Madurastatin B1

The alignment of them realized in the tetrapetide allows for a simultaneous inhibition of the proteolytic activity of trypsin-like serine proteases, papain-like cysteine proteases, and pepsin-like aspartyl proteases. Therefore, this unique compound represents a blueprint for the design of protease class-spanning inhibitors [85, 86]. The capability of (**59**) to inhibit proteases belonging to different classes for trypsin, cathepsin B, cathepsin L, and papain was reported (see Table 30.3). Miraziridine A [85] also inhibited cathepsin B with an IC_{50} value of 1.4 μg/mL. Aziridine-2,3-dicarboxylic acid (**14**) is a rare natural product, reported from a *Streptomyces* [36], and vArg has never before reported as a natural product.



(i)

Inhibition of papain-like cysteine proteases

(ii)

Inhibition of pepsin-like aspartyl proteases

(iii)

Inhibition of trypsin-like serine proteases

59 Miraziridine A

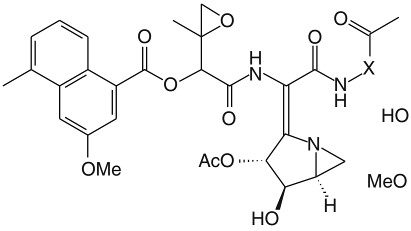
Miraziridine A (**59**) isolated from the marine sponge *Theonella* aff. *mirabilis* unifies within one molecule three structurally privileged elements: (a) (2*R*,3*R*)-aziridine-2,3-dicarboxylic acid, (b) (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (statine), and (c) (E)-(*S*)-4-amino-7-guanidino-hept-2-enoic acid (vinylogous arginine). The alignment of them realized in the tetrapetide allows for a simultaneous inhibition of the proteolytic activity of trypsin-like serine proteases, papain-like cysteine proteases, and pepsin-like aspartyl proteases. Therefore, this unique compound represents a blueprint for the design of protease class-spanning inhibitors [85, 86]. The capability of (**59**) to inhibit proteases belonging to different classes for trypsin, cathepsin B, cathepsin L, and papain was reported (see Table 30.3). Miraziridine A [85] also inhibited cathepsin B with an IC_{50} value of 1.4 μg/mL. Aziridine-2,3-dicarboxylic acid (**14**) is a rare natural product, reported from a *Streptomyces* [36], and vArg has never before reported as a natural product.

Anticancer antibiotics, azinomycin A (**60**) and B (**61**), were isolated from the culture broth of *Streptomyces griseofuscus* S-42227 [87, 88]. Azinomycin A and

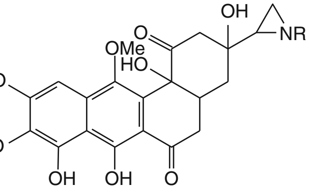
Table 30.3 Inhibitory properties of miraziridine A [60]

Protease class	Protease	Affinity
Serine protease	Trypsin	6×10^{-5} M
Cysteine protease	Cathepsin L	1×10^{6}/M/s
	Cathepsin B	1.5×10^{4}/M/s
Aspartyl protease	Pepsin	1.4×10^{-8} M

B expressed antitumor activities against P388 leukemia, P815 mastocytoma, B-16 melanoma, Ehrlich carcinoma, Lewis lung carcinoma, and Meth A fibrosarcoma, and it was markedly effective against i.p. inoculated tumors such as P388 leukemia, B-16 melanoma, and Ehrlich carcinoma [89]. Both compounds were active against Gram-positive and Gram-negative bacteria and L5178Y cells in tissue culture [87]. Azicemicin A (**62**) and B (**63**) were isolated from *Amycolatopsis sulphurea*, and its physicochemical properties and antimicrobial activity were defined [90]. It was also isolated from *Amycolatopsis* sp. (MJ126-NF4) cultures and showed MIC of 50 μg/mL against *Escherichia coli* NIHJ in vitro [91, 92]. Antimicrobial activities of azicemicin A and B were shown in Tables 30.4 and 30.5.



60 Azinomycin A, X = CH_2
61 Azinomycin B, X = C = CHOH



62 Azicemicin A, R = Me
63 Azicemicin B, R = H

A carboxylic acid antibiotic, carzinophilin, active against Gram-positive bacteria and tumor cells, was isolated from the broth filtrate of *Streptomyces sahachiroi* in 1954 [93, 94]. The structure of carzinophyllin (or carzinophilin) was similar to azinomycin B, whose partial structure was previously reported [95]. Lown and Hanstock reported the complete structure (**64**) [96]. It has a twofold symmetry axis and consists of a dimer of a substituted 1-naphthoic acid attached to a 4-amino-hydroxyvaline linked to an *N*-methyl-aminohexose moiety. It is the first naturally occurring *bis*-intercalative (macrocyclic polyoxide) bisalkylator (aziridine), and the mode of its antitumor antibiotic activity is attributed to the reactive moiety (**64**) [96].

Maduropeptin (**65**) is a chromoprotein antitumor antibiotic isolated from the fermentation broth of *Actinomadura madurae* [97]. Maduropeptin consists of a 1:1 complex of an acidic, water-soluble carrier protein (32 kD) and a 9-membered ring enediyne chromophore possessing potent antibacterial and antitumor properties [97]. It exhibits potent inhibitory activity against Gram-positive bacteria and tumor cells and strong in vivo antitumor activity in P388 leukemia and B16 melanoma implanted mice [98]. The biosynthetic gene cluster for the enediyne antitumor antibiotic maduropeptin (MDP) from *Actinomadura madurae* ATCC 39144 was cloned and sequenced. Cloning of the mdp gene cluster was confirmed by heterologous

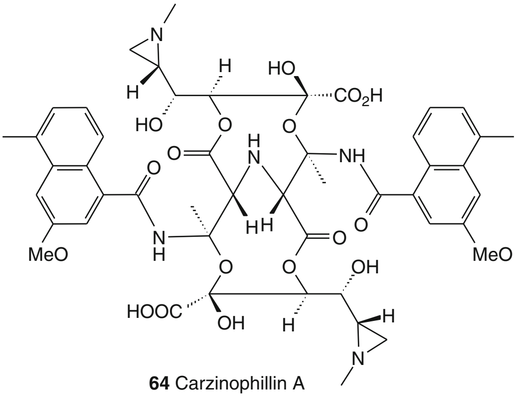
Table 30.4 Anti-mycobacterial activities of azicemicins (MIC, µg/mL) against the genus *Mycobacterium*

Tested organism	A (**62**)	B (**63**)
M. smegmatis ATCC 607	50	12.5
M. vaccae ATCC 15483	50	6.25
M. smegmatis ATCC 607 rifamycin-resistant	50	25
M. smegmatis ATCC 607 paromomycin resistant		6.25
M. smegmatis ATCC 607 capreomycin resistant		12.5
M. smegmatis ATCC 607 streptothricin resistant		25
M. smegmatis ATCC 607 streptomycin resistant		6.25

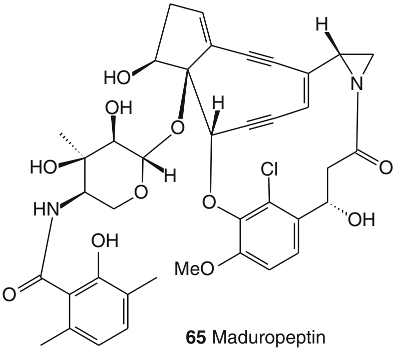
Table 30.5 Antibacterial activities of azicemicins A and B (MIC, µg/mL)

Tested organism	A (62)	B (63)
Bacillus anthracis	100	100
B. cereus ATCC 10702	>100	100
B. subtilis NRRL B-558	>100	100
B. subtilis PCI 219	>100	100
Corynebacterium bovis 1810	25	6.25
Escherichia coli NIHJ	50	25
E. coli K-12	>100	100
E. coli K-12 MLI629	>100	100
E. coli BEM11	100	100
E. coli BE1126	100	100
E. coli BE1186	100	100
Klebsiella pneumoniae PCI602	100	100
Proteus vulgaris OX19	>100	100
P. mirabilis IFM OM-9	>100	100
Providencia rettgeri GN311	>100	100
P. rettgm GN466	>100	100
Pseudomonas aeruginosa A3	>50	>50
P. aeruginosa GN315	>100	100
Staphylococcus aureus FDA209P	>100	100
S. aureus Smith	>100	100
S. aureus MS9610	>100	100
S. aureus No. 5 (MRSA)	>100	100
S. aureus No. 17 (MRSA)	>100	100
Micrococcus luteus FDA16	50	6.25
M. luteus IFO 3333	12.5	1.56
M. luteus PCI 1001	12.5	1.56
Salmonella typhi T-63	100	100
S. enteritidis 1891	100	>50
Shigella dysenteriae JS1 1910	100	25
S. flexneri 4bJS11811	50	100
S. typhi JS11746	100	100

complementation of enediyne polyketide synthase (PKS) mutants from the C-1027 producer *Streptomyces globisporus* and the neocarzinostatin producer *S. carzinostaticus* using the MDP enediyne PKS and associated genes [99].



64 Carzinophillin A



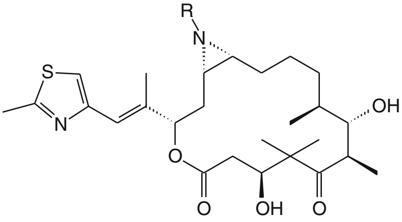
65 Maduropeptin chromophore

3 Selected Semisynthetic and Synthetic Aziridine Alkaloids as Analogues of Natural Products

The epothilones are a relatively new class of cytotoxic molecules identified as potential chemotherapeutic drugs which were originally identified as metabolites produced by the myxobacterium *Sorangium cellulosum* and/or *Streptomyces coelicolor* CH999 [100]. These compounds inhibited the growth of a broad range of human cancer cell lines in vitro with low nM or sub-nM IC_{50}s. A series of 12α,13α-aziridinyl epothilone derivatives as anticancer agents (**66**–**70**) were synthesized in an efficient manner from epothilone A. The final semisynthetic route involved a formal double inversion of stereochemistry at both the C12 and C13 positions. All aziridine analogues were showed cytotoxicity against cancer cell lines. Thus, (**67**) had IC_{50} value of 4.3 nM against KB cells. The obtained results indicate that the aziridine moiety is a viable isosteric replacement for the epoxide in the case of epothilones [101].

Several derivatives (**71–76**) of amide aziridine-2-carboxylic acid (also known as leacadine, **71**) were prepared as neoplasm inhibitors [102]. Leacadine has been used for treatment of multiple sclerosis [103]. The antitumor efficacy of azimexon (**72**) in experimental animals and humans was described with respect to its various immunological parameters [104]. Two synthetic aziridine-2-carboxylic acid (**2**) (**71** and **76**) showed antitumor activity against a mammary gland tumor in rats [105].

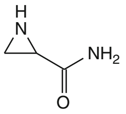
Treatment of (**77**) with KOH in MeOH at 50°C resulted in a 60% yield of an isomerization product (putative structures **78**,**79–81**) which in physiological saline solution converts to *N*-carboxyisoserine. This compound has which had cancerostatic and immunostimulating properties [106].



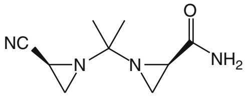
66 R = H
67 R = CH_2CH_2OH
68 R = CONHEt
69 R = COPh
70 R = CH_2Ph

Imexon (**78**) is an immunosuppressant which selectively suppresses B-lymphocyte activation and can be used in the treatment of B-cell or plasma cell leukemias or neoplasias. Thus, imexon inhibited the proliferation of stimulated human B-lymphocytes in vitro and inhibited the growth of methylcholanthrene-induced fibrosarcoma cells in vitro. It was also active against certain autoimmune disorders and infection with Rauscher leukemia virus [107], and also, imexon perturbs cellular thiols and induces oxidative stress leading to apoptosis in human myeloma cells (human 8226) [108]. More recently, (5*R*) and (5*S*) imexons (**78a** and **78b**) have been prepared and used in the treatment of cancer [109]. More details about activity of imexon, and their derivatives, have recently been reviewed [110]. Injection of 10–100 mg BM 06 002 (**78**) increased immune responses, as indicated by delayed cutaneous hypersensitivity and lymphocyte blastogenesis tests in vitro and also in cancer patients participating in clinical experiments [111].

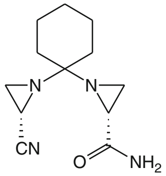
Two isomeric aziridine-containing analogues of the polyamine spermidine were synthesized and evaluated for cytotoxic activity against cancer cell lines. Replacement of one of the primary amino groups of spermidine with an aziridinyl functionality yielded either (**82**) or (**83**). N^1-Aziridinylspermidine (**82**) was cytotoxic in vitro against L1210 murine leukemia cells (IC_{50} 0.15 μM) and HL60 human leukemia cells (IC_{50} 0.11 μM). N^8-Aziridinylspermidine (**83**) was slightly less potent against L1210 (IC_{50} 0.31 μM) and HL60 (IC_{50} 0.30 μM) cells. Both compounds inhibited incorporation of radiolabeled thymidine, uridine, and valine into tricholoracetic acid-precipitable material by L1210 cells [112].



71 Leacadine



72 Azimexon



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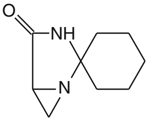

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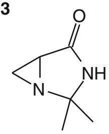

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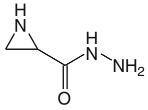

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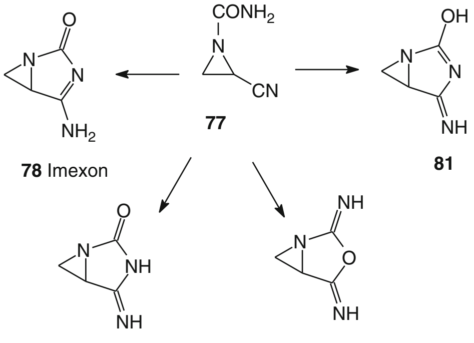

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78 Imexon

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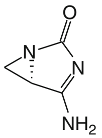
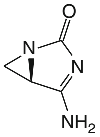




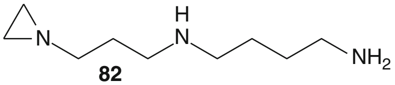
78a (5*R*)-Imexon **78b** (5*S*)-Imexon

Neoplasms inhibitor, 3,5-bis(1-aziridinylmethyl)-2,6-dimethyl-pyridine (**84**), was prepared and showed antitumor activity against spindle cell sarcoma 45 and Ehrlich muscle tumor in white rats [113]. Markofane (**85**), an oncostatic agent, was synthesized, and its properties and effect on hepatic lipids of rats with sarcoma M-1 were investigated [114]. The body weight of rats with sarcoma M-1 and given a 20% LD_{50} dose of markofane was slightly higher than that of non-treated, sarcomatous rats. Markofane proved quite toxic, and a daily dose of 40% LD_{50} resulted in 25% mortality. It exerted insignificant tumor-inhibiting effect on sarcoma M-1 in daily doses of 20% and 40% LD_{50}. Neither sarcoma M-1 nor markofane had any statistical significance on the content of lipids in dry liver. Markofane, 20% LD_{50}, administered to rats with sarcoma

M-1, increased the liver content of phosphatides. Preparations of (**86**) were less toxic, had a lower cumulative index, and did not produce profound leukopenia in treated animals and showed more antitumor activity than known aziridine derivatives. When tested clinically on 80 patients with chronic myeloleukemia, leukocyte counts decreased 30–80% on administration of between 60 and 80 mg daily doses of A95 [115].

All four prepared compounds (**87**–**90**) of the paramagnetic urethane phosphoric acid diethyl-enimides inhibited growth of the ascitic form of sarcoma 180 in rats 93–100%, and the three (**87**–**89**) inhibited Ehrlich ascites tumor growth by 90–98%. Two compounds (**87**,**88**) inhibited growth of erythromyelosis and Walker carcinosarcoma 100%, while (**89** and **90**) were essentially ineffective. Only compound (**87**) prolonged the survival of animals with leukemia La [116].

Several bioactive phospholipids (**91**–**96**) have been synthesized. Putative neoplasm inhibitors (**91**–**94**) showed significant activities in the Walker carcinosarcoma 256 and leukemia L1210 assay systems [117]. The low-melting cytotoxic phospholipids with aziridine groups (**95** and **96**) capable of forming stable dispersions in aquatic glycerol solutions containing 1% egg lecithin were prepared [118].



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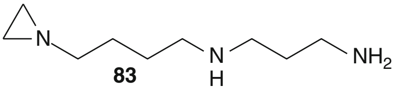

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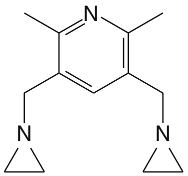

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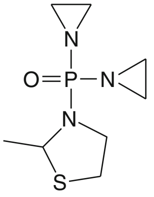
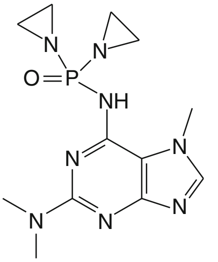


85 Markofane



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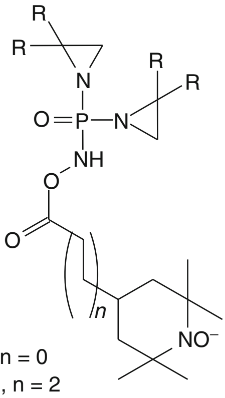


87 R = H, n = 0
88 R = Me, n = 2
89 R = H, n = 2
90 R = Me, n = 0

Fatty acid derivatives (**97–101**) containing an internal aziridine group were prepared by reaction of base with Me iodocarbamates obtained by addition of INCO to a natural fatty acid derivatives followed by treatment with MeOH [119]. Preparation of epimino-stearates (**97**) has also been reported [120].

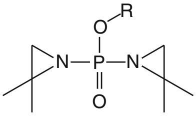
Synthetic monoglycerides (**102**) with epimino fatty acids showed antimicrobial activity against Gram-positive bacteria and yeasts [121]. Laboratory preparations of 2-ethyl-*1*-oleoyl-aziridine (**103**) showed a wide spectrum of antifungal and antimicrobial activity [122].

Certain arachidonate aziridines such as 13-(3-pentyl-2-aziridinyl)-5,8,11-trideca-trienoic acid (**104**) and its methyl ester (**105**) have been synthesized [123] which are inhibitors of arachidonate epoxygenase [124]. Preparation of the fatty acid aziridines (**106–115**) has been described [125]. Bis(aziridine) Me *cis*-9,10;*cis*-12,13-diepiminooctadecanoate, derived from linoleic acid, and tris (aziridine) and Me *cis*-9,10;*cis*-12,13;*cis*-15,16-triepimino-octadecanoate, both derived from linolenic acid, showed cytotoxic and antimicrobial activity as well as remarkable antitumor-promoting and useful neuroprotective effects [126].

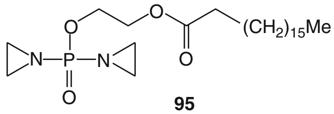
24(*RS*),25-epiminolanosterol (**116**) was a potent noncompetitive inhibitor ($K_i = 3.0$ nM) of the *S*-adenosyl-L-methionine-C-24 Me transferase from sunflower embryos [127]. Cholesteryl ester of 1-aziridine acetic acid (**117**) showed excellent inhibition of a dimethyl-benzanthrene induced and transplantable mammary adenocarcinoma [128].

Four steroidal alkylating agents (**118–121**) with an aziridine grouping at the C-16 position were synthesized. They were shown anticarcinogenic (oncolytic) activity against implanted mammary carcinoma (milk factor) in C3H/An mice. The steroids 16-(1-aziridinyl)-3β-hydroxy-pregn-5-en-20-one (**118**), 16-(1-aziridinyl)-3-methoxyestra-1,3,5(10)-trien-17-one (**119**), 16-(1-aziridinylmethyl)-3β-hydroxy-androst-5-en-17-one acetate (ester) (**120**), and 16-(1-aziridinyl)pregn-4-ene-3,20-dione (**121**), each injected (intraperitioneal, i.p.) at 0.5 mg/mouse/day for 14 days, inhibited tumor growth by 61%, 17%, 32%, and 55%, respectively. None of the compounds were toxic to the host [129].

Potentially cytotoxic estrogen derivatives (**122** and **123**) were prepared [130]. Aziridine derivatives demonstrated a high binding affinity for receptors but substitution of a bromoacetate group for the aziridine moiety in the same position decreased the binding affinity. Growth of MCF-7 and Evans-T cells from human breast cancer was inhibited by the nitrogen mustards, the mono-nitrogen derivative being the more potent analogue. This inhibitory action was unaffected by estradiol or 11 β-chloromethylestra-1,3,5(10)-trien-3,17-β-diol (ORG 4333). Aziridine derivatives either stimulated or inhibited cell growth depending on the concentration. Apparently, the antitumor action of cytotoxic-linked estrogens may be mediated through a mechanism involving estrogen receptors.



91 R = Et **93** R = n-Bu
92 R = n-Pr **94** R = $C_{12}H_{25}$



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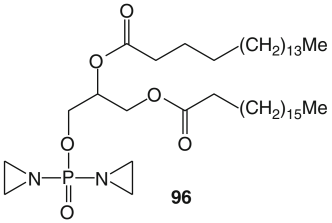
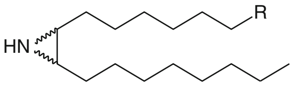


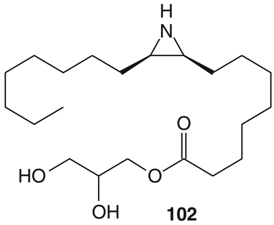
96

Mitomycin C is used extensively to treat various neoplasms and has led to the discovery of two aminoethylene disulfides: KW-2149 (**124**) and BMS-181174 (**125**). These new compounds differ from mitomycin C only in the C(7) substituent. Novel mechanisms for BMS-181174 and KW-2149 differ from the bioreductive activation pathway commonly accepted for mitomycin C, in that the C(7) aminoethylene disulfide unit undergoes thiol-mediated disulfide exchange to give a mitomycin C thiol derivatives [131].

The cell growth inhibitory activity, antitumor activity, and toxicity of M-16 and M-18, the major metabolites of a new mitomycin C (MMC) derivative, KW-2149 (**124**), in both mice and humans were compared with those of KW-2149 and MMC in vitro and in vivo. The growth inhibitory activity of M-18, a symmetric disulfide dimer, active against human uterine cervix carcinoma HeLa S3 cells was almost equivalent to that of KW-2149, and their IC_{50} values were about tenfold smaller than that of MMC. The activity of M-16, a Me sulfide form, was almost equivalent to that of MMC.



97 R = *cis*-COOH
98 R = *cis*-CH_2OH
99 R = *trans*-CH_2OH
100 R = *cis*-Me
101 R = *trans*-Me



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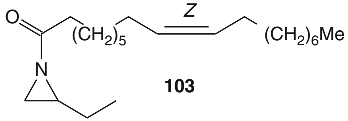

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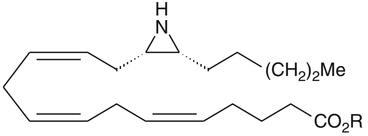
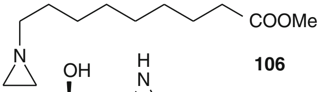


104 R = H
105 R = Me

The cell-killing activity of MMC and M-16 was augmented under hypoxic conditions, whereas that of KW-2149 and M-18 was reduced. M-16 also exhibited almost equipotent activities to MMC in vivo in terms of various biological parameters, i.e., antitumor activity against murine P388 leukemia, ascitic or solid B16 melanoma or human lung carcinoma xenograft L-27, and bone marrow toxicity in mice.

These results in vitro and in vivo indicate that the antitumor activity and toxicity of KW-2149 might not be mediated by M-16 in mice. On the other hand, M-18 exhibited almost equivalence activities to KW-2149 in this respect, implicating the involvement of M-18 in the biological activities of KW-2149 [132]. Introducing the mercaptoethyl group at the N-7 position of mitomycin C led to the formation of N7, N′7′-dithio-diethylene-dimitomycin C (**126**). It showed excellent antitumor activity against sarcoma 180 and leukemia P388 in mice. Among the various synthetic compounds, the water-soluble conjugate with Et γ-L-glutamyl-L-cysteinyl-glycinate side chain was far more effective against sarcoma 180 and leukemia P388 than mitomycin C [133].



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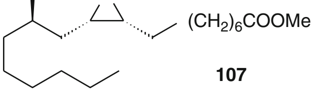

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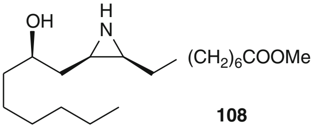

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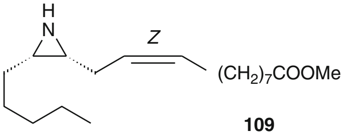

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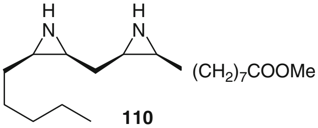
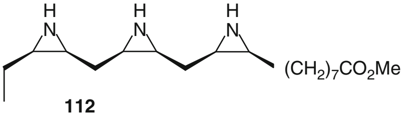
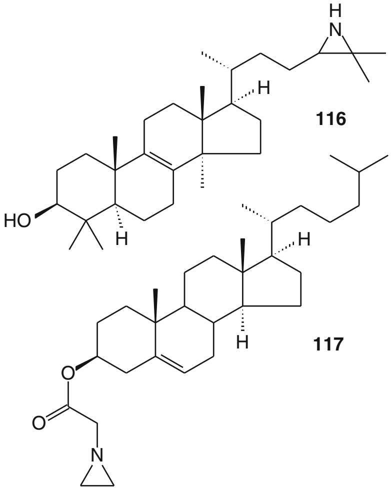


110
111 *anti*-diastereomer



112
113 all *anti*-diastereomer
114 *syn-anti*-diastereomer
115 *anti-syn*-diastereomer

The three dimers (**127**, **128**, and **129**) of mitomycin C (MC), of the aforementioned natural antibiotic and cancer chemotherapeutic agent, were synthesized in which two MC molecules were linked with -$(CH_2)_4$-, -$(CH_2)_{12}$-, and -$(CH_2)_3N(CH_3)(CH_2)_3$- tethers, respectively [134]. The dimeric mitomycins were designed to react as polyfunctional DNA alkylators, generating novel types of DNA damage. To test this design strategy, their in vitro DNA alkylating and interstrand cross-linking (ICL) activities were studied using MC, which is itself an ICL agent. Evidence was presented that (**127**–**129**) multifunctionally alkylate and cross-link extracellular DNA and form DNA ICLs more efficiently than MC.

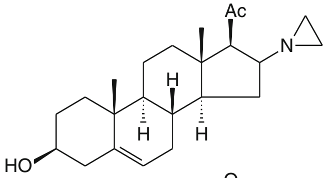


116

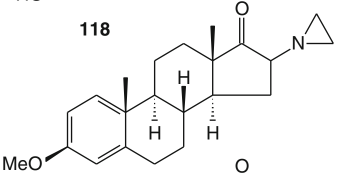
117

Biological activity depends upon reductive activation which is catalyzed by the same reductases and chemical reductants that activate MC. Dimer 5, but not MC, cross-linked DNA underwent activation by low pH environments. Sequence specificities of cross-linking of a 162-bp DNA fragment (tyrT DNA) by MC, (**128**), and (**129**) were detected using DPAGE.

The dimers and MC cross-linked DNA with the same apparent CpG sequence specificity, but (**129**) exhibited much greater cross-linking efficacy than MC. Greatly enhanced region selectivity of cross-linking to GC-rich regions by (**129**) relative to MC was observed, for which a mechanism unique to dimeric MCs was proposed. Covalent dG adducts of (**129**) with DNA were isolated and characterized by their UV and mass spectra. Tri- and tetrafunctional DNA adducts of (**129**) were also detected. Although the dimers were generally less cytotoxic than MC, dimer (**129**) was highly and uniformly cytotoxic to all 60 human tumor cell cultures of the NCI screen [134]. Its cytotoxicity to EMT6 tumor cells was enhanced under hypoxic conditions. These findings together verify the expected features of the MC dimers and warrant further study of the biological effects of dimer (**129**).



118



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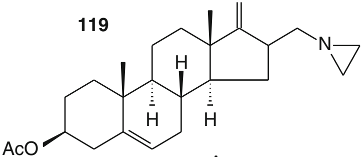

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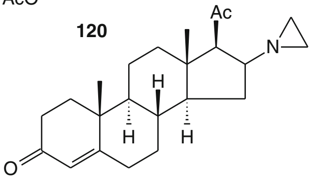

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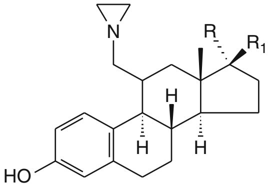
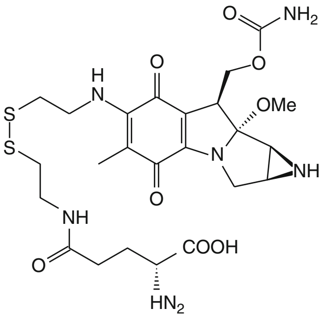


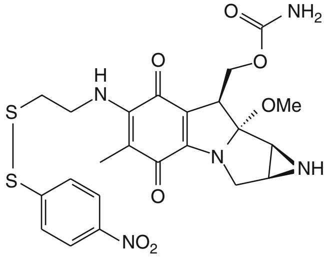
122 R = H, R_1 = OH
123 R,R_1 = O

PNU-159548 (4-demethoxy-3′-deamino-3′aziridinyl-4′-methyl-sulfo-nyldaunorubicin, **130**), a synthetic derivative of the anticancer idarubicin, has a broad spectrum of antitumor activity both in vitro and in vivo attributable to its DNA intercalating and alkylating properties [135]. This study was designed to determine the cardiotoxic activity of PNU-159548 relative to doxorubicin in a chronic rat model sensitive to anthracycline-induced cardiomyopathy. PNU-159548 caused a dose-dependent myelotoxicity, with the dose of 0.5 mg/kg per week being equimyelotoxic to 1.0 mg/kg per week doxorubicin. PNU-159548 also caused an increase in liver weight that was reversible. However, it caused a nonreversible testicular atrophy but, unlike doxorubicin, had no effect on kidney weight. The cytotoxic antitumor derivative, PNU-159548, was significantly less cardiotoxic than doxorubicin at equimyelo-suppressive doses. The combination of intercalating and alkylating activities within the same molecule without the cardiotoxic side effects of anthracyclines makes PNU-159548 an excellent candidate for clinic development in oncology. It also showed an $IC_{50} = 2.7$ ng/mL against LoVo colon adenocarcinoma cells [136]. A synthetic preparation of (**131**) showed an IC_{50} of 9.0 μg/mL against mouse L 5178Y tumor cells, and compound (**132**) had IC_{50} of 0.004 mM against 12 ovarian tumors in the tumor Salmon colony formation test [137].

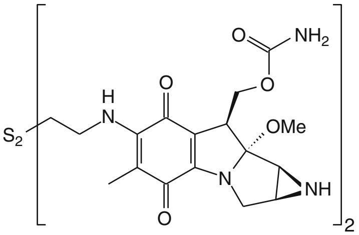
Semisynthetic aziridine derivative of colchicine (**133**) have been synthesized by the direct interaction of colchicine with chloroethylamine hydrochloride and also via the mono- and diethanolamine derivatives. These compounds had an increased radiomodifying and antitumoral activity and a decreased toxicity compared with the initial colchicine.



124 KW 2149

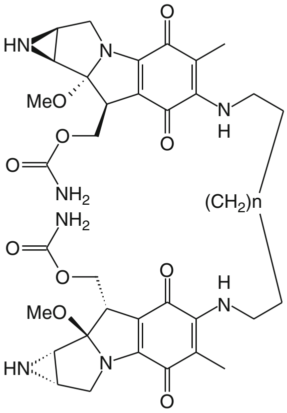


125 BMS 181174

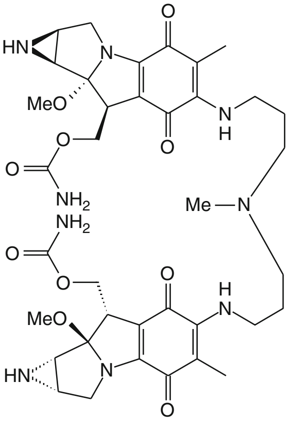


126

Results obtained in the National Cancer Institute of the USA from the study of the cytostatic activity of the (**133**) and bis(chloroethyl)amino derivatives on 60 tumor lines were reported [138]. Originally colchicine a soluble alkaloid was extracted from *Colchicum autumnale* also known as autumn crocus, meadow saffron or itkuchala in Uzbekistan which means "dog poison" [138].


HN
N
O
O
MeO
N
H
O
O
NH2
(CH2)n
NH2
O
O
O
H
MeO
N
HN
N
O

127 n = 0
128 n = 8


HN
N
O
MeO
N
H
O
O
O
NH2
Me—N
NH2
O
O
O
H
N
MeO
HN
N
O

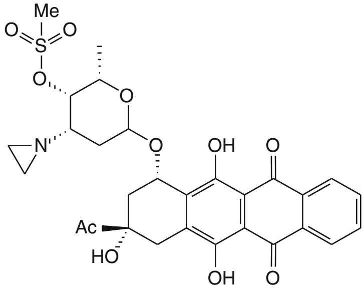
129

Neoplasm inhibitors at C-4 aziridine-bearing paclitaxel (taxol) analogues (**134–136**) were synthesized. The key step in the synthesis is the aziridine ring formation at the C-4 position via an intramolecular Mitsunobu reaction [139]. Biological activity of paclitaxel analogues is shown in Table 30.6.

Table 30.6 Biological activity of paclitaxel analogues (IC_{50}, nM)

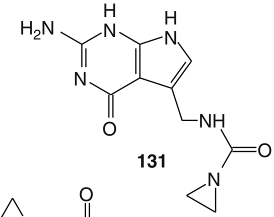
Compound	HCT-116[a]
134	15.6
135	6.9
136	2.0

[a] Human colon carcinoma



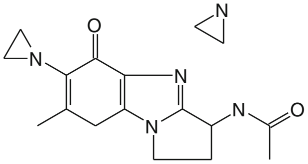
Me
O
S
O
O
O
N
O
OH
O
Ac
HO
OH
O

130 Ladirubicin

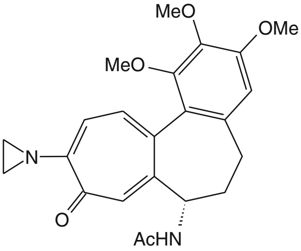


H
H
H_2N
N
N
N
O
NH
O
N

131

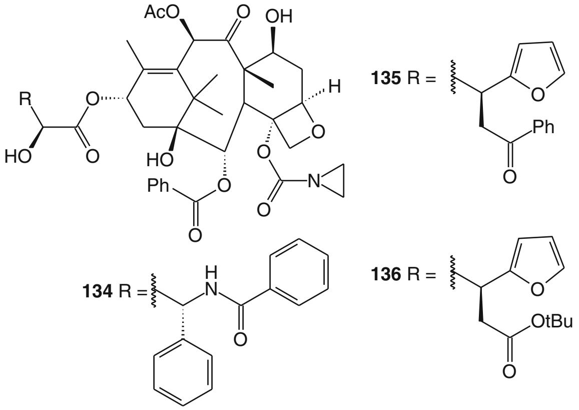


132



MeO
OMe
MeO
N
O
AcHN

133



4 Conclusion

Aziridine alkaloids comprise a rare group of natural products. They are mainly isolated from either microorganisms or plants. They have also been detected in some marine species. Reported activities for purified alkaloids have shown strong antitumor, antibacterial, antimicrobial, and other activities. A wide spectrum of pharmacological activities is associated with this type of alkaloid which extends to selected synthetic derivatives.

A priori, one should avoid rash conclusions that any of the reported effects of hundreds aziridines are due to their alkylating activity. Quite complex compounds (**21**–**65**) may display additional antioxidant properties; some of them would serve better substances for proteins assuring multidrug resistance, such as P-glycoprotein (MDR-1). It is generally accepted that this protein binds its substrates directly from the lipid bilayer rather than from the aqueous cytoplasmic phase. Binding sites of this protein thus in cancer cells characterized by increased expression of the gene mdr-1, these hydrophobic compounds would be more readily exported than such hydrophilic molecules as **1**–**20**.

Natural and/or synthesized aziridine-containing compounds, lipids, steroids, amino acids, as well as their peptide derivatives have shown to be promising candidates for the development of new drugs toward several diseases, especially neoplasms. No doubt incorporation of an aziridine warhead will allow development of interesting new synthetic and semisynthetic compounds with clinical utility.

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Part III

Alkaloids: Methods of Analysis

Methods of Analysis: Tropane Alkaloids from Plant Origin

31

Philippe Christen, Stefan Bieri, and Strahil Berkov

Contents

1 Introduction 1012
1.1 Occurrence, Numbering, and Chirality 1012
1.2 Pharmacology 1014
2 Methods of Sample Preparation 1015
2.1 Conventional Methods 1015
2.2 Microwave-Assisted Extraction 1016
2.3 Pressurized Liquid Extraction 1017
2.4 Supercritical Fluid Extraction 1018
2.5 Solid Phase Microextraction 1019
3 Methods of Analysis 1020
3.1 TLC 1020
3.2 Liquid Chromatography 1022
3.3 Gas Chromatography 1028
3.4 Capillary Electrophoresis 1032
3.5 Non-Chromatographic Methods 1034
4 Conclusion 1038
References 1039

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Abstract

Tropane alkaloids are natural compounds with a prominent impact in medicine since ancient times. Due to their importance in chemotaxonomy, physiology, phytochemistry, toxicology, and forensic on the one hand, and the tremendous development of analytical technologies on the other hand, numerous articles have been published in the field of tropane alkaloid analysis in the recent years. In this chapter, we review the latest achievements in sample preparation (extraction and purification), separation sciences (thin-layer chromatography, gas chromatography, high-performance liquid chromatography, and capillary electrophoresis), and hyphenation with different detectors (ultraviolet, evaporative light scattering, mass spectrometry) for the analyses of this class of compounds in various biological matrices. Emerging non-chromatographic methods such as immunoassays, optical sensors, as well as standalone nuclear magnetic resonance and mass spectrometry have also been reviewed as powerful complementary alternatives.

Keywords

Chromatography • Hyphenation • Sample preparation • Separation and detection techniques • Tropane alkaloids

Abbreviations

ASE	Accelerated Solvent Extraction
CE	Capillary Electrophoresis
CEDIA	Cloned Enzyme Donor Immunoassay
CLS	Chiral Lanthanide Shift Reagent
CZE	Capillary Zone Electrophoresis
DART	Direct Analysis in Real Time
DESI	Desorption Electrospray Ionization
DFT	Density Functional Theory
DMAE	Dynamic Microwave-Assisted Extraction
EA	Elemental Analysis
ECL	Electrochemiluminescence
ED	Electrochemical Detection
EI	Electron Impact
ELSD	Evaporative Light Scattering Detector
ESE	Enhanced Solvent Extraction
ESI	Electrospray Ionization
FID	Flame Ionization Detector
FMAE	Focused Microwave-Assisted Extraction
FTIR	Fourier-Transformed Infrared Spectroscopy
GC	Gas Chromatography
HILIC	Hydrophilic Interaction Chromatography

HPLC	High-Performance Liquid Chromatography
HPTLC	High-Performance Thin-Layer Chromatography
HRMS	High-Resolution Mass Spectrometry
ICH	International Conference on Harmonization
Irm	Isotope Ratio Measurement
IS	Internal Standard
KIE	Kinetic Isotope Effect
LC	Liquid Chromatography
LLE	Liquid–Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
MAE	Microwave-Assisted Extraction
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NACE	Nonaqueous Capillary Electrophoresis
NMR	Nuclear Magnetic Resonance
NPD	Nitrogen Phosphorus Detector
NPMAE	Nitrogen-Protected Microwave-Assisted Extraction
PDMS	Polydimethylsiloxane
PHWE	Pressurized Hot Water Extraction
PLE	Pressurized Liquid Extraction
PSE	Pressurized Solvent Extraction
QD	Quantum Dot
qTOF	Quadrupole Time of Flight
RI	Refractive Index
ROC	Receiver Operating Characteristic
RTP	Room Temperature Phosphorescence
SFE	Supercritical Fluid Extraction
SIM	Single Ion Monitoring
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
SRM	Single Reaction Monitoring
TCM	Traditional Chinese Medicine
TIC	Total Ion Current
TLC	Thin-Layer Chromatography
TOCSY	Total Correlation Spectroscopy
TOF	Time of Flight
UFLC	Ultrafast Liquid Chromatography
UHPLC	Ultrahigh Performance Liquid Chromatography
UMAE	Ultrasonic Microwave-Assisted Extraction
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet
VCD	Vibrational Circular Dichroism
VMAE	Vacuum Microwave-Assisted Extraction

1 Introduction

1.1 Occurrence, Numbering, and Chirality

Among the oldest medicinal plants known to man are those containing tropane alkaloids. The solanaceous plants of the genera *Atropa*, *Brugmansia*, *Datura*, *Duboisia*, *Hyoscyamus*, and *Scopolia* were already well known in ancient times. The term "tropane" originates from the name of the genus *Atropa*, which was named after one of the three Greek Fates, namely, Atropos (literally without turn) who was the Goddess that cut the thread of life and chose the manner of each person's death. Thus, she reflected the toxicity and lethality of the species *Atropa belladonna* [1]. At the beginning of the 19th century, extracts of *A. belladonna* were recommended to ophthalmologists to induce mydriasis, but it was in 1833 that Geiger isolated atropine from the roots of this species. *Scopolia carniolica* was studied by the Italian botanist Giovanni Antonio Scopoli, who isolated scopolamine, another tropane alkaloid with sedative properties. Fresh leaves of *Erythroxylum coca* have been traditionally chewed as a mild stimulant for many centuries. The active component, cocaine, was used as local anesthetic in surgical practice, but it has been replaced by synthetic local anesthetics because of its widespread abuse in western countries [2].

Tropane alkaloids are a group of tertiary or quaternary (*N*-oxides) bases comprising *ca*. 250 compounds that have the 8-azabicyclo[3.2.1]octane structure originating from L-ornithine and/or L-arginine in common, and bear at least one hydroxyl group in position 3 (Fig. 31.1A) [3]. The nitrogen atom in position 8 of the tropane skeleton is frequently methylated, but the methyl group may also be absent (*nor* derivatives). In most textbooks, tropane alkaloids are divided into two main groups according to the position of the substituent on carbon 3. 3α-Hydroxytropanes (tropine) carrying the hydroxy group in the axial position are the most frequently encountered derivatives, whereas 3β-hydroxytropanes (pseudotropine) with the hydroxy group in the equatorial position occur in Erythroxylaceae in particular. A typical alkaloid of this family is cocaine, which is distributed exclusively among the *Erythroxylum* genus. 3α- and 3β-hydroxytropanes are biosynthesized from tropinone, catalyzed by two enzymes, tropinone reductase I and tropinone reductase II, respectively (Fig. 31.1B). The majority of these alkaloids are carboxylated and benzoylated derivatives of mono-, di-, and tri-esters. They are distributed in various clades in the angiosperms, namely, in Solanaceae, Erythroxylaceae, Rhizophoraceae, Proteaceae, and Convolvulaceae. Occasionally, they have been found in Brassicaceae [4], Euphorbiaceae [5], Moraceae [6], Lamiaceae [7], and Olacaceae [8]. There are also structures with relatively restricted distribution among the tropane alkaloids bearing families such as pyranotropanes and C4 substituted tropanes [3]. Monocyclic analogues (pyrrolidinyl alkaloids) are considered side reactions of tropane alkaloid biosynthesis [9]. More than 30 acids, such as formic, acetic, hydroxyacetic, propionic, tiglic, methylbutyric, phenyllactic, tropic, senecioic, angelic, mesaconic, cinnamic, and benzoic acids, form esters with the alkamines. Substituents have been found at positions C1 to C4, C6, and C7 of

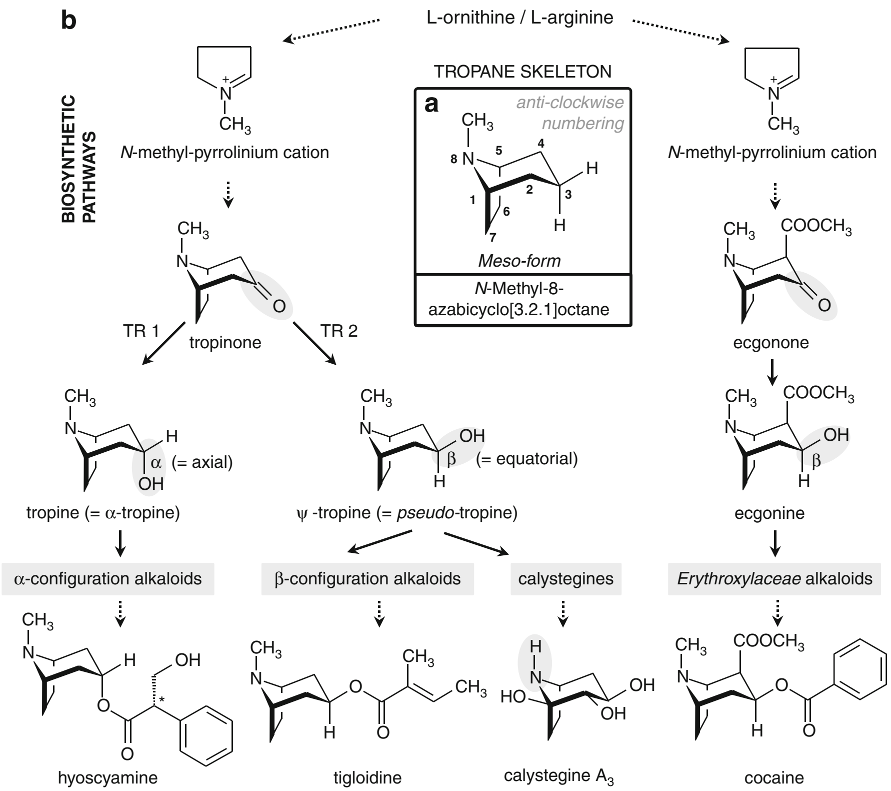
b
L-ornithine / L-arginine
BIOSYNTHETIC PATHWAYS
N-methyl-pyrrolinium cation
TROPANE SKELETON
a
anti-clockwise numbering
CH3
8 N
1 2 3 4 5 6 7
H
Meso-form
N-Methyl-8-azabicyclo[3.2.1]octane
N-methyl-pyrrolinium cation
tropinone
TR 1
TR 2
ecgonone
COOCH3
α (= axial)
OH
tropine (= α-tropine)
β (= equatorial)
ψ -tropine (= pseudo-tropine)
ecgonine
α-configuration alkaloids
β-configuration alkaloids
calystegines
Erythroxylaceae alkaloids
hyoscyamine
tigloidine
HO
calystegine A3
cocaine

Fig. 31.1 (**a**) Structure and numbering of the tropane skeleton (**b**) Biosynthetic pathways of tropane alkaloids via the action of the two tropine reductases and the ecgonine route

the tropane skeleton [3]. Dimeric esters appear in the Erythroxylaceae (truxillines and mooniines), the Solanaceae (schizanthines, belladonnines, scopadonnines), and the Convolvulaceae (convolvidine and subhirsine) families, while trimeric tropane esters (grahamines) have so far only been found in the Solanaceae [3, 10, 11].

Mono-, di-, or tri-esters of tropine are typical for the Solanaceae, although they have also been found in the other above-mentioned families. Hyoscyamine and scopolamine are the most characteristic alkaloids for this group. Still today, they are extracted from the solanaceous genera *Datura*, *Brugmansia*, *Hyoscyamus*, *Scopolia, Atropa*,and *Duboisia* for the pharmaceutical industry [12]. Hyoscyamine is a chiral molecule, whose chirality is introduced by the tropic acid moiety. Only the *R* form of the tropic acid occurs in nature. Racemization may easily occur during the isolation procedure yielding (*R*,*S*)-hyoscyamine, which is known as atropine [13]. Scopolamine, also known as hyoscine, racemizes in solution. Calystegines, a recently characterized *nor*tropane group with three to five hydroxyl groups (Fig. 31.1B), have been found in various Solanaceae, Convolvulaceae, Moraceae, Brassicaceae, and Erythroxylaceae [14, 15].

It has recently been demonstrated that the biosynthesis of tropane alkaloids follows separate pathways in the Solanaceae and the Erythroxylaceae, suggesting that the entire biosynthetic route has evolved more than once in the evolution of the angiosperms [16]. Such independent evolutions explain the vast structural diversity found for tropane alkaloids in the plant kingdom.

There is a lot of confusion in the literature about the numbering of tropane alkaloid molecules. Some authors used the anticlockwise numbering, making their compounds 3,6-disubstituted; others used the clockwise numbering resulting in 3,7-disubstituted derivatives (Fig. 31.1B). In some cases, both 6- and 7-substituted compounds have been described but are probably actually the same compound. The same confusion is encountered for 3,6,7-trisubstituted alkaloids. Frequently, the notation of 3,6/7-disubstituted derivatives has been chosen arbitrarily without taking into account the absolute configuration of these two positions [17]. Within the chemical class of tropane alkaloids, many compounds are 3α,6β-tropanediol derivatives that can exist as two stereoisomeric (3*R*,6*R* or 3*S*,6*S*) entities. This stereoisomerism is not well documented, and most of these molecules do not have a defined absolute configuration. Recent papers dealing with the determination of absolute configuration report on the use of vibrational circular dichroism (VCD) spectroscopy in combination with density functional theory (DFT) calculations [18, 19], electronic circular dichroism, also in combination with DFT calculations [20], or by the use of ^{1}H-NMR anisochrony (Δδ) induced by Mosher's chiral auxiliary reagents (*R*)-(−)- and (*S*)-(+)-α-methoxy-α-trifluoromethyl-phenylacetyl chlorides (MTPA-Cl) [17].

1.2 Pharmacology

Tropane alkaloids are competitive antagonists of acetylcholine, essentially by blocking its binding to muscarinic receptors. Some of them are widely used for their gastrointestinal, bronchial, genitourinary, and mydriatic effects. For these reasons, they are classified as parasympatholytic agents [21]. In addition, these compounds (those with a tertiary amine) also have pharmacological activities on the central nervous system, which may explain why some species of the Solanaceae family (*Brugmansia, Datura, Mandragora* spp.) have been used for centuries for their narcotic, hallucinogenic, and toxic properties. Biologically, cocaine acts as a serotonin–*nor*epinephrine–dopamine reuptake inhibitor. It is an addictive drug that increases alertness, feelings of well-being and euphoria, energy and motor activity, and feelings of competence and sexuality. Anxiety, paranoia, and restlessness are also frequent [22]. South American indigenous people have chewed the leaves of *Erythroxylum coca*, one of the four cultivated species that contains cocaine, for over a 1,000 years [23].

(−)-Hyoscyamine, its racemate atropine, (−)-scopolamine, and cocaine are among the oldest drugs used in medicine or as drugs of abuse. Furthermore, the purplish-black berries of *Atropa belladonna* L. (Deadly nightshade) and the beautiful red-to-pink, yellow, or white flowers of *Brugmansia* spp. (Angel's trumpet) are particularly attractive for children, causing serious intoxication in cases of ingestion. Because of their acute toxicity, it is of prime importance to have fast analytical methods able to unambiguously detect these alkaloids.

Tropane alkaloids are still of significant interest for the pharmaceutical industry, as well as for pharmacology, toxicology, and phytochemistry, which is evident from the number of articles on the topic which have been published since our previous review [15]. Thus, this chapter will essentially cover the evolution of analytical methods for this class of compounds over the past few years. Calystegines and other polyhydroxylated compounds will not be further discussed herein as they have been thoroughly reviewed by Dräger [14, 24].

2 Methods of Sample Preparation

2.1 Conventional Methods

Comprehensive reviews have been published on the classical and nonconventional extraction methods of tropane alkaloids [15, 24], and a brief overview of the different approaches will be mentioned here. Fresh or dried plant material may be used for the extraction. However, the drying process may change the chemical composition of the tissue, in particular, enzymatic degradation of the alkaloids may occur. It is therefore strongly recommended to freeze-dry the fresh plant and/or to store the plant material in a deep freezer (−18 °C). The classical procedures are based upon the physicochemical properties of alkaloids. The free bases are soluble in organic solvents, e.g., diethylether, chloroform, and dichloromethane, whereas the salts are soluble in water supplemented with inorganic acids. In most analytical experiments, tropane alkaloids are extracted by decoction, percolation, maceration, ultrasonication, turbo extraction, or hot continuous extraction (Soxhlet). These techniques have recently been summarized by Tiwari et al. [25]. However, they suffer from major drawbacks including the consumption of large volumes of polluting solvents and prolonged extraction time with alkali, which may lead to the formation of degradation products or artifacts (e.g., the racemization of hyoscyamine to atropine). Furthermore, these conventional methods often suffer from a lack of selectivity and allow only crude extracts to be obtained, which necessitates further cleanup procedures prior to chromatographic analysis. Liquid–liquid extraction (LLE) or solid phase extraction (SPE) using conventional sorbents (C_{18}, diatomaceous earth) are commonly used for this purpose [26].

With the advent of fast analytical methods (fast gas chromatography (GC), capillary electrophoresis (CE), ultrahigh performance liquid chromatography (UHPLC)) [27–29], it appears that the extraction methods are the bottleneck for most of the analytical processes. Thus, there is a need for appropriate fast and efficient extraction steps for plant samples.

2.2 Microwave-Assisted Extraction

Microwave-assisted extraction (MAE) is a technique which uses microwave energy to heat a solid sample immersed in a solvent to extract/desorb analytes from the matrix. Microwaves are electromagnetic radiation with a frequency ranging from 300 MHz to 300 GHz. They possess two oscillating magnetic and electric fields that are perpendicular to each other. The latter is responsible for the transformation of electromagnetic energy into calorific energy via two simultaneous mechanisms occurring into the sample, namely, dipole rotation and ionic conduction. This fast oscillation generates heat through collisions and friction between surrounding molecules. Therefore, microwave heating depends on the presence of polar molecules or ionic species. Unlike classical conductive heating methods, microwaves heat the whole sample simultaneously, thus leading to very fast and efficient extractions. It generates a rapid delivery of energy to the total volume of solvent, which leads to an extremely rapid heating process. The effect of microwave energy is strongly dependent on the nature of both the solvent and the solid matrix. Depending on the dielectric constants of the solvents used, the extracting selectivity and the ability of the medium to interact with microwaves can be modulated by using mixtures thereof. In some cases, the matrix itself, which contains residual water, interacts with microwaves, while the surrounding solvent remains cold as it possesses a low dielectric constant. This latter situation presents some obvious advantages in the case of thermosensitive compounds [30].

Microwave ovens can operate either under controlled pressure and temperature (PMAE) or at atmospheric pressure, which is also known as focused microwave-assisted extraction (FMAE). Most of the applications described so far have been used for digestions or acid mineralizations or for the recovery of pollutants from environmental matrices under drastic conditions, and very few studies have involved the use of the more moderate FMAE. Modified MAE, such as vacuum microwave-assisted extraction (VMAE), nitrogen-protected microwave-assisted extraction (NPMAE), ultrasonic microwave-assisted extraction (UMAE), and dynamic microwave-assisted extraction (DMAE), have been developed and reported by Chan et al. [31]. Only a few review articles deal with the application of MAE to the extraction of natural products [31–34]. Numerous papers have been published on the extraction of constituents from plants; however, very few articles reported on the extraction of alkaloids in general and on tropane alkaloids in particular. FMAE has been used to extract cocaine and benzoylecgonine from coca leaves [35]. Different parameters affecting the efficiency of the extraction of cocaine, such as the nature of the extracting solvent, the particle size distribution, the moisture of the sample, the applied microwave power, and the radiation time, have been investigated. A central composite design has been used to optimize microwave power and radiation time and to evaluate the robustness of the method. Results showed that methanol was the most appropriate solvent. It was found that moistening the leaves with water slightly improved cocaine recovery, while the addition of triethylamine or sodium bicarbonate significantly decreased cocaine recovery due to degradation of the latter in methanol. FMAE generated extracts similar to those obtained by conventional solid–liquid extraction but in a more efficient manner, as 30 s was sufficient to extract cocaine quantitatively from leaves. Cocaine distribution in the leaves of 51 wild *Erythroxylum* species was investigated by Bieri et al. [36]. Extractions have been performed in methanol within 30 s by FMAE on 100 mg of slightly hydrated leaves. After filtration, all samples were analyzed by GC-MS without any further purification. Cocaine was detected in 23 of the 51 species examined.

2.3 Pressurized Liquid Extraction

Pressurized liquid extraction (PLE) is also known as pressurized solvent extraction (PSE), enhanced solvent extraction (ESE), pressurized fluid extraction (PFE), or accelerated solvent extraction (ASE™) in the literature. PLE is considered an environmentally friendly extraction technique because it requires only small volumes of solvents. PLE was primarily used for the extraction of environmental samples, such as soils and sediments. Elevated temperatures (usually between 50 and 200 °C) and pressures (between 10 and 15 MPa) are used in closed vessels, which allow extractions to be completed in a very short time. High pressure allows the solvent to remain in its liquid state even at temperatures above its boiling point, and forces it into the matrix pores. High temperatures decrease the solvent viscosity and increase metabolite solubilization, the diffusion rate, and mass transfer kinetics, thus facilitating desorption of the analytes from the plant material. Most PLE applications reported in the literature employ the same organic solvents as those commonly used in conventional solid–liquid extraction techniques. When water is used as the extraction solvent, the technique is referred to as pressurized hot water extraction (PHWE). Extractions are carried out in stainless steel extraction cells of various volumes (typically 1–250 mL). One extraction cycle is generally applied for 5–20 min at temperatures ranging from 50 to 140 °C in the vast majority of applications.

There are two main setups for conducting PLE: static and dynamic modes. In the static mode, the extraction cell is filled with the dried powdered plant material and the solvent, placed in an oven, and then heated which results in the pressure increasing in the cell. After a specified period of time, the system is flushed into the collecting vial with a pressurized nitrogen flow. Static extraction is preferred for compounds that are strongly bonded to the matrix. In the dynamic mode, a few milliliters (typically 0.5–2.0 mL min^{-1}) of fresh solvent are continuously percolated through the cartridge under pressure at a constant flow rate for a fixed period of time. Dynamic extraction is preferentially used for easily extractable compounds. ASE instruments from Dionex are currently the only commercially available systems that allow static and dynamic extractions in the same run.

PLE also offers the possibility of performing extractions in an inert atmosphere that is protected from light, which represents an advantage for compounds that are very sensitive to these two factors. Some PLE devices offer the possibility of extracting up to 24 samples sequentially.

Numerous applications have been developed for a wide variety of compounds from different matrices, but surprisingly, only a few reviews dedicated to the extraction of medicinal plants have been published in the last few years [37–39]. ASE of cocaine and benzoylecgonine from coca leaves has been reported by Brachet et al. [40]. The influence of several extraction parameters such as the nature of the extracting solvent, the addition of alkaline substances, the pressure, the temperature, the extraction time, and the sample granulometry on cocaine recovery was systematically investigated. Methanol was found to be the most suitable solvent. Critical parameters were found to be pressure, temperature, and extraction time. A central composite design has been used to optimize these 3 parameters and to assess the robustness of the extraction method. The optimal conditions for the quantitative extraction of cocaine from leaves were the following: 20 MPa, 80 °C, 1 mL min^{-1}, 10 min extraction time, with a particle size distribution between 90 and 150 μm.

2.4 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) represents an interesting alternative to conventional methods. Supercritical fluids have interesting properties, such as a low viscosity and a high solute diffusivity, which increase the mass transfer and the extraction time. Carbon dioxide is the most frequently used solvent. It has many advantages such as a low critical temperature (31.1 °C) and pressure (73.8 bar/ 7.38 MPa), no toxicity, and is environmentally acceptable, cheap, and easily available. Extractions can be performed under mild conditions, thus reducing the risks of the thermal degradation of compounds. Its low solvating properties may be enhanced by the addition of 10–20 % of a polar modifier such as methanol or ethanol. Higher concentrations would lead to subcritical conditions.

Surprisingly, very little has been published on the extraction of tropane alkaloids by SFE. Even if hyoscyamine and scopolamine are soluble in pure CO_2, they are not extractable from plant material because target alkaloids are in the form of salts in the vacuoles of plant cells. Choi et al. [41] demonstrated that supercritical CO_2 supplemented with basified methanol as a polar modifier can extract hyoscyamine and scopolamine in the form of their free bases. Hyoscyamine, scopolamine, and cocaine have been extracted using SFE [42, 43]. The first two alkaloids were isolated from genetically transformed hairy roots of a *Datura* hybrid while cocaine was isolated from coca leaves. In both cases, a central composite design was used to optimize SFE parameters such as pressure, temperature, nature, and percentage of the polar modifier and to evaluate their influence on extraction recoveries. The quality of the models was verified and confirmed by the good agreement between experimental and predicted responses.

2.5 Solid Phase Microextraction

Solid phase microextraction (SPME) was introduced by Arthur and Pawliszyn over 20 years ago [44]. It is a straightforward, solvent-free, and fast sample extraction method. SPME has become a widely used technique in many areas of analytical chemistry, such as food analysis, environmental sampling, forensics/toxicology, and biological analysis. Recent reviews have been published showing the latest development of this versatile extraction method [45–48]. SPME is based on the partition of the analyte between the extraction phase and the matrix. The technique can be used to monitor analytes in liquid samples or in the headspace and is basically compatible with HPLC and CE, but most applications are made by GC. As indicated by its name, it is not an exhaustive extraction technique and only a fraction of the target analyte is actually extracted. The quantity of analyte extracted is proportional to its concentration, as long as the equilibrium between the analyte in the fiber and the sample is reached. It provides linear results for wide concentrations of analytes (typically from levels of parts per million to parts per billion).

SPME is realized with a fiber incorporated into a syringe and coated with a liquid (polymer), a solid (sorbent), or a combination of both. Extraction can be conducted as a direct extraction in which the coated fiber is immersed in the liquid sample or in the headspace configuration by exposing the fiber to the volatile fraction above the liquid sample. The SPME process consists of exposing the sorbent to the sample for a specified period of time. In a second step, analytes are desorbed by exposing the fiber in the hot injection port of a GC or in the desorption chamber of an HPLC interface. The selection of the fiber is determined according to the volatility and polarity of the analytes. SPME fibers are available in a variety of phases such as polydimethylsiloxane (PDMS), polyacrylate, Carbowax, polydimethylsiloxane/divinylbenzene, divinylbenzene/carboxen, carboxen/polydimethylsiloxane, divinylbenzene/carboxen/polydimethylsiloxane, polypyrrole, or molecularly imprinted polymers, as well as with different film thicknesses [49]. Reports on alkaloid analysis by SPME are scarce and mainly concern cocaine in biological fluids [50, 51] and human hairs [52, 53]. SPME has been used for the extraction of cocaine from leaves of *Erythroxylum coca* [54]. The plant material was first extracted by MAE in an open vessel for 30 s with methanol. A 7 μm PDMS fiber was immersed in the organic solution supplemented with cocaethylene as internal standard for 2 min, and the fiber was withdrawn and further inserted in the injection port of the fast GC for thermal desorption during 12 s. In this way, quantitative determination of cocaine was achieved in less than 5 min. Another study using a combination of MAE and SPME prior to gas chromatography was reported by Bieri et al. [55] for the analysis of cocaine in coca leaves. SPME was carried out in the direct immersion mode with a 100 μm PDMS fiber. A significant gain in selectivity was obtained with the incorporation of SPME in the extraction procedure. The analysis time was reduced to 6 min compared to 35 min with conventional GC.

3 Methods of Analysis

Recent advances in hyphenated analytical techniques, where a separation device is coupled online with detectors generating spectral information, have remarkably widened the analysis field of complex biological matrices. During the last few years covered by this chapter, a number of papers describing the application of TLC, GC-MS, HPLC-UV, HPLC-UV/MS, CE-MS, and NMR to the qualitative and quantitative analysis of tropane alkaloids in toxicological, physiological, forensic, phytochemical, and chemotaxonomical studies have been published.

3.1 TLC

Even though it seems to be an out-of-date technique, planar chromatography (TLC or HPTLC) still enjoys great popularity in phytochemical studies in general and in alkaloid chemistry in particular. Interesting reviews on the analysis of tropane alkaloids by TLC have been reported [24, 56]. The technique is typically used for the initial screening of plant extracts (Fig. 31.2), preparative purification, monitoring fractions after preparative column chromatography, or chemical synthesis. It is the method of choice for routine alkaloid analysis before more sophisticated instrumental chromatography. It can also be used for confirmation of the identity of natural products determined by other spectroscopic methods. Among the main advantages of TLC are its simplicity and versatility due to the large number of stationary phases available on the market, as well as to the numerous solvent systems reported in the literature [56]. Furthermore, many samples can be spotted on a single plate and developed simultaneously making (HP)TLC a very convenient method to control the separation process of individual compounds.

Because only a few alkaloids contain an aromatic ring which makes them detectable by UV light, tropane alkaloids are usually monitored around 205 nm. However, at this unspecific wavelength, other co-eluting compounds may also absorb and be detected. This is the reason why the addition of Dragendorff's, Mayer's, or iodoplatinate reagents is frequently used as a visualization agent of alkaloids. Reliable identification of the analyte is obtained by the combination of its *Rf* value and color after spraying a visualization reagent that is specific to a chemical class and comparison with authentic reference material [26]. TLC is an official assay in the *European Pharmacopeia* for *Atropa belladonna* and *Datura stramonium* monographies. Compound identification can be ascertained by using different separation conditions (i.e., different chromatographic supports or elution solvent mixtures).

Quantitative evaluation is carried out by densitometry, which requires additional instrumentation such as an automatic sample applicator, an automatic chamber for development, and a TLC scanner. Furthermore, an extensive calibration process is needed. TLC-densitometry, although less sensitive than HPLC and GC methods, can be regarded as an efficient quantitative tool for tropane alkaloids analysis [56].

The conditioning of the solid silica layer by mobile phase or ammonia vapor with respect to retention efficiency, peak symmetry, and separation selectivity of alkaloids

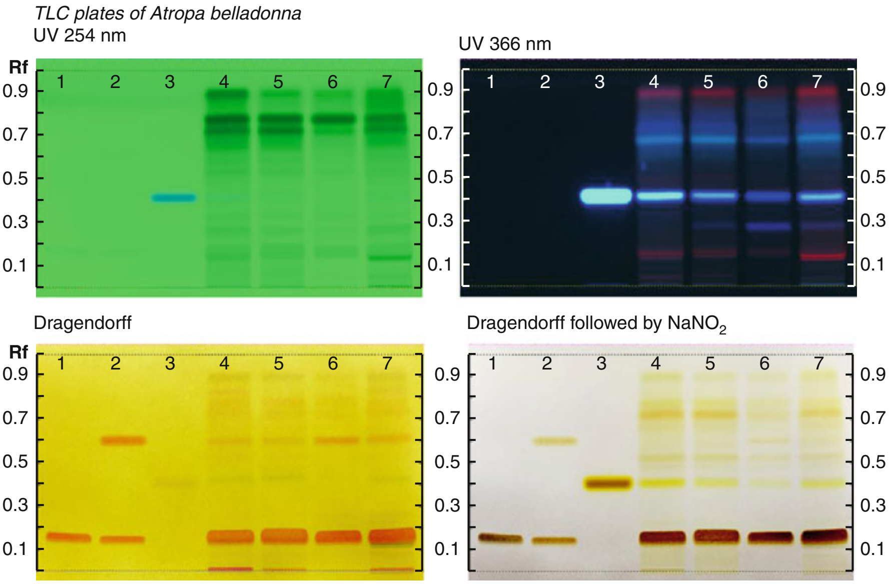
TLC plates of Atropa belladonna
UV 254 nm
UV 366 nm
Dragendorff
Dragendorff followed by $NaNO_2$
Rf
0.9
0.7
0.5
0.3
0.1
1
2
3
4
5
6
7

Fig. 31.2 TLC plates on silica gel with a developing solvent composed of: ammonia 28 %, water, acetone 3:7:90; (1) Atropin, (2) Hyoscyamin HBr (Rf 0.16) and Scopolamin HBr (Rf 0.62), (3) Scopoletin, (4–7) Various *Atropa belladonna samples* (Courtesy of Dr Eike Reich, CAMAG AG, Muttenz, Switzerland)

has been optimized by Gadzikowska et al. [57]. The most effective and selective systems were applied for separation of the alkaloid fraction of *Datura innoxia*. A simple TLC method with Dragendorff's reagent as the visualization agent and image analysis was applied for nutrient medium optimization of hyoscyamine production, as well as in an assay of hyoscyamine biosynthesis in hairy root cultures from *Datura stramonium* with different ploidy levels [58, 59]. An interesting application of TLC has been demonstrated in the work of Abdel-Motaal et al. [60], who determined the antifungal activity of hyoscyamine and scopolamine by TLC-bioautography against 40 isolated fungal strains associated with *Hyoscyamus muticus*.

In recent years, high-performance HPTLC became an attractive alternative to HPLC due to the significant improvement of the stationary phases, automation for sample application, plate development, detection reagent applications, and densitometric scanning. The plates used for HPTLC are characterized by smaller particle size (≤10 μm), thinner layers (≤150 μm), and smaller plates (≤10 cm developing distance) than for conventional TLC layers, resulting in an improved resolving power per unit of distance, faster development times, and reduced solvent consumption. An increasing number of articles demonstrate the application of this technique for qualitative and quantitative analysis of different analytes. Like HPLC, GC, and CE, HPTLC allows method validation corresponding to the ICH criteria and achieving for some given applications a precision accuracy and trueness comparable to that of HPLC [61].

A HPTLC fingerprinting method on a pre-coated silica gel HPTLC $60F_{254}$ has been applied for identifying three wild morphotypes of *Datura metel* based on chemical markers, such as hyoscyamine and scopolamine [62]. The use of a CAMAG HPTLC system equipped with an automatic TLC sampler ATS 4, TLC scanner 3, and integrated software winCATS allowed the validation and quantification of the above-mentioned alkaloids. After development of the plates with chloroform to acetone to diethylamine (50:40:10) as the mobile phase and visualization of the alkaloids with Dragendorff's reagent, the quantitative evaluation was performed in the absorption–reflection mode at 530 nm. The calibration curves were linear in the range of 1,000–4,000 ng for hyoscyamine and 500–2,000 ng for scopolamine. The correlation coefficients of 0.9997 and 0.9996 for hyoscyamine and scopolamine, respectively, were similar, but the limits of detection (LOD, 60 ng and 25 ng) and quantification (LOQ, 1,000 ng and 500 ng) were much higher as compared to a previously validated method with UV densitometry at 205 nm [63]. HPTLC was also used for the quantification of hyoscyamine and scopolamine in in vitro experiments with leaf-derived root cultures. A mobile phase of toluene to ethyl acetate to diethylamine (7:2:1) was used, and quantifications at 290 nm and 230 nm for hyoscyamine and scopolamine, respectively, were applied [64].

3.2 Liquid Chromatography

When looking at the number of papers that have been published during the last 4 years, it seems that liquid chromatography (LC) is currently the most frequently used separation technique for the analysis of tropane alkaloids. An excellent review on the analysis of this class of alkaloids has been published by Aehle and Dräger [27]. It is the method of choice in many laboratories working with natural products because it offers the possibility to analyze thermosensitive, polar, and high molecular weight compounds. LC has an advantage over GC for the analysis of polar compounds such as *N*-oxides, polyhydroxy alkaloids, alkaloid salts, and glycosides because it does not require derivatization before analysis. However, classical HPLC coupled with UV detector is not suitable for alkaloids lacking chromophore groups, like the polyhydroxy tropane alkaloids called calystegines. In this case, pre- or post-column derivatization is necessary. An evaporative light scattering detector (ELSD) or a refractive index (RI) detector may represent an alternative solution [26].

Numerous methods for high-performance liquid chromatography (HPLC) of tropane alkaloids have been developed. The chromatographic conditions depend on the variability of the analyzed matrices (extracts from different plant tissues, pharmaceutical preparations, clinical, and forensic probes) and analytes (pure compounds or alkaloid mixtures with different composition). Most often, columns packed with reverse-phase C18 stationary phase are used for the separation of tropane alkaloids. Gradient or isocratic elution generally involves buffered mixtures at the acidic pH of water–acetonitrile or acetonitrile–methanol, such as acetonitrile–triethylammonium phosphate buffer (25:75) at pH 6.2 [65] acetonitrile–50 mM phosphate buffer at pH 2.95 (10:90 and 20:80) [66], methanol–0.05 M ammonium acetate at pH 4.6 (58:42), and 0.0025 M sodium dodecyl sulfate (SDS) [67]. Cardillo et al. [68, 69] separated tropane alkaloid fractions isocratically with a 0.01 M octanesulfonic acid at pH 3-methanol (65:35) mixture at 40 °C. An exception was the work of Andreola et al. [70], who achieved the separation of tropane alkaloids in basic conditions (pH 8) on a Phenomenex Hypersil 5 C_{18} column with 25 mM phosphate buffer–acetonitrile (70:30).

A novel isocratic chiral method was developed for the separation of *S*-hyoscyamine (t_R 31.1 ± 0.2 min) and *R*-hyoscyamine (t_R 33.4 ± 0.2 min) on two consecutively coupled α-glycoprotein (AGP) chiral columns (150 × 2.0 mm, 5 μm) at 40 °C with 0.01 M ammonium formate at pH 8.0 and 0.01 M ammonium formate in 25 % acetonitrile (85:15) [13]. The flow rate was set at 300 μL min^{-1} for 50 min.

In 2004, the separation science was revisited with the introduction of ultra-performance liquid chromatography (UPLC). Significant advances in instrumentation and column technology were made to achieve dramatic increases in separation power, resolution, speed, and sensitivity in liquid chromatography. Until recently, LC separation technology has not been able to benefit of reducing particle size of stationary phase aiming better efficiency due to the limited working pressure range. New UPLC instruments are able to withstand ultrahigh pressures (up to 1,000 bar) which allow operation with columns packed with sub-2 μm particles and with high mechanical resistance. UPLC offers high-throughput separations with reduced column lengths or high resolution with the same column of an appropriate length. Russo et al. [29] were the first who applied UPLC technology for the analysis of tropane alkaloids achieving separation of scopolamine, 6β-hydroxyhyoscyamine, hyoscyamine, and littorine with a RP C_{18} Acquity BEH (ethylene bridged hybrid, 50 × 2.1 mm, 1.7 μm) column in less than 4 min with a gradient from 10 to 15 % ACN in an aqueous buffer of 0.1 % TFA at a flow rate of 500 μL min^{-1}.

Hydrophilic interaction chromatography (HILIC) is a separation process involving polar stationary phases with mobile phases made of highly polar aprotic organic solvents (>80 %) and a small amount of aqueous solvents. It is particularly suitable for the separation of very polar analytes. HILIC coupled to mass spectrometry may offer a significant increase in sensitivity. The isocratic separation of three calystegines (A5, B4, and C1) on an Acquity HILIC (50 × 2.1 mm, 1.7 μm) column with MeOH–10 mM aqueous ammonium acetate buffer at pH 5 (95:5) and a flow rate of 400 μL min^{-1} was possible in less than 1.5 min [29].

LC is frequently coupled with UV, diode arrays, ELSD, MS, and more rarely with NMR for the peak detection. The tropane nucleus itself has no chromophore and the sensitivity of UV detection and diode array detectors (DAD) depends on the esterified moiety. UV and DAD are suitable for the detection of alkaloids with chromophore-bearing moieties such as hyoscyamine and scopolamine. For such alkaloids, routine analytical work and quantitation are mostly performed between 210 nm and 220 nm [66–68, 71], but an analysis at a wavelength of 254 nm was also reported [70]. Hosseini et al. [72] reported limit of detection (LOD) and limit of quantification (LOQ) values of 5.15 and 17.4 ppm for atropine and 1.92 and 6.4 ppm for scopolamine, respectively, for a validated HPLC method with UV detection at 215 nm, which is comparable with previous reports [15].

HPLC-UV is routinely applied for the quantitation of hyoscyamine and scopolamine produced by in vitro tissue cultures [73, 74], including hairy roots, different plant organs [71, 72], and regenerated [75] or transgenic plants [67]. The products of biocatalytic reactions of transformation of hyoscyamine by hyoscyamine 6β-hydroxylase was also studied by HPLC-UV [69]. Screening of scopolamine and hyoscyamine content of four *Hyoscyamus* species having grown at different geographical locations has been investigated by Bahmanzadegan et al. [65]. It was found that among the four species, scopolamine was the predominant tropane alkaloid in *H. pusillus*, *H. niger*, and *H. kurdicus*, while *H. reticulatus* contained a higher amount of hyoscyamine. Ashtiania et al. [66] analyzing *A. belladonna* and *A. acuminata* from Iran by HPLC reported a surprisingly high concentration of 8.06 % total alkaloids in the roots, 2.88 % in the leaves, and 1.42 % in the stem of a wild *A. belladonna*, whereas the concentration in the same cultivated species reached 1.76 % in the leaves, 3.3 % in the roots, 1.42 % in the stem, and 4.82 % in the seeds. The total alkaloid content of the leaves of *A. acuminata* was found to be 5.88 %. These results, if they are confirmed, would be the highest concentrations of tropane alkaloids ever found in the genus *Atropa*.

For tropane alkaloids lacking UV-adsorbing moieties or with a low sensitivity to UV detection, an evaporative light scattering detector (ELSD) is a good alternative because of its quasi-universality, versatility, low cost, and acceptable sensitivity [76]. In the work of Russo et al. [29], UV at 210 nm and ESLD detection were compared, and the results showed a two to threefold higher sensitivity with the ELSD compared to UV detection in terms of signal-to-noise ratio for hyoscyamine, littorine, 6β-hydroxyhyoscyamine, and scopolamine. The ELSD also showed excellent sensitivity for calystegines, which are chromophore-lacking polyhydroxytropane derivatives.

The use of detectors other than UV for the analysis of tropane alkaloids has been reviewed previously [15, 24, 27]. In contrast to conventional detectors such as UV, RI, fluorescence, and ELSD coupled with LC, the MS detector provides information on the molecular structure of the separated compounds (Fig. 31.3). Over the past decade, liquid chromatography coupled with mass spectrometry (LC-MS) has undergone tremendous technological improvement and is now one of the most powerful tools for the investigation of natural compounds.

Powerful new technologies of ion analyses (tandem MS, time-of-flight MS (TOF MS), and ion-trap MS) substantially increased the capability of MS analyzers with respect to specificity and the extent of data handling. The range of applications of LC-MS in the field of natural products is constantly increasing, especially with the introduction of tandem MS (MS/MS) for quantification and high resolution MS (HRMS) for unequivocal structural elucidation using mass accuracy and the elemental composition of precursors and product ions.

There are numerous key advantages of using a mass detector with HPLC. Among them are (1) the selectivity by means of which mass spectra of co-eluting peaks can be deconvoluted; (2) the peak assignment for which a unique mass spectrum for the compound of interest is generated, ensuring correct peak assignment in the presence of complex matrices; (3) the molecular weight information

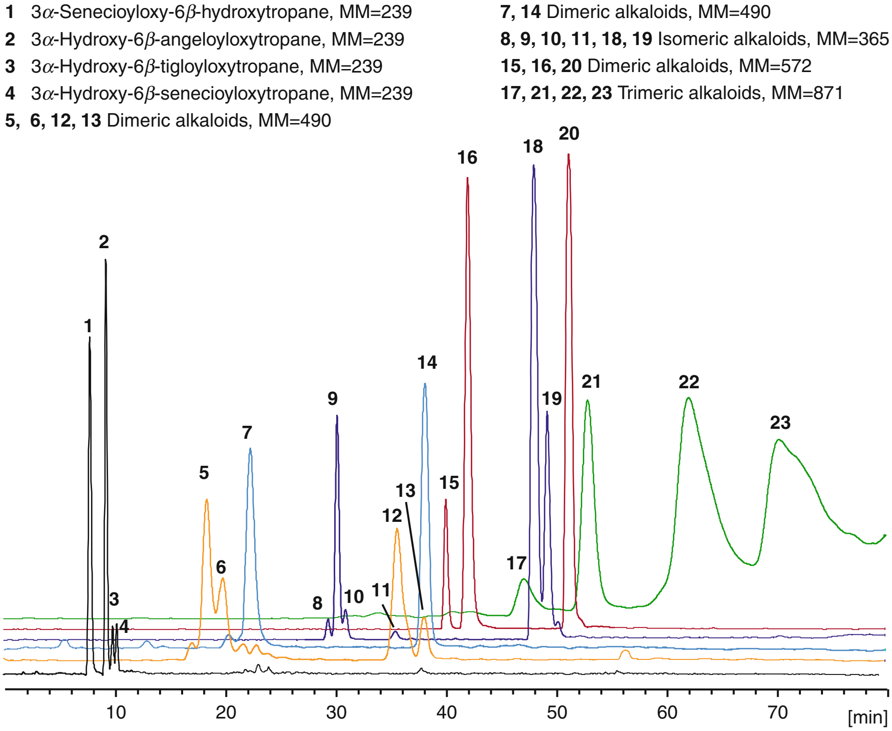

Fig. 31.3 Iontrap LC-MS profile (selected pseudo-molecular ions) of an alkaloid extract from *Schizanthus grahamii* (stem bark). Separation of isomeric series is achieved on a porous graphitic carbon column (Bieri, PhD thesis)

allowing confirmation of known and partially unknown compounds; (4) the structural information given by the controlled fragmentation of the compounds; and (5) the rapid method development allowing easy identification of eluted analytes with less tedious and time-consuming sample preparation procedures.

Among the different types of ionization techniques, electrospray ionization (ESI) is most frequently used in the analysis of tropane alkaloids. As easily protonated weak bases, the alkaloids are analyzed in positive mode. For this, 0.1 % formic acid or acetic acid is usually added to the mobile phase to enhance protonation and increase sensitivity. Only volatile buffers such as ammonium acetate can be used, as buffers such as phosphate may interfere with the ESI process. The latter is a soft ionization technique producing very little fragmentation, which provides information on the molecular mass of the compounds.

Tandem mass spectrometry typically examines mass transitions from precursors to fragment ions. The precursor ions of interest are isolated by a first analyzer according to their characteristic *m*/*z* values, which then undergo fragmentation achieved either spontaneously or by some activation technique to yield product ions and neutral fragments. These are then analyzed by a second mass analyzer. The main advantages of the MS/MS experiment are its enhanced sensitivity and specificity in both single reaction monitoring (SRM) and multiple reaction monitoring (MRM) modes. This is useful in isomer distinction, sequencing of biopolymers, and more particularly in the analysis of complex mixtures. Tandem mass spectrometry experiments eliminate or greatly reduce background signals due to other matrix components or instrumental noise. Due to its sensitivity, LC-ESI-MS/MS is an attractive approach for the analysis of tropane alkaloids and drugs of abuse, like cocaine in body fluids and forensic samples. These compounds are rapidly metabolized in the organism, and sensitive methods are needed for their accurate detection and measurement. A sensitive method was validated for the analysis of atropine, scopolamine, ipratropium, littorine, *N*-butylscopolamine, and cocaine by RP LC-ESI-MS/MS in MRM mode allowing quantification within a linear range of three orders of magnitude between 0.05 and 50 ng mL^{-1} of plasma [77]. The method, which proved to be precise (CV of 2–13 %) and accurate (87–122 %), was applied to stability studies in phosphate-buffered saline, human, and rabbit sera. In contrast to other tropane alkaloids used in medicine, such as atropine, scopolamine, ipratropium, or *N*-butylscopolamine, cocaine was found to degrade rapidly in human serum. Badoud et al. [78, 79] developed a method for the screening and confirmation of 103 doping agents including cocaine in urine samples by UPLC-qToF/MS. The separation was realized in less than 9 min on an Acquity column (BEH C_{18}; 100 × 2.1 mm, 1.7 μm) with a mixture of 0.1 % formic acid in water and 0.1 % formic acid in ACN. The automatic identification of analytes was based on retention time and mass spectra accuracy, with an automated tool for peak picking. The method was validated according to the International Standard for Laboratories described in the World Anti-Doping Code and was selective enough to comply with the World Anti-Doping Agency recommendations. The mass accuracy and the elemental composition of precursor and product ions were used to confirm compound identification.

Liu et al. [80] established liquid–liquid and solid phase extraction protocols which were coupled to LC-ESI-MS/MS using a 1.8 μm particle size analytical column operated at 50 °C for the analysis of 800 drugs and toxic compounds. Gradient elution of the analytes was conducted using a solvent system composed of methanol and water containing 0.1 % formic acid. Positive-ion ESI-MS/MS spectra and retention time of each compound were first established using standard solutions. These spectra and retention times were then transferred into the library and searched by the identification algorithm based on retention time matches and scores of fit, reverse fit, and purity resulting from the searching process for the confirmation of compounds found in test specimens. The established method was found to be highly effective when applied to the analyses of postmortem specimens (blood, urine, and hair).

Analysis of total cocaine residues in wastewater has been validated using LC-ESI-MS/MS [81]. After an alkaline hydrolysis step, the total cocaine was estimated by the amount of the degradation products ecgonine, anhydroecgonine, and *nor*ecgonine detected in the samples.

The pharmacokinetics of *S*- and *R*-enantiomers of hyoscyamine in humans have been examined by LC-ESI-MS/MS [13]. Plasma supplemented with atropine was incubated with human serum (not containing atropinesterase (AtrE)) and with rabbit serum possessing AtrE (EC 3.1.1.10), which stereospecifically hydrolyzes *S*-hyoscyamine into tropine and tropic acid while leaving *R*-hyoscyamine unaffected. The estimation of the differences between the total hyoscyamine content in the aliquots incubated with human and rabbit sera allowed the determination of the remaining *R*-hyoscyamine and hydrolyzed *S*-hyoscyamine. Both enantiomers were detected in the MRM mode. The method proved to be reproducible, precise (RSD 2–9 %), accurate (93–101 %), and selective. The enantioselective assay was applied to the analysis of atropine degradation in rabbit serum in vitro as well as to human in vivo plasma samples from a pesticide-poisoned patient treated with atropine. The method was also applied for kinetic studies of atropine administered to swine, where no obvious stereoselective elimination was found [82].

A rapid method for the determination of the most abundant *Datura* alkaloids atropine and scopolamine in leaves, flowers, stems, and seeds by LC-ESI-MS technique was developed and validated by Jakabova et al. [83]. After an extraction procedure using 30 min ultrasonication with a methanol–water (3:2) mixture followed by filtration, the analytes were separated within 5 min on a new generation of core-shell particle-packed columns. The use of a reversed-phase column (Ascentis Express C_{18}) packed with this type of sub-3 μm particles allows high speed and generates high efficiencies. Detection was performed in the more sensitive single ion monitoring (SIM) mode using the pseudomolecular ions at *m*/*z* 290 and 304 for hyoscyamine and scopolamine, respectively. The method was found to be selective, precise, repeatable, and very sensitive with LOD at 100 and 50 pg mL^{-1} and LOQ at 333 and 167 pg mL^{-1} for scopolamine and atropine, respectively.

During crop gathering, some parts of Solanaceae plants may be occasionally included. A method for the simultaneous determination of tropine, atropine, scopolamine, homatropine, anisodamine, α-solanine, and α-chaconine in grains and seeds (wheat, rye, maize, soybean, linseed) has been reported by Jandric et al. [84]. The analytes were separated by isocratic HPLC on a Chirobiotic V column and detected by ESI-MS/MS detector operating in the MRM mode. The method performances were presented in terms of linearity in the range 5–80 ng g^{-1}, specificity, selectivity, accuracy (recoveries from 61 % to 111 %), precision (CV <5 %), and ruggedness. The limits of quantitation (LOQ) were in the range 2.2–4.9 ng g^{-1}.

Boros et al. [85] investigated the floral nectar of different *Datura* species as a potential source of intoxication. Atropine and scopolamine were quantified by HPLC on an Ascentis Express C_{18} column (50 × 2.1 mm) packed with 2.7 μm fused-core particles and monitoring using the SIM acquisition mode by ESI-MS/MS detector.

Hou et al. [86] developed a method using a short C_{18} column for the separation of atropine, anisodamine, and scopolamine in Huashanshen dripping pills used in traditional Chinese medicine (TCM) in less than 1.5 min. The method showed linearity in the range of 250–4,000 ng mL^{-1} for atropine and anisodamine and 62.5–1,000 ng mL^{-1} for scopolamine. The intra- and inter-day precisions were less than 3.0 %, and the mean recoveries were more than 98.0 % for all analytes. This method is rapid, reliable, reproducible, and suitable for the routine use to control the quality of Huashanshen dripping pills.

The applicability of LC-MS for the structural elucidation of tropane alkaloids has been demonstrated in recent reports where the exact mass measurements providing elemental composition of the ion fragments are combined with fragmentation pattern recognition from MS/MS experiments. Jousse et al. [87] performed alkaloid profiling of hydroponic *Datura innoxia* plants by UFLC-ESI-HR/MS and LC-MS/MS. In total, 21 known compounds and four non-elucidated alkaloids were detected on the basis of their HRMS data and fragmentation pathway. Furthermore, by comparing the alkaloid profiles, the authors found that the alkaloid biosynthesis and particularly that of 3-acetoxy-6-hydroxytropane and 3-hydroxylittorine is higher in non-sterile conditions. Later, during the routine isolation of tropane alkaloids by preparative column chromatography, de Oliveira et al. [88] tentatively identified 2 possibly new tropane alkaloids by LC/ESI-MS/MS in fractions from the stem of *Erythroxylum caatingae*. Al Balkhi et al. [89] applied LC-MS to study the biosynthetic pathway of tropane alkaloids in *Datura innoxia* hairy roots. Fourteen alkaloids were separated by an isocratic method on an ODS AQ-C18 column and identified by LC–qToF/MS and LC–MS/MS analyses. Semi-preparative LC was used for the separation of *nor*littorine and *nor*hyoscyamine, which were shown to be induced under stress conditions by the conversion of littorine and hyoscyamine by isotope labeling experiments.

3.3 Gas Chromatography

Gas chromatography is a commonly used analytical technique in many research laboratories. A broad variety of samples can be analyzed as long as the compounds are sufficiently thermally stable and volatile enough. Pyrrolidine, pyrrolizidine, isoquinoline, piperidine, indole, and tropane alkaloids are routinely analyzed by GC [90]. A prerequisite is, however, that the alkaloids occur as bases, hydrochlorides, or picrates, and not as *N*-oxides, because of the extremely high boiling points of the latter. Tropane alkaloids had been analyzed by GC long before HPLC. Since the work of Hartmann et al. [91], the prevailing analytical approach for screening, identification, and quantification of tropane alkaloids was capillary GC combined with electron impact mass spectrometry (GC-EI/MS). It became widely recognized because of its sensitivity, accuracy, and versatility. The main advantages of GC-MS are the high resolution power of the capillary columns and the structural information provided by the mass detector. EI mass spectra collected under standard conditions (70 eV) are highly specific and reproducible, which allows their organization in mass spectral databases. The utilization of such libraries in combination with retention data (retention index or retention time locking) allows the automatic and quick identification of known components in an alkaloid mixture without the use of reference compounds. The mass fragmentation of tropane alkaloids is well known for about 50 years [92], and it has been possible to tentatively assign the structure of a number of new tropane alkaloids by applying the fragmentation rules [93, 94]. The development of gas chromatography for the analysis of tropane alkaloids has been described in several comprehensive reviews [15, 24, 27]. Here, we will mainly focus on the recent articles concerning the application of GC in the analysis of this class of alkaloids.

The limits and advantages of GC-MS for the separation and structural elucidation of complex mixtures of tropane alkaloids are fully demonstrated in the work of El Bazaoui et al. [93]. In alkaloid extracts of different organs of *Datura stramonium* cultivated in Morocco, the authors detected more than 70 alkaloids in less than 55 min. Among them, 67 tropane alkaloids were identified in the organs (roots, stems, leaves, seeds, and flowers) of the plant. Nine new tropane alkaloids (3,7-dihydroxy-6-propionyloxytropane, 6,7-dehydro-3-tigloyloxytropane, 3-tigloyloxy-6,7-epoxytropane, 3,7-dihydroxy-6-(2′-methylbutyryloxy) tropane, 6,7-dehydroapoatropine, 3-(3′-methoxytropoyloxy)tropane, 3-tigloyloxy-6-isobutyryloxy-7-hydroxytropane, 3-tropoyloxy-6-isobutyryloxytropane, and 3β-tropoyloxy-6β-isovaleroyloxytropane) were tentatively identified. In a similar important work [95], the authors reported 53 alkaloids in *D. innoxia*, including four new tropane esters, which were tentatively identified as 3-acetoxy-6,7-epoxytropane (acetylscopine), 3-acetoxy-6-propionyloxy-7-hydroxytropane, 6,7-dehydro-3-phenylacetoxytropane, and 3-(2′-phenylpropionyloxy)-6,7-epoxy*nor*tropane (dihydroapo*nor*scopolamine). The new alkaloids contributed less than 0.1 % each of the total alkaloid content. The configuration of the isomeric 3α- and 3β-tropane derivatives was deduced on the basis of their retention data and their relative abundances by comparison with those from the literature considering that the 3α-isomers occur in considerably higher amount than the corresponding 3β-isomers of the homologous tropine esters [96]. The unambiguous distinction between tigloyl and its isomeric angeloyl and senecioyl derivatives cannot be established upon mass spectral data only. Thus, the identity of the different tigloyl derivatives in the above-mentioned papers should be considered as a suggestion, only because it was based exclusively on their mass spectral fragmentation and retention data.

In the genus *Schizanthus* (Solanaceae), tigloyl, senecioyl, and angeloyl tropane derivatives have been identified using a combination of GC with MS, nitrogen phosphorus detector (NPD), and Fourier-transformed infrared spectroscopy (FTIR) for the resolution of four compounds of the same nominal mass in an alkaloid mixture extracted from the stem bark of *Schizanthus grahamii* [97]. The data obtained by GC-FID/NPD and GC-EIMS were insufficient for an unambiguous structure assignment. GC-NPD indicated the number of alkaloids in the sample, while GC-MS allowed their tentative identification as well as the position (C3 or C6) of the substituent. Four isomeric compounds possessing a molecular ion at *m*/*z* 239 were revealed from the GC-MS data. Among them, 3α-senecioyl-6β-hydroxytropane was easily distinguished by its base ion peak at *m*/*z* 94 and identified by cochromatography with a previously isolated reference compound. The 3 other alkaloids, however, displayed similar mass fragmentation with a base ion peak at *m*/*z* 113, which is characteristic for 3-hydroxy tropane derivatives and indicates a substitution at C6 with a tigloyl, senecioyl, or angeloyl moiety. The relative ion abundances of diagnostic fragments at *m*/*z* 55, 82, and 83 from the mass spectra of the four isomeric alkaloids displayed a characteristic pattern for each isomer, which allowed their tentative structure assignment as 3α-hydroxy-6β-angeloyloxytropane, 3α-hydroxy-6β-senecioyloxytropane, and 3α-hydroxy-6β-tigloyloxytropane. An unequivocal distinction between these isomers was obtained through IR spectra. Angelates are characterized by two absorption bands near 1,232 cm^{-1} and 1,156 cm^{-1}, while the corresponding tiglate absorption bands presented a specific band at 1,258 cm^{1} and another at 1,140 cm^{-1}. The contribution of each absorption band and their corresponding intensity made possible the assignment of the correct structures. As a result, the combined use of GC-MS (Fig. 31.4) and GC-FTIR rendered the analysis of complex mixtures and the direct identification of structurally similar tropane alkaloids more efficient and unambiguous. Additionally, the retention indices of the isomers, recorded on four stationary phases, were found to be different, which further assisted in the peak assignment and facilitated the identification of these isomers.

The sensitivity and selectivity of GC-MS was used for the development and validation of a novel analytical method for the rapid and simultaneous detection of scopolamine and atropine in buckwheat (*Fagopyron esculentum*) samples and related food products [98]. Low detection and quantification limits were achieved in the selected-ion mode (SIM) by derivatization (silylation) of the analytes. The LOD for atropine and scopolamine were found to be 0.3 and 1 μg kg^{-1}, respectively, while the LOQ were 1 and 6 μg kg^{-1}, respectively, which corresponds to less than one *D. stramonium* seed per million buckwheat fruits.

The application of internal standards (IS) is essential for recovery experiments in method development and validation, as well as for the accurate quantification of target analytes. When a mass spectrometer is used, the most appropriate IS is an isotope-labeled derivative of the analyte, as it has the same physicochemical properties but a different nominal mass. Isotope-labeled derivatives of drugs of abuse are preferred in clinical, toxicological, and forensic analyses, but they are very expensive and often difficult to obtain. Shaikh and Chen [99] reported on the synthesis of deuterium-labeled controlled drugs, including tropane alkaloids, having a main focus on GC-MS analyses.

The combination of gas chromatography and an isotope ratio measurement mass spectrometer (*irm*-GC-MS) was used to study the detoxification and degradation pathways of tropane alkaloids involving *N*-demethylation to the respective *nor*-compounds. Molinie et al. developed protocols for sample preparation, including liquid–liquid and SPE extraction, and analysis of the ^{15}N kinetic isotope effects (KIEs) with *irm*-GC-MS and *irm*-elemental analysis (EA)-MS [100]. Degradation experiments were performed with a *Pseudomonas* strain that uses tropane alkaloids as sole source of carbon and nitrogen. The authors found that liquid–liquid extraction from the aqueous medium in combination with *irm*-GC-MS was the most convenient method for measurement of the $^{15}N/^{14}N$ ratio. Later, Kosieradzka et al. [101] exploited derivatization of the alkaloids by reaction with ethyl chloroformate (ECF) in aqueous medium and their quantitative extraction. The

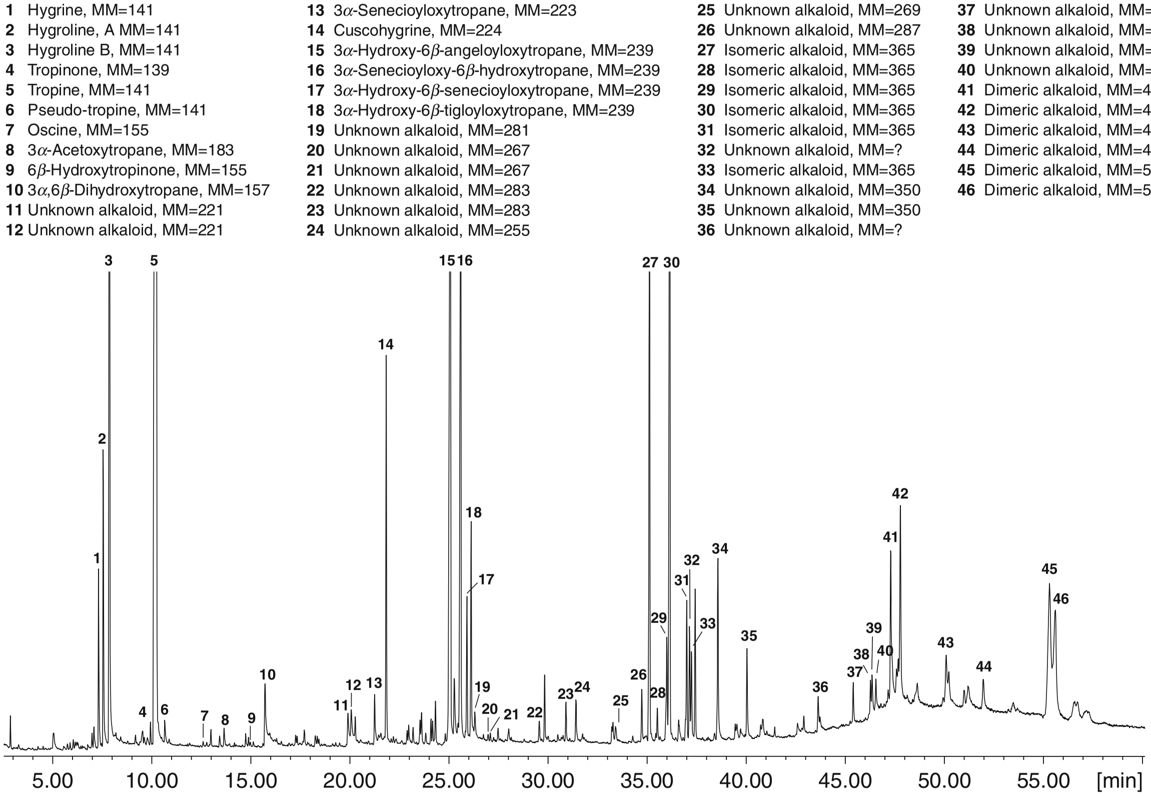

Fig. 31.4 GC-MS profile of an alkaloid extract from *Schizanthus grahamii* (stem bark). Tentative identification based upon characteristic mass fragmentation and authentic reference substances (Bieri, PhD thesis)

improved chromatographic properties of these derivatives resulted in better separation of the isomeric *nor*tropine and *nor*pseudotropine for measurement of their $\delta^{15}N$ (‰) values by *irm*-GC-MS. The achieved repeatability and precision were sufficient to measure the differences (>0.8‰) in the $\delta^{15}N$ values, with a standard deviation routinely *around* 0.3‰. The method was applied for the measurement of changes in the $\delta^{15}N$ values of *nor*tropine and *nor*pseudotropine during fermentation experiments with cell suspension cultures of a *Pseudomonas* strain expressing a specific capacity for tropine catabolism.

Conventional GC-MS has also been applied to study in vitro the biochemical response, namely, tropane alkaloid production, to minerals and elicitors in *D. stramonium* hairy roots [102], as well as in chemoecological experiments where the acaricidal activity of *Hyoscyamus muticus* extract against phytophagous mites *Tetranychus urticae* was attributed to the presence of high tropane alkaloidal content [103].

3.4 Capillary Electrophoresis

Capillary electrophoresis has been widely used in pharmaceutical and biomedical analysis due to its efficiency, accuracy, and high resolution [104]. It is a highly versatile alternative and complementary method to HPLC generating high efficiencies in a fast analysis time. Furthermore, the volume of organic solvent used for CE separations is dramatically reduced and can be restricted to only a few milliliters per day. It is a valuable technique for the quality control and analysis of medicinal plants [105]. When considering the number of reports published within the last 10 years, the analysis of alkaloids is among the most popular applications of CE for natural product separations. Its application in alkaloid analysis appears very attractive because these compounds are easily protonated and therefore easily separated by an electrophoretic process. Applications of capillary electrophoresis for analysis of tropane alkaloids in different matrices have been reviewed [15, 27]. One of the main advantages of CE in tropane alkaloid analysis that can be highlighted is its straightforward applicability for enantioseparation of atropine and other related chiral compounds [27, 106]. Despite all of its advantages, the practical implementation of capillary electrophoresis in the analysis of tropane alkaloids is relatively limited. This could be explained by the lower sensitivity of the most widely used UV detectors, the small injection volume also affecting the sensitivity and the intensive sample preparation required for reducing the influence of the complex biological matrices due to the lack of specificity when using UV. In addition, CE hyphenated with MS detectors is not as good as generally observed with HPLC-MS in terms of the reproducibility of peak area and retention times [107].

Various strategies have been applied during recent years to overcome the drawbacks associated with the analysis of tropane alkaloids by CE. Using atropine and scopolamine as model analytes, a comparison of UV and electrochemical detection (ED) in conjunction with non-aqueous CE (NACE) has been performed for capillaries with different internal diameters by Blasco et al. [108]. The results showed a significant increase in relative LODs with UV detection for capillaries with an internal diameter smaller than 25 μm. The absolute LODs remained nearly constant over a wide range of capillary dimensions. In comparison, ED showed some increase in relative LODs for capillaries smaller than 10 μm in diameter, but the absolute LODs decreased over the complete range of capillary diameters studied. Absolute LOD for scopolamine ranged from 2.4×10^{-15} to 7.9×10^{-18} mol in capillaries with an i.d. from 50 to 2 μm with electrochemical detection. With UV light detection, capillaries with an i.d. from 75 to 5 μm provided slightly improved absolute LOD for scopolamine of 213–120 nmol.

Ren et al. [109] established a method for the simultaneous determination of anisodamine, scopolamine, atropine, and anisodine by capillary electrophoresis with electrochemiluminescence detection (CE-ECL). The concentration and pH of the running buffer as well as the methanol content additive were investigated for the improvement of selectivity and sensitivity. These four alkaloids were separated within 6 min under optimal conditions in a buffer containing 20 mmol L^{-1} phosphate and 7 % methanol at pH 8.0. The method, showing RSD of the migration time less than 1.1 % and recoveries between 97.8 % and 102 %, was applied for the determination of anisodamine and scopolamine in an extract of *Przewalskia tangutica*.

The hyphenation of CE with MS detectors certainly offers the best opportunities for the qualitative analysis of natural compounds in general and of tropane alkaloids in particular, which is evident from the increasing number of articles published in recent years. Methods applying CE-MS for analysis of tropane alkaloids have been developed mainly in clinical and forensic laboratories. With this respect, HRMS detectors, enabling the identification of a compound through accurate mass measurement and isotopic pattern, are preferred for combination with CE. A comprehensive overview of principles and applications performed by CE coupled to TOF/MS detection has been published by Staub et al. [110].

A “metabolomic” approach using high resolution data was used to identify candidates with identical chemical formulae in urine, blood, and hair samples by CE-TOF/MS [111]. In this case, the detected peaks with their exactly determined mass were searched against a database with *ca* 50′500 pharmaco-toxicologically relevant compounds. Then, starting from the mass of the unknown, mass shifts corresponding to predefined biotransformations (e.g., demethylation, glucuronidation) were calculated, and the corresponding mass chromatograms were extracted from the total ion current (TIC) in order to search for metabolite peaks. The presence of metabolites in the TIC was correlated with functional group data in order to exclude candidates with structures that were not compatible with the observed biotransformations (e.g., loss of a methyl group from a structure not bearing methyls). The procedure was tested on 108 pharmaco-toxicologically relevant compounds, and their metabolites were detected in real positive samples resulting in a reduced list of possible candidates.

A method assessing forensic drugs including cocaine and benzoylecgonine by capillary zone electrophoresis (CZE) coupled with TOF-MS was developed by Gottardo et al. [112]. The separation was achieved by applying a constant voltage of 15 kV during 25 min in an uncoated fused-silica capillary (75 μm × 100 cm) using a 25 mM ammonium formate electrolyte solution (pH 9.5). LODs were in the range of 2–10 ng mL^{-1} and LOQs of 10–30 ng mL^{-1} for all analytes. The CVs, tested in biological matrices, were below 2.97 % for migration times and below 14.6 % for peak area. The analytes were identified in blood samples (real cases) based on matching measured exact mass and isotopic pattern.

Da Costa et al. [113] developed and validated a method for the simultaneous determination of cocaine, benzoylecgonine, cocaethylene, anhydroecgonine, anhydroecgonine methyl ester, and ecgonine methyl ester in human urine using CE-ESI-MS/MS. The electrolyte was 1 mol L^{-1} formic acid, whereas 0.05 mol L^{-1} formic acid in methanol to water (1:1) was used as the composition of the coaxial sheath liquid at the ESI nozzle. LODs were 100 ng mL^{-1} for cocaine and cocaethylene and 250 ng mL^{-1} for the other metabolites, whereas LOQs were 250 ng mL^{-1} for cocaine and cocaethylene and 500 ng mL^{-1} for all other compounds. Precision and recovery tests showed an RSD lower than 10 % (except for anhydroecgonine, 18 %) and recoveries ranged from 83 to109 % for all analytes. In real samples, cocaine and its derivatives were confirmed by MS/MS experiments.

NACE appears to be ideally suited for online coupling with mass spectrometry due to high volatility and surface tension of most of the organic solvents used [114]. Carefully optimized NACE-MS method was applied to a very large number of alkaloids in different psychoactive plant extracts, as demonstrated by Posch et al. [115]. The method allowed the separation of structurally closely related substances, such as diastereomers and other isobaric compounds, as well as to separate members of different alkaloid classes including tropane alkaloids without modification of the procedure.

CE-ESI-TOF-MS was successfully applied to analyze mixtures of unknown tropane alkaloids. Seven alkaloids (tropine, belladonnine, *nor*hyoscyamine, apoatropine, hyoscyamine, 6β-hydroxyhyoscyamine, and scopolamine) were simultaneously identified in a pharmaceutical preparation of *Atropa belladonna* leaf extract by TOF-MS by matching their mass accuracy and true isotopic pattern. CE-ESI-IT-MS was used to discriminate the putative presence of littorine. Optimal separation conditions were achieved with 60 mM ammonium acetate buffer at pH 8.5 containing 5 % isopropanol [116].

3.5 Non-Chromatographic Methods

There have been significant progresses in methods that hyphenate separation techniques with spectrometric or spectroscopic detectors for the unequivocal identification of target compounds. These hyphenated methods can provide the most complete information on the analyte distribution and even their chemical structures. However, they can be time-consuming, costly, and difficult to apply in routine analyses. Non-chromatographic procedures can sometimes provide sufficient information to identify the compounds of interest in a less time-consuming and more cost-effective way with competitive limits of detection. Thus, non-chromatographic target analysis continues to be a promising research area and has been applied to the development of several methodologies that facilitate this type of analytical approach. In the recent years, immunoassays and optical sensors, as well as standalone NMR and mass spectrometry, have been used as non-chromatographic analytical platforms dedicated to the analysis of tropane alkaloids. The advantages of immunoassay technology over other analytical techniques include low detection limits, high analyte selectivity, high sample throughput, reduced sample preparation, versatility for target analytes, cost-effectiveness for a large number of samples, and adaptability to field use. The limitations are matrix effects, cross reactivity with structural analogues of the target analyte, poor suitability for multi-analyte applications, low availability of reagents, and longer individual response times for assays compared to classical analytical methods. Recently, immunoassay methods for the analysis of cocaine and its derivatives have been applied in forensic and clinical samples. Cloned enzyme donor immunoassays (CEDIAs) for drugs of abuse screening were compared with chromatographic methods (GC-MS or LC-MS) to check and optimize the immunoassay cutoff values in blood serum using drug of abuse reagents in combination with a Hitachi 912 autoanalyzer [117]. Serum samples were measured directly or, in cases of hemolysis, after dilution with blank serum. Tests were performed for various amphetamines, cocaine, cannabinoids, methadone, benzodiazepines, and opiates. Sensitivity, specificity, positive and negative predictive values, and overall misclassification rates were evaluated by contingency tables and compared to receiver operating characteristic (ROC) analyses and Youden indices. Ideal cutoffs were statistically calculated on the basis of sensitivity and specificity as decisive statistical criteria with focus on a high sensitivity (low rate of false-negatives), i.e., using the Youden index. The LOD for benzoylecgonine was found to be 75 ng mL^{-1}.

Drugs of abuse, such as cocaine, cannabinoids, and amphetamines, were detected in hair by immunoassay followed by GC-MS for confirmation and quantitation [118]. Immunochemical screening was done with the Siemens EMIT® II Plus assays for opiates, amphetamines and ecstasy, and Microgenics CEDIA® assays for cocaine. All samples with positive results in the screening test for any drug and/or metabolite were analyzed by GC-MS for confirmation and quantitation. The cutoff value for cocaine was 0.20 ng mg^{-1}.

Wenger et al. [119] presented a fast and sensitive method for the detection of cocaine in liquid samples. The authors designed a system for the systematic monitoring of luggage at airport security controls. The system consisted of a waveguide grating sensor chip functionalized with cocaine antigen–protein conjugates. Drug molecules were covalently grafted onto immunoglobulin G carrier proteins. The detection was based on the changes of the effective refractive index of a waveguide grating upon adsorption of biomolecules. The sample which may contain cocaine is mixed with a fixed amount of monoclonal anti-cocaine antibodies and injected into the flow cell. The antibodies can either bind to free cocaine in solution or to the cocaine analogues present on the surface-bound carrier proteins. When the concentration of free analyte increases, fewer antibodies can bind to the surface. Thus, the instrument response decreases when cocaine is present in the sample. The sensitivity was substantially enhanced by three orders of magnitude using antibodies conjugated to gold nanoparticles. This effect is mainly attributed to the dielectric properties of the composite particles composed of gold and antibodies that amplify the observed changes of effective refractive index. The results obtained under optimized conditions showed that the working range was substantially shifted toward lower concentrations of cocaine. Its presence could be identified shortly after injection of the sample. Under optimized conditions, a lowest detectable concentration of 0.7 nM was reached within only 1 min.

Luminescence-based optical sensors have experienced significant growth during the past decade. Dispersed colloidal semiconductor nanoparticles, known as quantum dots (QDs), have attracted intensive research interests in scientific and technological applications. Because of their unique optical properties, such as broad excitation spectra, narrow and symmetrical emission peaks, enhanced photobleaching resistance, and tunable spectra, QDs have been applied in a variety of research areas as fluorescent species for cell labeling, clinical diagnosis, and tumor imaging.

Wu and Fan [120] developed an effective method for the quantitative determination of raceanisodamine (= 6-hydroxyatropine ?) hydrochloride and atropine in biological fluids by room temperature phosphorescence (RTP) detection applying Mn-doped ZnS quantum dots. Mn-doped ZnS QDs exhibit a phosphorescence spectra emission peak at 590 nm. ZnS is a wide gap semiconductor. Its conduction band and valence band can provide a wide range of energy levels for the doping ions. As Mn^{2+} and Zn^{2+} have equal electric charges and similar ionic radii, Mn^{2+} can be better incorporated into the ZnS crystal lattice without producing a greater impact. The alcoholic hydroxyl of raceanisodamine and atropine can be ionized to form a complex with a divalent cation under physiological conditions. Thus, it was possible to form the complex between the alkaloids and Mn^{2+}/Zn^{2+} on the surface of the Mn-doped ZnS QDs, and this combination significantly quenched the phosphorescence intensity of the Mn-doped ZnS QDs. To perform the analysis, 1 mL of a 50 mg L^{-1} Mn-doped ZnS QDs solution, 1 mL Tris–HCl (pH 7.5), and a range of concentrations of the drugs' standard solution or water sample solution were mixed, and RTP measured at an excitation wavelength of 305 nm and an emission wavelength of 590 nm. No pretreatments such as deproteinization or centrifugation were needed, except for a 100-fold dilution of urine and serum samples before analysis. The selectivity of the method was unaffected by excess of Na^{+}, K^{+}, Mg^{2+},Ca^{2+}, and amino acids. The method showed linearity in the range 0–9 μM, RSD below 2 %, and recovery from 95 to 104 %. It was shown to be rapid, easy to implement, selective and inexpensive, and displayed relatively high sensitivity. The LOD values were 0.11 and 0.09 μM for raceanisodamine hydrochloride and atropine, respectively.

Among the different techniques utilized for developing chemical sensors, those based on molecular fluorescence are the most sensitive. Oliveri and Di Bella [121] developed a powerful fluorescent probe based on an amphiphilic Schiff-base zinc (II) complex for the sensitive detection of alkaloids, including tropane and atropine. This complex exhibits fluorescent enhancement upon formation of 1:1 adducts with alkaloids. Its binding interaction, related to Lewis basicity, is strongly influenced by the steric characteristics of the coordinating alkaloid, leading to high selectivity in the micromolar range and sensitivity for pyridine-based alkaloids. The fluorescence emission wavelength was constant for all adducts except for cinchonine and tropane adducts and was independent of the excitation wavelength. LOQ for tropane and atropine were 4 and 15 μM, respectively.

NMR spectroscopy is of fundamental importance for the structural elucidation of natural compounds. In the past decade, NMR has been applied for metabolomics (qualitative and quantitative) studies in different matrices such as plant extracts and body fluids. The only variables in NMR spectroscopy are the solvent used and the magnetic field strength. The main characteristics of this nondestructive approach for quantification of target compounds are high reproducibility, high throughput, easy sample preparation, and a wide range of targets. The main drawback is, however, the low sensitivity as compared with other analytical detectors and the overlap of many signals when analyzing complex mixtures. The use of 2D NMR spectroscopy and 2D *J*-resolved spectra in particular can considerably improve the peak resolution. With the increasing field strength of the NMR magnets and the improved resolution, the application of NMR for quantification of target compounds became feasible. In contrast to the above-described non-chromatographic methods, NMR allows identification and quantification of several metabolites simultaneously [122].

Using an airlift system made from a NMR tube, Bartholomeusz et al. [123] performed in vivo quantitative ^{1}H NMR studies of tropinone metabolism in *Pseudomonas* AT3 cultures. In the range 1.8–4.2 ppm, tropinone was easily distinguished from the other compounds not only by its methyl peak but also by the H1, H2, H3, and H5 peaks. Tropine was distinguished by its H1 and H2 peaks, while pseudotropine was characterized by its H1, H2, and H6 peaks. Quantification of the metabolites by 1D NMR only proved to be difficult as a result of large overlaps in the ^{1}H spectrum due to similar metabolite structures. Good resolution of all target metabolites was obtained by using 2D TOCSY NMR. After optimization of the TOCSY mixing time with an equimolar solution, the 2D method was calibrated with solutions containing tropine, tropinone, and pseudotropine. The method showed a good linearity ($R^2 > 0.999$) between the concentration ratios and the peak ratios with a sensitivity of *ca*. 0.5 mM.

Lanfranchi et al. [124] developed a protocol using a ^{13}C NMR method to investigate the enantiomeric differentiation of hyoscyamine in plant extracts. ^{13}C NMR spectra are more suitable for that purpose because the signals are singlets and offer a large spectral width as compared to ^{1}H NMR. A chiral lanthanide shift reagent combined with trifluoroacetic acid (CLSR/TFA) was used. The addition of CLSR to a racemic compound having a Lewis basic site provokes deshielding of the signals of most carbons of the molecule and splitting of the signals for some of them. The enantiomeric differentiation of atropine free base was investigated with various CLSR, but only tris[3(heptafluoropropylhydroxymethylene)-(+)-camphorato] ytterbium(III), known as Yb(hfc)$_3$, resulted in weak deshielding and splitting of some carbons. For that reason, the free base was protonated with TFA. At molar ratios [Yb(hfc)$_3$]:[atropine-TFA] between 0.20 and 0.40, all the signals of the aromatic ring split. The method was optimized with various mixtures of (+) and (−)-hyoscyamine ranging from 50:50 (racemic mixture, i.e., atropine) to 98.5:1.5. The enantiomers were best differentiated by NMR using the split signals of aromatic C12 and C15, which were in agreement with the gravimetrically prepared samples. The method was applied to an extract of *Datura stramonium* revealing that *S*-(−)-hyoscyamine was the unique enantiomer.

A recent innovation in mass spectrometry is the ability to record mass spectra on samples in their native environment, without sample preparation or pre-separation by creating ions outside the instrument. A stream of ionizing or at least ion-desorbing fluid medium is directed onto a solid sample surface from which analyte ions are withdrawn and transported through air into the mass analyzer via a standard atmospheric pressure ionization interface. Direct analysis in real time (DART) and desorption electrospray ionization (DESI), two recently introduced mass spectrometric ionization techniques, have been found to be extremely useful for the direct detection of chemicals on surfaces without requiring any sample preparation such as solvent extraction. These techniques are rapid, providing the results within minutes, but can be regarded as semiquantitative only, as the detection of a compound largely depends on the matrix. Selectivity and accurate elemental composition assignment through exact mass measurement and MS/MS experiments are possible when coupled to a high resolution or tandem mass analyzer. The application of DESI-MS/MS for the analysis of tropane alkaloids *in Datura stramonium* and *Atropa belladonna* [125] have been discussed in a previous review [15].

Recently, the DART technique has been utilized for chemical profiling of hairy root cultures of *Atropa acuminata* [126]. The intact hairy roots were directly analyzed by holding them in the gap between the DART ion source and the mass spectrometer. Applying a resolving power of 6,000, two alkaloids were detected by their elemental composition and characterized as atropine and scopolamine on the basis of exact mass measurements.

4 Conclusion

Tropane alkaloids and the various plants containing them have attracted the interest of people for centuries due to their pharmacological effects and application in medicine. For these reasons, they continue to be in the research focus of many scientists working in the field of pharmaceutical, forensic and clinical analysis, plant physiology, biotechnology, and obviously in phytochemistry. Since the first analyses of tropane alkaloids by GC and LC in the early 60s, the analytical techniques have evolved remarkably with respect to both separation and detection capabilities. Basically, two main tendencies can be distinguished: continuous improvements of modern hyphenated techniques and the development of innovative non-hyphenated methods for the analysis of target molecules as well as screening of unknowns. Although a century-old technique, TLC is in its renaissance with the development of HPTLC instrumentation. This regained interest is primarily related to crude extract analysis and rapid plant profiling. The introduction of narrow bore columns (i.d. <0.25 mm) for GC and UPLC instruments capable of operating with columns packed with sub-2 μm particles considerably improved the separation power (increase in efficiency) or, alternatively, enabled the dramatic reduction of analysis time (reduction of column length) while maintaining initial resolution. Thus, manufacturers of both techniques introduced new terms and acronyms supporting the potential to considerably speed up analysis time. Simultaneously, the advance in benchtop mass spectrometers resulted in detectors coupled with a separation instrument, which provide structural rich information with extremely high sensitivity and selectivity. Consequently, GC-MS and LC-MS have become routine methods for the qualitative and quantitative analysis of tropane alkaloids, and researchers now have a wide choice of analytical techniques to solve almost any specific problem. The application of LC coupled with high resolution and tandem MS on the one hand, and NMR detectors on the other, is now expanding rapidly for the analysis of natural compounds, including tropane alkaloids. Such hyphenation reveals a new era of phytochemistry, where the isolation of the unknowns will not be a prerequisite (dereplication) for their complete structural elucidation. The innovative DART, DESI, and other mass spectrometry technologies allowing direct analysis of samples, without or with minimal sample preparation, are in their infancy, and it is expected that their applications are going to expand. Optical sensors and immunoassay methods are also in development due to their extreme sensitivity and applicability in forensic and clinical investigations. The future will certainly offer many exciting opportunities for scientists to continue to analyze, identify, and discover new tropane alkaloids while exploring the field of accessible natural products.

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Methods for Analysis of Pyrrolizidine Alkaloids

32

Colin Crews

Contents

1 Introduction 1050
2 Methods of Analysis 1051
2.1 Sample Preparation 1051
2.2 Extraction Procedures 1052
2.3 Clean Up 1055
2.4 Solid Phase Extraction (SPE) 1055
2.5 Chromatographic Methods 1056
2.6 Quantification 1061
2.7 Other Methods 1062
3 Conclusion 1064
References 1065

Abstract

Pyrrolizidine alkaloids poison animals grazing on toxic wild plants and those fed contaminated feed, causing economic losses. They poison humans through deliberate consumption of certain foods and herbal medicines and through consumption of food contaminated by wild plants, such as via transport of the toxins by bees into honey. Analytical methods are required for different purposes – to detect the presence of pyrrolizidine alkaloids, to quantify the total level of the toxins, or to measure the quantity of individual compounds. The task is made more challenging by the variety of PAs, their widespread nature and their different forms. Analytical methods are based on color reactions, enzyme linked immunosorbent assays (ELISAs), spectroscopy, and the full range of chromatographic techniques. A lack of reference standards and

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validated methods has hampered the application of available methods to food surveys, but has not prevented the development of a number of very useful methods as are described in this chapter.

Keywords

Comfrey • *Echium* • gas chromatography • honey feed • immunoassay • liquid chromatography • mass spectrometry • pyrrolizidine alkaloids • ragwort • *Senecio* • solid phase extraction

Abbreviations

ASE	Accelerated solvent extraction
EFSA	European Food Safety Authority
ELISA	Enzyme linked immunosorbent assay
GC	Gas chromatography
GC-MS	Gas chromatography with mass spectrometric detection
HPLC	High performance liquid chromatography
LC (High performance)	Liquid chromatography
LC-MS	High performance liquid chromatography with mass spectrometric detection
LC-MS/MS	Liquid chromatography with tandem mass spectrometric detection
LC-UV	Liquid chromatography with ultra-violet detection
LOD	Limit of detection
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
NMR	Nuclear magnetic resonance spectroscopy
PA	Pyrrolizidine alkaloid
PANO	Pyrrolizidine alkaloid *N*-oxide
SCX	Strong cation exchange
SCX-SPE	Strong cation exchange with solid phase extraction
SIM	Selected ion monitoring
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TLC	Thin layer chromatography
TOF	Time of flight mass spectrometry
UV	Ultra-violet

1 Introduction

Pyrrolizidine alkaloids (PAs) are toxins produced as secondary metabolites by a very large number of plants widespread across the plant kingdom. They are ingested by animals as a results of direct grazing in the absence of preferred grasses, or by inadvertent contamination of hay and silage, or of composite feeds. PAs affect humans mostly through consumption of herbal supplements and teas, and of honey derived from *Echium* or contaminated with pollen or nectar from *Echium vulgare*, *E. plantagineum* or ragwort (*Jacobaea vulgaris*). Lesser routes include milk and animal tissues. PAs are used by insects to afford protection from predators and limited chemical studies have been carried out into these relationships.

PAs cause veno-occlusive disease of the liver after significant consumption and are associated with other ill effects. Comprehensive knowledge of the occurrence of PAs in food and feed is required for the calculation of exposure affecting animals and humans, and to support legislation formulate advice to consumers. Current research into improving or replacing older analytical methods has been prompted by advice to consumers from Australian and New Zealand authorities related to contaminated honey, and by requests from the European Commission via the European Food Safety Authority (EFSA).

PAs have a large variety of related structures, and include both tertiary (free base) compounds and their corresponding *N*-oxides (PANOs), which behave differently in many analytical systems. The analytical requirements are for methods for detecting PAs in wild and cultivated plants, in plants and plant preparations used for dietary supplements and herbal medicines, hay, silage and compound animal feeds, foods including honey, milk, and teas.

There is a need for rapid field tests that are either qualitative or give a measure of the total PA content, and for instrumental techniques for providing profiles of the PAs content of samples and accurate quantification of low levels. The techniques applied include colorimetric tests immunoassays, gas chromatography, liquid chromatography, and mass spectrometry. Extraction methods mostly follow classical procedures but clean-up methods have evolved from repeated liquid-liquid partitioning to the currently favored solid phase extraction (SPE) techniques. The following review concentrates on a range of methods that are likely to continue to be used to meet all requirements, and discusses their relative merits and disadvantages. Analytical methods have been reviewed [1–3]

2 Methods of Analysis

2.1 Sample Preparation

PAs are very widespread in plants and vary with species and variety, and also geographically, therefore analyses that are targeted at particular alkaloids should be carried out with positive identification of the plant species where possible. This is particularly important where the variations in the PA composition are to be related to the species. Consideration should be given to the relevance of misidentification of plants, for example PA toxins have been measured in herbal products labeled as being derived from *Senecio aureus*, which is generally believed to contain few PAs. Pollen analysis of honey can be used as a guide to the expected PA composition and to verify results of analysis. As some parts of plants such as seeds or root fragments might contain very high levels of PAs it is important to ensure that the sample is sufficiently large and also that it is homogenous, for which the familiar grinding and mixing techniques are appropriate.

Plant samples can be blended in their fresh state, in which case whole portions can be homogenized in the extracting solvent. In other cases it might be more convenient to freeze the sample and blend it with dry ice or with a pestle and mortar with liquid nitrogen. Plants can be dried in air or under vacuum, or heated in an oven or freeze dried. Unfortunately there have been no comparative studies into any changes in the PA composition induced by these drying methods. Quite severe methods such as oven drying at elevated temperature do not appear to produce gross changes to tertiary PAs such as decomposition or oxidation to a prominent degree. Senecionine was stable in pollen during extended heating at 56 °C under which conditions about 50 % of its PANO was lost [4].

2.2 Extraction Procedures

Early classical extraction methods for PAs were applied without much consideration of the range of chemical structures of the alkaloids and in particular were not optimized to obtain the maximum yields of both the tertiary and oxide forms. In fact few publications even today describe the recovery achieved with the extraction procedure used. PAs have either a reduced tertiary nitrogen atom with a characteristic basic function or an oxidized form (PANO). Both forms have little solubility in non-polar solvents such as hexane and are best extracted with polar solvents such as methanol or with aqueous dilute acid. These solvents dissolve both the PAs and the PANOs with relative ease.

Several approaches have been used to extract PAs from plants. The plant material, fresh or dried, can be steeped in solvent, blended, or placed in Soxhlet type extractors. The solvent can be left at ambient temperature or heated to boiling. Methanolic extractions in particular have been enhanced by the addition of acid. Ultrasonic agitation can speed up the extraction process. Many solvent systems have been applied, ranging from mid-polarity solvents such as chloroform, diethyl ether or ethyl acetate, or to mixtures, to polar organic solvents (methanol) and acidified water. Methanol and dilute aqueous sulfuric or hydrochloric acids are both good solvents for the PA tertiary alkaloids and PANOs. A 30 min single step extraction with 2 % aqueous formic acid can be used for PAs in freeze-dried powdered plant material prior to filtration, dilution and analysis by high performance liquid chromatography with mass spectrometric detection (LC-MS). The use of formic acid is advantageous in LC-MS as it forms volatile products in combination with ammonium salts added to the mobile phase [5]. Earlier methods more often used dichloromethane or chloroform alone or mixed with methanol but the use of chlorinated solvents is today considered unacceptable when alternatives are available.

There have been a few comparisons of extraction solvent systems. These have found that refluxing dry plant material in a relatively high proportion (1 l per 10 g) of methanol and aqueous citric acid (1:1) for just 15 min gave the highest yield of free PAs, but the performance with PANOs was not measured. Stirring in water at neutral pH and room temperature was unsatisfactory, as was Soxhlet extraction with methanol [6].

The extraction conditions used can affect the yield of PAs, their relative proportions, and the ratio of the tertiary alkaloid to PANO. For all extraction methods higher proportions of PANOs are obtained by extracting at room temperature than by methods that involved heating. Prolonged Soxhlet extraction causes a marked loss of PAs and a decrease of the proportion of PANO. An unidentified factor present in the plant material is suspected of being responsible for this loss. The best yields are obtained by relatively short treatments with acidified water or methanol, or their mixtures, at elevated temperature. The choice between water and methanol can be influenced by the sample. Some starchy products can swell and form a gel in hot water but methanol extracts more chlorophyll from green plants and lipid from e.g., animal feed samples.

The quantity of PAs extracted will vary with the sample to solvent ratio. It would be expected that using a high proportion of solvent to sample would extract analytes from the sample more thoroughly, but this is not always the case, and may not be desirable. Limits of detection may be lower for dilute solutions measured by LC-MS as the suppression effect of the matrix is reduced. In practice, limited comparisons have shown that whereas extraction of PAs from comfrey with a high proportion of methanol (500 mL per 5 g comfrey) gave high yields of PAs whereas when a 2 g comfrey sample was extracted with 200 ml methanol the percentage yield was lower.

Extraction with methanol at 100 °C for a short 2 h gave good yields, and these were increased by the addition of 1 % tartaric acid [7]. However this acid is not really sufficiently volatile for use in LC-MS. Increasing the extraction times to in excess of 6 h (up to 18 h) gives no advantage.

PAs can be extracted with organic solvents from aqueous systems and solid samples made basic with ammonia. In this case dichloromethane or chloroform have often been used as the organic phase. PAs can conveniently be transferred from an aqueous extract into an organic solvent by means of solid phase extraction (SPE) using columns packed with inert diatomaceous earth such as Extrelut® [8]. The aqueous extract is supported on the column matrix and the PAs eluted by passing organic solvents through. The solid matrix prevents the formation of emulsions. This method is much less satisfactory for the more polar PANOs.

Yields obtained by all methods are best improved by carrying out successive extractions, in which following a first extraction the solvent is separated from the sample by centrifugation and then replaced by a second volume. The extraction is repeated and the extracts combined. The process may be repeated, however in practice repeated extractions are rarely applied.

Particular extraction methods are required for more challenging matrices such as honey and milk. There is great interest in the analysis of honey as bees transfer toxic PAs from plants into this human food. The contamination of milk and eggs through animals and hens is much less of a problem, and their analysis has not be the subject of much scrutiny.

Honey has been extracted by shaking or blending with organic solvents but today it is almost always analyzed by means of SPE after dissolution in water or methanol, which effects a simultaneous clean-up and is detailed in following paragraphs.

A larger proportion of the PA content of plants and contaminated foods is in the PANO form. The *N*-oxides of PAs are less soluble than the tertiary alkaloids in non-polar solvents and were not regularly determined in the early days of monitoring. Before the widespread availability of LC-MS methods measurement of the PANOs depended on their conversion to the tertiary alkaloid and measurement of the tertiary alkaloids in duplicate samples, one with and one without conversion to the tertiary alkaloid form, from which the PANO content could be determined by difference [9]. The PANOs are best extracted into relatively polar solvents such as methanol, or mixtures of alcohol and acidified water, they can be extracted into the same medium as the tertiary alkaloids with ease.

Reduction of the PANO to the tertiary alkaloid has classically been carried out using zinc powder with dilute aqueous acetic, hydrochloric or sulfuric acid, but it can also be achieved with sodium or potassium dithionite or metabisulfite. The dithionite can be used in solution or adsorbed on to a redox resin [10]. Zinc reduction is efficient and can be checked by using commercially available PANOs, but a comparison of reduction conditions [11] found that the quality of zinc and the reaction time affected performance. The often used overnight reduction with zinc gave lower yields than treatment for 0.5–4 h. Dithionite in water is suspected of failing to reduce senecionine NO or of destroying it, and the reduction reaction with dithionite in formic acid is slow. Zinc reduction in the presence of sulfuric acid gave much higher yields of tertiary PA from many PANO than sodium bisulfite in aqueous formic acid [5].

Today, the ease of the determination of both tertiary alkaloid and oxide forms in the same extract in a single LC-MS experiment has led to a decline in the application of the oxide reduction procedures except when used for the GC-based summation methods described later.

Two plants that contain PAs, echium (*Echium plantagineum*) and borage (*Borago officinalis)*, each provide a valuable oil that is nutritious on account of its fatty acid profile. There is some concern that PAs might be present in oils from these plants, an issue addressed by analysis using simple liquid–liquid extraction processes in which a solution of the oil in a non-polar solvent is shaken with dilute aqueous acid. The method had an LOD of 0.6 μg L^{-1} when applied to borage oil, including a PANO reduction step [12, 13]. The procedure has had little application, and has not revealed a health problem.

An alternative sample preparation is based on the QuEChERS (Quick Easy Cheap Effective Rugged Safe) technique [14]. In this the sample is extracted into acetonitrile and magnesium sulfate, sodium chloride and citrate buffer salts at pH 5–5.5 are added. the mixture is shaken and then centrifuged to separate the phases. When applied to honey [15] the samples, containing isoproturon-d6 as an internal standard, were diluted with water and homogenized before addition of the QuEChERS salts described above, and acetonitrile. After extraction, centrifugation and concentration the extract was analyzed using LC-MS with an on-line SPE cleanup step.

Some determinations have been made of the occurrence of PAs by insects, which assimilate the alkaloids from toxic plants to use in their own defense or to produce pheromones. PAs have been extracted from insects, using acidic methanol, followed by limited clean-up [16]. There is also interest in the PA content of pollen, for which it has been shown that careful washing with cyclohexane and grinding with sand is necessary to obtain pure PAs quantitatively [17].

For preparative isolation of PAs, useful in providing analytical standards, the most successful approach has been countercurrent chromatography [18, 19]. This procedure, which is based on the partition of target compounds between a pair of immiscible solutions, has been enormously improved in efficiency in recent years, however no modern applications to PAs have been published. Column chromatography and preparative Thin Layer Chromatography (TLC) are alternate approaches, and preparative high performance liquid chromatography (HPLC) should be useful for separation of the minor alkaloids.

2.3 Clean Up

Clean up procedures are considered essential for most plant and food matrices analyzed for PAs, although extraction and clean-up can be combined for some samples such as honey.

Both the tertiary PAs and their oxides are relatively polar, and solutions in acidified water or water:methanol mixtures can be cleaned of non-polar material (fats, waxes, terpenes etc.) by washing with hexane or petroleum ether. The PA bases can be cleaned thoroughly by making the resulting aqueous phase strongly basic and extraction of the alkaloids into a moderately polar organic solvent such as ethyl acetate, diethyl ether or chloroform. Further partitions achieved by shaking the organic solution with dilute aqueous acid generally remove all non-basic substances. However this procedure is lengthy and consumes large volumes of solvent, it is therefore best reserved for preparative isolations of PAs from bulk materials or to extract tertiary PAs following the reduction of PANOs. These techniques are inapplicable to unreduced PANOs, and for both tertiary PAs and PANOs clean-up can be achieved by simpler means on a smaller scale. SPE has rapidly become the preferred method for samples to be analyzed by LC-MS, but careful cleaning by other means has been used for certain samples. For the determination of PAs in cow's milk the fats were precipitated by cooling and freezing in methanol and centrifugation [20].

2.4 Solid Phase Extraction (SPE)

SPE can be used to extract PAs from aqueous solution and simultaneously clean-up the sample and concentrate the extract. In its simplest form SPE is used without chromatographic separation. An alkaline aqueous extract (or urine sample) is held on column of inert diatomaceous earth commercially available as Chem Elute® or Extrelut®. A water-immiscible organic solvent, preferably also alkaline, is passed through the column and dissolves the PAs in a single application without risk of emulsion formation. The method has now been largely superseded by cation-exchange methods.

The more usual SPE method involves an interaction of the PA with the stationary phase which comprises non-polar phases such as octylsilane or octadecylsilane (C8 or C18), or cation-exchange materials packed into small cartridges [21]. Cation-exchange materials are preferred as non-polar sorbents do not effectively separate PAs from co-extracted materials that could interfere with subsequent chromatographic separations. Polymer-based Strong Cation Exchange (SCX) SPE traps both tertiary alkaloids and PANOs, removing many potentially interfering compounds [22], the SCX phase probably retaining PANOs by binding to the active sites of the sorbent rather than by a cation-exchange process [7]. In practice the cartridges are wetted with methanol and conditioned with dilute (e.g., 0.05 mol L^{-1}) hydrochloric or sulfuric acid. The sample extract is applied in dilute acid, and the cartridge washed with methanol before elution of the PAs with methanol containing gaseous or aqueous ammonia [10]. PAs can also be eluted from SCX columns under acid conditions, such as with mixtures of methanol with aqueous hydrochloric acid [23]. The method has been applied to test the urine from cows that had been fed on ragwort [20].

SPE based on SCX resins have been much used for the determination of PAs in honey [24–27]. The technique requires some care to avoid blockage of the cartridges. The honey needs dilution with dilute acid and centrifugation, and the cartridges sometimes require warming. The sample on the column is washed with water and the PAs eluted with water–methanol mixtures made acidic [23] or containing ammonia [10]. The performance of SCX-SPE varies with cartridge type, size and manufacturer and preliminary recovery experiments are advised before sample analysis takes place.

2.5 Chromatographic Methods

2.5.1 Thin Layer Chromatography (TLC)

TLC with both silica and aluminum oxide adsorbents has been used to separate and isolate tertiary PAs, sometimes with ion-pairing using lithium chloride, potassium chloride, or sodium iodide [28]. Unsaturated PAs can be detected on TLC plates after spraying with Erlich's reagent [28–30]. Calibration is obtained by comparison of spot color intensity with external reference standard alkaloids. PAs can be isolated from TLC plates by excising the PA spot or band and dissolving the alkaloids from the stationary phase with polar organic solvents. Spots on TLC plates can be scanned with instruments (densitometers) that can compare their intensity with those of standards of known concentration. By this means quantities of about 1 μg can be detected reasonably quickly.

2.5.2 Gas Chromatography (GC)

GC is limited in being unable to determine PANOs without prior reduction to the tertiary alkaloid, requiring a duplicated analysis with considerable manipulations. However it does have the considerable advantages of much higher resolution, which separates and reveals minor and isomeric compounds, and of providing detailed mass spectra from which structures can be elucidated. The detection methods used most often are flame-ionization detection (FID) and mass spectrometry (MS) but nitrogen–phosphorus detection (NPD) has also been used [5, 9, 17]. The latter is particularly useful as in nitrogen mode where nitrogen containing molecules such PAs are detected selectively and can be quantified with reference to a standard nitrogen containing compound. A wide range of GC stationary phases have been used including non-polar and polar types [16, 31]. On non-polar stationary phases retention times are approximately in order of molecular weight. Saturated PAs elute after unsaturated ones and esters substituted in the 9-position elute after those substituted in the 7-position. GC profiles and mass spectral data allow many PAs to be identified, and allow distinctions to be made between plant varieties of different species and chemotypes of the same species [32].

2.5.3 Gas Chromatography-Mass Spectrometry (GC-MS)

MS is the GC detection method most commonly used to identify and quantify PAs following GC separation. The electron impact (EI) mass spectra of PAs contain ions unique to the necine base and characteristic and specific fragments [33]. Many PAs have the same molecular mass and weak molecular ions, and so the fragmentation pattern is useful in identification and the monitoring of specific fragment ions is required for quantification with good sensitivity. Unsaturated PAs with a retronecine base have characteristic fragment ions at m/z 93, 120, 136, 137 and 138. PAs with an otonecine base have characteristic fragment ions at m/z 110, 151, and 168 with saturated-base PAs having ions two mass units higher.

Chemical ionisation (CI) in positive or negative mode can be used to reduce fragmentation and allow detection of the molecular ion [34, 35]. Reagent gases used in CI have included methane, *iso*-butane and ammonia. An EI-GC-MS method in Selected Ion Monitoring (SIM) mode able to separate and quantify most of the 1,2-unsaturated retronecine type PAs has been reported [25].

Most PAs have several polar hydroxy groups which produced problems of poor peak shape and resolution in the early days of capillary GC, which were addressed by reactions to cap the hydroxy function (derivatisation). With modern bonded and end-capped stationary phases PAs can be chromatographed without derivatisation, but it can help with identification and with ensuring resolution. The hydroxyl groups can be derivatised with silylating reagents to form trimethylsilyl ethers [35] or with alkylboronates (methyl, butyl, or phenyl boronic acids) which form bonds across 1–2 and 1–3 diol groups [36, 37]. The remaining single hydroxyl groups can be silylated after the boronate reaction, but this is not always necessary.

2.5.4 Summation Methods

Retronecine has been detected as a metabolite of PAs in body fluids using GC following derivatisation with heptafluorobutyric anhydride [38], and its relatively easy measurement forms the basis of recent methods to measure the sum of the individual PAs in plants, feed and foods. The total PA content of a sample can be determined by hydrolysis of all of the PAs to the retronecine base and measurement of this by GC after derivatization, but more recently alternative methods have been used. In one approach [15], following sample extraction and clean-up using SCX-SPE the PANOs are reduced with zinc dust and the necine base is released from its esterifying acids by reaction with lithium aluminum hydride ($LiAlH_4$). After reaction with the $LiAlH_4$, which does not reduce the necine double bond, the reagent is decomposed with alkali and the base extracted into dichloromethane. The base is then derivatised with *N*-methy-*N*-(trimethylsilyl)trifluoroacetamide to form trimethyl silyl ethers which are analyzed by GC-MS. An alternative method, which avoids the difficult handing issues associated with $LiAlH_4$, is to carry out a transmethylation of the PAs isolated by SCX-SPE with tetramethylammonium hydroxide in dichloromethane, which converts the -OH functions into methyl ethers which can be analyzed by GC-MS. Recoveries using this technique varied with PA and for echium PAs the total levels determined were lower than for LC-MS/MS methods [39].

Summation methods have only so far been applied to 1,2-unsaturated PAs and not to otonecine type PAs such as senkirkine or supinicine PAs having an additional oxygen molecule attached to the necine ring and a methyl group attached to the nitrogen. For these PAs an additional reference standard having the otonecine or supinicine ring structure would be required. Heliotrine has been used as an internal standard for retronecine, the sample treatment converts this into di-TMS-heliotridine, a diastereomer of di-TMS-retronecine. The isomers are well separated in the final chromatographic step [15].

LC-UV measurement has been applied to the summation methodology by pre-column derivatization of retronecine to (7-methoxy-1-methoxymethyl-6,7-dihydro-5 H-pyrrolizine). Photodiode array detection (DAD) was used and the derivative had an absorption maximum at 223 nm. The LOQ for standard solutions was about 1 ng mL^{-1} [40].

GC-MS based summation methods have been applied to honey, pollen and foods that contained honey. They have LODs equivalent to 1–10 μg kg^{-1} retronecine, or about 2–20 μg kg^{-1} PA in the food [15]. They do not provide the useful information on the relative proportions of PAs and PANOs.

2.5.5 Liquid Chromatography (LC)

High Performance Liquid Chromatography (HPLC, abbreviated to LC when used with MS) has a great advantage in allowing the simultaneous detection of PAs and PANOs without prior reduction of the oxides. Thus the procedure is simpler than for GC methods with fewer manipulations. Reverse phase columns are generally used and there is a gradual movement from HPLC to columns with a smaller particle size (<2 μm) which is referred to as UHPLC (Ultra High Performance Liquid Chromatography). UHPLC has a higher resolving power and sensitivity than

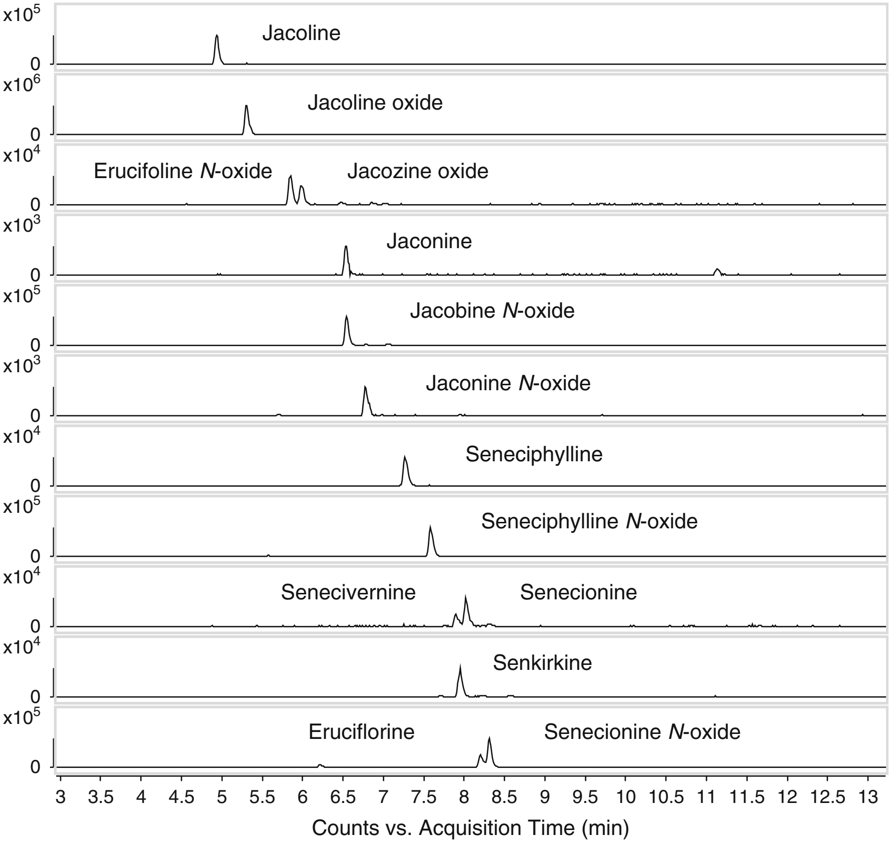

Fig. 32.1 Separation of ragwort PAs using UHPLC with LC- Time of Flight mass spectrometry

HPLC and retention times and correspondingly analysis duration are therefore much reduced [20, 26, 41].

The resolving power of LC-MS methods is not good enough to separate the many stereoisomers having the lycopsamine structure, including lycopsamine, echinatine, indicine, intermedine, and rinderine; or the stereoisomers of symphytine and symlandine. Figure 32.1 shows the elution of the major ragwort PAs using UHPLC with Time of Flight mass spectrometry.

Primary solvents of water, methanol and acetonitrile modified with acids (formic or acetic acid) or bases (ammonium hydroxide or diethylamine) have been most widely used. With acid solvents the PA tertiary alkaloids elute before their PANOs, whereas in basic conditions this order is reversed. Basic solvents at high pH (10–11) often give good peak shapes but at the expense of longer retention times. Peak shapes are however also good with acid solvents and retention times are shorter [42].

PAs have a non-specific UV absorption maximum of 214–220 nm and so simple UV detection has not been used very frequently. It has been applied to best effect in the purification of PAs, such as in the separation of the isomers intermedine and lycopsamine [43, 44].

Separation of tertiary PAs and of PANOs has been carried out on a C8 HPLC column with the aid of ion pairing with hexanesulfonic acid and detection by UV [7], but this approach has not been widely adopted, partly because the ion pair reagents are non-volatile and cannot be used with LC-MS. Some extracts have been analyzed with minimal clean-up simplified and less elaborate protocols [45]. Simple dilution and filtration of extracts and filtration can reduce matrix effects in LC-MS methods and the lack of coeluting compounds improves the background signal and the signal to noise ratio, therefore the sensitivity.

2.5.6 Liquid Chromatography- Mass Spectrometry LC-MS

LC-MS and LC-MS/MS methods have become the most popular approaches to the identification and quantification of PAs and PANOs as they combine reliability and high sensitivity with ease of sample preparation, and good data can be obtained for both tertiary alkaloids and PANOs as thermal decomposition is avoided. A range of mass spectrometer types have been used including ion traps, triple quadrupoles, and time of flight instruments. High sensitivity is provided by the ease with which the ring nitrogen is ionized in positive electrospray ionisation modes.

Ionisation methods investigated earlier, including thermospray, fast atom bombardment, and particle beam interfaces have been replaced by electrospray ionization (ESI) interfaces. Atmospheric pressure chemical ionisation (APCI) has also been applied but is less sensitive towards the PANOs. A combination of ESI with reversed-phase HPLC using acid mobile phases to protonate the PA molecules has most often been used [41].

Fragmentation is limited in single-stage LC–MS. This is a problem for the analysis of the many PAs that have the same empirical formula and are structurally similar, especially where authentic standards are not available to check the retention times and confirm peak identification. This drawback can be overcome for many PAs by the use of LC-MS/MS.

High resolution Time-Of-Flight (TOF) MS gives protonated molecular ions of most PAs with very high mass accuracy. This allows the determination of empirical sum formulae and easy searching for PAs using databases from empirical formulae with accurately calculated masses. Although less sensitive than LC-MS/MS methods, spectra are obtained for all ionizable compounds and the raw data can be searched retrospectively.

The limit of quantification (LOQ) for the individual PAs and their PANOs in dried plant material extracted with aqueous formic acid analyzed by LC-MS without SPE clean-up was between 0.2 and 0.5 mg kg^{-1} [5].

2.5.7 Liquid Chromatography- Tandem Mass Spectrometry LC-MS/MS

In LC-MS/MS methods collision-induced dissociation (CID) of the PA molecular ion provides fragment ions that can be used in single reaction monitoring (SRM), or multiple reaction monitoring (MRM). CID produces characteristic ions at m/z 94, 120 and 138 for 1,2-unsaturated retronecine base PAs and ions at m/z 122, 150 and 168 for 1,2-unsaturated otonecine-type PAs, which have an additional oxygen atom. Saturated ring platynecine-type PAs have characteristic fragment ions at m/z 122 and 140.

The retronecine PANOs have characteristic ions at m/z 118, 136, and 154. PANOs can form dimers in certain ESI interfaces which are not observed in the mass spectra of their precursor PAs [10].

MRM of the transitions from the protonated molecular ion to the fragments described above gives the highest sensitivity and specificity in PA analysis and has become a preferred method in recent years [15, 26, 27, 41]. The use of precursor ion scans can detect PAs in samples and give molecular weight information where the identity of the alkaloids is unknown. In this mode the (unknown) protonated molecular ion from which a known fragment ion is derived can be identified, indicating the molecular mass of the parent compound. LC-MS/MS measurements are conveniently carried out using ion-trap instruments, which have the capability for multiple dissociation experiments (MSn) and sensitivity in full scan mode. Certain TOF instruments also have a collision sector enabling high resolution detection of both parent and fragment ions. LC-MS/MS methods typically have LODs of 1–3 μg kg^{-1} for the common PAs.

Screening and quantification have been carried out simultaneously by LC-MS/MS with MRM of the characteristic fragment ions of 1,2-unsaturated PAs, which allows the detection and quantification of both known or targeted PAs and unknown ones in a single run [26]. The method had LOQs for the MRM approach of 0.8–36 pg ml^{-1} for standard solutions. UHPLC-MS/MS methods applied to *Senecio* plant material, including hay and silage typically can achieve a LOD of 3–10 μg kg^{-1} and a LOQ of 10–30 μg kg^{-1} [46].

2.6 Quantification

There are a very large number of PAs in the plant kingdom that can potentially contaminate feed and food. Although many are very minor components their determination is useful in plant classification. A considerable number of PAs that occur in sufficient quantity to be important in human and animal health and their determination has been recognized as a priority. These target PAs can be identified as senecionine, seneciphylline, jacobine, jaconine, jacoline and jacozine, erucifoline and acetylerucifoline (and their *N*-oxides) derived from ragwort and its relatives; lycopsamine/intermedine, acetyllycopsamine/acetylintermedine, symphytine, echimidine and their PANOs, derived from comfrey and *Echium.*

Certified pure reference standards are essential for accurate quantitation, particularly in LC-MS and LC-MS/MS. Standards of monocrotaline and retrorsine have long been commercially available at relatively low cost. The poor availability of most other PAs has handicapped identification of sample extracts and made quantification less accurate than desired. Additional PA standards are now becoming available in quantities of a few milligrams but are expensive. These include senecionine, seneciphylline and their oxides and lycopsamine. The PANOs are less easy to procure but can be synthesized from the tertiary alkaloid by oxidation with hydrogen peroxide or *m*-chloroperbenzoic acid [47] if loss of some material is tolerated. The isolation of bulk mixtures of PAs from plant material in quite high quantity is simple, as is the removal of non-PA material, but the separation of individual PAs on preparative scale is challenging. Quantities of a few mg of some PAs has been achieved with countercurrent chromatography.

In the absence of full sets of standards PA concentrations in food or plant samples can be estimated from calibration curves constructed from one or a few available PAs, or less frequently PANOs. Other PAs can be measured against these external standards and described in terms of equivalents of the standard alkaloid. This approach is compromised without correction for the different response factors. In GC-MS the quantification of PAs against a different external standard is possible using flame ionization detection, although response factors differ between open-chain and cyclic esters, and the use of nitrogen specific detectors is preferred.

Stable isotope labeled internal standards are used in most quantitative chemical investigations but no suitable standards are yet available for PAs, although radiolabeled and deuterium labeled PAs have been prepared for tracer studies by culture of PA-synthesizing plants in media containing labeled putrescine precursors [16]. In the absence of labeled internal standards, PAs that are believed not to occur in the plant or food sample have been used, including monocrotaline [41], heliotrine [5, 17], and (for *Echium* PAs) senecionine [24]. As a safety factor in this approach duplicate analyses with and without the presence of the internal standard, can confirm its absence in the native sample. A major problem associated with the lack of stable isotope labeled standards is that their use offers the best solution to the problem of matrix effects in LC-MS, in which the analyte signal is increased or more usually suppressed by coeluting compounds in the sample extract. Matrix effects can be overcome by quantification by the standard additions procedure where a calibration line is plotted by analyzing samples with different levels of added PA calibrants, or by the use of extracts of a non-PA containing plant as a medium for the calibration standards, for which purpose tansy (*Tanacetum vulgare*) has been used [20].

For the summation methods a single standard compound is required as a calibrant, and for internal standardization in the absence of a stable isotope labeled PAs, a base compound of a different structure is needed. To determine retronecine, heliotrine can be used.

2.7 Other Methods

2.7.1 Colorimetry

PAs give color reactions with numerous reagents typically applied to the detection of alkaloids. These methods have been developed to detect 1,2-unsaturated PAs and PANOs [30]. Tertiary PAs are oxidized to their PANOs, dehydrogenated to pyrroles with aqueous nitroprusside and coupled with Ehrlich’s reagent (4-dimethylaminobenzaldehyde) forming a red-violet colored dye that absorbs at 563 nm. Tertiary PAs plants are detected with *ortho*-chloranil followed by Ehrlich’s reagent. The reagent only reacts with unsaturated pyrrole rings and is therefore quite specific for PAs. The protonated PAs also give colored products on reaction with methyl orange, although this reaction is not specific to PAs.

2.7.2 Nuclear Magnetic Resonance (NMR)

NMR methods have been applied to PA analysis mainly for structural identification and identity confirmation of novel PAs [48–51]. A comprehensive review is available [48]. Proton NMR gives qualitative information more rapidly and from a smaller sample than C-13 NMR, whilst the latter gives more structural information. The necine base induces distinctive proton shifts showing the unsaturation and oxygenation of the PA molecule. Proton shifts due to the necic acid are less distinctive as the acids do not differ sufficiently in structure. Modern high-field NMR have much better performance characteristics, and linked with metabolomics can identify PAs in plant extracts [52].

2.7.3 UV–Visible Spectroscopic Methods

In spectroscopic methods Erlich’s reagent has been used with spectrophotometric detection to measure PAs quantitatively in wheat and flour. An alternative approach is to use aqueous methyl orange, which forms a yellow complex that is very soluble in organic solvents. Methyl orange can be released from the organic solution on treatment with strong acid, and measured with a spectrometer. These methods are not able to quantify individual PAs but allow determination of the total unsaturated PA content by construction of calibration series using the readily available standards such as monocrotaline and retrorsine.

2.7.4 Immunological Methods

The application of immunoassays to PA determination is relatively infrequent, the status last being reviewed in 2001 [53]. The technique is handicapped by the presence of some cross-reactivity precluding the easy detection of target PAs but more importantly a limitation to the degree of cross reactivity that prevents application to general screening. There is also cross-reactivity between the PANO and tertiary alkaloid forms. Sensitive quantitative ELISA techniques for detecting retrorsine and monocrotaline produced from immunogens based on quaternary pyrrolizidinium salts have shown variable cross reactivity to other PAs.

Antibodies raised to a retrorsine hemisuccinate-BSA conjugate have been used to determine total alkaloids including PANOs in reduced *Senecio* extracts [54]. Antibodies to a conjugate between the retronecine part of PAs and bovine serum albumin (BSA) detected retronecine and monocrotaline [55]. Antibodies that are specific to monocrotaline but which did not cross-react with retrorsine, retrorsine-*N*-oxide, riddelliine or retronecine have been reported [56]. Antibodies prepared to date react only to PAs that have related 12-membered macrocyclic structures closely related PAs but not to open chain or 11- or 13 membered macrocyclic PAs [57]. Polyclonal antibodies with a high specificity towards retrorsine, monocrotaline or retronecine were developed from immunogens based on ‘quaternary pyrrolizidinium salts linked to BSA or chicken egg ovalbumin [58].

Antibodies have been produced to both retronecine and monocrotaline and detected using an avidin-biotin antibody. Retronecine was obtained from the hydrolysis of monocrotaline, succinylated and coupled to bovine serum albumin or ovalbumin. Recently an ELISA lateral flow device (dipstick test) based on gold colloidal polyclonal antibodies has been developed for jacobine and lycopsamine in honey and animal feed [59].

2.7.5 Capillary Electrophoresis (CE)

CE has had only a few applications to PA determination, and reports of its performance have been mixed [6, 60]. Resolution if good when the concentrations of solvent and modifiers (buffer, sodium dodecyl sulfate, and methanol) have been optimized. The sensitivity has been improved by the use of preconcentration techniques.

3 Conclusion

Almost all methods for the determination of PAs and PANOs have complicated sample preparation stages. They require care and practice and a good understanding of the chemistry of the alkaloids and the extraction and clean-up procedures.

Qualitative screening methods can be based on colorimetry, TLC, or ELISA. They are suitable for analysis of fresh plant material containing relatively high levels of PAs, but they are not generally sufficiently sensitive or selective for quantification of PAs present at low levels. NMR methods are similarly not sensitive enough to determine the low levels of PAs in many food samples.

LC-MS and LC-MS/MS methods are much more suitable, combining a wide scope including PAs and PANOs of varying structures, with sensitivity, the ability to obtain confirmation of results, and reliable quantification at low level. GC-MS and GC-NPD methods remain useful in studies where high resolution of very similar PAs is required, and for the determination of necines in the summation methods that are becoming increasingly popular. GC–MS and LC–MS methods for the analysis of PAs can supplement each other where detailed knowledge is required [23]. LC-MS/MS methods can now measure about 70 PAs (tertiary bases and PANOs) encompassing monoesters, diesters and macrocyclic diesters, and the major PAs of interest in all of the more important plant families that produce them [46].

All methods are hindered by the poor availability of PA reference standards, the lack of interlaboratory validation of methods and proficiency tests, and the lack of certified reference materials.

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Methods of Analysis of Opium Alkaloids 33

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Contents

1 Introduction 1072
2 Methods of Analysis 1074
2.1 Analysis of Opium Alkaloids by Gas Chromatography 1074
2.2 Analysis of Opium Alkaloids by Liquid Chromatography 1085
2.3 Analysis of Opium Alkaloids by Capillary Electrophoresis 1102
3 Conclusions 1112
References 1113

Abstract

The control of drugs is an important task and will continue to be the subject of many publications. Many drugs have medical uses but a great number have recreational purposes with significant health risks which makes necessary both national and international control. While drugs remain controlled, it will be necessary, within the legal context, for the forensic scientist to carry out their analysis.

Among the numerous families of drugs, in this chapter, we have focused our attention on the most widely used methods of analysis found in the literature for the determination of six of the major alkaloids of the poppy plant (*Papaver somniferum*) named morphine, codeine, thebaine, papaverine, narcotine, and narceine. The choice of method will depend on the resources and equipment available to the analyst in the forensic laboratories, the legislative system in which the analyst is working, and the different forensic samples where

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the analysis of these substances needs to be carried out. Many techniques are already available for the quantification of opiates and their derivatives. Most of them employ separation techniques, such as gas chromatography (GC) and high performance liquid chromatography (HPLC), with a great variety of detection methods, especially mass spectrometry (MS). More recently, capillary electrophoresis (CE) mostly coupled with MS is being an attractive alternative for the analysis of numerous drugs of abuse in samples of forensic interest.

Keywords

Analytical techniques • environmental analysis • food and plant analysis • forensic and clinical analysis • opium alkaloids • pharmaceutical analysis • sample preparation

Abbreviations

6-AC	6-acetylcodeine
6-AM	6-acetylmorphine
6-MAM	6-monoacethylmorphine
ACE-CI	1-chloroethylchloroformate
AM	Amphetamine
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
ATPS	Aqueous two-phase system
Au-NPs	Gold nanoparticles
β-CD	β-cyclodextrin
BGE	Background electrolyte
BSTFA	*N*,*O*-bis(trimethylsilyl)trifluoroacetamide
BuAc	Butyl acetate
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CF	Caffeine
CI	Chemical ionization
CLD	Chemiluminescence detection
COD	Codeine
CP	Codeine phosphate
CSEI	Cation-selective exhaustive injection
DAD	Diode array detection
DBS	Dried blood spots
DDT	1,4-Dithiothreitol
DLLME	Dispersive liquid-liquid microextraction
DPX	Disposable pipette extraction
EC	Electrochemical detection
ECD	Electron capture
ECL	Electrochemiluminiscence

EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EI	Electron impact ionization
EME	Electromembrane extraction
ESI	Electrospray ionization
FASI	Field-amplified sample injection
Fe_3O_4/SiO_2/ poly(MAA-co-EDMA)	Magnetite/silica/poly(methacrylic acid-co-ethylene glicol dimetha-crylate)
FID	Flame ionization
FITC	Fluorescein isothiocyanate
FLC	Fast liquid chromatography
FLD	Fluorescence detection
FPD	Flame photometric
GC	Gas chromatography
HCOD	Hydrocodeine
HFBA	Heptafluorobutyric acid anhydride
HFIP	Hexafluoroisopropanol
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HS-SPME	Head-space solid-phase microextraction
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
MA	Methamphetamine
MAA-EGDMA	Poly(methacrylic acid-ethylene glycol dimetracrylate) monolith microextraction
MBTFA	Methyl-bis(trifluoroacetamide)
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxyethylamphetamine
MDGC	Multidimensional gas chromatography
MDMA	3,4-methylenedioxymethamphetamine
ME	Methylephedrine hydrochloride
MeCN	Acetonitrile
MEEKC	Microemulsion electrokinetic chromatography
MLC	Micellar liquid chromatography
MO	Morphine
MS	Mass spectrometry
MSPE-CZE	Magnetic solid-phase extraction coupled with capillary zone electrophoresis
MSTFA	*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide
MWCTs	Multi-wall carbon nanotubes
NICI	Negative ion chemical ionization
NM	Normorphine

NPD	Nitrogen-phosphorous
NP-HPLC	Normal-phase high performance liquid chromatography
NS	Noscapine
OF	Oral fluid
OR	Oripavine
PA	Polyacrylate
pCEC	Pressure-assisted capillary electrochromatography
PCI	Positive chemical ionization
PDMS	Poly(dimethylsiloxane)
PFPA	Pentafluoropropionic anhydride
PFPOH	Pentafluoropropanol
PH	Pholcodine
PV	Papaverine
QQQ	Triple quadrupole
RP-HPLC	Reverse-phase high performance liquid chromatography
SDME	Single-drop microextraction
SDS	Sodium dodecyl sulfate
SHT	Society of hair testing
SIM	Single reaction monitoring
SME	Solvent microextraction
SPE	Solid phase extraction
SPME	Solid-phase microextraction
TB	Thebaine
TLC	Thin-layer chromatography
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl derivative
TOF	Time of flight
UHPLC	Ultra high performance liquid chromatography
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
UV	Ultraviolet detection
WCOT	Wall-coated open-tubular columns

1 Introduction

Natural products and their derivatives have been a valuable source of therapeutic agents throughout history. The alkaloids of the poppy plant (*Papaver somniferum*) are important natural products [1, 2] from which more than 40 active chemicals could be extracted. They fall into general categories, each producing much different effects. The six opium alkaloids which occur naturally in the largest amounts are morphine, codeine, thebaine, papaverine, narcotine, and narceine.

In relation to their chemical structure and action, they can be classified into two categories. The first are phenanthrene alkaloids and are under international control: morphine (MO), codeine (COD), and thebaine (TB), which act on the central nervous system and are used as analgesics, narcotics, and potentially addicting compounds (pain relievers). Heroin is synthesized from MO. The second group is isoquinoline alkaloids: Papaverine (PV) and narcotine (also known as noscapine). Narcotine acts only to relax involuntary smooth muscles, for which it is considered an antitussive, and lacks addictive, analgesic, respiratory, narcotic, depressant, and sedative properties. Next to MO, which constitute about 10% by weight of raw opium, is the second most abundant alkaloid present in opium. The three last alkaloids (PV, narcotine, and narceine) are not under international control; specially, narcotine and narceine which have scarcely any medical or other uses. Consequently, the five economically significant alkaloids of opium are MO, COD, TB, PV, and narcotine.

Being contraband substances, the detection and identification of trace amounts of opium alkaloids in various samples including gum opium and many other biological samples of considerable health and forensic importance (urine, blood, hair) is a major analytical issue. Nowadays, many techniques are already available for the quantification of opiates and their derivatives [3]. As an example, Pothier and Galand have recently investigated the analysis of opium alkaloids still using thin-layer chromatography (TLC) [4] but numerous more sophisticated and sensitive separation techniques are the most used in this type of analysis. These include gas chromatography (GC) [5, 6], high performance liquid chromatography (HPLC) [7], and capillary electrophoresis (CE) [8]. Most of these methods can be applied for the analysis of the major opium alkaloids avoiding most of the important problems associated with the analysis of these compounds, such as the separation between them and matrix constituents.

GC is the method of choice for bioanalysis of opium alkaloids [9, 10], but for the analysis of plant extracts it may be less convenient due to the many less volatile substances co-extracted [11]. The major constrain of the GC methods is the commonly used prior derivatization of the analytes in order to volatilize them, avoid broadening of the peaks in order to achieve easier quantification. This makes the sample preparation complex and increases the costs for the GC method. The coupling GC-MS is often used because of its sensitivity, but the necessity of sample derivatization and the cost of the technique itself are restricting its applicability.

On the other hand, HPLC appears as a technique that could separate a wide range of analytes without any chemical pretreatment. As such, it has become the preferred technique in most applications, using a variety of detection methods [12, 13]. Reflecting the large polarity range of the major opium alkaloids, most applications apply reversed-phase liquid chromatography with gradient elution and ion-pairing agent. Such methods can be very sensitive to minor changes in chromatographic conditions, contributing to prolonged duration of analysis, and narrow the column lifetime [14].

In recent years, CE has emerged as a promising technique with great potential for separation and analysis of anionic, cationic, and neutral organic molecules but since the first applications reported for the separation of opium alkaloids in opium tincture [15, 16], there has been quite little progress with respect to the other separation techniques previously mentioned. Nevertheless, the methodology most widely used, capillary zone electrophoresis (CZE) with UV detection, together with different preconcentration procedures, present similar sensitivity compared to the earlier reported chromatographic methods, such as GC and HPLC and are comparable to that of radioimmunoassay [17] having the advantage over HPLC wherein peak asymmetry and poor resolution was observed, while derivatizations of alkaloids are necessary for GC analysis. For such a reason, the CE methodologies may find application for the routine analysis of the six major alkaloids present in gum opium samples and biological samples of forensic importance.

2 Methods of Analysis

2.1 Analysis of Opium Alkaloids by Gas Chromatography

GC uses a gaseous mobile phase to transport sample components through either packed columns or hollow capillary columns containing the stationary phase. In most cases, GC columns have smaller internal diameter and are longer than HPLC columns. In GC analytes, samples must be volatile although derivatization can be applied to increase volatility. The most usual carrier gases are inert such as helium, argon, hydrogen, and nitrogen, depending in part on the detector and having weak intermolecular interactions with solutes. The gas can be fed through a molecular sieve to remove trace of water.

Separation columns are placed inside the oven to control temperature and can be produced from stainless steel, glass, or mainly fused silica tubes, coated with a sheath of polyimide, which provides a protective layer and allows a maximum operating temperature of around 360°C. It is possible to distinguish between packed columns and open tubular or capillary columns. Packed GC columns are typically manufactured from glass or stainless steel. They are packed with pellicular silica particles (typically between 30/40 mesh and 100/120 mesh) onto which the stationary phase is coated. The smaller the particle size the higher the column efficiency. A typical packed GC column will be between 2 and 4 m in length with an internal diameter of between 2 and 4 mm and with efficiencies in the order of 1,000–2,000 plates per meter. Today, the most part of GC applications are developed by using capillary columns. Open tubular capillary columns consist of a long narrow tube of silica coated on the inside surface with a very thin film of immobilized polymeric liquid, gum, particulate, or zeolite stationary phase. These columns are known as wall-coated open-tubular columns (WCOT) and provide the highest resolution and efficiency (usually in the order of 3,000–5,000 plates per meter). This is mainly due to the length and homogeneously thin stationary phase films that can be achieved with capillary column technology. Fused silica is the most popular material of construction for WCOT (capillary) columns as it is flexible, inert, and produces very high efficiency columns. The inner surface of fused silica tubing is chemically treated to minimize interactions of the sample with the tubing. The reagents and process used depend on the type of stationary phase being coated onto the tubing. A silylation process is used for most columns. The selection of the correct stationary phase is one of the most critical parameters in GC separations. As the interaction of the analyte molecules with the mobile phase is almost negligible, the column temperature and the interaction of the analyte with the stationary phase will govern the selectivity of the separation. A wide variety of liquid stationary phases have been employed for different applications in GC in the field of forensic analysis, being polysiloxanes the most common ones, because they are stable, robust, and versatile. Standard polysiloxanes are characterized by the repeating siloxane backbone. Each silicon atom contains two functional groups, and the type and amount of the groups distinguish each stationary phase and its properties.

Temperature is one of the two most important variables in GC and affects the retention capacity (k), selectivity (α) and, to a lesser extent, the efficiency (N) of the separations. Two modes of operation can be considered in relation to oven column temperature changes during the analysis: isothermal or gradient temperature GC. In isothermal GC, the temperature of the GC oven and the column remains constant during the course of the analysis, being adequate for the analysis of compounds whose boiling points do not differ significantly. In this mode, when analytes differ significantly in relation to retention behavior, to achieve a reasonable analysis time, a fairly high temperature has to be used. This has the disadvantage that poorly retained analytes elute with very low capacity and the separation cannot be so adequate. In temperature programming or gradient temperature programmed mode, the oven starts at a low temperature to assist with the separation of early eluting peaks, is then ramped and usually held for a specified time at an upper temperature. The well-resolved, highly volatile solutes are removed from the column at the lower temperatures before the compounds of low volatility leave the origin at the column inlet. This mode allows the analytes with significant differences in boiling point to elute within a reasonable time frame and to be sure that all analytes have eluted from the column. An important advantage is that all peaks elute with approximately the same width.

GC detectors respond to a physicochemical property of the analyte, amplify this response, and generate an electronic signal for the data system to produce a chromatogram. Many different types exist and the choice is based mainly on nature of analytes and required sensitivity. There are different detectors commonly used in drugs analysis: flame ionization (FID), electron capture (ECD), flame photometric (FPD), nitrogen-phosphorous (NPD), and MS detectors; the two last options being the most widely used. GC techniques produce fast analyses because of the highly efficient nature of the separations and by using a combination of oven temperature and stationary phase chemistry very difficult separations can be solved, being excellent for quantitative analysis of volatile compounds, mainly in combination with MS detectors for quantification and identification purposes. In fact, GC–MS is the long-accepted standard technique in confirmatory analytical methodologies for drug of abuse. GC–MS using EI ionization mode is the most widely used technique in drug of abuse analysis in biological matrixes, mainly urine. EI mode leads to a number of fragment ions providing more structural information. In addition, it allows identification of unknown compounds by comparison of their mass spectrum with reference mass spectra in commercially available libraries. However, due to the extensive fragmentation in the EI mode, the chemical ionization (CI) mode could provide more selectivity as this technique often gives molecular mass information. In addition, the sensitivity can be improved through the use of negative ion chemical ionization (NICI) ionization analytical methods. Both modes can be integrated as a multiple ionization mode approach of GC–MS, and this possibility has been applied in the simultaneous hair testing of common drugs of abuse, including opiates [18].

In GC, derivatization is usually performed after preconcentration and cleanup of the extracts. In order to decrease the polarity and increase the volatility of the analytes, derivatization of drugs of abuse and their metabolites prior to GC–MS analysis is required. The main requirements for a successful derivatization reaction are as follows: a single derivative should be formed for each compound; the derivatization reaction should be simple and rapid, and should occur under mild conditions; the derivative should be formed with a high and reproducible yield and should be stable in the reaction medium; in quantitative analyses the calibration curve should be linear [19]. Application of derivatization methods in recent years has been relevant, especially for silylation, acylation, alkylation, and the formation of cyclic or diastereomeric derivatives. The most common silylation procedure is trimethylsilylation. Higher alkyl homologs or halogen-containing analogs have been used to increase hydrolytic stability of the derivative, to improve detectability with some particular detectors, to improve resolution, or to obtain mass spectra of higher diagnostic value [20]. Trimethylsilyl (TMS) derivatives combine thermal and chemical stability and high volatility. They are easy to prepare, and show excellent GC behavior. A variety of trimethylsilylating reagents with different properties (such as volatility, reactivity, selectivity, by-product formation, etc.) have been developed including tri-methylhalosilanes [20]. The TMS amides, *N*,*O*-bis (trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) are the most commonly employed silylating reagents in analytical determination of opiates [19]. The addition of a catalyst has been used to increase the silylating power of these reagents to derivatize sterically hindered functions or to enhance reaction rates, TMCS being one of the most usual. BSTFA with 1% TMCS as a catalyst has been widely used to analyze drugs of abuse and their metabolites. Acylation is another commonly used derivatization method in GC–MS. It consists of the introduction of an acyl group in a molecule holding a reactive hydrogen. Acylated derivatives can be obtained from a great variety of functional groups. Acylation reactions can be performed using three main types of reagents:

acyl halides, acid anhydrides, or reactive acyl derivatives such as acylated imidazoles [19]. Haloalkylacyl derivatives are the most popular acyl derivatives. These derivatives increase the electron affinity of the compounds and make possible highly sensitive analyses using NCI-MS. Perfluoroacyl derivatives, including heptafluorobutyric acid anhydride (HFBA), are the most widely used in practice.

MDGC and especially comprehensive two-dimensional gas chromatography (GC × GC) is a recent trend that has advantages over classic one-dimensional GC in many areas of analysis by offering improved peak capacity, often enhanced sensitivity and, especially in the case of GC × GC, the unique feature of "structured" chromatograms. A recent paper reviews recent advances in MDGC and GC × GC in drug analysis with special focus on ecstasy, heroin, and cocaine profiling [21].

2.1.1 Main Applications of GC to the Analysis of Opium Alkaloids

Table 33.1 shows selected and relative recent applications of GC in the monitoring of opiates in different matrices, including sample treatments, derivatization procedures, detection modes, and sensitivity.

Blood and urine as the most important samples in forensic analysis by using GC. The differentiation between legal and illegal opiate product use through the detection of marker compounds in urine is one of the most important subjects in forensic toxicology. As example, TB can be detected by GC-MS and has been suggested as a good urinary marker of poppy seeds use in differentiation from that of illicit heroin. Although TB lacks the conventional functional group, it was found to form a trimethylsilyl derivative making possible to simultaneously detect TB together with MO and COD in the same urine sample to confirm opium use, thus saving time and cost of analysis [25].

There are other possibilities for monitoring drug of abuse, and specifically opiates. For example, oral fluids, which has some advantages such as its collection is easy and noninvasive and there is a lower risk of infection than in drawing blood, being sample volumes smaller. Also samples can be collected under supervision making more difficult to adulteration. Detection times of drugs in oral fluids (5–48 h) are similar to that in blood (1–2 days) whereas the detection times in urine can be much longer, from a couple of days up to 3 months. Due to short detection times, this is a feasible matrix for confirmation analysis of driving under the influence of drugs cases. However, some common habits, such as smoking or using mouthwash, and also some drugs or diseases may reduce salivation, limiting the sample volume available for analysis. Recently, this sample has been used for analysis of 50 drugs of abuse including opioids by GC-MS (COD, MO, 6-MAM) [41].

Other different sample is vitreous humor, a biological fluid frequently used in postmortem toxicological analysis since all substances present in blood are also present. This matrix is relatively protected from postmortem degradation and contamination and its analysis can provide information in those cases where other biological samples have suffered extensive chemical changes during the postmortem interval. This sample has been analyzed by GC-MS for controlling

Table 33.1 Selected applications of GC for the analysis of opiates in different matrices

Analytes	Sample/Preparation	Derivatives	Method characteristics	Sensitivity	References
COD, MO, 6- MAM	Oral fluid Extraction at pH 6 with Toxitube A	BSTFA + 1% TMCS (100°C for 20 min)	GC-PCI-MS (capillary column 12 m × 200 mm i. d, 0.33 mm thick film of 5% phenylmethylsiloxane) SIM	LOD: 0.7 ng/mL (COD), 2.0 ng/mL (MO), 0.6 ng/mL (6-MAM)	[22]
Opiates (COD, MO, 6-MAM) cocaine, benzoylecgonine	Hair Enzymatic hydrolysis overnight at 37°C: DTT + Pronase E in Tris–HCl (pH 7.2). SPE at pH 9.2 (Oasis HLB)	BSTFA + 1% TMCS (100°C for 20 min)	GC-EI-MS (HP-5 capillary column 30 m × 250 mm i.d.,5% phenylmethylsiloxane, film thickness 0.25 mm) SIM	LOD : 0.02 μg/g (COD), 0.04 μg/g (MO), 0.05 μg/g (6-MAM)	[23]
Opiates (MO and COD) and cocaine and its metabolites	Pericardial fluid Enzymatic hydrolysis at pH 5.2. SPE at pH 9.0 (Bond-Elut)	MSTFA (60°C for 10 min)	GC-EI-MS HP-Ultra 1 capillary column (cross-linked methylsilicone, 0.2 mm × 25 m) SIM	Low ng/mL levels	[24]
TB, MO and COD	Urine Enzymatic hydrolysis and extraction with Toxi-A Tube	BSTFA + 1% TMCS	GC-EI-MS	LOD: several nanograms on-column	[25]
MO, dihydrocodeine, COD, 6-MAM and other drugs	Urine Enzymatic hydrolysis at pH 5 and extraction with diethyl ether: chloroform (4:1)	PFPA + PFPOH (70°C for 40 min)	GC-MS (HP-5MS capillary column 30 m × 0.25 mm i.d., 0.25 μm film thickness) SIM	LOD: 250 ng/mL (MO), 50 ng/mL (MO, dihydrocodeine, 6-MAM)	[26]
COD, MO, 6-MAM, cocaine, benzoylecgonine	Meconium Extraction with MeOH and SPE at pH 9.2 (Oasis HLB)	BSTFA + 1% TMCS (100°C for 20 min)	GC-EI-MS (HP-5 capillary column 30 m × 250 μm i.d. with film thickness 0.25 μm) SIM	LOD: 30 ng/g (COD), 20 ng/g (MO, 6-MAM)	[27]
Opiates (MO, 6-MAM, dihydrocodeine, COD) and other drugs	Hair 0.1 M HCl (50°C overnight) and SPE (Bakerbond Narc-2 mixed-mode)	MSTFA + 1% TCMS (80°C for 1 h) + MBTFA (80°C for 30 min)	GC-EI-MS (capillary column Zebron ZB-5, 30 m × 0.25 mm i.d. × 0.25 μm d.f.) SIM	LOQ : 10 ng for all the opiates	[28]

COD, MO, 6-MAM and other drugs	Hair Ultrasound assisted enzymatic hydrolysis: DTT + Pronase E in Tris–HCl (pH 7.3). Sonication (30°C for 30 min). SPE at pH 9.2 (Oasis HLB)	BSTFA + 1% TMCS (100°C for 20 min)	GC-EI-MS (HP-5 capillary column (30 m × 0.22-mm i.d., 0.33-μm film thickness of cross-linked 5% phenyl methyl silicone)	LOD: 0.02 μg/g (COD), 0.04 μg/g (MO), 0.05 μg/g (6-MAM)	[29]
Opiates (MO, COD, 6-MAM), amphetamines, ketamine and metabolites	Hair Incubation overnight at 25°C in methanol/TFA SPE (Bond Elut)	HFBA (70°C for 30 min)	GC-EI-MS and GC-NCI-MS (HP-5MS, capillary column 5% phenyl methyl siloxane, 30 m × 0.25 mm i.d., 0.25 μm film thickness)	LOD: 2 pg/mg (MO and COD), 10 pg/mg (6-MAM)	[18]
Multianalyte (150 components controlled in doping) including MO, 6-MAM, ethylmorphine, and diacetylmorphine	Urine Enzymatic hydrolysis at pH 7 and extraction with diethyl ether and tert-butyl methyl ether at pH 9.5 and 14, respectively	MSTFA/NH_4I/ ethanethiol (640/1/2; v/w/v) (80°C for 1 m.)	GC-MS (J&W-Ultra 1 column, 17 m, 0.2 mm i.d., and 0.11 μm film thickness), SIM/full scan (screening method)	Linear range (MO): 250–2000 ng/mL	[30]
Opiates (MO, COD, 6-MAM), amphetamines, ketamine	Hair Incubation with methanol/TFA (9:1) overnight at 45°C. Extract with phosphate buffer at pH 6.0 and SPE (Bond Elut) eluting with a dichloromethane/isopropanol/ ammonium hydroxide (80:20:2, v/v/v)	HFBA (70°C for 30 min) and BSTFA (90°C for 20 min)	GC-EI-MS (HP-5MS capillary column, 5% phenyl methyl siloxane, 30 m × 0.25 mm i.d., 0.25 μm film thickness), SIM	LOD: 0.05 ng/mg (MO, COD), 0.08 ng/mg (6-MAM)	[31]
COD, MO, 6-MAM, 6-AC and other drugs and metabolites	Sweat Extraction from patches with 0.5 M acetate buffer (pH 4.0). SPE elution with methylene chloride:2-propanol:ammonium hydroxide (78/20/2, v/v/v)	BSTFA + 1% TMCS (60°C for 20 min)	GC-MS (HP-5MS capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness), SIM	LOD : 1.25 ng/patch (COD, MO), 2.5 ng/patch (6-MAM), 5 ng/patch (6-AC)	[32]

(*continued*)

Table 33.1 (continued)

Analytes	Sample/Preparation	Derivatives	Method characteristics	Sensitivity	References
COD, MO and other drugs of abuse	Serum LLE	MSTFA	GC-MS (screening method)	LOQ: 0.025 μg/mL	[33]
COD and other anesthetic and analgesic drugs	Urine HS-SPME (fibers: 100 μm bonded PDMS, 85 μm PA)	non	GC-NPD	LOD: 1.5 ng/mL (COD)	[34]
COD, MO, 6-MAM, cocaine, benzoylecgonine	Meconium Extraction with MeOH and SPE at pH 6 (Bond Elut)	BSTFA + 1% TMCS (100°C for 20 min)	GC-EI-MS (HP-5 column 30 m × 250 μm, 0.25 μm film thickness), SIM	LOD: 5 ng/g (COD), 10 ng/g (MO and 6-MAM)	[35]
Multianalyte (128 compounds) including TB	Urine SPE	BSTFA + 1% TMCS	GC-EI-MS (screening method)	nr	[36]
COD, MO, and 6-MAM	Hair HS-SPME (fibers: 100 μm bonded PDMS, 85 μm PA)	BSTFA + 1% TMCS	GC-EI-MS (capillary column 30 m, 0.25 mm, i.d. 0.25 film thickness). Full scan	LOD: 0.002 ng/g (COD), 0.005 ng/g (MO, 6-MAM)	[37]
MO and other drugs	Adipose tissue HCl (1 h.). Extraction of the aqueous phase at pH 10–11 with SPE (Extrelut® NT3)	BSTFA (70°C for 20 min)	GC-MS (capillary column EVDX-5MS 5% PH ME Siloxane, 12.5 m × 0.20 mm i.d., 0.33-μm, film thickness). Full scan	LOD: 0.010 μg/g (MO)	[38]

MO, COD, and dihydrocodeine	Urine, serum Monolithic spin column	TMS	GC-MS	Linearity 10-2,500 ng/mL (urine) and 5-1,200 ng/mL (serum), LOD at ppb levels	[39]
MO, COD, and 6-MAM	Vitreous humor 25 mL HCl 0.1 M and 25 mL CH_3CN to precipitate collagen. Vortex and centrifugation. Disposable pipette extraction	MSTFA	GC-NPD	LOD: 0.50, 0.16, 1.25 μg/mL, respectively	[40]
50 drugs of abuse including opioids (codein, MO, 6-MAM)	Oral fluid LLE (3 mL BuAc) and SPE (Oasis MCX)	75% BuAc:MeCN 1:1 (v:v) + 25% MSTFA (80°C for 30 min)	GC-EI-MS	LOQ: 7 ng/mL (6-MAM), 59 ng/mL (codein), 12 ng/mL (MO)	[41]
Opiates (MO, COD, 6-MAM), tetrahydrocannabinol, cocaine, and analogs	Urine Enzymatic hydrolysis and LLE (3 mL mixture of chloroform/ isopropanol 9/1)	MSTFA + 1% TMCS (70°C for 20 min)	GC-MS (5% Ph Me silicone capillary column, 10 m length × 18 mm i.d)	LOD: 10 ng/mL (MO, COD, 6-MAM)	[42]
MO, 6-MAM, COD, 6-AC, tramadol	Hair Incubation with MeOH at 65°C and SPE (Oasis MCX)	MSTFA + 5% of TMCS (80°C for 25 min)	GC-EI-MS (capillary column 30 m × 0.25 mm i.d., 0.25 μm film thickness) with 5% phenylmethylsiloxane (HP-5 MS)	LOD: 0.05 ng/mg (all compounds)	[43]

heroin abuse [40], considering that heroin is quickly metabolized to 6-monoacethylmorphine (6-MAM), which is in turn de-acetylated to MO. The presence of 6-MAM is therefore a marker of heroin abuse and is often used to discriminate between heroin and MO exposure, since when MO is detected; one cannot know whether MO originates from heroin, COD, or MO itself. Since 6-MAM is rapidly converted to MO, it is regarded as an indicator of recent heroin intake. Because of its rapid conversion to MO, 6-MAM may not be present in detectable quantities in blood following heroin exposure. Nevertheless, it has been shown that 6-MAM persists in cerebrospinal fluid and vitreous humor and that these alternative specimens may be useful in proving heroin abuse.

In a similar way, pericardial fluid may be an alternative sample to blood for toxicological examinations in drug-related deaths. This body fluid is an ultrafiltrate of plasma with an extremely similar amount of proteins and is located within a tight compartment so that it is almost free of contamination by pathogens. The usual volume of 5–20 ml is enough for analytical purposes. In 2006, the first study reporting levels of drugs of abuse (opiates such as MO and COD, cocaine, and its related metabolites) in this body fluid was carried out by using GC-MS [24]. Also, several illicit drugs have the capability to be stored within the body for long time, being possible to use lipidic tissues. Postmortem adipose tissue can be used to detect absorbed drugs while living, provided that the drugs involved are highly lipophilic and bind to tissues. This is also an optimal sample in cases with extended postmortem time lag and whenever body fluids cannot be obtained, providing long-term information about an individual's drug use, which is especially useful when the history of drug abuse is not easily obtained. In 2010, the first extraction method for the detection of cocaine and metabolites, methadone, and MO was validated for this matrix, using GC-MS [38].

Over the past two decades, the use of hair in the field of toxicology has gained more and more relevance. This is due not only to the well-known advantages of this specimen over the more traditional blood or urine samples, for example, low invasiveness of the collection procedure, difficult adulteration, and wider detection window (months to years), but also to the evolution in chromatographic techniques. However, a difficulty in interpreting hair testing results is that the concentration of drugs in hair can be influenced by factors such as ethnic differences in hair pigmentation, more specifically, the natural melanin content in hair. Furthermore, it has been observed that drug concentration declines dramatically after cosmetic treatment (bleaching, dyeing, and permanent waving) as well as after UV exposure or exposure to water, soil, or natural weathering. One important issue regarding hair analysis for opiates is the real possibility of heroin or 6-acetylmorphine (6-AM) conversion to MO as an artifact, which is likely to occur under uncontrolled acidic or alkaline conditions. This spontaneous conversion would impair the reliable identification of heroin abuse, since the detection of 6-AM in hair is the definite proof of heroin consumption. This issue can also be dealt with if 6-acetylcodeine (6-AC), a specific marker of illicit heroin synthesis, is detected [44]. An application of this matrix was the qualitative and quantitative analysis of several opiates (MO, 6-MAM, COD, 6-AC) and tramadol [43]. The extracts were derivatized with

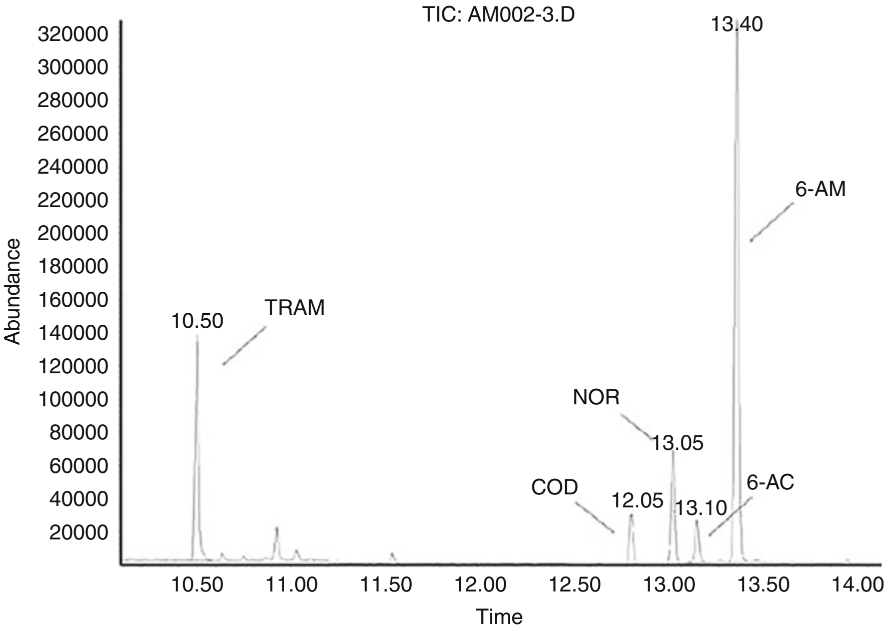


Fig. 33.1 Total ion chromatogram of an authentic hair sample (*TRAM*: 9.94 ng/mg; *COD*: 4.13 ng/mg; *MOR*: 20.45 ng/mg; *6-AC*: 15.38 ng/mg; *6-AM*: 102.45 ng/mg) (With permission, from Ref. [43])

MSTFA in presence of 5% TMCS and analyzed by GC-MS in the selected ion monitoring mode, accomplishing the cutoff values proposed by the society of hair testing (SHT) for the detection of these substances in hair (0.1 ng/mg). Figure 33.1 shows a total ion chromatogram of an authentic hair sample for the detection of these compounds.

Meconium is another important optimal matrix for identifying in uterus the consumption of toxic substances during pregnancy as it is considered to be static once deposited in the fetal intestine, thus being a preserved record of the ultimate exposure by the fetus [45]. In spite of increased sample preparation time relative to blood and urine, meconium's the long metabolite history, coupled with the ease and wide window of collection make meconium the ideal matrix for determining fetal drug use. A GC–MS method was proposed to analyze cocaine and opiates in meconium and validated according to internationally acceptance criteria, providing a wide window for the detection of fetal exposure to cocaine and opiates [35]. The analysis of meconium demonstrated the exposure to cocaine and COD during the last 20 weeks of gestation whereas urine was only able to prove that these drugs had been consumed in the last hours prior to birth.

Other alternative matrix in forensic analysis by GC is sweat. For this matrix, drug detection windows may be longer (up to 1–2 weeks) and sweat testing offers a cumulative picture of drug use over an extended time frame and noninvasive specimen collection. Another advantage includes a reduction in the potential for specimen adulteration; homeostatic mechanisms preclude the dilution (a method commonly used in urine testing to produce false negative results) of drug concentrations in sweat. As example, a method using GC-MS was validated for the simultaneous quantification of methadone, heroin, cocaine, and metabolites: 6-MAM, frequently encountered in sweat and also 6-AC and anhydroecgonine. 6-AC is an effective biomarker of illicit street heroin abuse for patients in pharmaceutical heroin treatment and although 6-AC is only present in small quantities in street heroin, its lipophilic nonpolar structure make it an interesting analyte to monitor in sweat [32].

2.1.2 Typical Sample Pretreatments Prior GC for the Analysis of Opium Alkaloids

For GC, the most part of reported methods have employed extraction protocols, typically liquid-liquid extraction (LLE) or solid phase extraction (SPE), which has become very popular in the pretreatment of forensic samples despite the fact that it requires relatively large volumes of solvents. SPE has several advantages over LLE: decreased solvent volumes, resulting in decreased solvent disposal costs; reduced operator time; high recovery of analytes; and low limits of detection and quantitation for the analytes. Most importantly, SPE yields clean extracts and minimizes the appearance of endogenous peaks, thereby increasing analyte sensitivity and specificity.

In recent years, solid-phase microextraction (SPME) offers an attractive alternative method for the sample preparation of biological samples. SPME integrates sampling, extraction, preconcentration and sample introduction in a simple single-step procedure. For the sampling and pretreatment of complex biological specimens, head-space (HS)-SPME provides a powerful tool especially for the extraction of volatile or semivolatile compounds [46]. Important parameters controlling SPME has to be studied: selection of SPME fiber, type and amount of salt added, preheating and extraction time, extraction temperature, sample volume, and desorption time. HS-SPME, in combination with GC-NPD, afforded a wide linear range, satisfactory detection sensitivity and repeatability for the analysis of COD and other anesthetics and analgesics in the form of the parent compounds rather than their metabolites, using directly urine samples [34]. The method was applied to the determination of the anesthetics and analgesics in human urine from patients that had undergone coronary bypass surgery operations. The proposed protocol can function as an attractive alternative for clinical acute intoxications and medicolegal cases.

Disposable pipette extraction (DPX) is a new approach in sample pretreatment, which has proved to be valuable in the analysis of drugs of abuse in biological matrices, such as urine and blood. DPX requires low-cost equipment and consumables and uses smaller volumes of samples and solvents, producing less waste. In contrast to SPE columns, DPX uses loosely packed sorbent and therefore does not require vacuum for elution. The sample is aspirated into the tip, where it is actively mixed with the sorbent to form a suspension. Furthermore, it minimizes the necessary conditioning steps and therefore speeds up the procedure of sample pretreatment being possible easy automation. This strategy has been performed for the monitoring of heroin abuse in postmortem samples such as vitreous humor by detecting MO, COD, and 6-MAM [40]. Recoveries obtained with DPX ranged from 61% to 74% for MO, from 78% to 85% for COD and from 80% to 99% for 6-MAM.

In relation to hair analysis, the most critical and time-consuming step in analysis for opiates is their quantitative extraction from within the matrix. This extraction can be performed in several ways, employing mild acidic hydrolysis [47] or methanol extraction [48], being the latter compatible with almost all drug substances [49]. The problem is that aqueous acid solutions can hydrolyze some analytes, for instance, partial hydrolysis of 6-MAM to MO. Recently, a method was described for the analysis of COD, MO, 6-MAM, 6-AC, and tramadol in hair samples by GC-MS, after a simple overnight incubation with methanol at 65°C, followed by sample cleanup using mixed mode SPE. Using these incubation conditions, the conversion of 6-MAM to MO was found to be minimal, with excellent repeatability and reproducibility. This is advantageous since there is no need to control strictly the sample pH to avoid MO production. In addition, this procedure uses a small amount (20 mg) of sample, which does not limit the usefulness of the technique since low limits of quantitation have been obtained [43]. Enzymatic hydrolysis is another common reported pretreatment for isolating illicit drugs from hair. These methods are more expensive than methanol- and acid-based treatments. An important drawback of enzymatic hydrolysis is the long time required for completing the hydrolysis process, commonly in the range of 6–24 h. This fact, together with the use of an efficient cleanup method, makes enzymatic hydrolysis of hair to be a tedious and time-consuming methodology. In this sense, ultrasound energy has been successfully applied for speeding up the Pronase E enzymatic hydrolysis of human hair for extracting illicit drugs, including opiates [29]. Variables affecting this ultrasound-assisted hydrolysis, such as hydrolysis temperature, hydrolysis time, enzyme concentration, catalyzer (1,4-dithiothreitol) concentration, ionic strength, pH, and ultrasound frequency, were simultaneously evaluated by experimental design. The most statistically significant variables were ionic strength and pH, which means that analyte extraction is mainly attributed to Pronase E activity. The optimization of all the factors allows completing the hydrolysis in 30 min.

2.2 Analysis of Opium Alkaloids by Liquid Chromatography

Liquid chromatography (LC) is an analytical method widely employed for the separation, identification, and determination of the chemical components of complex mixtures. The separation is based on the speed that a certain component passes through a stationary phase, swept along by a mobile liquid phase. In the "chromatography in column" the stationary phase is located inside a tube and the mobile phase is forced to pass through it by gravity or external pressure. Basically, HPLC is an improved form of the traditional chromatography in column. In HPLC, the mobile phase is pressure-driven through the column by an external pump at pressures up to 400 bar, which enables a faster separation. The use of high pressure pumps allows the use of column packing material of smaller particle size, which implies a huger surface area for interaction, with the subsequent improvement of the separation efficiency.

Two variants of HPLC can be distinguished according to the relative polarity of the solvent and the stationary phase: normal-phase high performance liquid chromatography (NP-HPLC) and reverse-phase high performance liquid chromatography (RP-HPLC), respectively. Polar stationary phases (typically, bare silica or silica derivatized with small organic ligands) and nonpolar solvents are employed in NP-HPLC. The elution power of the mobile phase in NP-HPLC increases with its polarity. In the case of RP-HPLC, the silica of the stationary phase is modified by long hydrocarbon chains (normally C8 or C18) attached in its surface, which makes it nonpolar. The elution in RP-HPLC is carried out by polar solvents or mixtures of them (e.g., water/methanol or water/acetonitrile) while in RP-HPLC, the eluting power of the mobile phase decreases with its polarity. On the other hand, the hydrophilic interaction liquid chromatography (HILIC) is sometimes the technique of choice for the analysis of hydrophilic and polar compounds. In HILIC, compounds are retained on a hydrophilic stationary phase (e.g., silica- and polymer-based), and a water miscible, normally MS compatible mobile phase is used to carry out the elution. Also, two elution modes can be employed in LC: isocratic or gradient elution. Under isocratic elution, the composition of the mobile phase is maintained during the whole run. In gradient elution, the elution power of the mobile phase is gradually increased along the run. Gradient elution implies the re-equilibration of the system to the initial mobile phase composition, which sometimes supposes longer analysis times. Nevertheless, gradient elution is a must to achieve the complete separation in a reasonable time of complex mixtures.

The last but not least part of an HPLC system is the detector. There are several ways to detect when a certain compound elutes from the column. Detection systems based on molecular absorption (UV, Visible, or DAD), fluorescence (FLD) or chemiluminescence, EC or MS are the most popular. It is worthy to highlight the high sensitivity and selectivity provided by detection systems based on fluorescence or chemiluminescence.

RP-HPLC (i.e., hydrophobic stationary phase/polar aqueous mobile phase) is the more usual mode to separate and analyze opium alkaloids. The most attractive advantage of RP HPLC is the ability to determine a wide variety of compounds, which have different molecular structures, polarity, and acidity/basicity. Analysis of opium alkaloids by NP-HPLC (i.e., polar stationary phase) is not common and no references have been found in the scientific literature. As it is reflected in Table 33.2, octadecyl columns (C18) are the most commonly employed. When monolithic columns are used, high flow rates are allowed, which gives rise to very short separation times [57] with no loss of resolution. MLC sometimes is an interesting alternative to RP-HPLC since it allows separating analytes with different hydrophobicity without gradient elution and with acceptable peak symmetry. MLC has also been successfully applied to the analysis of opium alkaloids [60, 63]. Although it has not been much exploited yet for opioids analysis, HILIC has been scarcely used for the determination of intact MO glucuronide conjugates in plasma [86, 87] and urine [74].

Ultra high performance liquid chromatography (UHPLC) or ultra performance liquid chromatography (UPLC) is a quite novel technique, but it is one of the most popular nowadays, due to the fact that it is much more rapid than conventional LC. UHPLC systems can work at very high pressures and are compatible with stationary phases with particle sizes of around two micrometers or even smaller. Thus, the development of UHPLC methods [69, 77, 80] or the conversion of HPLC to UHPLC methods is a current trend, due mainly to the speed of analysis and low solvent use.

For forensic applications, elution is normally carried out in gradient, especially when a high number of compounds are intended to be separated in a reasonable time. However, isocratic elution has also been successfully carried out for opioids analysis, as reflected in Table 33.2. Methanol and acetonitrile are the most common organic components of the employed mobile phases. Regarding the acidity, acid mobile phases are the most common for the analysis of opium alkaloids. Although more scarcely, neutral [72] and basic [77] mobile phases have been also employed.

Regarding the detection systems, MS has been the most employed in recent years. MS offers universality (it detects the most organic compounds), selectivity, and sensitivity. LC-MS has been widely used for the structural characterization of opium alkaloids, as well as for their quantitative analysis in a variety of matrices such as biological fluids, water, food, etc. Some authors refer to MS as the best detection system for LC. The role of LC-MS in the determination of opioids in biological fluids was exhaustively reviewed by Pichini et al. in 1999 [88]. Diode array detection (DAD) is the second detection system of election for the LC analysis of opium alkaloids, after MS. Ultraviolet wavelengths between 240 and 300 nm are the most commonly employed. Fluorescence detection (FLD) has been employed mainly to the analysis of MO and COD [60, 66]. Also, chemiluminescence (CLD) with tris (2,2′-bipyridil)ruthenium(III) and acidic potassium permanganate provide remarkable sensitivity and selectivity for the analysis of many opium alkaloids and it has been employed as detection system for the LC-analysis of MO, COD, oripavine (OR), ethylmorphine and TB [56, 57]. The use of CLD for the detection of opium poppy alkaloids was reviewed by Francis et al. in 2008 [89].

2.2.1 Main Applications of LC to the Analysis of Opium Alkaloids

LC has been widely employed for the analysis of opium alkaloid in several matrices. Applications within the clinical, forensic, environmental, food control, vegetal, and pharmaceutical analysis fields can be found in the scientific literature, as reflected in Table 33.2.

Table 33.2 Selected applications of LC for the analysis of opiates in different matrices

Analytes	Separation	Detection	Sample	Sample treatment	Notes	References
17 opium alkaloids	"high-speed" phenyl column Gradient elution with A: water:methanol 90:10 (v:v); B: methanol, both containing 25 mM triethylammoniumformate pH 4.5	DAD and FLD	Blood and urine	SPE with weak cation exchange (Bond Elut CBA)		[50]
PV, narcotine and narceine (among other alkaloids)	Zorbax Extend-C18 Mobile phase: Organic modifier (methanol, acetonitrile, tetrahydrofuran): ion-ion interaction reagents (sodium hexafluorophosphate, perchlorate, and trifluoroacetate): phosphate buffer at constant pH (2.7)	DAD				[51]
PV, narceine		UVD: 254 nm			Basic study	[52]
Pholcodine and its five structural analogs (treated like impurities): MO, COD, TB, oripavine, and PV	LiChrospher C-8 column Gradient elution with 2% ammonium hydroxide in water and acetonitrile	DAD	Pharmaceutical preparation	Direct dissolution in sample solvent	LOD 0.50 μg/mL LOQ 1.0 μg/mL	[53]

MO, COD, TB, PV, and noscapine	H5-ODS C18 column Isocratic elution with a mobile phase consisted of 55% buffer containing 10.0 mM sodium phosphate monobasic and 0.70 mM sodium dodecyl sulfate and 45% acetonitrile	UVD: 285 nm	Urine	Dispersive liquid-liquid microextraction (DLLME)	LODs in the range 0.2–10 μg/L	[54]
TB	ODS-3 column The mobile phase consisted of 10 mM phosphate buffer, pH 3.8, and methanol (70:30)	UVD: 285 nm	water, urine, poppy capsule, street heroine, and COD tablet	Electromembrane extraction (EME)	LOD < 15 μg/L Analysis time 10 min Recovery 44–54%	[55]
MO, pseudomorphine, COD, oripavine, ethylmorphine and TB	Hybrid FIA/HPLC system Monolithic column: Chromolith Flash RP-18e (25 mm length, 4.6 mm i.d.)	Chemiluminiscence detection (tris(2,2′-bipyridyl)ruthenium (III)/acidic $KMnO_4$	Urine	Dilution		[56]
MO, COD, oripavine, and TB	Chromolith SpeedROD RP-18e (50 × 4.6 mm i.d.) column	Chemiluminiscence detection (tris(2,2′-bipyridyl)ruthenium (III)/acidic $KMnO_4$	Process streams		Analysis time <2 min LODs < 10^{-9} M	[57]
MO and analogs	Winchrom-GP C18 (5 μm, 250 × 4.6 mm i.d.) column Mobile phase: methanol/ 0.05 M NaH2PO4, 17:83 v:v	UVD: 240 nm MS (API)	Compound liquorice	Aqueous two-phase system (ATPS) of poly(ethylene glycol)/K_2HPO_4	Recoveries: 91.7–100.3%	[58]

(*continued*)

Table 33.2 (continued)

Analytes	Separation	Detection	Sample	Sample treatment	Notes	References
MO, COD, oripavine, TB, PV, noscapine, and sanguinarine	Column: TSKgel ODS-120 Gradient elution with acetonitrile (solvent A) and 10 mM sodium 1- heptanesulfonate in water (adjusted to pH 3.5 with phosphoric acid) (solvent B)	UVD: 284 nm	Papaver plants	Extraction by 5% acetic acid by sonication followed by SPE with reversed-phase cation-exchange cartridges.	Recovery percentages between 100 and 112%	[59]
COD, MO and TB	Micellar LC: C18 column mobile phase 0.15 M SDS-7% (v/v) butanol buffered at pH 7	UVD (FLD for COD and MO)	Serum	Direct injection		[60]
COD and PV			Pericarpium papaveris	Ionic liquid-based aqueous two-phase system. (Ionic liquid: 1-butyl-3-methylimidazolium chloride)	Recoveries 90.0–100.2% for COD and 99.3–102.0% for PV	[61]
MO, COD, 6-acetylmorphine, and other drugs	Column: X-Terra RP8 Gradient elution with acetonitrile-phosphate buffer pH 6.53	DAD (200-400 nm) (285 nm for detection of MO, COD, and 6-acetylmorphine)	Plasma	SPE: Bond Elut Certify cartridges	LODs ranged between 0.010 and 0.055 μg mL^{-1}	[62]

MO, COD, PV, and noscapine	Micellar LC	UVD (280 nm)	“Omnopon, solution for injection”		LOD 0.45–1.94 μg mL^{-1}	[63]
	Column: reversed-phase Kromasil C18 (150 × 4.6 mm, 5 μm)					
	Isocratic elution with 0.10 mol L-1 SDS with 5% (v/v) of 1-butanol, adjusted to pH 2.5 by phosphoric acid					
PV and its photooxidation products (papaverinol, papaveraldine, and pyrrocolonium ion)	Column Hypersil Duet C18/ SCX (250 × 4.6 mm, 5 mm particle size)	DAD				[64]
	Isocratic elution with phosphate buffer (pH = 3.80)/acetonitrile (40:60, v:v)	(UVD 230 nm, 278 nm and 300 nm)				
PV	Column: μ-Bondapak C18 column (10 μm, 300 × 3.9 mm i.d.)	UVD at 250 nm	Pericarpium papaveris	Two-phase system (ATPS) of poly (ethylene glycol) (PEG)-$(NH_4)_2SO_4$	Extraction efficiencies for PV: 93–96%	[65]
	Isocratic elution with 0.5% ammonium acetate:1% triethylamine:methanol (v/v, 49:1:50)				LOD 2 ng/mL	

(*continued*)

Table 33.2 (continued)

Analytes	Separation	Detection	Sample	Sample treatment	Notes	References
MO	Column: μBondapak C18 (300 × 4 mm i.d., 10 μm) Isocratic elution with 5% methanol, 3% acetonitrile, 0.5 mmol/l sodium acetate, 0.012 mol/l potassium dihydrogen orthophosphate and 0.148 mmol/l phosphoric acid in distilled water	FLD (excitation wavelength of 235 nm and a 349 nm emission)	Plasma	Deproteination of plasma with zinc sulfate and bicarbonate buffer (pH = 10.5) and extraction with tetrahydrofuran prior to SPE with Zeolite Y column	LOD 10 ng mL^{-1}	[66]
MO and its 3- and 6-glucuronide metabolites, COD, 6-monoacetylmorphine	Column: C18 Atlantis Gradient elution with 20 mM ammonium formate buffer (pH 2.8) (solvent A) and acetonitrile/solvent A (90:10 v/v)	MS/MS (ESI+)	Dried blood spots	SPE (Oasis HLB cartridges)		[67]
MO, COD, 6-acetylmorphine, and other drugs of abuse	nano-HPLC-Chip-MS/MS Analytical column: ZORBAX 80 SB-C18 with 5 μm particle size Gradient elution by water with 0.1% formic acid (Eluent A) and acetonitrile with 0.1% formic acid (Eluent B)	QQQ/MS (ESI+)	Hair	Sonication. Extraction solution: methanol: acetonitrile:20 mM ammonium formate in a ratio of 25:25:50 (v/v/v)	LODs and LOQs from 0.1 to 0.75 and 0.2 to 1.25 pg/mg, respectively Separation time 15 min	[68]

MO, COD, oxycodone, buprenorphine, and other drugs of abuse	Column: Acquity HSS T3 Gradient elution with methanol (solvent A) and 10 mmol/L aqueous ammonium formate, pH 3.1 (solvent B)	MS/MS (ESI+)	Whole blood	LLE: ChemElute columns with Ethyl acetate/heptane (4:1)	LOQ ranging between 0.1 and 521 ng/mL	[69]
MO, COD, their metabolites 6-acetylmorphine dihydrocodeine, and drugs of abuse	Column: Fused-CoreTM Ascentis Express C18 (4.6 × 50 mm) with a 2.7 μm particle size Gradient elution with Milli-Q water with acetic acid (pH 2.8) (solvent A) and acetonitrile (solvent B)	MS/MS (ESI+)	Waste and surface waters	SPE: Oasis MCX sorbent	LODs for MO, COD, and their metabolites 0.5 ng/L (surface water) and 2-5 ng/L (wastewater)	[70]
MO, COD, norcodeine, oxycocodone, oxymorphone, normorphine, dihydrocodeine, buprenorphine, norbuprenorphine, among other drugs of abuse.	Column: ACQUITY UPLC BEH C18, 1.7 μm Gradient elution with 79.7% water, 20% methanol, 0.3% acetic acid (solvent A, pH 2.9) and 99.7% methanol, 0.3% acetic acid (solvent B, pH 3.30)	MS/MS (ESI+)	Wastewater and surface water	SPE: Oasis MCX sorbent	LODs for opioids ranged from 0.025 to 1 μg/L SPE recoveries >60%	[71]
MO, COD, 6-acetylmorphine, among other licit and illicit drugs	Column: Zorbax extend C18 (50× 2.1 mm i.d., 3.5 μm particle size) Gradient elution with 2 mM ammonium acetate at pH 6.6 (solvent A) and methanol (solvent B)	MS (ESI+)	Breast milk	SPE: Bond Elut Certify cartridges	LOD 1.5 ng/mL for MO and 1.0 ng/mL for 6-acetylmorphine and COD Recovery ranged between 51.6% and 86.5%	[72]

(*continued*)

Table 33.2 (continued)

Analytes	Separation	Detection	Sample	Sample treatment	Notes	References
MO, COD, 6-acetylmorphine, 6-acetylcodeine	Column: Synergi Polar-RP 80A (75 × 2 mm, 4 μm) Gradient elution with 0.1% formic acid and acetonitrile	MS/MS (ESI+)	Sweat (patches)	SPE: Extraction of sweat patches acetate buffer at pH 4.5; isolation of the supernatant and SPE on Strata-XC-cartridges	LODs 0.75 ng/patch Separation time: 15 min	[73]
MO, COD, morphine-3-glucuronide, morphine-6-glucuronide, and codeine-6-glucuronide	HILIC Column: Zorbax Hilic Plus column 100× 2.1 mm (3.5 μm) Gradient elution with 10 mM ammonium formate, pH 6.4 (solvent A) and 10 mM ammonium formate, pH 6.4 in 90% acetonitrile (solvent B)	TOF/MS (ESI+)	Urine	SPE on Sep-Pak C18 (50 mg) cartridges		[74]
MO, COD, and other 15 drugs of abuse	Varian Pursuit 3 C18, 3-μm particle size column Gradient elution with 2 mM ammonium formate buffer/ 8% acetonitrile pH 5.3 (solvent A) and 100% methanol (solvent B)	MS/MS	Whole blood	SPE: mixed-mode columns (Isolute Confirm HCX)	LOQs ranged from 0.0005 to 0.01 mg/kg	[75]

MO, morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), COD, dihydrocodeine, noscapine, PV, and 6-acetylmorphine	Column: Phenomenex Synergi Polar Gradient elution with 1 mM ammonium formate and 0.1% formic acid – pH 2.7 (solvent A) and 70% acetonitrile, 1 mM ammonium formate, and 0.1% formic acid – pH 3.8 (solvent B)	Hybrid quadrupole linear ion-trap mass spectrometer	Plasma, whole blood and postmortem blood	SPE: Varian Bond Elut C18, 6 mL, 200 mg SPE column	Deuterated labeled internal standards Recovery in blood was greater than 90% and ion suppression/ enhancement was shown to be less than 15% LODs ranged between 0.34 and 3.33 μg L^{-1}	[76]
MO, COD, 6-monoacetylmorphine (6-MAM), pholcodine, oxycodone, ethylmorphine	UPLC Column: Acquity UPLC BEH C18 column (2.1mmID × 50 mm, 1.7 μm particles) Gradient elution with a basic mobile phase consisting of 5 mM ammonium bicarbonate buffer, pH 10.2 (solvent A), and methanol (solvent B)	MS/MS (ESI+)	Urine	SPE: mixed mode cation exchange (MCX) cartridge	Deuterium labeled internal standards Total run time, including injection and equilibration time was 5.7 min LODs ranged between 0.0010 and 0.0026 μg mL^{-1}	[77]

(*continued*)

Table 33.2 (continued)

Analytes	Separation	Detection	Sample	Sample treatment	Notes	References
MO, COD, 6-monoacetylmorphine, and amphetamines	Column: LiChroCART (125 × 3 mm i.d., particle size 5 μm) filled with Purospher RP 18 Gradient elution with 0.1% formic acid in water (solvent A) and acetonitrile (solvent B)	MS/MS (APCI)	human hair segments	Hair samples were pulverized and extracted with methanol	Good linearity was obtained for all compounds in the range of 0.2–20 ng mg^{-1} LODs ranged between 0.05 and 0.2 ng mg^{-1}	[78]
Morphine-3-glucuronide, codeine-6-glucuronide, ethylmorphine-6- glucuronide, and 6-acetylmorphine	Column: Phenomenex Luna C18 column (3-m particle size, 100-mm length, 2.0-mm i.d.) Gradient elution with 1% acetonitrile (solvent A) and 90% acetonitrile (solvent B), both with 25 mmol/L of formic acid	MS (ESI+)	Urine	SPE: Oasis HLB cartridges	LODs ranged between 5 and 30 ng mL^{-1}	[79]
MO, O-6-monoacetylmorphine, COD and other drugs of abuse	UHPLC Column: Kinetex C18 column (2.6 μm, 100 × 2.1 mm) Gradient elution with 5 mM ammonium formiate containing 0.05% formic acid (solvent A) and methanol/acetonitrile 1:1 with 0.1% of formic acid (solvent B)	MS/MS (ESI+)	Oral fluid	Direct injection	LODs 0.2–5 ng/mL	[80]

MO	Column: Synergi Polar (150× 3 mm, 4 μm particle size)	QQQ-MS	Urine	Acid hydrolysis and SPE with Bond Elut Certify SPE cartridge	Derivatization of MO contained in urine with dansyl chloride	[81]
	Gradient elution with 1 mM ammonium acetate and 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B)	LTQOrbitrap Spectrometer (+)			LOD 35 fmol/mL (10 pg/mL) and LOQ 87.5 fmol/mL (25 pg/mL)	
MO, COD, norcodeine, dihydrocodeine, 6-monoacetylmorphine, acetyl COD, methadone, and other drugs of abuse		MS/MS	Urine	Enzymatic hydrolysis and SPE	Run time: 6 min.	[82]
MO, COD, 6-acetylmorphine, cocaine, and benzoylecgonine	XBridge Ph column	QQQ-MS/MS (ESI+)	Hair	Pulverization and extraction with methanol. Direct injection of the extract into the LC system	Run time: 10 min.	[83]
	Mobile phase: methanol and 10 mM ammonium acetate adjusted to pH 4.00 with 99% formic acid (95:5, v/v). Isocratic elution				LOD 10 pg/mg for MO, COD, and 6- acetylmorphine	

(*continued*)

Table 33.2 (continued)

Analytes	Separation	Detection	Sample	Sample treatment	Notes	References
COD, MO, Hydrocodone, Hydromorphone, Oxycodone, and 6- Acetylmorphine	Column: Waters Nova-Pak CN HP analytical column (3,9 × 100 mm, 4 µm particle size). Isocratic elution with 15% acetonitrile and 85% 2 mM ammonium formate buffer at pH 3.0	MS/MS	Urine, serum, plasma, whole blood, and meconium	SPE. Direct elution into autosampler vials	LOD 1 ng mL^{-1}	[84]
MO and COD	Column: 150 × 2 mm i.d., 3 µm, RP18 Gemini Gradient elution with 20 mM ammonium hydrogen carbonate, adjusted with ammonia to pH 9 (solvent A) and water/methanol 5:95 (v/v), 20 mM ammonium hydrogen carbonate adjusted with ammonia to pH 9 (solvent B)	MS/MS (ESI+)	Poppy seed	Cold extraction with methanol/0.1% acetic acid		[85]

Many applications found in the literature are focused in biological fluids, mainly urine, plasma, and whole blood, as reflected in the selected works summarized in Table 33.2. The determination of the father alkaloids as well as their glucuronide metabolite(s) is of great importance in interpretive forensic and clinical toxicology. The estimation of metabolite/father compound ratios provides important information about route, dose, and time of exposure. Direct detection of glucuronide derivatives without previous enzymatic cleavage procedures and/or derivatization is the actual challenge of LC-methods. Opioids have also been LC-analyzed in dried blood spots (DBS) [67], which presents some advantages, such as it requires a less invasive sampling method, it offers a simple storage and easier transfer, and requires a smaller blood volume.

Monitoring residues of drugs in human breast milk is also a need. When drugs are administered to a lactating mother, a certain percentage of the drugs may be excreted into the breast milk, which can harmfully result in a transfer of the residues to the infant. Marchei et al. proposed in 2011 [72] a LC-MS/MS method to detect residues of opium alkaloids and other legal and illegal drugs in this type of sample, applying the validated methodology to screen samples from the largest Spanish milk bank.

Alternative matrices in forensic toxicology, such those also being analyzed by GC (oral fluid, sweat, and hair), are receiving increasing interest due to ease and noninvasiveness of collection and/or longer windows for drug detection, and LC has demonstrated to be a suitable analytical technique to monitor small amounts of opium alkaloids in them. The level of drugs in OF correlates well with plasma time courses of drug appearances/disappearances for which it can be employed as an alternative specimen to monitor the consumption of opium alkaloids having potential applications for roadside drug screening. UHPLC-MS/MS has been successfully employed to monitor the presence of opium alkaloids and other drugs of abuse in OF [80].

Sweat is collected with patches that are generally worn for 1 week, permitting drug monitoring over a relatively long period. Sweat patches are mainly used in treatment following and criminal justice monitoring programs, but on-site fast drug detection is also possible [90]. Concheiro et al. have recently proposed a sensitive and selective LC/MS-MS method to simultaneously quantify 14 drugs (among them, opiates) and metabolites in a single sweat patch [73].

Hair has been also an object of analysis to detect the consumption of opium alkaloids [68, 78, 83]. This matrix has attracted a lot of attention for the easy and noninvasive sample collection and difficult adulteration compared to the plasma, urine, and saliva specimens. In addition, hair samples are more stable in storage and can provide a wider detection time window, which can reveal the history of drug abuse.

Regarding the pharmaceutical analysis, it is important to point out that quality, safety, and effectiveness of drugs are essential concerns in pharmaceutical industry. Effective monitoring and controlling of impurities are a must to ensure the quality and safety of drugs intended for human consumption. Unequivocal analytical characterization of drug impurities is a challenge in modern pharmaceutical examinations. Pholcodine is an example of drug which must be submitted to this kind of control. Pholcodine is a semisynthetic drug and it might present residues which might come from impurities present in the MO starting material as well as from the manufacturing process itself. Thus, MO and possible MO-analogs impurities of pholcodine including COD, TB, and PV must be monitored, although the number of published LC-methods for pholcodine impurities determination is limited yet [53]. Another example of the applicability of LC into the pharmaceutical analysis is the preparation called "omnopon." "Omnopon" belongs to the group of narcotic analgesics and it is commercialized as solution for injection. It represents a mixture of opiate alkaloids: MO, COD, PV, and noscapine. In clinical medicine "Omnopon" is used as an analgesic in strong pain syndromes. Kulikov et al. [63] have recently proposed a rapid MLC method with isocratic elution, for the determination of MO, COD, PV, and noscapine.

Pericarpium papaveris, contains several bioactive alkaloids, such as PV, MO, and COD. It is a type of traditional Chinese herbal remedy commonly used to treat chronic cough, chronic lax, and cramp. The main benefits obtained from *P. papaveris* are due to the contained PV, which has the functions of relaxing smooth muscle and alleviating human suffering. However, the content of PV in *P. papaveris* varies with different growing conditions. Therefore, it is very important to determine PV accurately in *P. papaveris* for its quality control and LC has been employed for this purpose [61, 65].

Opium alkaloids have been also LC-determined in opium samples. In 2008, Remberg et al. [91] published a study which they claimed like the one dealing with the largest set of authentic opium samples analyzed in a work until 2008. They analyzed the content of MO, COD, TB, and papaverine and it allowed them assessing possible correlations between samples and selected external factors, such as region of origin within Afghanistan, year of harvest, or intra-batch variation. Compounds issued from the natural photooxidation of PV have been also investigated by LC. A solution of PV naturally photooxidized contains papaveraldine, papaverinol, pyrrocolonium ion, and PV. Due to the acid-based characteristics of these compounds, Badea et al. [64] developed a LC method a Duet C18/SCX column that ensured the separation of ionic and nonionic compounds in a single run, which enabled the determination of PV in the presence of its main oxidation products.

On the other hand, environmental analysis is an important field of application of opium alkaloids analysis. The presence of drugs of abuse in environmental samples is becoming a matter of global concern. The source of these residues in water is mainly from consumers, since unchanged drugs and their metabolites are excreted through urine. Drug residues reach natural surface waters primarily due to the insufficient removal of these compounds at wastewater treatment plants. The concentration of these residues in water is normally low and sensitive analytical methodologies including preconcentration stages and sensitive detection systems are needed. LC-MS/MS is normally the method of choice for the analysis of opium alkaloids in the aqueous environment due to the high signal-to-noise ratio and the selectivity offered. Pedrouzo et al. [70] and Baker et al. [71] have recently proposed LC-MS/MS methods to monitor the presence of opium alkaloids and other drugs of abuse both in waste- and surface-waters. Anyway, other detection systems have also demonstrated to be suitable to monitor such small concentrations of drugs, for example, Costin et al. proposed, in 2008, a rapid and sensitive LC method with chemiluminescence detection with two different reagents: acidic potassium permanganate and tris(2,2′-bipyridyl)ruthenium(III), for the detection of *Papaver somniferum* alkaloids in process streams [57].

Due to the high potential of LC coupled to MS or tandem MS, it is common practice to use the development of multiresidue methods to monitor the presence of opiates together with other families of illicit drugs or pharmaceuticals in general. Doping control [92, 93], forensic [94], and environmental [95, 96] applications can be found in the scientific literature. The presence of plant toxins in general is also very controlled in food and feed of vegetal origin and, for example, Mol et al. [97] published in 2011 a method of screening of a selection of 150 substances, among them opium alkaloids, in a variety of food and feed matrices using full-scan high resolution (Orbitrap) MS, obtaining excellent results.

2.2.2 Typical Sample Pretreatments Prior LC for the Analysis of Opium Alkaloids

In some of the methods summarized in Table 33.2, samples or sample extracts are directly injected into the chromatographic system without being previously treated [53, 60, 80, 83]. However, this is not usual, especially when dealing with complex samples. Normally, some sample treatment stages are carried out before LC, both for cleaning up and preconcentration of the target compounds.

SPE has been the most widely employed sample pretreatment prior to LC for the analysis of opium alkaloids, as it is reflected in Table 33.2. Reversed phase-cation exchange is the more common sorbent for SPE of opium alkaloids [59, 70, 71, 75, 77]. Øiestad et al. [69] employed supported LLE on ChemElute columns as sample treatment for the analysis of opiates in whole blood, prior to LC-MS/MS. However, LLE has not been that employed as SPE prior to LC for opiates analysis.

LLE and SPE require large volumes of toxic organic solvents, and can be highly time consuming. SME methodologies are the current trend in sample treatment being considered green pretreatment techniques, since they involve the reduction of organic solvent volumes, with the subsequent production of less toxic residues. Dispersive liquid-liquid microextraction (DLLME) can be cited within these green techniques. It is a novel SME technique, initially developed by Rezaee et al. [98], based on ternary solvents. Basically, this extraction procedure is based on the formation of tiny droplets of the extractant in the sample solution using water-inmiscible organic solvent (extractant) dissolved in a water-miscible organic dispersive solvent. Extraction of the analytes from aqueous sample into the dispersed organic droplets takes place, obtaining a high enrichment factor, high extraction recovery, simplicity of operation, and low cost. The use of DLLME prior to LC is not very exploited for the analysis of opium alkaloids yet. In 2011, Shamsipur and Fattahi [54] proposed a DLLME-HPLC-UV for the determination of MO, COD, TB, PV, and noscapine (NS) in urine samples. They compared their figures of merit with those of previously published SPE-FLC-DAD, SPE-CZE-DAD, SPE-HPLC-DAD methods, and concluded that RSDs was about the same with previous methods, but their LODs and extraction times were lower. Thus, they proved that DLLME prior to HPLC is a sensitive, fast, reproducible, and simple technique that can successfully be used for the preconcentration and determination of opium alkaloids in urine samples.

Aqueous two-phase system (ATPS) is another environmental-friendly sample pretreatment technique. It is formed by mixing two aqueous solutions of structurally different polymers or by mixing a polymer and a salt aqueous solution together when the concentrations of the components are higher than a critical value. Compared with conventional LLE or SPE, ATPS can be completed in one operation and it is less costly. ATPSs of poly(ethylene glycol)/K_2HPO_4 and poly(ethylene glycol)–$(NH_4)_2SO_4$ have been coupled with LC for the determination of MO in compound liquorice and PV in *P. papaveris*, respectively [58, 65]. An ionic liquid-based ATPS prior LC has been also employed for the determination of COD and PV in *P. papaveris* [61].

Electromembrane extraction (EME) has also been used as sample treatment prior to LC for the analysis of TB. In EME, an electric voltage is applied which facilitates the extraction of analytes across a hollow fiber membrane. The application of voltage helps to extract the analytes in a short time. Seidi et al. [55] proposed an HPLC-UV method, prior to EME for the determination of TB in water samples, biological fluids, the poppy capsule, and narcotic drugs. They compared the proposed method with different existing methods for extracting and determining TB and concluded that through selecting an appropriate organic solvent, EME can yield a high sensitivity as well as high cleanup after extraction in complex matrices. The employed device to perform EME is shown in Fig. 33.2 (with permission of Ref. [55]).

2.3 Analysis of Opium Alkaloids by Capillary Electrophoresis

In CE, electrophoretic or electrokinetic separations are carried out in tiny capillaries at high voltages (10–30 kV), thus achieving high efficiency ($N > 105$), resolution, power, and mass sensitivity (down to 10^{-18}–10^{-20} mol). The main characteristics of CE are versatility of application, use of different separation modes with different selectivity, low demands on sample volume, negligible running costs, the possibility of interfacing with different detection systems including MS, and the ruggedness and simplicity of the instrumentation.

For all of these characteristics, CE applications in the forensic sciences are now rapidly growing in the last years, particularly in forensic chemistry and toxicology [99, 100]. A recent paper briefly described the basic principles of CE and presents a selected review of its main applications to the analysis of illicit/controlled drugs in biological samples [101–103]. In these papers, it can be concluded that the peculiar separation mechanisms and the high complementarily of CE to chromatography make it a new powerful tool of investigation in the hands of forensic toxicologists.

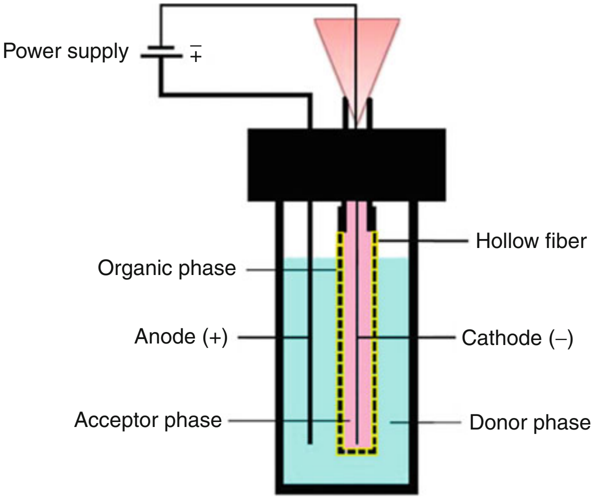


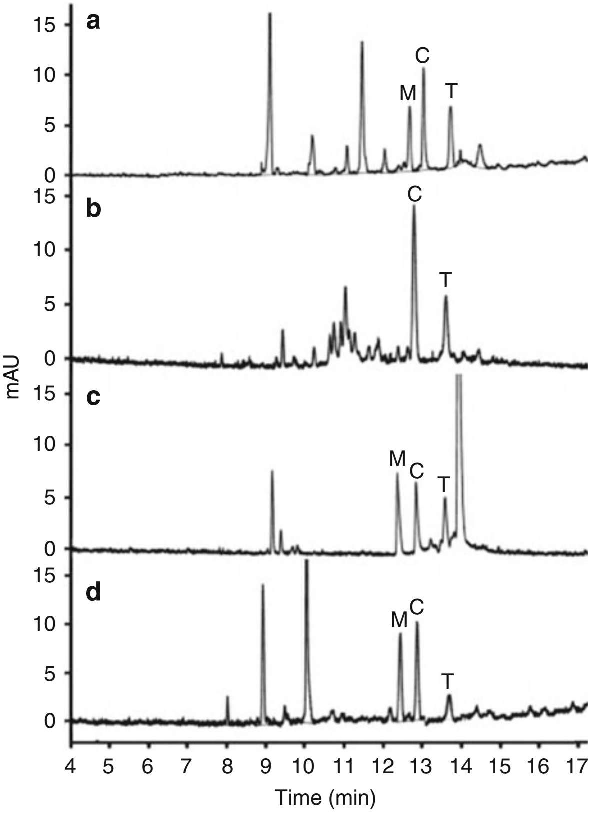
Fig. 33.2 Schematic illustration of the employed device for electromembrane extraction (EME) (With permission, from Ref. [55])

The exceptional power of separation and resolution, rapid analysis time, simplicity, versatility, low mass detection limits, economy of reagents, minimum sample requirements, and the possibility of direct sample injection without complex sample pretreatments make CE an attractive methodology to forensic scientists [104–106] where the sample matrix may be extremely complicated and contain a number of endogenous components which have to be resolved from the solute of interest. On the other hand, lack of sensitivity and “maturity” means that CE is not yet very well established in forensic laboratories as an additional tool to other analytical techniques.

Detection in CE has to face two challenging problems, namely, small amounts of analytes injected and tiny peak volumes. To the present, UV–vis detector is, by far, the most widely adopted despite the short internal diameter of the capillary limits the concentration sensitivity. Among the different modes of CE, CZE coupled with UV detection is the most widely found for the analysis of opium alkaloids in different matrices [107–111]. Nevertheless, to avoid the major disadvantage of CE related to its lack of sensitivity compared to HPLC, various approaches to this sensitivity limitation have been developed, mostly by the use of preconcentration procedures such as sweeping techniques [112, 113] or field-amplified sample injection (FASI) [107, 108].

Cyclodextrin (CD)-modified CZE with the same detection system has proven successful to increase both sensitivity and specially selectivity in this type of analysis [114, 115]. The most frequently background electrolyte (BGE) employed both in CZE-UV and CZE-CD-UV is sodium phosphate at acidic pH in the presence of a small percentage of organic solvents, such as methanol or acetonitrile (MeCN), to increase the selectivity of the CE separation. A good separation of morphine, codeine, and thebaine in different opium poppy plants can be seen in Fig. 33.3 [114].

Fig. 33.3 The typical electropherograms of the real samples: (**a**) *Papaver glaucum*, (**b**) *P. fugax*, (**c**) *P. dubium*, (**d**) *P. tenuifolium* morphine (*M*), codeine (*C*), and thabeine (*T*). Optimum conditions: 60 cm (50 cm to detector window) 50 μm id fused silica capillary operating at 25°C and 20 kV with UV detection at 214 nm and 100 mM phosphate buffer (pH 3) containing 5 mM α-CD (With permission, from Ref. [114])



The other mode of application widely used in CE for the analysis of the major opium alkaloids is micellar electrokinetic chromatography (MEKC). This mode can separate a wide variety of solutes (basic, acid, and neutral solutes) including compounds that are high polar, thermally labile, and/or nonvolatile, with high efficiency and selectivity, being an excellent technique for general drug screening and the analysis of substances, such as drugs of abuse and, among them, the opium alkaloids. Also, the UV detection system is frequently employed with the MEKC mode for the determination of different opiates in forensic samples [116, 117] but usually with the application of the preconcentration procedures such as cation-selective exhaustive injection (CSEI) together with sweeping [118–120]. These stacking CE methods could increase 2,500-fold sensitivity of the opium alkaloids being analyzed, when comparing with CZE. Most of them employ LLE or SPE as extraction procedures, which permit to obtain limits of detection in the ng/mL order when using a phosphate buffer at basic pHs as BGE in presence of a surfactant such as SDS and a percentage of an organic solvent, specially methanol or MeCN, between 7% and 30%.

Although UV is the most widely used detection technique found for the analysis of the major opium alkaloids in different matrices, another optical detection techniques have been found. Luminescence is also a very attractive detection system, due to its low sensitivity but it has been scarcely employed in the analysis of the major opium alkaloids of samples of forensic interest. FLD have been employed for the analysis of a binary mixture of MO and 6-AM based on the derivatization with diazomium salt of aniline at 0°C [121]. It was necessary to optimize all the conditions that affect derivatization (diazonium concentration and reaction time) and those related with the separation (electrolyte concentration, pH, β-CD concentration, organic additives, and separation potential) but good linearity was obtained in the range of 50–2000 ng/mL.

Due to the fact that laser induced fluorescence (LIF) allows high energy excitation, it is more sensitive but instrumentally more complex and expensive and has the limitation of the excitation wavelengths available with the different commercial lasers. LIF detection was also examined using a two-step pre-column derivatization procedure [122]. In this case, the drugs extracted from human urine were first subjected to an N-demethylation reaction involving the use of chloroethylchloroformate (ACE-CI) and then derivatized using fluorescein isothiocyanate (FITC) isomer I with excitation and emission wavelengths of 488 and 520 nm, respectively. The use of FLD/LIF in forensic analysis is mainly restricted to urine samples.

Also the ECL detection is an interesting alternative in the different detection systems based on optical methods. Two papers have been found that employ this detection system for the determination of opiate alkaloids [123, 124]. 1-butyl-3-methylimidazolium tetraborate and 1-buthyl-3-methylimidazolim tetrafluoroborate and 1-ethyl-3-methylimidazoliumtetraborate were used as ionic liquids in the BGE which resulted in significant changes in separation selectivity and obvious enhancement in ECL intensities for those alkaloids with similar structures. Under the optimal conditions, the four drugs being analyzed were well separated within 8 and 7 min, respectively.

Because of the importance of information provided by MS techniques, many efforts have been carried out for the development of interfaces between CE and MS, the electrospray ionization (ESI) being the most common interface, presenting enough sensitivity and selectivity for the determination of a wide range of analytes of clinical and forensic significance. For such a reason, one of the most potential techniques that can be use today in forensic laboratories is the hybrid analytical technique CE-MS [125]. The result is the possibility to separate and identify a wide variety of drugs of abuse, carrying out both as a screening and as a confirmatory method. The coupling of CE-MS, both with ESI or time of flight (TOF), combines efficiency and speed of separation with high mass accuracy and fast scanning capability for the analysis of drugs of abuse in forensic laboratories. CE with MS detection with direct electroinjection of different opioids from untreated urines has provided a rapid and simple approach for unambiguous urinary testing of drug of abuse [126]. A head-column FASI in presence of a water plug inserted at the capillary tip provides a more than 1,000-fold sensitivity enhancement when applied to real samples.

The coupling of CE-MS using ESI as interface has been employed for the simultaneous determination of stimulants and narcotics in urine samples [127] and different illicit drugs, including MO and cocaine in hair samples [128]. The buffers usually employed have to be considerably volatile, such as ammonium formate or acetate, working at basic pHs (9.0–9.5), with a sheath liquid composed by a mixture of isopropanol-water in presence of a small percentage of formic or acetic acid. Also, the analysis of hair samples have been carried out by CE-MS but, in this occasion using two different ionization procedures, ESI and TOF [129]. FASI stacking injection was employed to obtain detection limits below the cutoff values proposed by SHT for detecting these analytes in hair analysis (0.1 ng/mg) and a simple LLE procedure, allowing the detection of a broad spectrum of basic drugs and their major metabolites. The coupling of CE-TOF-MS was again employed for the analysis of illicit and controlled drugs in blood samples, using LLE extraction procedure and injected under FASI, applying the method to real samples [130].

The first application of charged AuNPs as semipermanent capillary coating in CE-MS is due to Zhang et al. [131]. The Au NPs enhanced the coating stability toward flushing by methanol which results in an improvement of the run-to-run and capillary-to-capillary repeatabilites, obtaining RDSs of migration time in the range of 0.43–0.62%.

The MS detector has been also coupled with another less employed mode of operation in CE, such as CEC in different occasions for the analysis of multiple drugs of abuse in human urine samples [132]. The coupling of packed CEC columns with MS as detector, using a liquid junction interface, provided several advantages such a better sensitivity, low dead volume, and independent control of the conditions used for CEC separation and ESI analysis. Also, pCEC with ESI-MS based on silica-based monolithic column have been developed for the five major opium alkaloids [133] and for the analysis of narcotics [134] combining the extremely high permeability and separation efficiency of silica-based monolithic columns with the high selectivity and sensitivity of pCEC-ESI-MS.

2.3.1 Main Applications of CE to the Analysis of Opium Alkaloids

Table 33.3 summarizes a representative selection of applications of CE to the analysis of opium alkaloids.

As it has been seen previously, forensic analysis of the six major opium alkaloids is usually carried out in biological samples, mainly in human urine, blood, and hair.

Urine is the most commonly used biological sample for the analysis of opiates in forensic laboratories by CE. It is generally used for the detection of these compounds in schools and workplaces because it is obtainable noninvasively, as compared to blood sampling. The main limitation in its use is that it provides direct evidence only for short-term use because they remain in urine no longer than for 10 days after use. Most of the applications in this type of samples by CE are carried out with the coupling CE-UV [107–112] obtaining limits of detection between 20 and 50 ng/mL.

Table 33.3 Selected applications of CE for the analysis of opiates in different matrices

Analytes	Mode of CE	Preconcentracion and extraction procedures	Experimental and instrumental parameters	Limits of detection	Matrix of application	References
MO, COD, PV, NS, TB, OR, reticuline, narceine	CZE-sweep-UV	Sweeping technique			Opium samples and poppy straws	[113]
Heroin, 6-MAM, MO, COD, PV, narcotine	CZE-UV	MAA-EGDMA Electrokinetic injection with FASI	0.1 M disodium hydrogen phosphate (pH 4.5 adjusted with 1 M hydrochloric acid) and 20% methanol (v/v). 25 kV at 25°C	6.6–19.5 ng/mL	Human urine	[107]
MO, COD, TB	CE-CD-UV	Ultrasound-assisted extraction procedure for 1 h, 60 kHz with water–methanol (80:20) at 40°C	Sodium phosphate buffer (100 mM, pH 3.0 with 5 mM β-CD	0.1 μg/mL	Iranian papaver plants	[114]
ME, TB, CP, AC	CE-ECL with ionic liquid (1-butyl-3-methylimidazolium tetrafluoroborate)			2×10^{-8} to 1×10^{-7} M	Human urine	[123]
Eight heroin related alkaloids	CE-MS with charged polymer-protected Au NPs		120 mM ammonium acetate (pH 5.2) with 13% methanol. 20 kV at 20°C	0.43–0.62% RSDs of migration time	Illicit samples	[131]
MO, COD, OR, TB	CZE-CD-UV		100 mM Tris (pH 2.8) with 30 mM hydroxypropyl-β-CD	2.5×10^{-6} M	Industrial process liquors	[115]
	MEEKC and MEKC-UV					[116]
TB, COD, NO, narcotine	CE-ECL	One-step extraction approach. FASI	Buffer (25 mM borax, 8 mM 1-ethyl-3-methylimidazolium tetrabflouoroborate	1×10^{-9} to 1×10^{-6} M	Chinese traditional medicine opium poppy samples	[124]

(*continued*)

Table 33.3 (continued)

Analytes	Mode of CE	Preconcentracion and extraction procedures	Experimental and instrumental parameters	Limits of detection	Matrix of application	References
			(EMImBF4) ionic liquid (pH 9.18). 15 kV			
8 illegal drugs (ketamine, amphetamines, opiates, and metabolites)	MSPE-CZE-UV	Hydrodynamic injection with FASI	30 mM phosphate buffer (PBS, pH 2.0) with 15% MeCN (v/v)	0.015–0.105 μg/mL	Urine samples	[108]
Amphetamine, ephedrine, methadone, pethidine, tetracaine COD, heroin	CE-ESI-MS		20 mM ammonium acetate (pH 9.0). 22 kV and sheath liquid of isopropanol/water (1:1 v/v) with 7.5 mM acetic acid with 3.0 μL/min flow rate	0.4–1.0 ng/mL		[127]
Amphetamines, cocaine, COD, heroin, MO	CEC-ESI-MS	SPE on a strong cation exchange cartridge	MeCN and 25 mM ammonium formate buffer (pH 3.0) (30:70 v/v). 12 kV	0.78–3.12 ng/mL	Human urine	[132]
MO, COD, 6-AM	CSEI-sweep-MEKC-UV	LLE Sweeping at -25 kV using phosphate buffer/20 mM, pH 2.5) and 80 mM SDS	Phosphate buffer (50 mM, pH 2.5) with 30% methanol and high condition buffer (100 mM phosphate, 41.3 kPa for 18 s). Electrokinetic injection (20 kV, 300 s)	10 ng/mL	Real urine samples from addicts	[118]
COD, ketamine, MA, MO, alprazolam, oxazepam, and metabolites	CZE-sweep-UV	Sweeping buffer (phosphate 75 mM, pH 2.5 and methanol (90:10 v/v with 65 mM SDS	Phosphate buffer (75 mM, pH 2.5) and methanol (70:30 v/v)	20–50 ng/mL	Urine samples from addicts	[112]

ME, MDA, MDEA MDMA, methadone, cocaine, MO, COD, 6-AM, benzoylecgonine	CZE-TOF-MS	LLE with FASI	25 mM ammonium formate electrolyte solution (pH 9.5) at 15 kV. Sheath liquid of isopropanol-water (1:1, v/v) with 0.5% formic acid	2–10 ng/mL	Blood samples of real cases	[130]
CF, TB, theophylline, paracetamol, propylphenazone, acetylsalicylic acid, salicylic acid, CP	MEKC-UVat 210 nm		20 mM phosphate buffer (pH 9.0), 80 mM sodium dodecyl sulfate and 7.5% acetonitrile (v/v). 25 kV at 25°C		Food, beverages, natural products, pharmaceuticals, and cosmetics	[117]
Narcotine, PV, TB, COD, MO	pCEC with monolithic column			1.5–6.0 μg/mL	Pericarpium papaveris	[133]
AM, MA, MDA, MDMA, ephedrine, cocaine, MO, COD, 6-MAM, bezoylecgonine	CE-ESI-MS and CE-TOF-MS	LLE with FASI	25 mM ammonium formate (pH 9.5)	0.1 ng/g	Hair samples	[129]
MO, COD, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	CE-UV		Nearly neutral pH phosphate buffer and poly (ethylene oxide) as coating agent		Urine specimens	[109]
MO, COD, ketamine, MA	CSEI-sweep-MEKC-UV	Sweeping at -20 kV using phosphate buffer (25 mM, pH 2.5) with 20% methanol and 100 mM SDS	Phosphate buffer (50 mM, pH 2.5) with 30% methanol and high condition buffer (100 mM Phosphate, 6.9 kPa for 99.9 s). Electrokinetic injection (10 kV, 500 s)	15 ng/mL (MO and COD) and 5 ng/mL (MA)	Real urine samples	[119]

(*continued*)

Table 33.3 (continued)

Analytes	Mode of CE	Preconcentracion and extraction procedures	Experimental and instrumental parameters	Limits of detection	Matrix of application	References
MO, 6-AM	CE-FLD ($\lambda_{ex} = 350$ nm, $\lambda_{em} = 500$ nm)		Derivatization with diazonium, in presence of β-CD	1.0–3.3 ng/mL	Human urine	[121]
MO, COD, NM, M3G, M6G	CSEI-sweep-MEKC-UV at 200 nm	SPE. Stacking using phosphate buffer (25 mM, pH 2.5) containing 22% methanol and 100 mM SDS	Phosphate buffer (75 mM, pH 2.5) with 30% methanol and high condition buffer (120 mM phosphate, 10.3 kPa for 99.9 s)	10–35 ng/mL	Urine samples	[120]
Five narcotics	pCEC-ESI-MS with silica-based monolithic column		2 mM ammonium acetate (pH 6.0) with 65% MeCN (v/v) at 8 bar, 25 kV and 3 μL/min of sheath liquid	2.0–80 nM		[134]
6-MAM, MO, AM, MA, MDA, MDMA, benzoylecgonine, ephedrine, cocaine	CZE-MS	LLE after overnight incubation with 0.1 M HCl at 45°C	25 mM ammonium formate (pH 9.5). 15 kV. Sheath liquid composed of isopropanol-water (50:50 v/v) with 0.5% formic acid at 4 μL/min	0.1 ng/g	Hair samples	[128]
Lidocaine, bupivacaine, noscapine, PV	CZE-UV	SPE using Oasis HLB cartridges		300 ng/mL	Urine samples	[110]
TB, COD, MO, PV, narcotine	CZE-UV				Opium samples from India	[111]
Heroin, amphetamines, ketamine	CE-UV	DLLME	Chloroform (organic extractant) and isopropanol (dispersive solvent)	0.05–0.20 μg/L	Banknot, kraft paper, silver paper samples	[135]

Plasma and serum are typically used for emergency testing in forensic and toxicology, and in therapeutic monitoring, as they are suitable as samples to determine the short-term use of drugs. Drawbacks are that blood is an invasive sample, difficult to handle and reflects the patient's dosages for some hours only. CZE with TOF ionization coupled with MS have been used for the analysis of opiates in blood samples, obtaining limits of detection between 2 and 10 ng/mL [130].

Although urine and blood are the most common and preferred matrices used for the toxicological studies involving this type of analysis, hair is gaining in importance as an alternative specimen for documentation of use or exposure to drugs because of its different advantages, especially its stability and ease for sampling and storage, compared with the other conventional biological samples. Hair analysis is a useful specimen to provide evidence for previous drug use in legal cases. It is currently applied in workplace drug testing, postmortem toxicology, doping control, and certification of physical fitness to drive vehicles. As a sample, it is obtainable noninvasively, it is easy to preserve, and it is the only one that provides information about the quantity and historic pattern of drug use, having a retrospective period of months or years, saving drugs and their metabolites unchanged. Nevertheless, a limited number of papers have described methodologies for hair analysis based on CE and always coupled with MS, both employing ESI or TOF ionization procedures and using LLE as sample extraction procedure [128, 129]. In both cases, the limits of detection are below the cutoff of 0.1 ng/g proposed by the SHT.

The other field of application of the different methods of analysis is the cultivated opium plant (*P. somniferum L.)* itself, which produces TB. It has a long cultivation history worldwide and there are many varieties having various colors and shapes of flowers, shapes of capsules, and alkaloid composition and content. Despite the strict control of the plants, seeds of *P. somniferum* have been imported and sold as an ornamental gardening flower for which to control illegal cultivation of the plants, rapid and reliable extraction methods of opium alkaloids from many plant samples are required. CZE-UV has been employed for the analysis of opium samples and poppy straws [111, 113] and in presence of β-CD by CE-CD-UV in Iranian opium poppy plants [114]. Other detection systems such as MS and ECL with the same separation technique have been employed for the analysis of illicit samples [131] and Chinese traditional medicine opium poppy samples [124], respectively.

2.3.2 Typical Sample Pretreatments Prior CE for the Analysis of Opium Alkaloids

CE presents potential advantages in forensic science to carry out the analysis of opium alkaloids, as it can be in the different applications being published. Nevertheless, the lack of the sensitivity required for this type of analysis is always a great problem. One of the most basic approach for sensitivity enhancement is based on increasing analyte mass loading via online sample preconcentration techniques. The most widely employed in the analysis of major alkaloids is FASI that basically consists in a mismatch between the electric conductivity of the sample and that of the running buffer. It is achieved by injecting the sample diluted in a solvent of lower conductivity than that of the carrier electrolyte. Upon the application of the voltage (electrokinetic injection), an enhancement of the electric field strength occurs in the low-conductivity zone, which induces an increase of electrophoretic velocities. When electrokinetic injection is performed in normal polarity mode, cations experience the high field strength, move rapidly and concentrate in sharp bands in the tip of the capillary at the boundary between the sample and the separation buffer. Once in the separation buffer, the injected components of the sample migrate in different zones according to their charge/mass characteristics. FASI has been successfully applied to the analysis of opium alkaloids in many different forensic samples such as human urine [107, 108], hair [129], blood [130], and also in Chinese opium plants [124].

The most important prerequisite for achieving high sensitivity associated with FASI is that samples have to be free of electrolytes. Biological samples have high salts and ion contents which seriously interfere in FASI. For such a reason, online or off-line LLE [118, 128–130] and SPE [110, 120, 132] are traditionally the most employed pretreatment methods. However, LLE usually requires some poisonous volatile organic solvents while SPE is a method with good purification and concentration effects, but it requires a solvent desorption step with traditional volatile organic solvents and the pretreatment processes are relatively time consuming and, sometimes, sample recoveries are not satisfactory. Therefore, the development of simple and environment-friendly pretreatment methods such as the previously cited DLLME is of great interest. Until now, DLLME is widely applied to the preparation of environmental water samples but it is rarely applied to the analysis of drugs of abuse by CE. To our knowledge, the first time to combine DLLME with CE using UV detection for the separation of heroin, amphetamines and ketamine in different samples of forensic interest have been carried out by Meng et al. [135].

3 Conclusions

Determination of opiate alkaloids in samples of forensic interest at very low levels of sensitivity will be always required, thus new analytical strategies will have to take this trend into account. Gas chromatography, liquid chromatography, and capillary electrophoresis mainly coupled with tandem mass spectrometry are the methods of choice for the quantification of these drugs of abuse and their metabolites in numerous samples such as opium plants, food, pharmaceutical preparations, environmental samples, and mainly biological samples such as blood, urine, hair, meconium, vitreous humor, and sweat. Online sample cleaning methods, which allow the sample preparation and introduction steps to be combined into one, have been thoroughly investigated. LLE and SPE are the most widely employed but require large volumes of toxic organic solvents and are highly time consuming. SME methodologies are the current trend. Recently, the sample preparation step before analysis is getting more and more simplified, SME methodologies, especially EME and DLME, being the current trend because they are considered green pretreatment techniques, obtaining high enrichment factors, high extraction recoveries, simplicity of operation, and low cost.

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Analysis of Catharanthus Alkaloids

34

Qinhua Chen and Zilin Chen

Contents

1 Introduction 1122
2 Method of Sample Preparation 1123
3 Analytical Methods 1124
3.1 High-Performance Liquid Chromatography 1124
3.2 Capillary Electrophoresis 1128
4 Conclusions 1129
References 1131

Abstract

Catharanthus roseus is an important dicotyledonous medicinal plant that contains various anticancer components. The most important constituents with biological activity in *Catharanthus roseus* are terpenoid indole alkaloids. The analytical methods for determining bioactive compounds in *Catharanthus* alkaloid are summarized in this chapter. HPLC and CE methods are mainly introduced; the advantages and limitations of each technique are also compared and discussed.

Keywords

Alkaloids • analysis • *catharanthus roseus*

Abbreviations

LLE	Liquid–liquid extraction
MIPs	Molecularly imprinted polymers

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SFE	Supercritical fluid extraction
VCR	Vincristine
VLB	Vinblastine

1 Introduction

Catharanthus roseus (L.) G. Don (family of Apocyanaceae) is a tropical perennial subshrub which is rich in terpenoid indole alkaloids. Indigenous to Madagascar, *Catharanthus* is now cosmopolitan in distribution, spreading to South-East Asia, India, Indonesia, Australia, and North and South America [1]. It is a worldwide known medicinal plant used for the treatment of tumor, hypotensive, and arrhythmic diseases [2]. The *Catharanthus* (or Vinca) alkaloids comprise about 130 terpenoid indole alkaloids, in which vincristine (VCR) and vinblastine (VLB) are the most important alkaloids for the pharmaceutical industry [3]. VLB and VCR were discovered as a result of a drug-screening program which performed in the late 1950s. VLB and VCR have been applied in the treatment of several kinds of cancer, such as Hodgkin's disease, lymphosarcoma, choriocarcinoma, neuroblastoma, carcinoma of the breast, and chronic leukemia [3].

The growing interest in secondary metabolites of plants leads to the requirement of the development on analytical method for the secondary product analysis. Chromatographic procedures for the determination of alkaloids have been well established. Based on the literatures published in past years, further improvement of the current methods for the analysis of C*atharanthus* alkaloids are needed [4]. Besides, the chemical complexity and unique bisindole alkaloid structure of the aforementioned molecules hindered their laboratorial synthesis. The isolation of VLB and VCR is laborious and costly, mainly due to their low contents in the plant and coexistence in a large number of other alkaloids [5]. Therefore, it is important for separation, identification, and quantification of these *Catharanthus* alkaloids. The methods of extraction and purification were focused on liquid–liquid extraction, solid-phase extraction, supercritical fluid extraction (SFE), and molecularly imprinted polymers (MIPs)-based extraction. For separation, GC is not suitable for the bisindole alkaloids due to their high boiling point. The major methods for analysis of *Catharanthus* alkaloids are liquid chromatography (LC) and capillary electrophoresis (CE).

Different methods for analysis of *Catharanthus* alkaloids are employed for different purposes. (1) Plant tissues are analyzed when investigating the quality of plant raw material for alkaloid extraction, purification, and isolation. Chromatographic methods for this purpose must be highly selective for large numbers of similar alkaloid metabolites and highly sensitive for minute quantities. (2) In pharmaceutical quality control, the composition of the samples is usually relatively simple, but extraction reproducibility and automation of a method are also required. Sensitivity is necessary as well to ensure the detection of byproducts or degradation of the product. (3) In clinical drug monitoring or in toxicological cases, the sample matrix is complex. In forensic analysis, there are similar samples. Thus, sample preparation becomes a major issue as to provide sufficient concentration of analytes. The detection is usually combined with identification methods such as MS [4, 6].

Chromatographic method is commonly use and powerful technique for the analysis of *Catharanthus* alkaloids. Therefore, this chapter will focus on practical application of chromatographic and electrophoretical methods for analysis of *Catharanthus* alkaloids.

2 Method of Sample Preparation

Extraction and sample preparation are of importance in plant analysis. Clinic and forensic analyses usually rely on appropriate sample preparation to achieve a sufficiently low limit of detection. The pretreatment procedure before the chromatographic measurement must ensure exhaustive extraction of the analyte and removal of matrix that may interfere with analysis. Sample preparation methods for analysis of *Catharanthus* alkaloids include liquid–liquid extraction (LLE), supercritical fluid extraction (SFE), and molecularly imprinted polymers (MIPs)-based extraction.

Sample preparation methods without any loss of the target compounds are important, especially when a quantitative assessment of all alkaloids in a given plant sample is required. For many alkaloids, the sample preparation procedure is based on the difference in the solubility of the alkaloids salt in comparison with the free bases. An acidic aqueous extraction must be basified to transfer alkaloids into the free base for extracting into organic solvents nonmiscible with water. Excessive use of alkali, however, is detrimental for the ester alkaloids.

When LLE is used, it is advisable to do either very quickly or from only a moderately alkaline aqueous solution, which was basified by sodium carbonate or ammonia. The *Catharanthus* alkaloids was prepared by LLE from the 75 % ethanol extract at pH 3.5, then adjusted the basic to pH 12 and finally extracted by chloroform [7]. Three major alkaloids, vinblastine and its monomeric precursors (vindoline and catharanthine), were monitored in transformed root cultures of *Catharanthus roseus*, after rapid sample preparation by LLE in our previous work [8]. The extraction method was the same as above. Other Catharanthus alkaloids, such as vindoline, catharanthine, and anhydrovinblastine, were prepared by LLE from the methanol extraction [9].

An alternative to LLE is supercritical fluid extraction (SFE). SFE technique has been considered as a good option for the extraction of natural products, particularly for food and pharmaceutical ingredients. Supercritical carbon dioxide is the most common fluid used because of its physiological compatibility, nontoxicity, inflammability, inexpensiveness, and availability. In addition, high selectivity, convenient critical parameters and environmental friendliness are significant advantages of using carbon dioxide as supercritical fluid [10].

The procedure is described as follows: supercritical fluid extractions were performed with an automated ISCO SFX™ 3560 instrument using 6 mL extraction vessels. The extraction vessels were filled with 100 mg of dried plant samples mixed with anhydrous sodium sulfate. The extracted analytes were collected into 10 mL of methanol. The internal standard was added to the collection vials immediately after extraction. The collection temperature was 5 °C. The SFE instrument was equipped with a 260 mL syringe pump for the addition of carbon dioxide at a flow rate of 1.5 mL/min and a manually controlled Jasco PU-980 HPLC pump for addition of the modifier (methanol) at flow rates of 0.04–0.1 mL/min (2.6–6.6 %). The restrictor temperature was set at 60 °C in all extractions [11]. In this paper, the extracts from SFE were found to be much cleaner in comparison with those obtained by solid–liquid extractions or Soxhlet extractions. The results showed that supercritical fluid extraction is a valuable alternative technique to traditional extraction methods of *Catharanthus* alkaloids from dried leaves.

Ionic liquids are a class of salts that exhibit surprisingly low melting temperatures. They are liquid at room temperature, which is remarkable when the high melting temperatures of classical inorganic salts such as NaCl are considered. Ionic liquids have recently attracted much interest in a variety of applications because of their unique chemical and physical properties. They benefit from extremely low vapor pressure, high specific solvent ability, chemical and thermal stability, and nonflammability.

Ionic liquid-based ultrasound-assisted extraction (ILUAE) was successfully applied to the extraction of three alkaloids, vindoline, catharanthine, and vinblastine from *Catharanthus roseus*. Twelve ionic liquids, with different cations and anions were investigated in this work. In addition, ultrasound extraction parameters, including soak time, solid–liquid ratio, ultrasound power and time, and the number of extraction cycles, were optimized. ILUAE offered short extraction times (from 0.5 to 4 h) and remarkable efficiency. Therefore, the use of ionic liquids in the ultrasound-assisted extraction of key chemicals from medicinal plants shows great potential [12].

3 Analytical Methods

3.1 High-Performance Liquid Chromatography

HPLC analysis for pharmaceutical preparations of alkaloids started about 30 years ago. Gas chromatography (GC) is suitable for volatile compounds and requires laborious derivatization. Thus, GC is unsuitable to analyze the bisindole alkaloids due to their high boiling point. HPLC can provide better analytical precision and higher sample loading capacity. HPLC methods coupled to UV and MS detection have been applied for the determination of the active compounds in *Catharanthus roseus*.

In a previous work, we developed a LC-UV method for simultaneous determination of five active alkaloids vinblastine, vindoline, ajmalicine, catharanthine, and vinleurosine in *Catharanthus roseus*. HPLC analyses were performed on an 1100 HPLC instrument equipped with a binary pump, a UV detector, an autosampler, and

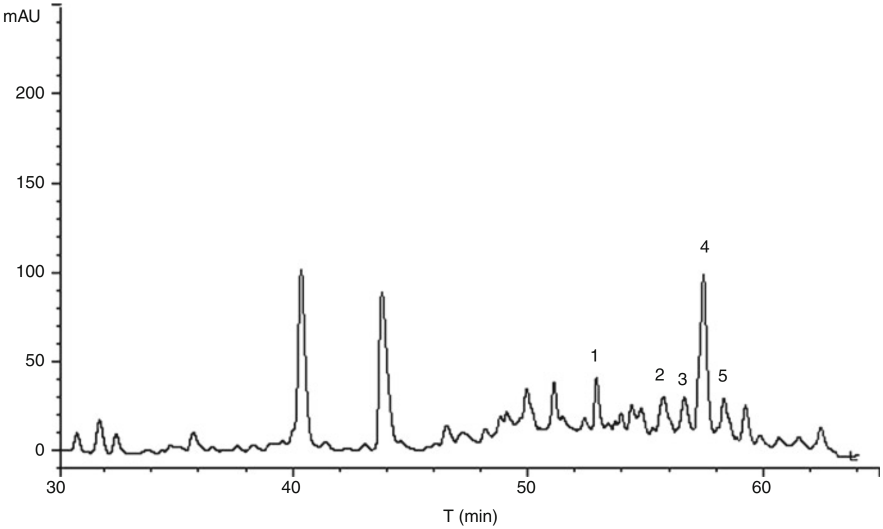


Fig. 34.1 Typical chromatograms of f sample by LC-UV. Peak identification: *1* vinblastine, *2* vindoline, *3* catharanthine, *4* ajmalicine, *5* vinleurosine

a column thermostat. Chromatographic separations were carried out on a DL-C_{18} column (5.0 μm, 250 × 4.6 mm, Japan) column at 25 °C. Elution was performed with a flow rate of 0.5 mL/min. Acetonitrile (A) and 10 mM ammonium acetate in water (B) were used as a mobile phase. Gradient elution was used as follows: 10 % A (2 min), 10–30 % B (10 min), 30–45 % B (30 min), 45–70 % B (50 min), 70–80 % B (65 min), 80–95 % (70 min). After the run, the gradient was set back to 10 % A and the system was allowed to equilibrate. The injection volume was 10 μL and the detection wavelength was 280 nm. The typical chromatograms of sample are shown in Fig. 34.1.

HPLC in combination with tandem mass spectrometry (MS) appears to be a more suitable technique for the screening of the active compounds in plant samples in terms of sensitivity and selectivity. In recent years, analytical techniques have made great advances. LC-MS and LC-tandem MS (LC-MS/MS) equipped with quadrupole time of flight (Q-TOF) and quadrupole-linear ion trap (Q-LIT) play a crucial role in plant analysis. Ion trap (IT) mass spectrometry is well used in the structure elucidation and in the identification of unknowns in complex matrices. Moreover, several reports show that IT mass spectrometry can also be very useful for quantitative analysis. The five alkaloids can be studied in positive-ion modes by IT-MS, due to their weak basicity from the azacyclo-group in the structure. More importantly, MS^2 can afford much more confidence for compound identification and much lower noise level for quantification. The fragmentation map of a target analyte is useful in performing MS^2 for either qualitative or quantitative analysis. The identical five active alkaloids were dissolved in methanol and analyzed by

ESI-MS^n in positive-ion mode. The protonated molecule ions of $[M + H]^+$ and major fragment ions were observed in the full scan MS and MS^2 spectra in positive-ion mode. We choose the protonated molecular ions and the highest intensity of product ions as the quantification ions. Thus, vinblastine, vindoline, ajmalicine, catharanthine, and vinleurosine were *m/z* 825 → 807, 457 → 397, 353 → 144, 337 → 144 and 809 → 748 were chosen by LC-IT-MS, respectively.

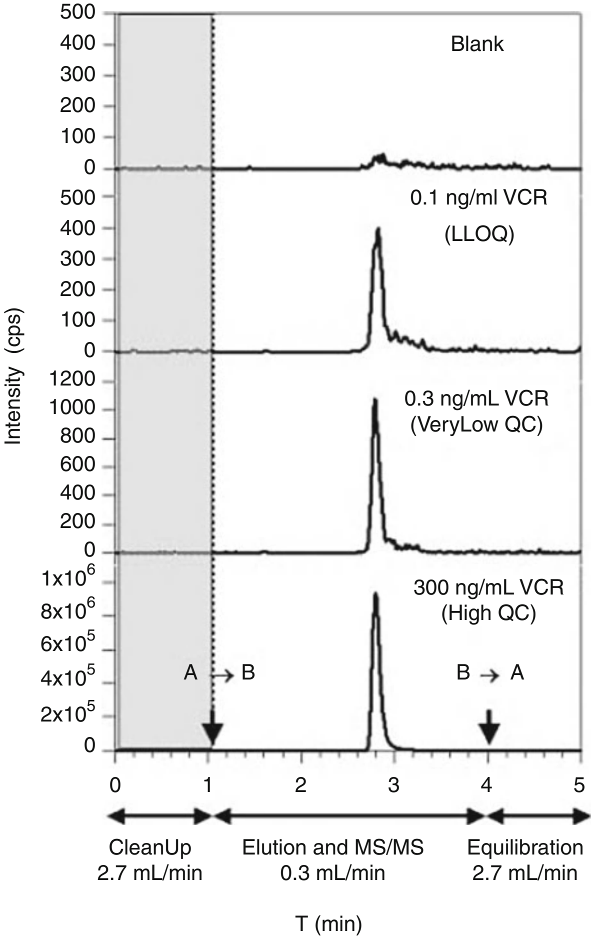
Recently, we developed a LC-MS method for simultaneous determination of these five active alkaloids. Comparing with the two methods, MS can supply a high selectivity and sensitivity for determination of the analytes in positive mode and UV can obtain an excellent repeatability for analysis of them in *Catharanthus roseus*. In conclusion, the two methods have a good linear, reproducibility, precision, accuracy and recovery, and could be used for quantitative analysis of the five active compounds in *Catharanthus roseus*.

For analysis of alkaloids in *Catharanthus roseus* roots, Ferreres et al. describe a phytochemical study on this species roots. Alkaloids in aqueous extracts, the usual form of consumption of this matrix, were studied by HPLC-DAD-ESI-MS/MS, which allowed the identification of 19-S-vindolinine, vindolinine, ajmalicine and an ajmalicine isomer, tabersonine, catharanthine, serpentine, and a serpentine isomer. Quantification of the identified compounds revealed that serpentine and its isomer were predominant (64.7 %) over the other alkaloids, namely, vindolinine and its isomer (23.9 %), catharanthine (7.7 %), and ajmalicine (3.8 %). The proposed procedures revealed to be simple, sensitive, and reproducible [13].

An ultra-performance liquid chromatography/mass spectrometry method for simultaneously quantifying vindoline, catharanthine, serpentine, and ajmalicine in *Catharanthus roseus* cell line C20hi was reported by He et al. Samples were extracted with 1 % acetic acid, basified to pH 10 with ammonia, then extracted with ethyl acetate, dried, reconstituted with methanol −1 % acetic acid water solution (1:1, v/v) and analyzed using an acetonitrile −0.1 % formic acid gradient as the mobile phase. Detection was carried out by electrospray ionization mass spectrometry in the positive-ion mode with selective ion monitoring. The analysis of one sample was achieved in 6 min. The limits of detection were 0.46–0.70 ng/ml in cell samples, and 0.10–0.16 ng/ml in medium samples. The linearity of detection was over the wide range of 1.00–6250.0 ng/ml. Intra- and inter-day accuracies (recovery 88.0–111.8 %) and precision (RSD 1.25–7.81 %) showed the performance of the assay. This method provides a more sensitive and high-throughput technique to quantify the four alkaloids in large amount of samples, and will be helpful in high-production cultivar screening [14].

Liquid chromatography coupled to mass spectrometry and tandem mass spectrometry (LC/MS and LC/MS/MS) lead to major breakthroughs in the field of quantitative bioanalysis due to high specificity, sensitivity, speed, and reliability, which becomes the preferred analytical tools for analysis of drugs and metabolites in biological matrices. A simple and rapid method has been developed and validated for the quantitation of vincristine in human plasma by LC/MS/MS with atmospheric pressure chemical ionization using on-line solid-phase extraction. The method uses vinblastine as internal standard and the sample preparation is

Fig. 34.2 Examples of MRM (m/z: 825.3 → 765.3) chromatograms for VCR. In a blank plasma sample, in plasma spiked with VCR concentration at 0.1 ng/mL (*LLOQ level*), 0.3 ng/mL (*Very-Low QC*), and 300 ng/mL (*High QC*). The *shadowed area* refers to the time section for sample loading and cleanup, the *A* and *B* are referred to switching valve positions (From Ref. 15)



limited just to a plasma protein precipitation step. Further sample cleanup is carried out on-line through a perfusion column preceding an analytical phenyl LC column, the latter directly connected to the mass spectrometer. Quantitation is performed in multiple reaction monitoring mode using the transitions of m/z 825.3 → 765.3 and 811.3 → 751.3 for vincristine and vinblastine, respectively. Figure 34.2 shows the typical chromatograms for a blank plasma sample, spiked plasma at LLOQ, and QC samples at very-low and at high concentrations. No peaks from interfering endogenous plasma components are present in the chromatograms, further supporting the high specificity of the 825 → 765 amu MRM transition.The assay was linear ($r^2 \geq 0.99$) in a concentration range from 0.1 to 500 ng/mL. Carryover, measured on the experimental setup, was less than 0.04 %. Recovery for vincristine and the internal standard was within 90–95 %. The intra-day and inter-day assay precision ranged from 1.2 % to 6.8 % RSD, while mean percentage deviation from nominal value ranged from 0.01 % to 6.1 %. The proposed assay was found suitable for pharmacokinetics investigations and clinical therapeutic drug monitoring especially in pediatric cancer patients [15].

3.2 Capillary Electrophoresis

CE appears well suited to alkaloid analysis because these compounds are natural cations if the appropriate acidic buffer is chosen. Migration of the analytes in the usual cationic mode is caused by the charged nitrogen atom. The sample volume in CE is very low, only a few nanolitres, and therefore very sensitive detection is required. The separation mechanism of CE fundamentally differs from HPLC and, therefore, CE can provide complementary or additional information on the composition of a sample. In general, CE separations can be achieved in a fast and highly efficient way without extensive sample pretreatment. Other advantages of CE include the very low or even the absence of organic solvent consumption, the small amount of reagents needed, and the use of simple fused-silica capillaries instead of the expensive LC columns. The relatively poor sensitivity of CE, resulting from the small loading volumes, could be circumvented by the incorporation of preconcentration strategies. While the advantages of MS detection are embodied in the improvement of detection sensitivity as well as the capability of both determining the exact mass of analytes and providing structural information, including the possibility to identify and determine co-migrating species in overlapping peaks.

Vincristine (VC) and vinblastine (VB) are dimeric catharanthus alkaloids isolated from the plant *Catharanthus roseus*. A capillary zone electrophoresis (CZE) was conducted for systematic and comprehensive study of the separation and quantification of two dimeric catharanthus alkaloids. Various separation parameters such as buffer concentration and pH, column internal diameter, and applied voltage were studied: column, 72 cm (57 cm effective length) × 75 μm I.D.; buffer, 0.2 M ammonium acetate solution, pH 6.2 and an applied voltage of 10 kV. Although the separation of VB and VC was the primary focus, the separation parameters determined in this study can be applied to the separation of other alkaloids as well. Separation of other alkaloids in the plant samples was observed under conditions presented in this work. A secondary objective of this study was to develop a method with experimental conditions which could be applied to electrophoresis-mass spectrometry. For this purpose, ammonium acetate buffers, which are more compatible with mass spectrometry than the widely used phosphate buffers, were used exclusively. Also, methanol–water-acetic acid was used as external buffer for the same reason [16].

An aqueous electrolyte solution was initially used to separate the indole alkaloids mentioned in the experimental. The electrolyte tested was potassium hydrogenphosphate at different pH values where poor resolution was observed. Efforts to improve the separation by adding different organic solvents such as acetonitrile and methanol were found to be invalid. The use of nonaqueous solvents as electrophoretic media offers many advantages, in comparison with aqueous media, such as low joule heating and provides different selectivity which can be used to improve separation. A rapid method for the determination of Vinca alkaloids by nonaqueous capillary electrophoresis with diode array detection has been developed. A group of 11 alkaloids (catharanthine, vinorelbine, anhydrovinblastine, vinflunine, vindoline, 4-*O*-deacetylvinorelbine, 4-*O*-deacetylvinflunine, vindesine, vinblastine, 49-deoxy-20′, 20′-difluorovinblastine, vincristine) could be readily separated within 10 min. The compounds were separated using a capillary of 38 cm effective length, a running buffer composed of 50 mM ammonium acetate and 0.6 M acetic acid in a methanol–acetonitrile (75:25, v/v) 3 mixture. A constant voltage of 25 kV with a ramp time of 1 min and a 344.7310 Pa pressure, applied simultaneously to inlet and outlet buffer vials, were used during sample analysis. Five of these alkaloids were selected for optimization of the separation and for validation studies with respect to specificity, linearity, range, limits of quantification and detection, and then accuracy. The feasibility of the assay was demonstrated by analyzing a commercial sample of vinorelbine (Navelbine, ampoule at 10 mg/ml of vinorelbine base). The results were compared to a high-performance liquid chromatography method [17].

In previous work, we describe simultaneous determination of VLB and its monomeric precursors by CE-IT-MS. A baseline separation for three components has been achieved by using a running buffer consisting of 20 mM ammonium acetate and 1.5 % acetic acid in less than 20 min. Quantification of three components was assigned in positive-ion mode at protonated molecular ion $[M + H]^+$. The CE-MS method was validated for linearity, sensitivity, accuracy, and precision, and then used to determine the content of the above components. The detection limits of vinblastine, catharanthine, and vindoline are 0.8, 0.1, and 0.1 μg/mL, respectively. The precision was no more than 4.54 % and the mean recovery of the analytes were 95.04–97.04 %. The CE-MS method has been successfully applied to determine vinblastine and its monomeric precursors in real sample *Catharanthus roseus* [8]. Under the optimized conditions, CE-ESI-IT-MS separations (shown in Fig. 34.3) were obtained for total ion chromatogram (TIC) and selected ion chromatograms of VLB, VLD and CTA. Separation of a standard mixture was shown in Fig. 34.3a under TIC mode by CE-MS. Within 20 min VLB, VLD, and CTA could be well separated. Figure 34.3b, c showed the extracted ion chromatogram (EIC) of a standard mixture and real sample *Catharanthus roseus* by CE-MS. A baseline separation for three components was obtained in the standard mixture and real sample.

4 Conclusions

HPLC with UV detection is the routine method for analysis of plant material, only if the intact ester alkaloids with an UV absorbing aromatic acid or other UV absorbing functionality are to be measured. HPLC methods are robust with widely available instrumentation and high sensitivity due to improved UV detectors. A wide range of other detectors is available for specific problems and more sensitive determination.

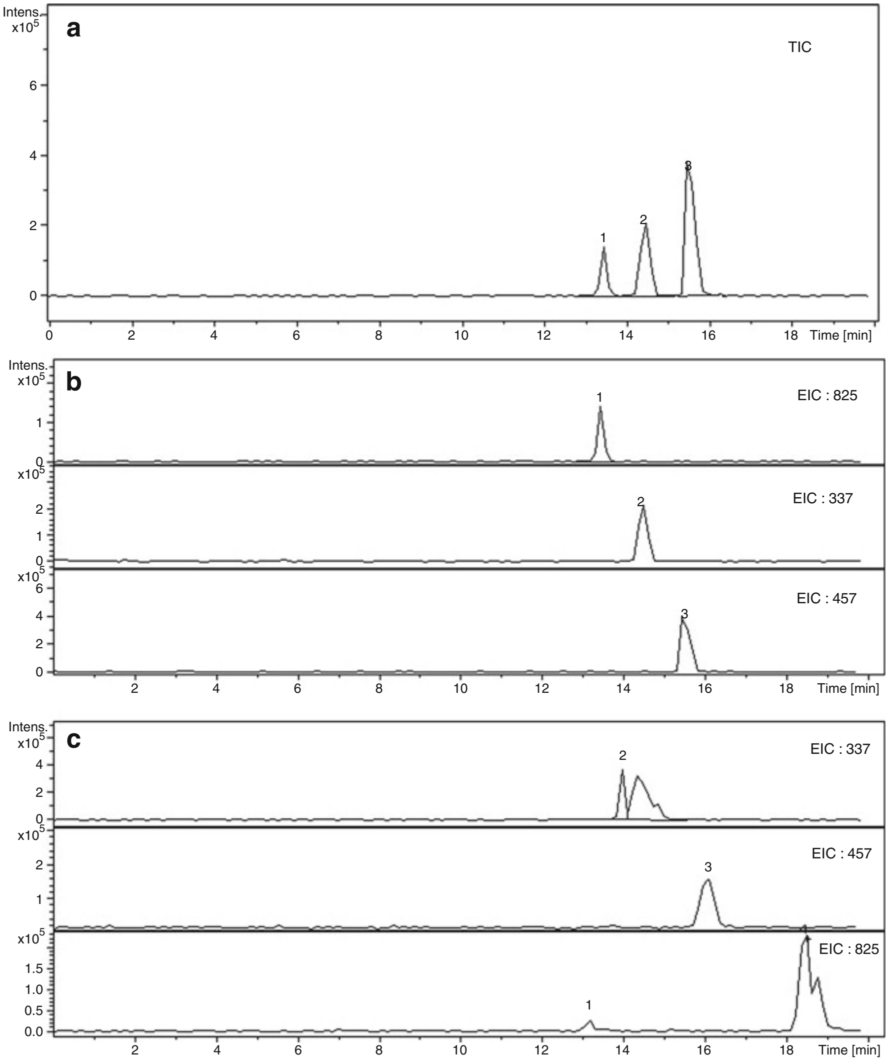


Fig. 34.3 Total ion chromatogram (**a**) and extracted ion chromatogram (**b**) of a standard mixture solution, and extracted ion chromatogram (**c**) of *Catharanthus roseus* extract by CE-MS. CE conditions: capillary, 65 cm length and 50 μm i.d, buffer: 20 mM s ammonium acetate aqueous with 1.5 % acetic acid, temperature: room temperature, sample, 50 mbar for 5 s injection; MS-conditions: ESI positive, sheath gas: 4 psi; dry gas: 6 L/min; dry temperature: 130 °C; capillary voltage: 3.5 kV; sheath liquid: methanol/water = 1/1, with 0.1 % acetic acid; flow rate of sheath liquid: 4 μL/min. Peak identification: *1* vinblastine; *2* catharanthine; *3* vindoline (From Ref. 8)

Usually, sample preparation for HPLC is relatively simple. For the sensitive measurement of *Catharanthus* alkaloids at low concentration, however, sample purification is required. HPLC, in contrast to GC or CE, can be scaled up to allow the preparative separation of alkaloids.

LC-MS for *Catharanthus* alkaloid analysis is a promising approach which will be increasingly used in the future, as interfaces are improved and highly selective solvent systems become available. The metabolites and catabolites of *Catharanthus* alkaloids can also be identified and determined by LC-MS conveniently.

CE possesses higher separation efficiency and could supply complementary information to HPLC. The LOD expressed as concentration in CE is higher than GC and HPLC because of the small sample volumes and the relatively low concentration for Catharanthus alkaloids. Using nonaqueous solvents, CE can be coupled to MS easily and would provide a powerful means for alkaloids separation and identification in the near future.

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Methods for Analysis of Ergot Alkaloids 35

Colin Crews

Contents

1 Introduction 1134
2 Methods of Analysis 1138
2.1 Sampling 1138
2.2 Spectroscopic and Related Methods 1138
2.3 Extraction Procedures 1139
2.4 Sample Cleanup 1140
2.5 Immunological Methods 1141
2.6 Thin Layer Chromatography 1142
2.7 Gas Chromatography (GC) and Gas Chromatography–Mass Spectrometry (GC-MS) 1143
2.8 Liquid Chromatography 1143
2.9 Liquid Chromatography-Fluorescence Detection (LC-FLD) 1145
2.10 Liquid Chromatography–Mass Spectrometry (LC-MS) 1146
2.11 Alternative Methods 1148
2.12 Multianalyte Methods 1149
2.13 Quantification Methods 1149
3 Conclusion 1150
References 1150

Abstract

The determination of contamination of feed and food by ergot alkaloids is of great importance in preventing poisoning of cattle and consumers and economic losses. Established but crude methods based on the gravimetric measurement of ergot fruiting bodies (sclerotia) after microscopy and manual selection are now being replaced by more sophisticated techniques. Enzyme linked immunosorbent assays (ELISAs) can rapidly indicate the total ergot contamination on several samples

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simultaneously but give no information on the individual alkaloids. Methods based on liquid chromatography with detection by fluorescence measurement or mass spectrometry permit the quantification of individual ergot alkaloids and their epimers, which is important as their toxicities vary. The merits and handicaps of these methods are described, and associated factors such as the availability and stability of standards, and the extraction procedures are discussed.

Keywords
Cereals • *Claviceps* • ergot • feed • fluorescence • grain • lysergic acid • liquid chromatography • mass spectrometry • sclerotia

Abbreviations

ASE	Accelerated solvent extraction.
EFSA	European Food Safety Authority
ELISA	Enzyme linked immunosorbent assay
GC	Gas chromatography
GC-MS	Gas chromatography with mass spectrometric detection
LC (High performance)	Liquid chromatography
LC-FLD	Liquid chromatography with fluorescence detection
LC-MS	High-performance liquid chromatography with mass spectrometric detection
LC-MS/MS	Liquid chromatography with tandem mass spectrometric detection
LC-UV	Liquid chromatography with ultraviolet detection
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Lysergic acid diethylamide
MRM	Multiple reaction monitoring
NIR	Near infrared
NMR	Nuclear magnetic resonance spectroscopy
PLE	Pressurized liquid extraction
PSA	Primary and secondary amine
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TLC	Thin layer chromatography
UV	Ultraviolet

1 Introduction

Ergot alkaloids are produced by various species of the fungus *Claviceps*, most notably by *Claviceps purpurea*. In cereals, the fungus replaces the developing grain with the alkaloid-containing sclerotium. Three major groups of ergot alkaloids are most commonly encountered, examples of which are shown in Fig. 35.1. The groups are clavines, water-soluble lysergic acid derivatives, and ergopeptides, which are lysergic acid derivatives insoluble in water.

Clavine alkaloids appear to be biosynthetic precursors of the other groups. The amount and pattern of these alkaloids vary between fungal strains and depend on the host plant and the geographical region, but the most frequently encountered alkaloids produced by *Claviceps* species are ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, and ergocornine. Related compounds having the lysergic acid structure, notably its diethylamide (LSD) are of medicinal and forensic interest.

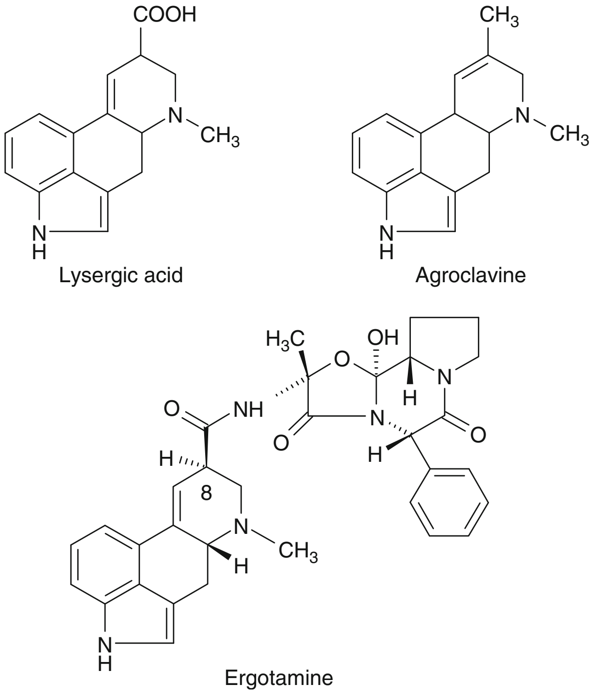
Ergot alkaloids that contain a C9 = C10 double bond readily undergo reversible epimerization at the asymmetric carbon atom in the 8 position in the lysergic acid part of the molecule to produce a mixture of left-hand rotational isomers with the C-8-(R) configuration which are called ergopeptines, and right-hand rotational (S)-isomers diastereomers called ergopeptinines, as shown in Fig. 35.2.

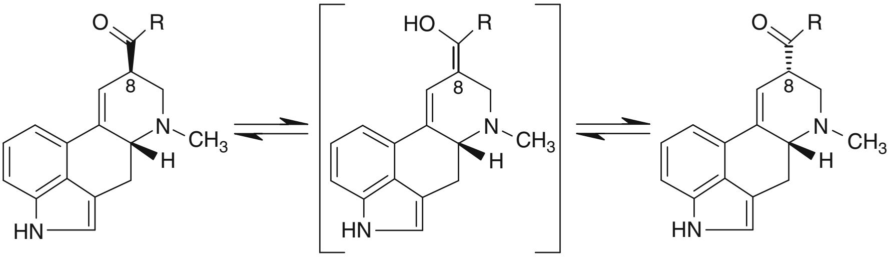
The epimers are reflected in the names, for example, where ergotamine is the ergopeptine form the ergopeptinine epimer is named ergotaminine. The C-8 epimers differ in biological and physicochemical properties, with the -ine forms being more biologically active. In nature the alkaloids always exist in both forms, and epimerization also occurs on storage of samples or during extraction from cereals.

A small proportion of -inine alkaloids, based on *iso*-lysergic acid, are commonly reported in extracts of sclerotia. These may be generated, at least in part, through the epimerization of lysergic acid during analytical extraction procedures.

Ergot alkaloids can cause adverse health effects in humans and animals. There are reports on human intoxications and on ergot poisoning of farm animals in particular cattle, horses, sheep, pigs, chicken, and even wild animals. Sclerotia will be harvested together with the cereal grains and can thus result in alkaloid contaminated cereal-based feed and food products. Sclerotia are most prevalent in rye and triticale, but they can also occur in wheat and other small grains. The average total alkaloid content of ergots found in central Europe varies between 0.01 % and 0.2 %. Normally, most intact ergot sclerotia are removed by sieving during the first cleaning process of grain in the mill. Grain can be rapidly checked for ergot before milling by examination under a stereo microscope, but more rapid and automated methods are desirable. Levels of ergot alkaloids in animal feed containing unground cereals are limited to a maximum of 0.1 % (1,000 mg kg^{-1}) under European Commission directive 2002/32/EC [1]. In 2012, the European Commission [2] recommended that the presence of ergot alkaloids in cereals and cereal products intended for human consumption or intended for animal feeding, in pasture/forage grasses for animal feeding and in compound feed and food be monitored, and that whenever possible, the sclerotia content in the sample should be measured simultaneously in order to be able to improve the knowledge on the relationship between the content of sclerotia and the individual ergot alkaloids. EFSA, the European Food Safety Authority stated in April 2005 that there is a special concern related to animal feed, and that validated analytical methods are required for the quantification of ergot alkaloids in feed material [3]. In 2012, EFSA issued an opinion on ergot alkaloids that included a call for laboratories to carry out

Fig. 35.1 Structures of the ergot alkaloids lysergic acid, agroclavine, and ergotamine


COOH
N
CH3
NH
Lysergic acid
CH3
N
CH3
NH
Agroclavine
H3C
OH
O
N
H
O
NH
N
H
O
H
8
N
CH3
H
NH
Ergotamine


O
R
8
N
CH3
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HN
HO
R
8
N
CH3
H
HN
O
R
8
N
CH3
H
HN

Fig. 35.2 Epimerization of ergot alkaloids containing a C9 = C10 double bond with respect to the center of symmetry at C-8 (From Krska and Crews [7]. Reprinted by permission of the publisher (Taylor & Francis Ltd, http://www.tandf.co.uk/journals))

a survey of ergot alkaloids in cereals intended for human consumption and animal feeding [4]. The EFSA Panel on Contaminants in the Food Chain has recommended that data on the variability of the ergot alkaloid patterns in feed materials should be collected in Europe and that validated analytical methods for the quantification of the major ergot alkaloids in feed materials should be developed.

EFSA has also indicated the procedures required for storing samples before and after pretreatment, milling, and other sample preparation steps. The preferred analytical methods are based on multianalyte liquid chromatography (LC).

LSD is used as a drug of abuse and has potential medical applications. There are therefore forensic requirements to measure LSD and its isomer *iso*-LSD in body fluids including urine. LSD is considered a semisynthetic drug, being made from some naturally occurring ergot alkaloids by hydrolysis to lysergic acid, which is then reacted with diethylamine to produce LSD.

Methods for the analysis of ergot alkaloids have been reviewed by several authors [4–7]. Sclerotia can be measured in grain by picking out the particles or by electronic imaging techniques. These are useful at the processing plant but to test for food and feed safety instrumental methods that measure the alkaloids in total or individually are preferred. The three major approaches of enzyme linked immunosorbent assays (ELISA), liquid chromatography with fluorescence detection (LC-FLD), high-performance liquid chromatography with mass spectrometry (LC-MS). Alternative methods such as gas chromatography with mass spectrometry (GC-MS) and thin layer chromatography (TLC) remain useful for certain applications. Nuclear Magnetic Resonance spectroscopy (NMR) is becoming more importance in analytical chemistry as instrument sensitivity increases. It has not been applied to the quantitative determination of ergot alkaloids in cereal products but has proven useful in structural determinations, particularly of products used as therapeutics.

Sclerotia have long been quantified in grain by visual inspection or microscopy. Today, more sophisticated and automated methods are available for counting the proportion of sclerotia in grains. For quantification of the total ergot alkaloid content methods based on color reaction tests, immunoassays, or NMR are available. For quantification of individual ergot alkaloids liquid chromatographic methods with fluorescence detection or mass spectrometry are required. The instrumental methods require effective extraction of the alkaloids, and some degree of clean up. Appropriate procedures are needed to avoid epimerization during the sample and extract handling stages.

The facile epimerization of ergot alkaloids is very important in their analysis. The -inine epimers are thought to be physiologically inactive but since they are readily converted to the –ine form it is greatly advantageous to determine both isomers. The active and inactive isomers cannot be differentiated by techniques that use the typical color reactions for ergot alkaloids, or by most methods that do not involve chromatographic stages. Epimerization affects standard solutions as well as sample extracts. It is influenced by temperature and solvent. For longer-term storage stock standards need to be kept free from solvent and frozen to at least −18 °C, being reconstituted immediately before use. For medium-term storage additives such as ethylene glycol, 1,2-propanediol, or tartaric acid dissolved in mixtures of ethanol and water have been recommended.

Ergopeptide alkaloids form water-addition products known as lumiergopeptines, and so analysis of plant materials and food needs to be carried out in subdued light. Another rearrangement occurs in the peptide part of ergot alkaloids, with isomers being slowly formed at room temperature. This reversible isomerization leads to rearrangement products (aci-alkaloids) that are practically inactive pharmacologically.

Although much progress has been made in this field in recent years method validation has been largely restricted to studies made in individual laboratories. The reported validations have included descriptions of the type of calibration, the limit of detection (LOD), and limit of quantification (LOQ), the recovery, repeatability, within-laboratory reproducibility, and ruggedness.

2 Methods of Analysis

2.1 Sampling

Ergot sclerotia are not distributed homogeneously throughout grain, and therefore sample sizes should if possible be fairly large (1–5 kg). Samples or subsamples may require drying, in which case the loss of weight should be recorded, and the measured alkaloid content corrected for the original weight.

2.2 Spectroscopic and Related Methods

Analytical methods based on visual and spectroscopic determination may be applied to both sclerotia (intact and broken) and to the total alkaloid content. Methods for detecting ergot bodies in cereals require passing a suitably sized (e.g., 250 g) sample through a range of sieves and collecting a fraction of 1–3.5 mm diameter. A portion of this is spread on a table, and the ergot sclerotia are picked out with tweezers and weighed.

In microscopy methods such as that for feed described by the International Association of Feedingstuff Analysis [8], sclerotia and sclerotia fragments with a particle size greater than 0.5 mm are selected and identified based on characteristic features and comparison to reference material. Confirmation can be achieved through color reaction. The sample is placed across a filter paper soaked with an ethanolic solution of sodium or potassium hydroxide. After about 5 min a red-violet halo appears around any ergot fragments, although this staining procedure is only applicable to fresh sclerotia material. The outer dark violet hyphae layers of sclerotia color solutions of chloral hydrate violet. The quantity of ergot is determined by weighing. Microscopy is rather slow, requiring 15–60 min to analyze 250 g cereal.

Ergot bodies can be quantified in cereals using an online near infrared (NIR) hyperspectral imaging system combined with chemometric tools [9]. This technique is more rapid than the classical microscopic method and enables a large quantity of material to be analyzed, thereby avoiding the sampling problems associated with representative sampling. NIR hyperspectral imaging allows fast (a 250 g sample can be analyzed in 1 min) and accurate inspection of food or feed in the laboratory or on the production line. This method scans a 30 cm wide conveyor belt width running at 100 mm/s and carrying up to about 100 kg grain per hour. Rapid chemometric-based data processing methods are required but the number of false negative and false positive results is low.

A large number of reagents react with ergot alkaloids to form colored products that can be detected visually, by colorimeters or by densitometers, they are thus used to show the presence of ergot alkaloids in test solutions and to identify them on TLC plates. The reagents act on all ergot alkaloids and therefore in quantitative analyses give a value for the sum of all of the ergot alkaloids present. The most widely used tests use van Urk's reagent, *p*-dimethylaminobenzaldehyde in acid solution, which reacts with two molecules of ergot alkaloids to give a blue color with an absorbance at 580 nm after exposure to light. The reagent has been refined by the addition of ferric chloride and sodium nitrite. Colorimetric tests cannot distinguish between ergot alkaloid epimers or between ergot alkaloids and some other alkaloids or amine-containing compounds such as tryptophan.

2.3 Extraction Procedures

Unlike the visual and spectroscopic techniques described above, instrumental analysis of the ergot alkaloids in food and feed samples and body fluids and tissues requires their extraction from the sample matrix. Ergot alkaloids can be extracted from cereals with nonpolar organic solvents under alkaline conditions or with polar solvents under acid conditions, with alkaline extraction conditions giving the highest yields. For modern chromatographic separations simple mixtures of acetonitrile with ammonium carbonate or ammonium hydroxide are preferred. When extraction solvents for the 12 major ergot alkaloids in cereals were compared the highest recoveries were obtained with an 84:16 v:v mixture of acetonitrile and ammonium carbonate buffer. Recoveries ranged from 91 % to 121 %. Basic conditions improve the solubility of ergot alkaloids. Alternatively mixtures of methanol or acetonitrile with dilute organic acids or buffers such as ammonium acetate can be used. Other solvent mixtures have included dichloromethane/ethyl acetate/methanol with ammonium hydroxide, and methanol/water/phosphoric acid where epimerization is less of a problem and when using HPLC with UV or fluorescence detection. Rather simple extraction procedures have been used, such as blending with acidified methanol, ethanol, or acetonitrile. Solid phase extraction using column-packed commercially available diatomaceous earth materials such (Extrelut®) as a support can be used to partition ergot alkaloids from aqueous medium into organic solvents. For the extraction of ergovaline from endophyte-infected grasses an improved performance in terms of sample losses and extraction times has been reported for the use of a mixture of 2-propanol and aqueous lactic acid solution [10].

For LC-MS methods where the LC solvent is evaporated in the instrument nonvolatile buffers must be avoided but for detection techniques such as ELISA there is no such problem and extraction solvents based on phosphate buffered saline solutions can be used alone or mixed with methanol.

Different ratios of sample to solvent might be expected to affect extraction recovery and sensitivity. The use of a high amount of solvent for a small sample size would give an extract that is relatively dilute in terms of its ergot alkaloid concentration, whereas extraction with a low solvent to sample ratio would give a more concentrated extract. A disadvantages of using a small sample size is that the chance of sampling errors is increased. However, the dilute nature of the analyte can bring increased sensitivity and mass spectrometer performance as matrix effects are reduced and there is less interference from the background signal. For ground cereals sample:solvent ratios (wt:vol) over the range 1:3 to 1:10 differ little in performance.

For LC-MS separation with highly specific detection methods simple extraction procedures and filtration can be followed by direct injection into the LC-MS. Extraction with methanol/water acidified with formic acid and subsequent ultrafiltration with a 30 kD filter has been used with success. Similarly simple approaches form the basis of most multi-mycotoxin methods which determine several ergot alkaloids along with other mycotoxins.

Solid phase extraction (SPE) is very often used to clean up sample extracts and provide more concentrated solutions of alkaloid analytes as described below but some ergot alkaloids can be extracted from comparatively clean sample matrices such as plasma or urine by direct liquid–liquid extraction or passing them through cationic SPE cartridges. LSD and *iso*-LSD have also been extracted from urine by making the sample basic and saturated with salt before shaking with 1-chlorobutane. Lysergic acid can be extracted from urine by adjusting the pH of the sample to 5.0–5.5 and passage through an SCX SPE column preconditioned with methanol followed by 0.1 M hydrochloric acid and a wash of water. After eluting unbound material with water the lysergic acid is eluted with dilute ammonium hydroxide in methanol and analyzed by LC-MS.

Another successful technique has been to use an immobilized primary and secondary amine (PSA), as dispersive PSA-SPE [11]. The PSA phase is a modified silica with ethylenediamine functional groups. It retains polar matrix compounds strongly and reduces matrix effects considerably.

Pressurized Liquid Extraction (Accelerated Solvent Extraction, ASE) has been used to extract multiple mycotoxins including ergotamine, ergocristine, ergocryptine, ergocornine, and ergonovine from grains [12]. Ground grain was mixed with drying matrix and packed into the sample cylinder. Mycotoxins were extracted with 90 % aqueous acetonitrile at 100 °C and a pressure of 10.3 MPa. Two sequential 5 min extraction steps were used. Following ASE the extracts were kept at +4 °C overnight when proteins were precipitated. An aliquot of the filtered and evaporated solution was dissolved in 50 % aqueous acetonitrile for LC-MS/MS.

2.4 Sample Cleanup

Solvent extracts usually need to be purified ("cleaned-up") prior to separation and detection. This can be achieved by liquid–liquid partitioning, column chromatography on silica gel, SPE typically using a strong cation exchange mode based on acid–base partition. Reasonably clean biological samples such as urine or plasma can be processed using SPE with considerably less prior treatment.

Cleanup can be achieved by partitioning of the alkaloids between immiscible solvents, including salting out techniques [13], following which dissolution in a polar solvent mixture and a simple wash with hexane produced a suitable sample for LC-MS/MS.

Various types of SPE have been applied, including the use of silica, basic alumina, cation ion exchange, C18 or mixed-mode cartridges [14, 15]. Ergot alkaloids can be isolated using short columns of silica gel or a commercially available chemically modified silica gel (Ergosil®) which will preferentially adsorb ergopeptine alkaloids such as ergotamine and ergovaline from plant extracts. However materials based on silica do not retain –inine isomers. Commercial “push-through” SPE cleanup columns are available for ergot alkaloids. They can reduce the time required for cleanup to less than 1 min.

2.5 Immunological Methods

Radioimmunoassays have had little use in ergot alkaloids analysis but ELISAs have been used quite extensively [16–19]. ELISA techniques are widely used in mycotoxin screening. An antibody specific for a particular antigen is immobilized on a solid support bound to another antibody specific to the same antigen. A multi-welled plastic microtiter plate is usually used. A detector antibody covalently linked to an enzyme (such as alkaline phosphatase) is added to form a complex with the antigen. The support is washed between steps to remove unbound material. The support is developed by adding an enzymatic substrate (such as *p*-nitrophenyl-phosphate) to produce a visible signal, which can be determined colorimetrically. For quantification standard curves are constructed using standards of the target analytes, preferably in a matrix extract based solution.

Both polyclonal and monoclonal antibodies have been used. Antibodies produced by exposing murine hybridoma cell lines to lysergol as the hapten have some cross-reactivity to most ergot alkaloids, except those such as dihydroergocornine and bromoergocryptine that have no substitution at the 9,10 double bond of the lysergic acid ring which changes the three-dimensional conformation of the ring.

ELISAs can screen many samples in one batch without the need for costly and complex instrumentation. They are therefore popular for screening agricultural crops and grains. They are fairly are nonspecific due to cross-reactivity, which can be high for one group of ergot alkaloids and low for another. Their specificity varies according to the antibody used. Polyclonal antibodies have been developed to bind to peptide ergot alkaloids such as ergotamine, ergostine, and ergocristine that have a phenylalanine substituent, but monoclonal antibodies have much more scope as they recognize the ergoline ring structure.

The lack of specificity is due in part to differences in the binding ability of different compounds to the antibody. Monoclonal antibodies generally bind to the lysergic acid ring of ergot alkaloids. An analysis targeted at a single compound such as lysergic acid or ergovaline is handicapped by the fact that other compounds of related structure can also bind and contribute to the nonspecific signal produced at the colorimetric measurement stage. Where classes of ergot alkaloids are targeted different binding affinities can give erroneous results. Ergocryptine, ergocristine, ergocornine, and ergotamine have the lysergic acid main ring structure but do not bind strongly to some antibodies due to the blocking effect of the large side groups on their molecules.

Competitive inhibition ELISAs are commonly used to detect ergovaline and clavine alkaloids in infected tall fescue grass [17]. Some comparisons have shown ELISAs to be in good agreement with LC-MS, but others have failed to do so. The lack of specificity means that the quantities of ergot alkaloids measured are often greater than the total amounts of ergot alkaloid content measured by chromatographic procedures in which individual compounds are quantified.

ELISA methods have LODs above 0.01 mg kg^{-1}, and reliable LOQs of 0.1 mg kg^{-1} or above. The method gives results for the “total alkaloids” and is best suited for providing a rough estimate of the contamination of food or feed. Some ELISA kits are available commercially today [20, 21]. They are often applied to the determination of ergovaline in tall fescue or in the urine of animals that have eaten infected fescue hay. The kit manufacturer claims a LOD of about 2 μg kg^{-1} total ergot alkaloids in plant tissue.

2.6 Thin Layer Chromatography

Early analyses of ergot alkaloids were carried out using paper chromatography, however, this was soon superseded by TLC methods based on silica gel or alumina supported on glass plates. TLC methods are inexpensive and relatively rapid, and they can often be carried out in the field, although they perform best with sclerotia or fungal cultures. Alkaloids can be quantified to some degree while on the plate, but are more often the spot and its supporting medium is excised from the glass plate and the alkaloids eluted from the medium and reacted with reagents that form colored derivatives that can be measured against a standard colorimetrically. TLC methods can separate isomers and have low limits of detection. Their major disadvantages are the inability for automation, a certain lack of specificity, and interference from other components of food and feed matrices.

Stationary phases of silica gel are most often used but alumina is a suitable alternative. A wide range of development solvents have been reported, many of which use chemicals (benzene, chloroform) considered undesirable today on account of their toxic properties. Mixtures of solvents are invariably required, such as chloroform/methanol; benzene/chloroform/ethanol; ethyl acetate/dimethylformamide/ethanol; benzene/acetone/diethyl ether; chloroform/acetone/acetic acid; and chloroform/ethanol.

Fluorescent ergot alkaloid spots can be identified by examination of the plate under UV light. The double bond at C9-C10 is responsible for the fluorescent properties enabling the detection of the major ergot alkaloids found in grain, but excluding clavine alkaloids. Excitation of the molecule is brought about by irradiation with light of 254, 313, 325, or 366 nm wavelength, and the fluorescence emission has a wavelength of 445 nm. Using automated detection and scanning instruments small quantities (15–100 ng of ergonovine and 50–200 ng of peptide alkaloids) can be measured. However, TLC plates are more often sprayed with Ehrlich's reagent (*p*–dimethylaminobenzaldehyde) or van Urk's reagent (described above) which produces a blue-colored product on reaction with the indole ring [22]. Examples of many other chemicals that can be used as spray reagents for ergot alkaloids can be found in the literature, including sodium 1,2-naphthoquinone-4-sulphonate in ethanol:water which gives red-purple spots on a light pink background, or 2-nitroso-1-naphthol followed by 10 % acetic acid which gives blue-black spots on a yellow background after heating.

TLC is restricted by its inability to separate the major alkaloids from each other, and is thus best used to get a picture of the overall contamination level where expense is an issue.

2.7 Gas Chromatography (GC) and Gas Chromatography–Mass Spectrometry (GC-MS)

Ergopeptide alkaloids decompose at the high injector temperatures required for common gas chromatographic (GC) split/splitless systems and the method has less value than LC methods for many applications [23].

LSD and *iso*-LSD can be measured in urine by GC-MS in electron impact (EI) mode [24]. Derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA) is required to reduce the polarity of the LSD and increase its volatility. After solvent extraction and SPE and solvent partition cleanup the LSD was reacted with BSTFA at 70 °C for 15 min and analyzed by GC-MS in selected ion monitoring mode. Some loss of derivatized LSD occurred on GC. The overall recovery was found to be 69 % and the LOD was 50 ng L^{-1}. The LOD of the procedure is 50 ng L^{-1}.

Limited experiments have used GC-MS to identify the amino acids of the ergot peptides following hydrolysis. GC is in practice restricted to the determination of clavine alkaloids and lysergic acid amides, as in the determination of LSD in forensic samples. LSD is derivatized at the N1 position with trifluoroacetic anhydride or trimethylsilylating reagents to decrease its polarity and increase volatility for GC-MS.

2.8 Liquid Chromatography

High-performance liquid chromatographic (HPLC) procedures using reversed-phase columns and detection by ultraviolet (UV) light, fluorescence detection (FLD),

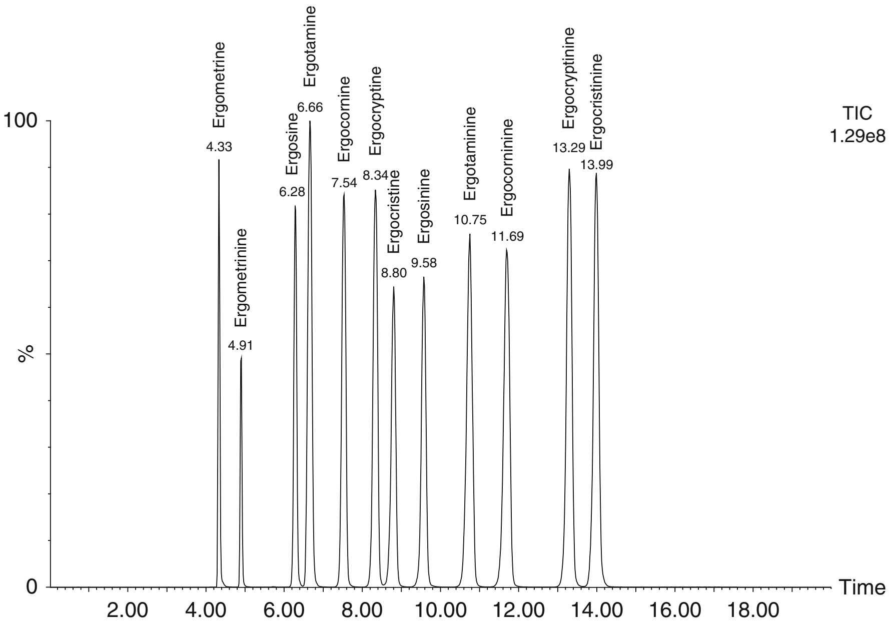

Fig. 35.3 LC-MS/MS chromatogram for the six major ergot alkaloids and their epimers (From Krska and Crews [7]. Reprinted by permission of the publisher (Taylor & Francis Ltd, http://www.tandf.co.uk/journals))

and mass spectrometry (MS) are ideally suitable for determination of ergot alkaloids in extracts of plant, cereal, and body tissues. The six major ergot alkaloids identified as being of concern by EFSA (ergometrine, ergotamine, ergocornine, ergocryptine, ergocristine, ergosine) and their respective -inine isomers are the major targets of analysis by LC methods. The epimeric forms are fairly readily separated by several mobile phase gradient programs. The ergot alkaloid content of food and feed samples can be reported as the sum of the individual alkaloids (the total alkaloid content) or as concentrations of the individual alkaloids. Figure 35.3 shows the separation obtained for the six major ergot alkaloids and their epimers.

Reversed-phase LC with C8, C18, or phenyl-hexyl stationary phases is universally used for ergot alkaloids, with isocratic or gradient mobile phases. The physical size and particle size of LC columns has decreased considerably in recent years, accompanied by an improvement in resolution and sensitivity and a reduction in analysis time. The use of ultrahigh-performance liquid chromatography columns (UHPLC) of short length and small particle size enables the confident separation of the major ergot alkaloids and their (R)- and (S)-epimers within in a run time of less than 10 min. Under alkaline mobile phase conditions the individual ergopeptines are eluted before the corresponding ergopeptinines.

The mobile phases used depend on both analytes and detection method. Those used most frequently are based on mixtures of water with methanol or acetonitrile made alkaline by the addition of ammonium carbonate, ammonium carbamate, ammonium hydroxide, or triethylamine. However, acidic mobile phases are preferred by some because many silica-based LC phases are degraded at high pH, but they do not permit the monitoring detection of both epimeric forms. Volatile weak acids are used in LC-MS to promote the ionization of basic compounds in electrospray positive mode.

Isocratic separation are preferred for UV detection to avoid drift in the baseline signal as the solvent changes. Solvents for this mode have included mixtures of acetonitrile with aqueous ammonium carbonate and of acetonitrile with a phosphate buffer. Ion pairing agent additives such as heptanesulfonic acid have been used. The nonvolatile components of some solvents used with UV detectors cannot be used in LC-MS methods where the solvent is fully evaporated in the instrument.

Epimeric conversion can take place during LC runs. This can be a real problem when large numbers of samples are stored in autosampler racks for continuous batch analysis at room temperature. In the usual solvents such as acetonitrile with aqueous ammonium carbonate epimerization from 4 % to 6 % of some ergot alkaloids over 18 h has been observed.

2.9 Liquid Chromatography-Fluorescence Detection (LC-FLD)

UV detection can be used to detect ergot alkaloids. Ergopeptines and ergopeptinines absorb at wavelengths of 310 nm and dihydroergopeptines at 280 nm, but LODs are relatively low (~0.1 mg kg^{-1}) compared to FLD, which is used far more frequently.

In LC-FLD, the analyte molecule is exposed to an excitation beam of light usually of wavelengths of 235–250 nm. The molecule absorbs a photon and is excited from its ground electronic state to higher vibrational states. Vibrational energy is lost in collisions with other molecules and the analyte molecule drops to one of several vibrational levels in the ground state, emitting a photon in the process. The photons emitted have a range of energies and thus frequencies can be detected by scanning in a spectrophotometer set to monitor specific emission wavelengths. LC-FLD has high sensitivity, selectivity, and repeatability [15, 25].

Response factors for different ergot alkaloids differ under fluorescence detection, that of ergometrine being about twice that of the other major ergot alkaloids, and so careful use of standards is required. Fluorescence detection used regularly for determining ergot alkaloids in sclerotia, endophyte-infected tall fescue grasses, cereal grains, and cereal-based foods.

Excitation wavelengths are in the range 235–250 nm or 310–360 nm, and emission wavelengths are in the range 410–420 nm for 9, 10-ergolenes. For 8, 9-ergot alkaloids and saturated D-ring ergot alkaloids the maximum fluorescence signal is obtained with an excitation wavelength of 272 nm and a detection wavelength of 371 nm. Lysergic acid has been measured in urine by LC-FLD with an excitation wavelength of 250 nm and an emission wavelength of 420 nm.

LC-FLD methods have good sensitivity with detection limits for individual ergot alkaloids in grains and grain foods of 0.01–0.5 mg kg^{-1}. The fluorescence response for ergometrine is about twice that of the other major grain ergot alkaloids. The identity of compounds measured as ergot alkaloids can be confirmed by heating the extract in dilute acetic acid, and monitoring the expected increase in the proportions of the ergopeptine –inine isomers.

2.10 Liquid Chromatography–Mass Spectrometry (LC-MS)

Sample preparation for LC-MS comprises cleanup of organic solvent extracts using the procedures described earlier, or simple dilution of the extracts and injection without cleanup, a procedure known as “dilute and shoot.”

The two major LC-MS techniques, single stage mass spectrometry (LC-MS) and LC tandem MS (LC-MS/MS), are used in ergot alkaloid determination, especially for animal feed and foodstuffs. Both LC-MS and LC-MS/MS have many advantages over other analytical methods for ergot alkaloids. They can be applied to all common ergot structures and unlike LC-FLD are unaffected by saturation at the C-10 position of the lysergic ring system.

The ionization mode most effective in ergot alkaloid analysis is positive electrospray ionization, which produces more intense protonated molecular ions than the deprotonated molecular ions that result from negative ionization, and this has become the preferred ionization method. When ergot alkaloids are ionized in positive electrospray mode the ions formed are predominantly the protonated molecular ions [M + H] + ions, with little evidence of adduct formation with ions of sodium, potassium, or ammonia, despite the ubiquitous presence of sodium in aqueous mobile phase solvents and the addition of comparatively high levels of ammonium salts.

LC-MS can be operated in scanning mode to obtain spectra of the alkaloids and is useful in identifying new compounds [26] but as the ionization methods are “soft” little fragmentation occurs. Focusing on a range of single ions (selected ion monitoring, SIM) increases sensitivity considerably and allows a number of alkaloids to be detected and measured, including coeluting compounds of different mass.

LC-MS/MS is more sensitive and selective than LC-MS. In tandem mass spectrometry a characteristic ion from the initial fragmentation is focused into a chamber where collisions with neutral atoms or molecules induce fragmentations to form product ions. Ergopeptides and lysergic acid ions form characteristic product ions at m/z 223 and 208 [27]. By selecting one or more product ions in the second mass analyzer and scanning in the first mass analyzer the precursor ions can be identified. This technique can be used be used to detect unknown ergot alkaloids in sample extracts. Studies of fragmentation patterns of ergot alkaloids have allowed the mass spectra of related compounds for which standards are not available to be predicted.

A mass spectrometric mode called selected reaction monitoring (SRM) is very useful for low level quantification of ergot alkaloids [28, 29]. In this approach, the first mass analyzer is set to monitor a specific ion derived from the target analyte, while the second mass analyzer measures a specific collision-induced transition of that ion. The resulting signal is very specific on account of the formation route, and the LOD is very low because of the lack of background signal. Ideally, further transitions are used (multiple reaction monitoring MRM) as one SRM transition or in some cases two transitions might not be sufficient to identify a compound with confidence. In MRM modes a principal transition is used for quantification and "qualifier" transitions for confirmation of identity. The relative intensities of the ions measured must agree within certain limits for the identity confirmation to be accepted. In a determination of LSD by LC-MS/MS in the SRM mode a minimum of three transitions were required for positive identification and elimination of a false positive, supported by the absence of *iso*-LSD in the sample.

Fragmentation experiments such as precursor ion scans using triple-stage quadrupole and ion trap mass spectrometers methods provide insights can identify common and characteristic product ions and allow the detection of minor ergot alkaloids not included in routine analyses today. Ion trap detection allows several fragmentations (MSn experiments) that can enhance the ability to elucidate the structures of unknown ergot alkaloid derivatives.

After epimerization at the C-8 position of the lysergic ring system the two epimers that are produced can be resolved chromatographically on LC systems, but on LC-MS the different epimers differ in the relative intensities of the two common ergot alkaloid fragments ions at m/z 223 and m/z 208. This fact can be used to distinguish between the epimers but it introduces an error requiring that both peaks be quantified if LC-MS/MS in SRM mode is used as a means of quantification. In addition, dehydration and hydrogenation of the analytes is suspected to occur during sample workup.

Mass spectrometric detection has a great advantage that all known ergot alkaloids can potentially be determined in one run and chromatographic coelution is not a problem for compounds having different MS/MS transitions. Furthermore, analogs not available as reference standards can be tentatively identified.

A currently remaining problem in resolution is the occasional coelution of some ergot alkaloids. β-ergocryptine and ergocristine coelute under some conditions, leading to errors in the quantification of both when they are detected by FLD. LC-MS/MS methods can distinguish between these compounds as they have different molecular masses. The situation is less satisfactory for the isomers α- and β-ergocryptinine, which are not separated on LC and have identical masses, and so LC-MS cannot tell them apart.

2.10.1 Matrix Effects

Matrix effects are frequently seen in LC-MS. These occur when compounds in the sample are extracted along with the ergot alkaloids and pass through any cleanup stages with the alkaloids. The co-extracted compounds are often of much higher concentration than the alkaloid, increasing the likelihood of coelution on chromatographic systems. Coelution leads to enhancement of ionization or more often to its suppression. Matrix effects can be reduced by dilution of the extract or by more specific cleanup such as ion exchange. Signal suppression in quantitative analysis is compensated for by preparing the calibration standards in a blank matrix extract known to be free of target analytes, such standards are known as “matrix-matched.”

LODs and LOQs are low for modern LC-MS and LC-MS/MS methods with LODs for the individual compounds measured in various cereal matrices ranging from 0.05 to 0.4 μg kg^{-1} and LOQs for the ergopeptine-isomers and ergometrine ranging from 0.2 to 2.8 μg/kg. Lower LOQ (0.01–0.06 μg kg^{-1}) have been achieved for five ergopeptines in wheat with UHPLC-MS/MS [12].

Very few comparisons based on the same samples have been made of LC-MS and ELISA methods, but for measurements of ergovaline in samples of seed and straw there was poor agreement with no consistent pattern [17]. The issue is important because some LC procedures determine ergovaline but do not detect simpler ergoline alkaloids such as lysergic acid, D-lysergic acid amides, lysergol, and ergonovine.

2.11 Alternative Methods

Capillary zone electrophoresis with UV detection has been used to separate ergometrine, ergotamine, and their epimers [30]. The addition of cyclodextrins in phosphate buffer at pH 2.5 was required to resolve five ergopeptine alkaloids and their epimers. Supercritical fluid chromatography has also been used with UV detection at 280 nm or mass spectrometry to identify clavine alkaloids in extracts of *Claviceps purpurea*.

Rapid lateral flow immunoassays for ergot alkaloids have been developed and are undergoing characterization and improvement [31]. The lateral flow devices are in the form of dipstick tests that can be used in the field. Following a rapid solvent extraction the stick is dipped into the extract, the solution migrates through a region containing the antibody conjugate, the bound sample-conjugate then flows to a “strip” area where dynamic control lines define positive and negative results by color changes, indicates the presence or absence of the alkaloid. A dipstick has been produced that reacts to ergotamine, ergocristine, and ergosine. The 50 % inhibitory concentration (IC_{50}) of the best polyclonal antibodies for ergotamine is 76 ng ml^{-1}, and cross-reactivity profiles determined by ELISA were ergotamine: 100, ergocristine: 70, ergosine: 5.

A novel approach to identifying the presence of ergot alkaloid has been to quantify the fatty acid ricinoleic acid, (R)-12-hydroxy-(Z)-9-octadecenoic acid [32]. The acid is not usually found in grain but is a major component of the lipid portion of *Claviceps*. The lipid extracted from sclerotia can be analyzed for the ricinoleic acid by GC with flame ionization detection after transesterification and derivatization of the hydroxy group by silylation. There is no correlation between the content of ricinoleic acid and the ergot alkaloids and so quantitative levels of ergot alkaloids cannot be provided by this method.

2.12 Multianalyte Methods

Multianalyte methods used to measure substantial numbers of mycotoxins in feed and food have often included ergot alkaloids [12, 33–35]. A multianalyte LC-MS/MS method without cleanup has been used to measure 87 different mycotoxins including 25 ergot alkaloids with LODs of 0.02–1.2 μg kg^{-1}. A similar approach has been used to perform a semiquantitative survey that included all 12 of the major *C. purpurea* ergot alkaloids and also another 12 other ergot alkaloids from different other Claviceps species.

These methods invariably have extraction and cleanup procedures that are not optimal for all analytes and for ergot epimerization, low recoveries, and higher LODs are more likely. They typically involve extraction with acetonitrile, evaporation of the solvent, and reconstitution in the LC mobile phase solvent. The detection method is by necessity LC-MS or LC-MS/MS.

2.13 Quantification Methods

Quantification methods can be based on standard additions or external calibration. Internal standards may be used to correct for some losses of material but for many analyses suitable compounds are unavailable. In LC-MS and LC-MS/MS the ideal internal standards are stable isotope analogs of the target compounds as these behave exactly as the native compound used but can be distinguished by their different masses. For LSD-related compounds with slightly different structures such as methysergide have been used, but trideuterated LSD is available commercially.

2.13.1 Purity and Stability of Standards

Currently commercially available standards have purity levels that can be considered good for mycotoxins [11]. LC-MS estimation of impurities in a typical batch of the six major ergot alkaloids and their epimers which did not account for non-ergot constituents such as salts showed purities considerably above 98 % apart from ergocristinine (94 %), ergosine (96 %), and ergosinine (96 %). Ergocristinine contained 1.7 % ergocristine, 0.8 % ergocorninine, and 3.3 % ergocryptinine. Ergosine contained 2.7 % ergotamine and 0.9 % ergocryptine. Ergosinine contained 2.0 % ergosine and 2.5 % ergotaminine.

Ergot alkaloids are more stable in solvents that do not participate in hydrogen bonding (aprotic solvents) whether these are organic, aqueous, or mixtures. Extracts of cereals can be kept refrigerated in the dark overnight without significant epimerization. After about 2 weeks under these conditions epimerization of ergocornine, ergocryptine, and ergocristine is apparent, with up to 50 % of ergocornine being altered. A decrease of the –ine forms is accompanied by an equivalent increase in the corresponding -inines. In practice it is advisable to store all extracts and solutions of standard frozen in the dark.

3 Conclusion

Analytical methods have so far concentrated mainly on ergot alkaloids from *C. purpurea* and few data are available for alkaloids from other *Claviceps* spp. infecting grain. Only a small number of all ergot alkaloids are well characterized, and several unknown compounds including bound forms might contribute to the health hazard posed by food and feed contamination.

There are as yet unresolved problems in the quantitative determination of ergot alkaloids in biological and environmental matrixes because of the reactivity of the alkaloids and the poor stability of suitable standards, especially in both isomeric forms. Similarly there are no certified matrix reference materials available, and only one proficiency study for the determination of ergot alkaloids is in regular use. The performance of many non-LC methods has not been well characterized. Only a single international ring trial of LC methods has been carried out [36]. In this study 27 participants analyzed 6 cereal flour test materials and 2 control solutions using a method based on extraction with ethyl acetate/methanol/25 % ammonia, cleanup using aluminum oxide SPE, and LC-FLD. The performance of the method was considered suitable for its proposal for use as an official method and its use in surveys. After elimination of 5 % of the results (mostly from four participants) as outliers, the relative repeatability for all analytes in flour was less than 10 % and the relative reproducibility was between 6.3 % and 18.9 %. LOQs ranged from 0.08 to 3.30 μg kg^{-1} [25].

The three major approaches of ELISA, LC-FLD, LC-MS are likely to continue to be applied to the currently increasing demand for information on occurrence. ELISA techniques are limited in the information provided and as they give variable between-run results, they are therefore best used as semiquantitative tools. Only LC-FLD and LC-MS/MS enable the identification and quantification of the major ergot alkaloids and their epimers. Mass spectrometry is required. LC-MS and LC-MS/MS methods provide unequivocal identification of the alkaloids, and the ability to obtain structural information through collision-induced fragmentation greatly enhances its usefulness for confirmation and elucidation of structure confirmation. LC-MS/MS methods therefore could be preferred to LC-FLD; however, the capital costs of equipment are higher.

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Analysis of Alkaloids by Capillary Electrophoresis

36

Roberto Gotti

Contents

1 Introduction to Capillary Electrophoresis: Background and Instrumentation 1155
1.1 Electroendosmosis 1156
2 Capillary Zone Electrophoresis in Analysis of Alkaloids 1157
2.1 Effect of pH of the Running Buffer in CZE 1165
2.2 Effect of Nature and Concentration of the Running Buffer in CZE 1168
2.3 Effect of the Presence of Organic Solvents in CZE Running Buffer 1169
2.4 Effect of Buffer Additives in CZE 1171
3 Nonaqueous Capillary Electrophoresis, NACE 1174
4 Micellar Electrokinetic Chromatography (MEKC) and Microemulsion Electrokinetic Chromatography (MEEKC) 1177
5 Capillary Electrochromatography (CEC) 1181
6 Capillary Electrophoresis–Mass Spectrometry (CE-MS) 1182
7 Detection Sensitivity Improvement in Analysis of Alkaloids by CE 1185
7.1 Chemiluminescence Coupled to CE 1185
7.2 Online Sample Preconcentration 1189
8 Conclusion 1191
References 1192

Abstract

Capillary electrophoresis (CE) is a useful tool in phytochemical analysis, and it meets the requirements for the quality control of herbal drugs because of its versatility and high separation power. The wide opportunity for selectivity tuning allows the analysis of molecules with a wide range of polarity and molecular weight; in addition like high-performance liquid chromatography (HPLC), the method is suitable for hyphenation with mass spectrometry (MS).

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In this chapter, an overview on the most relevant aspects of the analysis of alkaloids by electromigration methods, namely, capillary zone electrophoresis (CZE), nonaqueous capillary electrophoresis (NACE), micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC), and capillary electrochromatography (CEC), is given. Selected recent applications of CE in analysis of alkaloids are summarized in a table following the classification according to the chemical nature of the considered compounds.

Keywords
Capillary electrochromatography (CEC) • capillary zone electrophoresis (CZE) • mass spectrometry (MS) • micellar electrokinetic chromatography (MEKC) • microemulsion electrokinetic chromatography (MEEKC) • nonaqueous capillary electrophoresis (NACE)

Abbreviations

1B-3MI-TFB	1-butyl-3-methylimidazolium tetrafluoroborate
1E-3MI-TFB	1-ethyl-3-methylimidazolium tetrafluoroborate
ACN	Acetonitrile
BGE	Background electrolyte
CD	Cyclodextrin
CD-MEKC	Cyclodextrin-modified *MEKC*
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CZE	Capillary zone electrophoresis
DM-βCD	Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin
DTAF	5-(4,6-dichloro-*s*-triazin-2-ylamino)fluorescein
EC	Electrochemical detection
ECL	Electrochemiluminescence
ED	Electrochemical detector
EKC	Electrokinetic chromatography
EOF	Electroosmotic flow
ESI	Electrospray
FASS	Field-amplified sample stacking
FL	Fluorescence
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HP-βCD	Hydroxypropyl-β-cyclodextrin
LED	Light-emitting-diode-induced fluorescence
LIF	Laser-induced fluorescence
LOD	Limit of detection
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MS	Mass spectrometry
MS-SCX	Monolithic silica–strong cation exchange

NACE	Nonaqueous capillary electrophoresis
NBD-Cl	4-chloro-7-nitrobenzo-2-oxa-1,3-diazol
NBD-F	4-fluoro-7-nitro-2,1,3-benzoxadiazole
NIST	National Institute of Standard Technology
NMF	*N*-methylformamide
NMR	Nuclear magnetic resonance spectroscopy
PCA	Principal component analysis
PMT	Photomultiplier tube
SDS	Sodium dodecyl sulfate
SPE	Solid-phase extraction
SRM	Standard reference material
TCM	Traditional Chinese medicine
THF	Tetrahydrofuran
TOF	Time of flight
U.S. FDA	U.S. Food and Drug Administration
αCD	α-cyclodextrin
βCD	β-cyclodextrin

1 Introduction to Capillary Electrophoresis: Background and Instrumentation

Capillary electrophoresis (CE) is a separation technique that employs the basic concepts and separation mechanism of conventional electrophoresis in a capillary format. The core of the CE system is in fact a capillary, normally in fused silica, with an internal diameter of 50–100 μm that is filled with the buffer solution (defined as the background electrolyte (BGE)) to be used for separation of the charged molecules.

As in conventional electrophoresis, the separation occurs on the basis of the different rates of the ions through the capillary, resulting by their different charge-to-mass ratio. The electrophoretic mobility μ_{ep} describes the speed of movement of the charged molecule in the capillary, and it is given by

$$\mu_{ep} = q/(6\pi\eta r) \tag{36.1}$$

where q is the number of ionic charges in the molecule, η is the viscosity of the buffer solution, and r is the ionic radius. Therefore, when a mixture of ions possessing the same charge undergoes to the electrophoretic process, the smaller will migrate faster than the larger.

The electrophoretic mobility can also be expressed as a function of the zeta potential of the ion (ζ_i), the dielectric constant (ε), and viscosity of the solvent, according to

$$\mu_{ep} = 2\varepsilon\zeta_i/(3\eta) \tag{36.2}$$

Interestingly, the anticonvective properties of small internal diameter tube improve the resolution of the electrophoretic process because of the limited longitudinal diffusion of the analytical zones. Furthermore, in this format, high electric fields can be applied for fast separations, generating only modest Joule heating, and finally, small amounts of both analytes and buffer solutions are required.

The separated components can be conveniently monitored by UV on-capillary detection as they migrate in a portion of the capillary exposed to a monochromatic UV light beam. Using this approach the band broadening occurring in high-performance liquid chromatography (HPLC) as due to the post-separation mixing of the analytical zones eluting from the end of column is avoided. The UV absorption occurs according to the UV spectrum and concentration of the analytes; alternative detectors used in online CE analysis are fluorescence (FL), laser-induced fluorescence (LIF), and electrochemical detector (ED). In addition, the hyphenation of CE and mass spectrometry (CE-MS) is frequently used in CE analysis of phytochemical substances to achieve structural information on the separated analytes [1–3].

1.1 Electroendosmosis

The inner capillary fused-silica wall contains silanol groups (-Si-OH) that behave as weak acids; when the pH values of the background electrolyte used for CE separation are higher than 3, the dissociation of silanols generates a negatively charged inner capillary surface. The positive ions of the BGE are attracted to the fixed negative charge of the capillary to maintain the electroneutrality, and an electrical double layer is formed. The application of the high voltage for the electrophoretic run causes the migration of the hydrated cations of the double layer toward the cathodic end of the capillary thus resulting in a flow of the bulk solution contained in the capillary. The flow, defined as electroosmotic flow (EOF), is related to the charge of the inner capillary surface, and it is dependent on the pH of the buffer, its concentration, nature, and viscosity, as described by the following equation:

$$\mu_{\mathrm{eo}} = \varepsilon\zeta/(2\pi\eta \mathrm{d}) \qquad (36.3)$$

where μ_{eo} is the electroosmotic mobility (the rate of the EOF), ζ is the zeta potential (charge on the inner capillary surface), η is the viscosity of the running buffer, and d is the capillary diameter.

In the presence of a stable and strong EOF (generally obtained using alkaline buffers), the negatively charged molecules of a given sample are swept toward the cathodic end because their electrophoretic mobility is often lower than the electro-osmotic mobility. This condition allows for the simultaneous analysis toward the cathodic end of the capillary of both anionic and cationic species, whose differential migration driven by the difference in charge-to-mass ratio is still maintained [1–3].

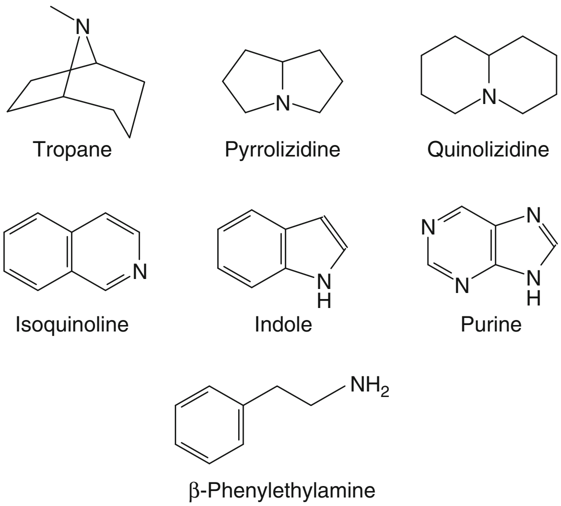

Fig. 36.1 Backbone structure of the principal alkaloids

The success of CE is due in large part to the wide range of separation modes available that allow for the application of different operating conditions often resulting in different separation selectivity. This feature makes CE suitable in analysis of molecules with a wide range of polarity and molecular weight, and because of its versatility and high separation power, the technique meets the requirements for the quality control of herbal drugs, phytopharmaceuticals, and natural products [4, 5]. The principal separation methods that have been applied in analysis of alkaloids are capillary zone electrophoresis (CZE), nonaqueous capillary electrophoresis (NACE), micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC), and capillary electrochromatography (CEC). Each of these approaches will be discussed with selected examples of their application to the analysis of alkaloids in plants, herbal drugs, and natural products. Based on their general structures, alkaloids are divided into several subgroups, and the most important of them are reported in Fig. 36.1. In the Table 36.1, selected examples of application of CE from the recent literature are summarized following a classification according to the alkaloids structure.

2 Capillary Zone Electrophoresis in Analysis of Alkaloids

Capillary zone electrophoresis, CZE, is the simplest CE mode in which the separation is based on differences in the charge-to-mass ratio of the analytes. This approach can be effective in a number of applications; in particular, quaternary alkaloids owing to the permanent charge are ideal solutes in CZE, regardless of the pH of the running buffer. Below, the most important parameters affecting the CZE separation will be considered with emphasis on the relevant aspects involved in analysis of alkaloids in herbal drugs and medicinal plants.

Table 36.1 Application of electromigration techniques to the analysis of alkaloids

Alkaloids	Sample	Separation conditions	Detection	References
Tropane alkaloids				
Calystegine	*Solanaceae*	20 mM histidine, 20 mM BES, 20 % methanol	Indirect UV at 300 nm	[22]
		80 mM tetraborate buffer, pH 9.2	UV at 191 nm	[21]
Atropine	*Cannabis sativa*	20 mM ammonium acetate, pH 8.5	CZE-ESI-MS	[109]
Atropine, belladonnine, norhyoscyamine, apoatropine, hyoscyamine, 6β-hyoscyamine, scopolamine	*Atropa belladonna*	60 mM ammonium acetate (pH 8.5), 5 % isopropanol	CE-ESI-TOF-MS	[9]
Atropine, scopolamine, anisodamine	*Flos daturae*	20 mM phosphate (pH 7.0), 4 mM βCD	ECL	[43]
		20 mM phosphate, pH 8.48	ECL	[8]
		NACE: 1 M acetic acid, 20 mM sodium acetate, 2.5 mM tetrabutylammonium perchlorate in acetonitrile:2-propanol (4:1, v/v)	Dual ECL/EC	[120]
3α-Senecioyloxy-7β-hydroxytropane	*Schizanthus grahamii*	NACE: 1 M trifluoroacetic acid, 25 mM ammonium formate in methanol:ethanol (40:60, v/v) or methanol:tetrahydrofuran (80:20, v/v)	UV at 220 nm and ESI-MS	[66]
3α-Hydroxy-7β-senecioyloxytropane				
3α-Hydroxy-7β-angeloyloxytropane				
3α-Hydroxy-7β-tigloyloxytropane				
Phenylethylamine alkaloids				
(−)-Ephedrine, (+)-pseudoephedrine	*Ephedra* supplements	25 mM phosphate-triethanolamine (pH 2.5), 7.5 % highly sulfated βCD	UV at 190 nm	[54]
(−)-Ephedrine, (+)-pseudoephedrine, (−)-*N*-methylephedrine	*Ephedra sinica* and phytopreparations	20 mM Tris–phosphate (pH 2.5), 20 mg/mL DM-βCD, 5 % tetrabutylammonium chloride	UV at 210 nm	[57]
(−)-Ephedrine, (+)-pseudoephedrine	SRM supplements	BGE: 25 mM phosphate buffer (pH 2.5)	UV at 210 nm	[56]
		Method A: 2.8 % sulfated βCD + 1.2 % DM-β CD		
		Method B: 4 % DM-βCD		
		Method C: 4 % HP-βCD		

(−)-Ephedrine, (+)-pseudoephedrine	*Ephedra* herb phytopreparations	NACE: 80 mM ammonium acetate, 3 % acetic acid in methanol	LIF ex 488, em 520	[64]
		MEKC: 25 mM tetraborate (pH 9.7), 20 mM SDS	LIF ex 488 em 520	[112]
		MEKC: 20 mM tetraborate (pH 9.8), 20 mM SDS, 15 % acetonitrile	LIF ex 488 em 520	[88]
(−)-Ephedrine, (+)-pseudoephedrine, (−)-methylephedrine, (−)-norephedrine, (+)-norpseudoephedrine, (+)-*N*-methylpseudoephedrine	SRM supplements	MEKC: 15 mM ammonium acetate, 35 mM polysodium *N*-undecenoxycarbonyl-L-leucinate, pH 6.0, 30 % acetonitrile	UV and ESI-MS	[91] [92]
(±)-Octopamine, (±)-synephrine, tyramine, *N*-methyltyramine, hornedine	*Citrus* species and related genera	70 mM phosphate (pH 3.1), 40 mM HP-βCD, 9 mM βCD, 8.2 mM DM-βCD	UV at 210 nm	[55]
Aconitine alkaloids				
Aconitine, mesaconitine, hypaconitine, benzoylaconine, benzoylmesaconine, benzoylhypaconine	*Aconitum* roots	200 mM Tris–150 mM perchloric acid, 40 % 1,4-dioxane (pH 7.8)	UV at 214 nm	[33]
Aconitine, mesaconitine, hypaconitine	*Aconitum kusnezoffii A., carmichaelii*	30 mM phosphate, pH 8.4	ECL	[118]
		20 mM 1E-3MI-TFB, pH 9.15	ECL	[121]
		35 mM 1B-3MI-TFB, pH 8.5	UV at 254 nm	[39]
		20 mM phosphate, 35 % acetonitrile, pH 9.5	UV at 235 nm	[32]
Quinolizidine alkaloids				
Matrine, sophoridine, sophocarpine, oxymatrine	*Sophora flavescens* roots and phytopreparations	60 mM borate, pH 8.5	UV at 204 nm	[11]
Sophocarpine, matrine, lehmanine, sophoranol, oxymatrine, oxysophocarpine, cytisine	*Sophora tonkinensis*	50 mM phosphate (pH 2.5), 1 % HP-βCD, and 3.3 % isopropanol	UV at 200 nm	[31]
Sophoridine, matrine, oxymatrine	*Sophora flavescens*	50 mM phosphate, pH 8.4	ECL	[12]

(*continued*)

Table 36.1 (continued)

Alkaloids	Sample	Separation conditions	Detection	References
Sparteine, lupanine, angustifoline, 13α-hydroxylupanine	*Lupinus* species	NACE: 100 mM ammonium formate in methanol/acetonitrile/water 70/20/10, 1 % acetic acid	UV at 210 nm and ESI-MS	[78]
Matrine, sophoridine, oxymatrine, oxysophocarpine, cytisine	*Sophora flavescens* roots	MEEKC: 98.2 % 1 mM tetraborate–2 mM phosphate (pH 6.5), 21 mM sodium cholate, 4 mM Mg^{2+}, 1.2 % 1-butanol, and 0.6 % ethyl acetate	UV-FASS at 200 and 214 nm	[97]
Isoquinoline alkaloids				
Berberine, hydrastine	*Hydrastis canadensis*	CZE: 100 mM ammonium acetate–acetic acid (pH 3.4) and methanol (20:80, v/v)	UV at 225 nm	[17]
Berberine, palmatine, jatrorrhizine, columbamine, epiberberine, coptisine, tetradehydroscoulerine, tetrahydrocheilanthifolinium	*Rhizoma coptidis*	NACE (I): 50 mM ammonium acetate (pH* 6.8) in acetonitrile–methanol (20:80, v/v)	UV at 230, 265, 350 nm	[29]
		CZE (II): 50 mM ammonium acetate (pH 6.8) in acetonitrile–water (50:50, v/v)	ESI-MS	
Berberine, jatrorrhizine, palmatine	*Rhizoma coptid*is, *Caulis mahoniae*, *Cortex berberidis*, *Cortex phellodendri*, *Herba chelidonii*	NACE: 50 mM ammonium acetate, 0.5 % acetic acid in acetonitrile–methanol (10:90, v/v)	UV at 214 nm	[67]
Berberine, jatrorrhizine, palmatine	*Rhizoma coptidis*, *Caulis mahoniae*	NACE: 35 mM ammonium acetate, 0.25 % acetic acid in acetonitrile–methanol (5:95, v/v)	LIF (native fluorescence)	[68]
			Ex 488 nm Em 520 nm	
Berberine, coptisine, palmatine, tetrandrine	*Coptidis rhizoma*	MEKC: 3 mM borate–10 mM phosphate (pH 7.3), 50 mM sodium deoxycholate, 30 % acetonitrile	UV at 270 nm	[95]

Berberine, coptisine, palmatine,	*Coptidis chinensis*	MEKC: 100 mM phosphoric acid, 15 mM SDS, 10 % tetrahydrofuran, pH 1.82	UV at 264 nm with sweeping	[127]
Berberine, jatrorrhizine, palmatine	*Coptidis chinensis*	CEC: strong cation exchange on monolithic silica column. Mobile phase: 12.5 mM phosphate (pH 7.4)–acetonitrile (40:60, v/v)	UV at 263 nm	[104]
Sanguinarine, chelerythrine	*Macleaya cordata*, *Chelidonium majus*	NACE: 40 mM ammonium acetate, acetonitrile–methanol (1:1), acetic acid pH* 5.6	LIF (native fluorescence)	[69]
Chelerythrine, nitidine	*Zanthoxylum nitidum*	NACE: 100 mM sodium acetate (pH 5.0) in methanol:water (4:1, v/v)	UV at 228 nm	[28]
Coptisine, palmatine, *N*-methyllaudanine, allocryptopine, protopine, corycavidine, glaucine, corydine, bulbocapnine, corydaline, corypalmine, tetrahydropalmatine, canadine, thalictrivacine	*Corydalis* species	NACE: 50 mM ammonium acetate, 1 M acetic acid, and 10 % methanol in acetonitrile	ESI-MS	[76]
Protopine, cryptopine, corydamine, sinactine, (+)-adlumine, (−)-α-hydrastine, (+)-corlumine, (−)-fumarophycine, (−)-*O*-methylfumarophycine, (+)-parfumine	*Fumaria officinalis* and phytopreparations	NACE: 60 mM ammonium acetate in acetonitrile–methanol (9:1) and 2.2 M acetic acid	ESI-MS	[75]
Fangchinoline, tetrandrine	Radix *Stephaniae tetrandrae* and phytopreparations	NACE: 50 mM ammonium acetate, 0.5 % acetic acid in acetonitrile–methanol (50:50, v/v)	UV at 214 nm	[70]
Fangchinoline, tetrandrine	Radix *Stephaniae tetrandrae*	Flow injection MEKC: 15 mM acetic acid–15 mM sodium acetate, 3 % Tween 20–5 % methanol at pH 5.5	UV at 254 nm	[93]
Protopine, chelidonine, coptisine, sanguinarine, allocryptopine, chelerythrine	*Chelidonium majus* L.	20 mM phosphate buffer, pH 3.1	UV-LED	[14]

(*continued*)

Table 36.1 (continued)

Alkaloids	Sample	Separation conditions	Detection	References
Pyrrolizidine alkaloids				
Senkirkine, senecionine, retrorsine, seneciphylline	*Kuan donghua* and *Qian liguang*	MEKC: 20 mM borate (pH 9.1), 30 mM SDS, and 20 % methanol	UV at 220 nm	[89]
Senecionine, seneciphylline	*Gynura segetum*	MEKC: 120 mM Tris–35 mM lauric acid (pH not reported) and 20 % methanol	UV at 220 nm	[94]
Senkirkine, senecionine, retrorsine, seneciphylline	*Kuan donghua*	MEKC: 20 mM borate (pH 9.1), 30 mM SDS, and 20 % methanol; analysis using dynamic pH junction-sweeping technique	UV at 220 nm	[131]
Indole alkaloids				
Strychnine, brucine	*S. nux-vomica*	NACE: 30 mM ammonium acetate, 1.0 % acetic acid, and 15 % acetonitrile in methanol	UV at 214 nm	[71]
	Shen jin huo luo wan	NACE: 25 mM Tris–boric acid (pH 4.0) and 40 % methanol in acetonitrile	UV at 214 nm	[72]
		MEKC: 50 mM phosphoric acid (pH 2.0), 100 mM SDS, and 20 % acetonitrile; analysis performed using sweeping technique	UV at 203 nm	[129]
Mesembrine, mesembrenone, Δ^7 mesembrenone, mesembranol, epimesembranol	*Sceletium tortuosum* tablets	CZE: 50 mM phosphate, pH 1.5	UV at 228 nm	[15]
Harmaline, tetrahydroharmine, harmine, harmane, harmol, norharmane	*Psychotria viridis* l	CZE: 200 mM formic acid and 7 mM ammonia in water with 10 % acetonitrile	LIF (ex 266 nm) and CE-MS	[30]
Yohimbine	*Pausinystalia yohimbe*	NACE: 30 mM ammonium acetate, 0.5 % acetic acid in methanol	UV at 220 nm	[65]
Methylxanthine alkaloids				
Theobromine, caffeine	Green tea	CD-MEKC: 25 mM borate–phosphate (pH 2.5), 90 mM SDS, and 25 mM HP-βCD	UV at 200 nm	[86]

Theobromine, caffeine	*Theobroma cacao*	CD-MEKC: 50 mM Britton–Robinson buffer at pH 2.5, 90 mM SDS, and 12 mM HP-βCD	UV at 220 nm	[87]
Theobromine, caffeine	Tea leaves at different fermentation degree	MEKC: 10 mM phosphate, 4 mM borate (pH 7.0), 45 mM SDS, and 0.5 % ethanol	UV-DAD at different wavelengths in the range 200–280 nm	[85]
Caffeine	Decaffeinated coffee	MEKC: 10 mM carbonate (pH 11.0), 50 mM SDS	UV at 206 nm	[83]
Caffeine, theobromine	Beverages (yerba mate, coffee, cocoa, tea)	MEKC: 90 mM borate buffer (pH 8.5), 50 mM SDS	UV at 200 nm	[84]
Caffeine, theophylline	Beverages	CEC: organic–inorganic hybrid silica monolith C18-sulfonic groups using a mobile phase containing 25 mM phosphate–borate (pH 8)–acetonitrile (40:60, v/v)	UV at 254 nm	[105]
Morphinane alkaloids				
Morphine, codeine, oripavine, thebaine	Industrial liquors (from extraction processes)	CD-CZE: 100 mM Tris–phosphate (pH 2.8) + 30 mM HP-βCD	UV at 214 nm	[47]
Morphine, codeine, thebaine	*Papaver* species	CD-CZE: phosphate buffer 100 mM (pH 3.0) + 5 mM αCD	UV at 214 nm	[48]
Morphine, codeine, thebaine, narcotine	Dried poppy samples	CZE: 25 mM borate–80 mM 1E-3MI-TFB, pH 9.18	ECL	[23]
Papaverine, narceine, noscapine, thebaine, reticuline, oripavine, codeine, morphine	Poppy straw samples	MEKC: 50 mM phosphate, 80 mM SDS, and 25 % methanol	UV at 200 nm	[130]
Morphine, thebaine, codeine, papaverine, narcotine	*Pericarpium papaveris*	CEC: hydrophilic/cation-exchange monolith; mobile phase, 5 mM phosphate (pH 4.0)–90 % acetonitrile (v/v)	UV at 224 nm	[103]

(*continued*)

Table 36.1 (continued)

Alkaloids	Sample	Separation conditions	Detection	References
Sinomenine: 7,8-didheydro-4-hydroxyl-3,7-dimethoxy-17–methylmorphinan-6-one	*Sinomenium acutum*	CZE: 50 mM phosphate, pH 5.0	ECL	[116]
	Sinomenium acutum	NACE: 80 mM ammonium acetate, 2.0 % acetic acid, 20 % acetonitrile in methanol	UV at 262 nm	[73]
Miscellaneous				
Vinblastine, catharanthine, vindoline	*Catharanthus roseus*	CZE: 20 mM ammonium acetate, 1.5 % acetic acid	ESI-MS	[111]
Galanthamine	*Bulbus Lycoridis Radiatae*	CZE: 18 mM phosphate at pH 9.0	ECL	[119]
Galanthamine, haemanthamine	*Narcissus* species	NACE: 90 mM ammonium acetate, 0.5 % acetic acid, 25 % acetonitrile in methanol	UV at 280 nm	[74]
Verticine, verticinone	*Fritillariae* species	CZE: 8 mM phosphate–40 mM 1B-3MI-TFB	ECL	[38]
Camptothecin, 9-methoxycamptothecin	*Nothapodytes foetida*	MEKC: 10 mM borate (pH 8.6), 90 mM SDS, and 20 % dimethyl sulfoxide	UV at 369 nm	[90]
Pyrido[1,2-a]azepines: stemofoline, oxystemokerrin, didehydrostemofoline, stemocurtisinol	*Stemona curtisii*, *S. collinsae*, *S. tuberosa*	NACE-MS: 50 mM ammonium acetate, 1 M acetic acid, 10 % methanol in acetonitrile	ESI-MS	[77]

BES = *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
1B-3MI-TFB = 1-butyl-3-methylimidazolium tetrafluoroborate-based ionic liquid
1E-3MI-TFB = 1-ethyl-3-methylimidazolium tetrafluoroborate-based ionic liquid
αCD = α-cyclodextrin
βCD = β-cyclodextrin
DM-βCD = heptakis(2,6-di-*O*-methyl)-β-cyclodextrin
EC = electrochemical detection
ECL = electrochemiluminescence detection
FASS = field-amplified sample stacking
HP-βCD = hydroxypropyl-β-cyclodextrin
UV-LED = UV light-emitting-diode-induced native fluorescence
SRM = standard reference material

2.1 Effect of pH of the Running Buffer in CZE

Alkaloids are cyclic organic compounds containing nitrogen in a negative oxidation state exhibiting pKa values above 5; thus, they can be easily protonated in a wide pH range, and CZE can be considered as the electrokinetic technique of choice for their analysis. In particular, using running buffers at pH values below the pKa, the molecules exist mainly in the protonated water-soluble form [4, 5]. In addition, according to the Henderson–Hasselbalch equation, it can be derived that the ionized base still exists also at pH values well above the pKa; for example, when pH of the electrolyte is pKa + 1, the fraction of the protonated base $[BH^+]/[B]$ is 1:10 [6]. Obviously, the presence of additional basic functions in the molecule of the alkaloid leads to higher charge density (thus higher electrophoretic mobility) whereas acidic functions, because of dissociation, can generate negative charge leading to a reduced mobility. Therefore, alkaloids containing both acidic and basic functions have to be dealt by considering the existence of a critical pH value corresponding to the average of the pKa values of acidic and basic function, i.e., the isoelectric point, p*I*.

Several examples of CZE methods for analysis of the main alkaloid groups show that often the separations are carried out under neutral/alkaline conditions (Table 36.1). A strong and stable EOF is in fact obtained using BGE at pH values higher than 8, thus allowing for fast and reproducible separations [1–3]. In addition, as theoretically demonstrated, the optimum separation selectivity is obtained if the pH value of the running buffer is close to the pKa of the analytes [7]. As an example, atropine and scopolamine, the two active tropane alkaloids found in *Flos daturae*, are characterized by pKa values of 9.7 and 8.2, respectively; their CZE separation could only be obtained at pH higher than 7.5, and the optimum condition was achieved using a BGE at pH 8.5 [8]. Similar conditions were also successfully applied to a more challenging separation of tropane alkaloids (among them atropine, belladonnine, apoatropine, hyoscyamine, scopolamine) in *Atropa belladonna* leaf extracts [9]. Alkaline BGE was used also for separation of the quinolizidine alkaloids oxymatrine, sophoridine, and matrine in *Sophora flavescens* Ait.; in particular, the isomers sophoridine and matrine (Fig. 36.2), exhibiting pKa values of 6.51 and 7.72, respectively [11], could be separated using a phosphate buffer at pH 8.5 [10, 12].

Excellent separation selectivity can be obtained using acidic running buffers as was shown by Stöckgit et al., in analysis of fully protonated alkaloids. The migration order of 15 indole alkaloids under CZE acidic conditions (100 mM ammonium acetate buffer, pH 3.1) was evaluated, and it was found that the separated compounds could be clustered in different groups with decreasing mobility according to the increased molecular mass. Interestingly, the migration velocity of β-methylajmaline despite its higher molecular mass (Mw 341) was found to be much higher than that of ajmaline (Mw 326). Since β-methylajmaline shows a quaternary amine function, it does not be involved in formation of the intermolecular forces between the nitrogen atom and solvent molecules of the acetate buffer used as the medium. This leads to lower solvation degree, and the mass-to-charge ratio decreases accounting for the higher electrophoretic velocity [13].

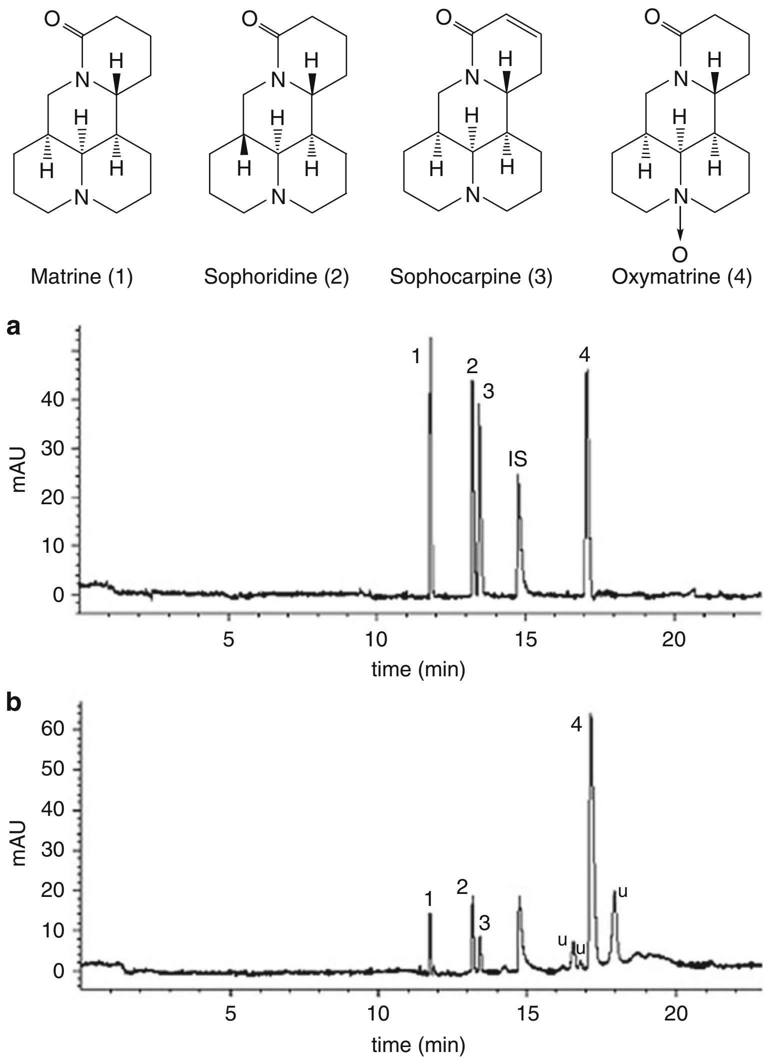

Fig. 36.2 Chemical structures of quinolizidine alkaloids in *Sophora flavescens* and their CZE separation in (**a**) standard solution and (**b**) real sample (Kushen crude drug). IS, jatrorrhizine (internal standard); u, unknown. CZE conditions: 60 mM borate at pH 8.5, applied voltage 12 kV, detection at 204 nm, capillary effective length 56 cm (i.d. 75 μm) (Modified from reference [10])

Recently, Kulp et al. observed a similar behavior in CZE analysis of isoquinoline alkaloids in extracts of *Chelidonium majus* L. [14]. These compounds exhibit distinct anti-inflammatory, antimicrobial, and antitumor activities, and the most effective alkaloid components of the plant are protopine, chelidonine, coptisine, sanguinarine, allocryptopine, and chelerythrine. Being strongly basic molecules with a heterocyclic-bound nitrogen, these compounds show pKa values above 5; thus, in acidic

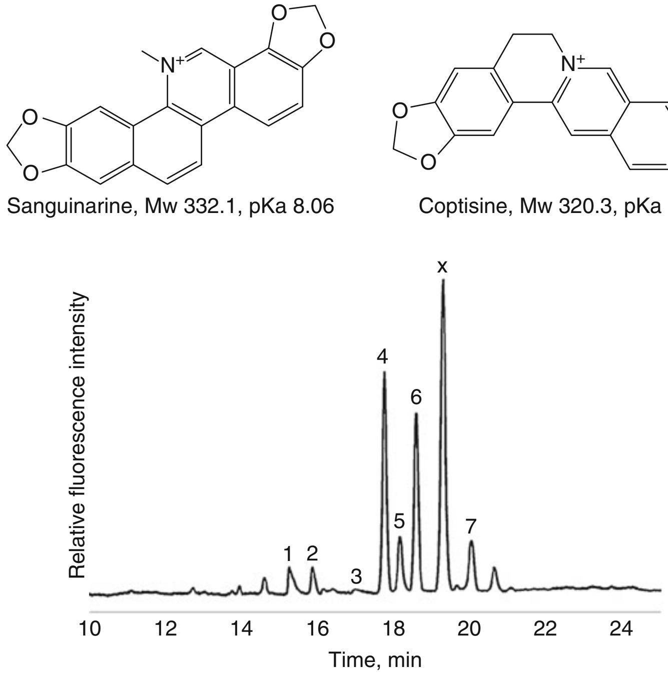

Fig. 36.3 Chemical structures of sanguinarine and coptisine and CZE separation of seven alkaloids in an extract of *Chelidonium majus* L. Peaks: (*1*) sanguinarine, (*2*) coptisine, (*3*) chelerythrine, (*4*) stylopine, (*5*) chelidonine, (*6*) protopine, (*7*) allocryptopine, and (*x*) unknown component. Conditions: 20 mM phosphate buffer, pH 3.1. Separation capillary: 75 μm, i.d. uncoated fused silica, 50 cm in length (40 cm effective length). Applied voltage 16 kV. Light-emitting-diode-induced fluorescence (LED) excitation wavelength 280 nm (Modified from reference [14])

electrolytes, they are protonated and migrate as cations. The best selectivity and resolution was obtained using 20 mM phosphate buffer, pH 3.1 (Fig. 36.3). According to their basic properties and their molecular masses, the isoquinoline alkaloids migrated in the predictable order: (I) strongly basic alkaloids such as sanguinarine, coptisine, and chelerythrine; (II) alkaloids with medium basic character such as stylopine, chelidonine, and protopine; and (III) allocryptopine, characterized by high molecular mass and very weak basic properties. Unexpectedly, the migration velocity of sanguinarine (Mw 332.1) was higher than the migration velocity of coptisine (Mw 320.3); according to the explanation provided by Stöckgit et al. [13], this is due to the presence of an additional methyl group at the nitrogen of sanguinarine, which prevents the formation of intermolecular forces between the nitrogen atom and the solvent molecules of the phosphate buffer medium. In the same study, the impressive separation of chelidonine and protopine, two compounds with the same mass and charge, was also shown. Apparently, slightly different basic properties and the presence of the electronegative oxygen atom, which shifts electron density from the nitrogen in protopine molecule, caused the difference in migration velocities of these two alkaloids [14]. These findings underline the importance of intermolecular interactions between functional groups of the alkaloids on their migration behavior.

A further interesting example showing, in this case, the influence of the molecular geometry on the electrophoretic velocity is given by the excellent selectivity of CZE performed under strongly acidic conditions of hydroindole alkaloids isolated from *Sceletium* species. These psychoactive compounds are mainly represented by (−) mesembrine (Mw 289) with a 3a-aryl-*cis*-octahydroindole skeleton; other congeners include the Δ^4 mesembrenone and Δ^7 mesembrenone with a double bond at either the position $4 - 5$ or $7 - 7a$, respectively (Mw 287). In addition, *Sceletium* phytopreparations may contain mesembranol and its stereoisomer epimesembranol (Mw 291) with the carbonyl function of mesembrine reduced to the corresponding hydroxy group. A simple CZE method based on phosphate buffer at pH 1.5, involving the full protonation of the basic nitrogen of the indole, was successfully applied to the separation of the alkaloids in real samples. The complete separation of the alkaloids was achieved, and the two isomers, namely, Δ^4 mesembrenone and Δ^7 mesembrenone, migrated respectively as the first and the last compound of the series [15].

2.2 Effect of Nature and Concentration of the Running Buffer in CZE

The running buffer selection is a key factor to the success of any CE separation. As a general rule, it has to be considered that the buffer system effectively works in a pH range approximately corresponding to two units centered to the pKa value. Phosphate and citrate having more than a pKa value show good buffering capacity in a wide pH range [1–3]. In particular, phosphate is often selected as a useful counterion for analysis of alkaloids in CZE, and as an alternative, tetraborate buffer, exhibiting a native pH of 9.2, can be used for analysis of strongly basic alkaloids. The low absorbance at the wavelength of detection (often in the UV range) makes these buffers as ideal for conventional CZE separations. In addition, phosphate and borate showed to be suitable for CZE coupled with a number of detection systems including electrochemiluminescence (ECL) detection and LIF detection; differently, the compatibility of phosphate and borate BGEs with MS detection is rather poor. The buffer concentration affects the electrophoretic behavior of the alkaloids by influencing the separation selectivity, the EOF strength, and stacking effect. In particular, by increasing the buffer concentration, the EOF is decreased because of the increased viscosity of the medium (see Eqs. 36.2 and 36.3). In addition, a stacking effect, leading to the reduction of the width of the sample zone before separation, occurs when the capillary is filled with a separation electrolyte with a higher ionic strength than that of the sample solution. Marsh et al. observed that the CZE separation of nicotine and nicotine-related alkaloids could be significantly improved by using a 100 mM phosphate running buffer (pH 2.5) instead of a diluted BGE (25 mM), used in previous experiments. In particular, at higher BGE concentration, the migration of cationic analytes was slackened and the separation enhanced. Furthermore, because of the sample stacking effect, narrowed peaks were obtained and detection sensitivity improved [16]. Running buffers at relatively high concentration showed to be useful also in preventing the adsorption of strongly basic alkaloids to the inner capillary wall, as it was observed in CZE analysis of berberine and hydrastine in root powder of *Hydrastis canadensis* performed using as the BGE, a 100 mM solution of ammonium acetate/acetic acid at pH 3.4 [17].

Borate buffers are often applied in CZE for their ability to give in situ complexation of polyhydroxy compounds such as carbohydrates and polyphenol aglycones. In particular, they form charged mobile five-membered-ring complexes (with 1,2-diols) and six-membered-ring complexes (with 1,3-diols), and as a consequence, an increased selectivity of separation can be achieved [18]. Interestingly, the interaction of borate ions with the polyhydroxy compounds results in UV absorbance enhancement; thus, the formed complexes can be detected using conventional spectrophotometric mode, despite the absence of significant chromophores in the original analyte molecule [19, 20]. A typical example of the described approach is given by the CZE analysis of calystegines, an interesting class of bioactive nortropane polyhydroxylated alkaloids isolated from different species belonging to the *Solanaceae*, *Convolvulaceae*, and *Moraceae*. Although calystegines possess a very low molar absorptivity, the CZE analysis approached using tetraborate solution (80 mM, pH 9.2) as the BGE allowed good UV detection sensitivity (limit of detection, LOD within 25–50 μg mL^{-1}). The migration behavior was found to be significantly dependent by the chemical structure of the analytes (i.e., number and position of hydroxy groups) and could be tuned by the proper choice of tetraborate concentration. Under optimized conditions, baseline separation of the selected compounds was achieved in less than 12 min [21]. As an alternative, indirect UV detection was applied using histidine as the UV marker [22]. The effect of using borate buffer to improve resolution was also shown in separation of opium alkaloids. Gao et al. observed that only partial separation was achieved by optimizing pH and concentration of phosphate buffer in separation of codeine, thebaine, morphine, and narcotine. Advantageously, by employing tetraborate as the BGE, the four alkaloids could be baseline separated [23].

2.3 Effect of the Presence of Organic Solvents in CZE Running Buffer

Under neutral or alkaline CZE conditions, most of the alkaloids are present as free bases showing low water solubility; therefore, the addition of organic solvents is a good choice to avoid precipitation of the solute during the electrophoretic run. Since the addition of organic solvents to the aqueous running buffer changes zeta potential and viscosity, a variation in electroosmotic velocity is then observed. In particular, by addition of organic solvent, the EOF is generally reduced even when the silanol groups of the capillary wall are fully ionized, i.e., at high pH. Furthermore, application of organic solvents as constituents of the buffer solution can lead variation of the pKa of the alkaloids with consequent effects on their absolute and actual mobility [24]. Methanol, ethanol, *n*-propanol, isopropanol, and acetonitrile (ACN) are the most used solvents because of their aqueous solubility and low UV absorption. It has been shown that using mixtures of water–alcohols (e.g., methanol and isopropanol), the EOF decreases compared to the pure aqueous buffer; differently, addition of ACN, because of its very low viscosity, decreases the EOF [24, 25]. Besides the described general effects, the separation selectivity can be altered by the presence of organic solvents, even though they are added in small percentage with respect to the aqueous components of the BGE. It can be concluded that the impact of the organic solvent addition on the electromigration of the analytes is the combination of a number of effects including the extent of interaction with the analytes through solvational, dissociative, or multimolecular complex formation phenomena; thus, the results in term of CE separation using aqueous–organic mixtures have to be experimentally determined. The separation of strongly basic isoquinoline alkaloids has been often performed using mixed aqueous–organic running buffers. Protoberberine alkaloids, namely, berberine and hydrastine, were analyzed in *Hydrastis canadensis* L. samples by optimizing the CZE separation through addition of methanol and ACN to the aqueous BGE. The electrolyte used was a solution of ammonium acetate, which proved to be suitable for preparation of both nonaqueous and mixed aqueous–organic buffers. Addition of methanol (80 %, v/v) increased the run time and improved the separation selectivity [17]. Similarly, methanol (70 % v/v) in 100 mM sodium acetate buffer was found to be the best choice for the separation of major alkaloids of gum opium (thebaine, codeine, morphine, papaverine, and narcotine) in real samples [26]; a similar mixed aqueous–organic buffer using ACN instead of methanol resulted in faster separation of the same compounds, but it could be only applied to standard mixture because of the interferences with the components of the matrix [27]. High methanol percentage (80 %, v/v) in 100 mM sodium acetate electrolyte was found to be necessary in obtaining the desired separation selectivity and to reduce peak tailing in analysis of the isomeric quaternary benzo[c]phenanthridine alkaloids such as chelerythrine and nitidine in *Zanthoxylum nitidum*, a common Chinese herbal medicine used to promote blood circulation and relieve pain [28]. Using a BGE composed of a 50:50 v/v water: ACN, containing 50 mM ammonium acetate at pH 6.8, Chen et al. achieved a fast separation of 8 isoquinoline alkaloids in real samples of *Rhizoma coptidis* [29]. Huhn et al. developed a mixed aqueous–organic CE method for the analysis of β-carbolines in extracts of *Psychotria viridis*. Using 200 mM formic acid and 7 mM ammonia in water with ACN 10 %, the baseline separation of norharmane, harmane, and tetrahydroharmane was accomplished. A dual detection system, LIF and electrospray mass spectrometry (ESI-MS), was developed; in particular, the use of optical fibers allowed the LIF detection to be set close to ESI source making easy the comparison of the LIF and MS electropherograms [30]. Finally, also isopropanol, whose behavior in term of effect on the EOF is similar to that of methanol, can be used for selectivity tuning as it was shown in some applications to the analysis of tropane alkaloids [9] and quinolizidine alkaloids [31].

In analysis of aconitine and the congeners mesaconitine and hypaconitine, the main diester-diterpene alkaloids of aconitum plants (*Ranunculaceae*), sample preparation involves extraction with nonpolar solvents (e.g., ether) under reflux of the pulverized roots. After evaporation of the organic solvent, the residue is reconstituted with ACN; thus, the CZE analysis can be conveniently carried out using mixed aqueous–organic buffers in order to improve the compatibility of the organic sample plug injected into the capillary [32, 33]. A BGE composed of 200 mM Tris buffer containing 150 mM perchloric acid and dioxane 40 % at pH 7.8 showed compatibility with the injected samples and allowed for the simultaneous separation of three diester-diterpene alkaloids (the native aconitine, hypaconitine, and mesaconitine) and related degradation products (benzoylaconine, benzoylmesaconine, and benzoylhypaconine). In particular, the study focused on the evaluation of the stability of the native aconitum alkaloids, which undergo to hydrolysis of the ester moiety. Monoester diterpenes, namely, benzoylaconine, benzoylmesaconine, and benzoylhypaconine, can occur in processed aconitum herbal drugs, and, interestingly, these artifacts showed less toxicity compared to the parent compounds. The analysis of crude and processed herbal preparations clearly showed that the manufacture process affected the content of aconitum alkaloids with a significant decrease of the native diester diterpenes [33].

2.4 Effect of Buffer Additives in CZE

2.4.1 Ionic Liquids

Ionic liquids are substances with melting points at or close to room temperature, and they are composed entirely of ions, most of them having organic cations, in particular alkyl imidazolium. The anions in ionic liquids could be inorganic, including often PF_6^- and BF_4^-, as well as organic anions such as trifluoromethylsulfonate and trifluoroethanoate. Their unique properties are wide liquid range, low volatility, thermal stability, electrolytic conductivity, noninflammability, and so on. Recent reviews dealing with different aspects of the use of ionic liquid in analytical separations have shown that they can be good candidates as additives to the CE running buffers because of the ability in creating pseudo-stationary phases as well as to behave as simple modifiers of the aqueous buffer. In particular, ionic liquids can interact with the silanols of the capillary, thus modifying the EOF and reducing adsorption of macromolecules on the inner capillary wall [34–36]. In addition, as widely reported in separation of polyphenols [37], alkyl imidazolium cations were found to be able in establishing hydrogen bonding interactions with the analytes. In case of CZE analysis of verticine and verticinone, two isosteroid alkaloids with a hexacyclic benzo[7,8]fluoreno[2,1-*b*]quinolizine nucleus isolated from plants belonging to *Liliaceae* family such as *Bulbus fritillariae*, these interactions were favorable and resulted in improved separation selectivity. In particular, the ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate (1B-3MI-TFB) 40 mM in a pH 8.0 phosphate solution allowed for the complete separation of the alkaloids in less than 10 min [38]. Addition of 1B-3MI-TFB to the aqueous BGE was found necessary to accomplish separation of aconitine, hypaconitine, and mesaconitine in *Aconitum* extracts [39].

2.4.2 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides composed of D-glucopyranose molecules bonded through α-1,4 linkages and produced by enzymatic reaction on starch. In details these macrocyclic compounds are the main end products of the glycosyltransferase enzyme, defined as αCD, βCD, and γCD. Because of their capacity to form inclusion complexes with a considerable number of analytes (host–guest interaction), CDs can modify the physicochemical characteristics of the guest molecule, resulting in altered retention and/or migration behavior. Several reviews deal with the use of CDs in capillary electrophoresis as selective agents for analytical separations of isomers since they can form inclusion complexes discriminating between positional isomers, molecular substructures, homologues, and enantiomers [40–42]. Examples of the application of CDs as additives to the CZE-BGE for improving resolution of strictly related alkaloids are given by analysis of tropane [43] and morphinane alkaloids. The latter are typically determined in *Papaver somniferum*, and earlier studies by Bjørnsdottir and Hansen showed the necessity to supplement the acidic running buffer (pH 4) with heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-βCD) to obtain the simultaneous separation of morphine, codeine, papaverine, thebaine, and noscapine, not only in crude opium but also in illicit drugs and cough mixtures [44]. Successively, other CZE systems involving different CDs were optimized for separation of different mixtures of opium alkaloids and derivatives in forensic samples [45, 46] and in the extracts produced during the industrial manufacture processes of alkaloids extraction (i.e., the process liquors). In Fig. 36.4 are reported the electropherograms related to the separation of opiate alkaloids using different CDs as additives to the BGE. Depending on the nature of the CD, different separation selectivity could be obtained. The optimum conditions were selected by evaluating parameters such as analysis time, resolution between adjacent peaks, peak symmetry, and separation of alkaloids from interferences by sample matrix. Suitable conditions for analysis of morphinane alkaloids in samples of different origins were achieved using acidic running buffer (pH 2.8–3.0) supplemented with hydroxypropyl-βCD (HP-βCD) 30 mM or α-cyclodextrin (αCD) 5 mM [47, 48].

Lurie et al. used a combination of HP-βCD and DM-βCD in a dynamically coated capillary (to increase the strength and stability of EOF) for separation of opium alkaloids in opium gum and latex [49].

Medicinal herbs containing phenylethylamine alkaloids (i.e., *Citrus* species and *Ephedra sinica*) are widespread used for their effects on human metabolism in particular by stimulating lipolysis and thus promoting the fat mass reduction in obese people. Specifically, ephedra extracts such as *Ephedra herba* (ma huang) contain alkaloids such as (1*R*,2*S*)-(−)-ephedrine, (1*S*,2*S*)-(+)-pseudoephedrine, (1*R*,2*S*)-(−)-norephedrine, (1*S*,2*S*)-(+)-norpseudoephedrine, (1*R*,2*S*)-(−)-*N*-methylephedrine, and (1*S*,2*S*)-(+)-*N*-methylpseudoephedrine. The possible adverse effects associated to the use of these products prompted the U.S. Food and Drug Administration (FDA) to ban ephedra-containing dietary supplements depending on the dosage [50]. In addition, the National Institute of Standards and Technology (NIST) issued standard reference materials with certified values for ephedra

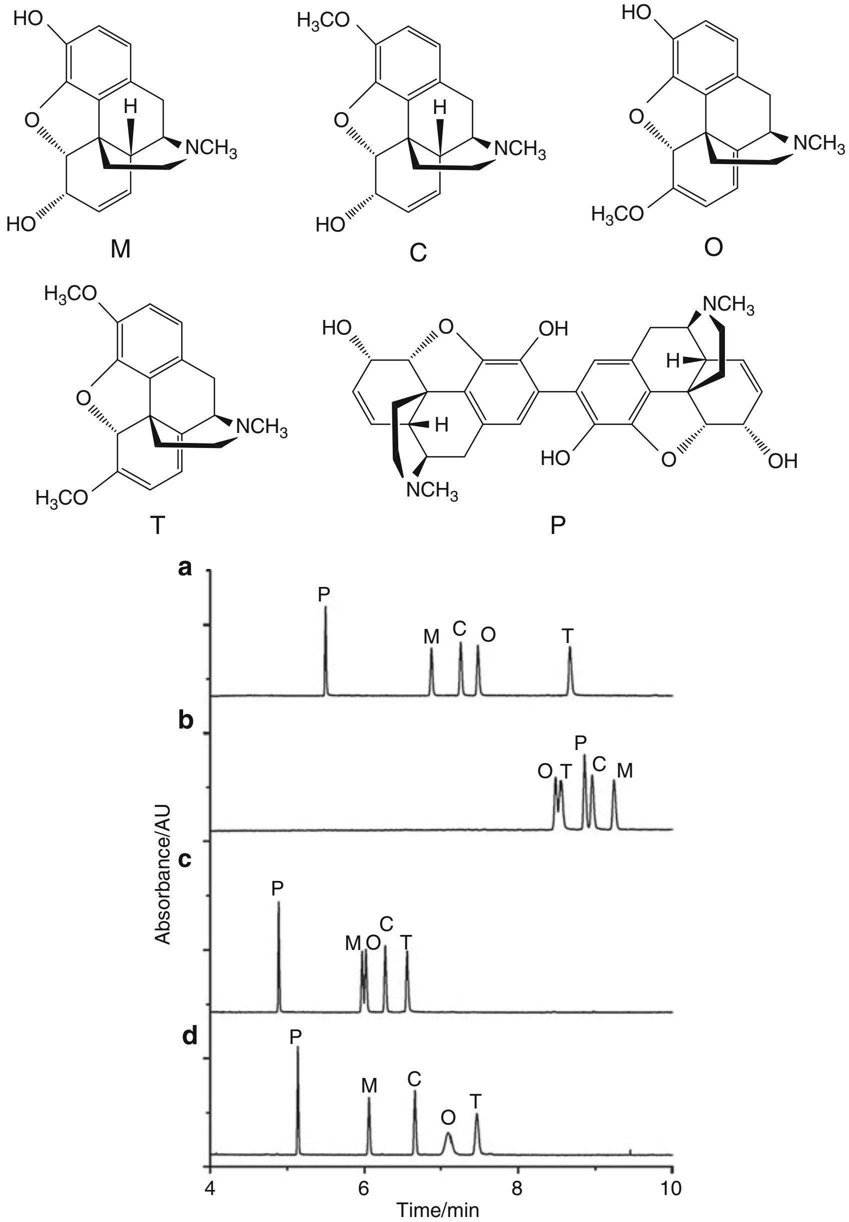

Fig. 36.4 Chemical structures of morphine (M), codeine (C), oripavine (O), thebaine (T), and pseudomorphine (P) and their separation by cyclodextrin-modified CZE using different CDs. Conditions: (**a**) HP-βCD, (**b**) γCD, (**c**) βCD, and (**d**) αCD. Buffer: 100 mM Tris–phosphate, pH 2.8; CD concentration of 30 mM except for βCD (10 mM due to limited solubility); applied voltage of 25 kV; 2 s injection at 50 mbar; 20 °C; capillary 50 cm × 50 μm i.d.; UV absorption detection at 214 nm (Modified from ref. [47])

alkaloids, synephrine, and caffeine [51]. As a consequence several analytical methods have been developed with the aim to provide reliable quantitation of ephedrine and pseudoephedrine in ephedra extracts and herbs [52]. Biosynthetic pathways involved in plant kingdom drive often to enantiomerically pure compounds. Furthermore, although several factors affect the chiral composition of the plant metabolites, detecting a distortion of the enantiomeric ratio can be considered as an acceptable hint of the natural origin of the compounds [53]. In characterization of *Ephedra sinica* and its medicinal phytopreparations, chiral analysis provides a useful tool because only (1*R*,2*S*)-(−)-ephedrine and (1*S*,2*S*)-(+)-pseudoephedrine enantiomers are found in nature. Simple CZE analysis of ephedrine and pseudoephedrine in herbal drugs (tablets) was carried out in a 25 mM triethanolamine phosphate buffer (pH 2.5) in condition of reversed EOF. Highly sulfated β-cyclodextrin was supplemented as the chiral additive to obtain the required enantioselectivity and to allow the separation of ephedrine and pseudoephedrine enantiomers from other herbal drug constituents [54]. A combination of three neutral cyclodextrins, namely, βCD, HP-βCD, and DM-βCD, under acidic conditions was used as a chiral system to achieve the simultaneous enantioresolution of octopamine and synephrine enantiomers in the presence of tyramine and *N*-methyltyramine in *Citrus* species dietary supplements. In particular, the chiral analysis was applied to evaluate the thermal racemization of *l*-synephrine. Although information on the kinetics of the conversion was not provided, the study demonstrated that the extraction procedure applied to the real samples did not lead to any chiral artifacts since the racemization of synephrine occurs at relatively high temperature (i.e., 100 °C) [55].

Phinney et al. developed three CE-UV methods that allowed the enantioseparation of ephedrine and pseudoephedrine using neutral HP-βCD, DM-βCD, and charged sulfated βCD as chiral selectors. The combination of negatively charged sulfated βCD (2.8 %) and DM-βCD (1.2 %) under acidic conditions (pH 2.5) provided the best separation of the two couples of enantiomers. Standard reference materials (SRMs) containing ephedra in development at NIST were analyzed by each of three CE methods. In the SRM samples, only the naturally occurring enantiomers (−)-ephedrine and (+)-pseudoephedrine were found; however, the method proved to be suitable in detecting product adulteration by its potential in identification of specific stereoisomers [56]. The neutral HP-βCD was also found to be a useful chiral selector in the presence of tetrabutylammonium chloride as additive, for the simultaneous enantioseparation of ephedrine, pseudoephedrine, *N*-methylephedrine, and norephedrine enantiomers in *Ephedra sinica* extracts [57].

3 Nonaqueous Capillary Electrophoresis, NACE

The term “nonaqueous capillary electrophoresis” (NACE) is used to describe the application of pure organic solvents, or their mixtures, in preparation of running buffers for CE experiments. The most important advantages of using nonaqueous running buffers are (1) the improved solubility of hydrophobic analytes and

(2) opportunity in gaining specific selectivity because of the effect of the solvent (or mixtures) on acid–base properties of the solutes. In particular, pKa values of alkaloids in organic solvents are notably different from those in water, thus allowing separations which are difficult to be obtained in aqueous running buffers.

NACE widens the set of physicochemical characteristics of the running buffers, which are known to affect the electrophoretic behavior of the solutes by influencing interactions with solvents, additives, and ion–ion interactions that are too weak or cannot take place in aqueous BGEs. In particular, the dielectric-constant-to-viscosity ratio (ε/η) of an organic solvent affects both the electrophoretic mobility of ions and the electroosmotic mobility (see Eqs 36.2 and 36.3); therefore, mixing organic solvents in different ratios is an effective strategy in optimization of the CE separation. Furthermore, NACE appears to be ideally suited for online coupling with mass spectrometry due to the high volatility and low surface tension of many organic solvents [58–62].

Acetonitrile and methanol can be considered as the best solvents for NACE of alkaloids [63]. In particular, methanol possesses a favorable dielectric constant (33.7 at 20 °C), ability in intermolecular hydrogen bonds, and autoprotolysis; therefore, it is suitable to dissolve the supporting electrolytes necessary to the CE experiments. A few of recent applications have dealt with NACE of alkaloids in pure methanol; this choice is mainly driven by the necessity for compatibility of the BGE with the solvent used in samples preparation and to achieve the desired separation selectivity [64, 65]. In most of the applications, however, methanol is mixed with different organic solvents such as ethanol, isopropanol, and tetrahydrofuran (THF). In a study by Humam et al., the potential of NACE by approaching the challenging separation of four isomeric tropane alkaloids that was unsuccessful in aqueous BGEs was shown. Mixtures of methanol with either ethanol or THF led to interesting results. Using ethanol, because of the higher viscosity compared to that of THF, the analysis time was longer; however, for complex plant extract samples, a mixture methanol/ethanol (40:60, v/v) was selected as a compromise between analysis time and resolution of the alkaloids. In Fig. 36.5, the separation achieved under optimized conditions of the four strictly related solutes (3α-senecioyloxy-7β-hydroxytropane, 3α-hydroxy-7β-senecioyloxytropane, 3α-hydroxy-7β-angeloyloxytropane, and 3α-hydroxy-7β-tigloyloxytropane) is reported [66].

The presence of ACN in NACE BGEs is useful to increase both the electrophoretic and electroosmotic mobility because of the high (ε/η) of this solvent. Therefore, in separation of protonated alkaloids, the percentage of ACN in methanol can be adjusted to tune analysis time and separation selectivity [67–78]. An interesting example was given by Sturm et al., in analysis of isoquinoline alkaloids in *Fumaria officinalis* and *Corydalis* species [75, 76] and pyrrolo- and pyrido[1,2-*a*]azepine alkaloids in *Stemona* [77]. The authors developed a system to be hyphenated with ESI-MS, and because of the requirement of the instrumental setup for a 90-cm-long capillary, ACN was used as major component (90 % v/v) of the solvent mixture to achieve fast separations. In these applications quaternary alkaloids showed the shortest migration time; then, the migration order was found to follow the pKa values with some exceptions related to the structural subtypes of the compounds.

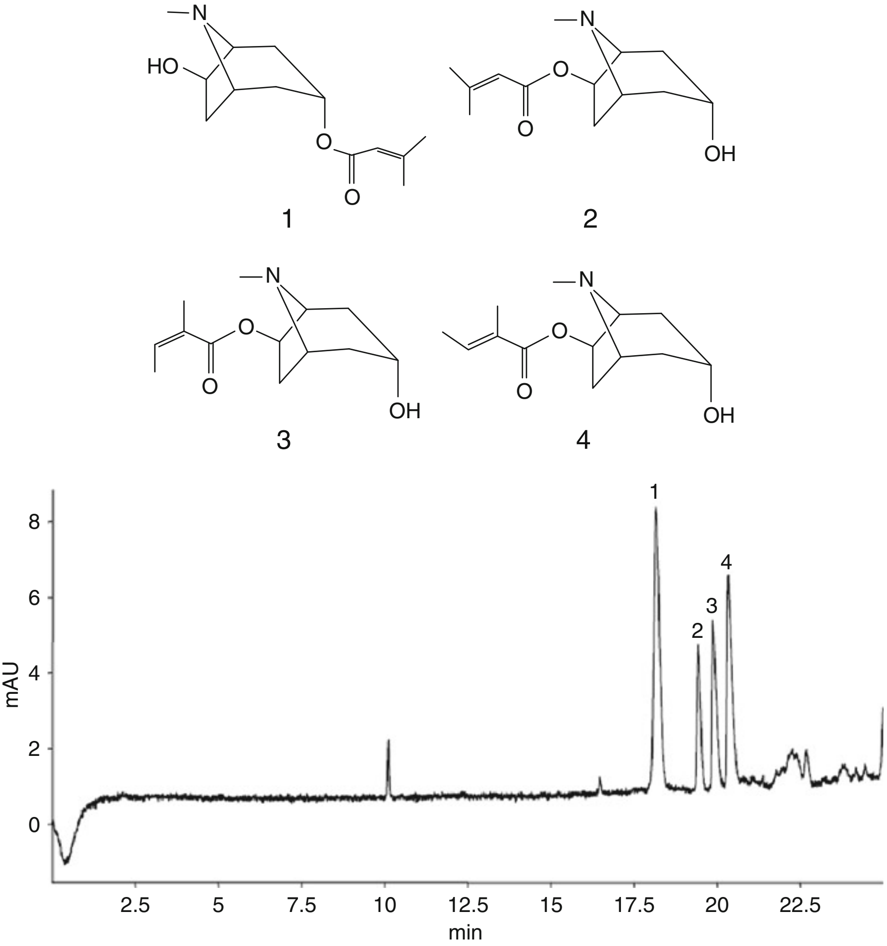

Fig. 36.5 Chemical structures of 3α-senecioyloxy-7β-hydroxytropane (**1**), 3α-hydroxy-7β-senecioyloxytropane (**2**), 3α-hydroxy-7β-angeloyloxytropane (**3**), and 3α-hydroxy-7β-tigloyloxytropane (**4**) and their NACE separation using a fused-silica capillary (64.5 cm total length, 50 μm i.d.) and BGE of 1 M trifluoroacetic acid, 25 mM ammonium formate in methanol/ethanol (40:60, v/v). Electrophoretic conditions: 25 kV; T, 25 °C; injection, 25 mbar (8 s); UV detection, 220 nm (Modified from ref. [66])

Liu et al. developed NACE methods for analysis of quaternary protoberberine alkaloids in Chinese herbal medicines by using LIF detection. It was found that the native fluorescence signal of the analytes decreased in mixtures of ACN: methanol compared to that observed in pure solvents; thus, it was suggested that the optimization of solvent system composition for sensitive LIF detection has to be carried out by considering this effect [68, 69].

The analysis of toxic quinolizidine alkaloids like lupanine, angustifoline, and 13α-hydroxylupanine in *Lupinus* species was carried out in nonaqueous buffer systems to obtain high compatibility with MS analysis. The effect of the most relevant separation parameters was carefully evaluated; interestingly, it was observed that without addition of a small percentage of water (10 %) and acetic acid (1 %) to the electrolyte solution, lupanine and angustifoline could not be resolved. This was explained invoking a different electrophoretic mobility of the alkaloids in charged versus neutral state. On the other hand, when the content of water and/or acetic acid was too high, a general decline in resolution was observed [78].

Because of their solubility in organic solvents mixtures, the most used supporting electrolytes in NACE are ammonium acetate, ammonium formate, and sodium acetate. The migration time of the alkaloids was found to increase with increasing electrolyte concentration; this effect is explained by the diminished EOF due to the decreased zeta potential caused by reduced thickness of the electric double layer on the inner capillary wall. High concentration levels of supporting electrolytes lead enhanced resolution ascribed to the effect of the interaction between the protonized alkaloids with the acetate co-ion in nonaqueous environment. In most of the applications, the supporting electrolyte concentration was within 25–100 mM; higher concentration should be avoided because of the negative effect on analysis time that was attributed to the increase in the mass transfer resistance of the analytes with the increase of ionic strength and viscosity [60, 61, 68, 74]. The acidity of nonaqueous buffers affects the alkaloid protonization extent; thus, by lowering the pH of the BGE, the migration of the alkaloids is expected to be faster. However, it has to be underlined that in organic solvents, the common pH measurement carried out using the glass electrode provides the apparent pH (pH*) that is not correlated to the H^+ activity. Acetic acid in the concentration range within 0.5–3 % is often used for the acidity control of nonaqueous BGEs [60, 61].

4 Micellar Electrokinetic Chromatography (MEKC) and Microemulsion Electrokinetic Chromatography (MEEKC)

Electrokinetic chromatography (EKC), and in particular micellar electrokinetic chromatography (MEKC), has been introduced in 1984 by Terabe and proved to be not only the method of choice in analysis of neutral compounds but also one of the most versatile separation approach among the electromigration methods. In MEKC a surfactant (often sodium dodecyl sulfate, SDS) is introduced into the BGE at concentration above the critical micelle concentration in order to generate the so-called pseudo-stationary phase constituted by the anionic SDS micelles in which the hydrophilic head groups form the outer shell and the hydrophobic tails create the core. In most of the MEKC applications, alkaline BGEs are used, and the electroosmotic velocity is faster than the electrophoretic velocity of the micelles; thus, the latter also migrates in the same direction of the EOF. The neutral analytes that are driven toward the cathode by the EOF can be incorporated into the micelle depending on their hydrophobicity; therefore, the separation mechanism is a combination of chromatographic partitioning of solutes between the pseudo-stationary phase and continuous phase. In analogy, the charged solutes undergo to the same process; however, in such a situation, the separation is the result of the combination of the chromatographic partitioning to the pseudo-stationary phase and the electrophoretic behavior of the ionic molecule. In MEKC the separation selectivity can be modulated not only by variation of BGE type, pH, and concentration but also by the proper selection of the surfactant as well as by optimizing its concentration [79–81].

Because often alkaloids have to be analyzed in plant materials containing neutral compounds, MEKC is a useful method providing the full characterization of complex extracts. Typical examples are EKC methods developed for analysis of methylxanthines in a variety of sources. These alkaloids, characterized by a purine backbone, are represented by caffeine, theophylline, and theobromine, and because of their stimulant effects on cardiovascular and nervous system, methylxanthine-containing plants are sometimes used as phytopharmaceuticals and dietary supplements. Methods for quantitation of the active compounds are necessary because, depending on the country, the level of the methylxanthine alkaloids could be limited. The U.S. FDA sets the limit of 0.02 % [82]; however, the so-called energy drinks when classified as "supplements" may contain more. Further, natural caffeine levels are exempt from these regulations; thus, coffee-based drinks may have more caffeine than 0.02 % without being classified as a supplement. Methylxanthine content is also used as an index of the quality of edible products such as coffee, tea, and cocoa; however, in order to achieve a comprehensive information on the biological value of these products, the quantitative determination of antioxidant polyphenols (mainly catechins) is required. The simultaneous CE analysis of methylxanthine alkaloids and catechins is conveniently accomplished by MEKC. Alkaline BGEs can be used [83, 84]; however, at high pH values, Pomilio et al. observed a peak splitting of theobromine and caffeine due to the keto-enolate tautomers, which could have different partitioning into the SDS micelle [84]. Thus, neutral or acidic running buffers could be conveniently used also to improve the stability of catechins [85–87]. Under these conditions, the addition of cyclodextrins to the micellar solution (CD-MEKC) showed to be a useful approach to obtain highly chemoselective and stereoselective systems that were successfully applied to the analysis of catechins and methylxanthines in tea and *Theobroma cacao* samples [86, 87]. Selectivity modulation in EKC methods can also be achieved by addition of organic solvents as it was shown in MEKC of ephedrine and pseudoephedrine [88] and pyrrolizidine alkaloids [89]. In particular, alkaloids such as senecionine and seneciphylline contain the 4-azabicyclo[3.3.0]octane system, and they differ only for the presence in seneciphylline of the C_{13}–C_{23} double bond. Owing to the same pKa values (5.44), these strictly related compounds could be separated only by MEKC. The addition of methanol (20 %) to the BGE was necessary to achieve the required selectivity [89]. In analysis of camptothecins, a valuable family of quinoline alkaloids from *Camptotheca acuminata*, *Nothapodytes foetida*, *Ophiorrhiza pumila*, and *Tabernaemontana heyneana*, the main issue is their poor water solubility. Extraction of active alkaloids from plant material was optimized using dimethyl sulfoxide; subsequently, an MEKC method was developed for the separation of camptothecin and 9-methoxycamptothecin. In order to achieve compatibility of the separation medium with the extraction solvent, dimethyl sulfoxide was added to a borate-based BGE. Interestingly, borate ($pH > 8$) running buffer was found to be effective in increasing the solubility of camptothecin and derivatives by promoting the equilibrium of the lactone forms to the carboxylate as a consequence of the complexation with borate ions. Owing to the characteristic UV spectrum, the selective detection in real samples was performed at the wavelength of 369 nm and the LOD could be assessed as about 2 $\mu g\ mL^{-1}$, thus adequate to this application [90].

Although SDS is the most versatile and useful micellar agent, different surfactants can be introduced in MEKC to gain specific selectivity. Interestingly, Hou et al. performed the simultaneous MEKC enantioseparation of four pairs of ephedrine related compounds, namely, (±)-ephedrine, (±)-pseudoephedrine, (±)-norephedrine, and (±)-*N*-methylephedrine, using a polymeric chiral surfactant, i.e., polysodium *N*-undecenoxycarbonyl-L-leucinate. Because of the high molecular mass of the surfactant, the BGE showed to be compatible with the online ESI-MS detection [91]. In Fig. 36.6, the effect of the chiral surfactant concentration on the separation of the studied analytes is shown.

The method was applied for assays of ephedra products in dietary supplements in an effort to possibly reduce health risks, which are associated with the use of these products [91, 92].

The separation and determination of tetrandrine and fangchinoline, two bioactive alkaloids found in Radix *Stephaniae tetrandrae*, was accomplished under MEKC conditions using an acidic running buffer supplemented with Tween 20 as a nonionic surfactant which was found to enhance the solubility of the hydrophobic cationic alkaloids [93]. Lauric acid was used as a convenient alternative to SDS in MEKC of seneciphylline and senecionine, two hepatotoxic pyrrolizidine alkaloids in *Gynura segetum* extracts; in particular, it was found that micelles from lauric acid exhibited lower conductivity compared to those from SDS, thus allowing for application of high electric field resulting in fast analysis and limited Joule heating [94]. Using sodium deoxycholate 50 mM as the surfactant, MEKC separation of coptisine, berberine, and palmatine was achieved simultaneously to that of neutral bioactive compounds such as flavonoids (wogonin, baicalein, baicalin) and anthraquinones (sennosides A and B, emodin, rhein, physcion, chrysophanol, and aloe emodin). The method showed excellent selectivity and was applied to the analysis of *Coptidis rhizoma*, *Scutellariae Radix*, *Rhei Rhizoma* (rhubarb), and phytopreparations containing the three herb mentioned above. The sample preparation was carried out by exhaustive extraction using methanol–water (70:30, v/v) in ultrasonic bath. The extracts were concentrated under vacuum and dissolved in acetonitrile–water (70:30, v/v). The method was validated for linearity, precision, and accuracy (recovery values were in the range 98.12–107.58 %, RSD % inter-assay < 5.21, $n = 9$), and the detection limits in analysis of protoberberine alkaloids were $<0.65\ \mu g\ mL^{-1}$ [95].

Microemulsion electrokinetic chromatography (MEEKC), similarly to MEKC, utilizes as the separation medium a pseudo-stationary phase defined as

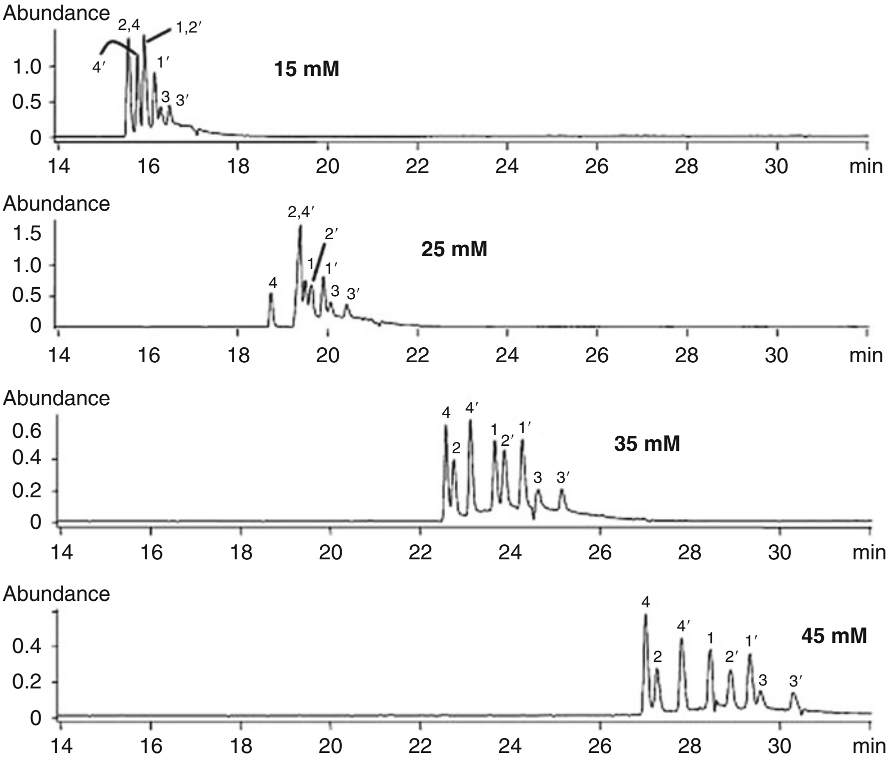

Fig. 36.6 Optimization of MEKC conditions for enantioseparation of ephedrine alkaloids using polysodium *N*-undecenoxycarbonyl-L-leucinate (poly-L-SUCL) as chiral surfactant. In the electropherogram, the effect of surfactant concentration on the separation is reported. Conditions involved the use of 35 mM ammonium acetate – 30 % ACN at pH 7.0 and ESI-MS detection. Peak identification: (1) (1*R*, 2*S*)-(–)-ephedrine, (1′) (1*S*, 2*R*)-(1)-ephedrine, (2) (1*R*, 2*R*)-(–)-pseudoephedrine, (2′) (1*S*, 2*S*)-(+)-pseudoephedrine, (3) (1*R*, 2*S*)-(−)-Norephedrine, (3′) (1*S*, 2*R*)-(+)-Norephedrine, (4) (1*R*, 2*S*)-(–)-*N*-methylephedrine, and (4′) (1*S*, 2*S*)-(+)-*N*-methylephedrine (Modified from ref. [91])

microemulsion. Typically the microemulsions are composed of nanometer-sized oil droplets suspended in aqueous buffer (oil-in-water microemulsions, O/W). These systems are stabilized by the presence of a surfactant, i.e., SDS and a cosurfactant, which is a short-chain alcohol such as butanol. The oil droplets are obtained by dispersion of *n*-octane or other types of hydrophobic solvents [80, 81, 96]. In MEEKC separation selectivity can be modulated not only by using different concentration and type of surfactant but also by the proper selection of cosurfactant and the oil phase. Yu et al. used sodium cholate (the surfactant), 1-butanol (the cosurfactant), and ethyl acetate (the oil phase) to obtain a microemulsion for analysis of quinolizidine alkaloids in *Sophora flavescens* preparations [97]. Pomponio et al. investigated the effect of different cosurfactants on the MEEKC separation of methylxanthines and green tea catechins. It was found that while the migration order and resolution of the hydrophilic catechins could be changed by using different and unconventional cosurfactants, the migration order of methylxanthines remained unchanged. However, the proper selection of cosurfactant allowed for the complete separation of caffeine and theophylline (the internal standard) from the other neutral components of real samples (tea from different countries) [98].

5 Capillary Electrochromatography (CEC)

Capillary electrochromatography (CEC) is included among the electrokinetic techniques because it combines the electromigration of charged solutes with chromatographic partition in capillary format. Differently to MEKC and MEEKC, the chromatographic partition is established between a liquid mobile phase and a solid stationary phase (small particles, ~3 μm), packed in a fused-silica capillary and fixed by frits prepared by silica sintering. CEC can also be performed in monolithic stationary phases formed by the in situ (in-capillary) polymerization of monomers. The CEC column is inserted in the cartridge of the conventional CE instrumentation, and the mobile phase is then driven through the stationary phase by the EOF generated by the inner capillary wall as well as by the charged groups of the solid stationary phase [99].

Specific CEC applications on natural products have been recently extensively reviewed [100, 101]. In general, the CEC experiments are carried out using stationary phases commonly prepared in laboratory by application of specific procedures that, in case of packed column, include the critical frit fabrication. This step of the preparation often limits the analytical reproducibility; in addition the frits have been considered the source of frequent bubble formation in the mobile phase with a consequent lost of sensitivity and system stability during the chromatographic run. The separation of opiate compounds was achieved by CEC using a reversed-phase packed column (1.5 μm particles) in about 2 min with very high separation efficiency. It was found that bubble formation could be limited by addition of a small concentration of SDS to the mobile phase [102]. The advances in column technology have led to an increasing preference for application of CEC monolithic column consisting of porous structure fabricated inside the fused-silica capillary tube and forming a continuous bed of chromatographic material, thus avoiding frits. A monolith consisting of hydrophilic/cation-exchange mixed stationary phase was found to be suitable to perform CEC separation of narcotine, papaverine, thebaine, codeine, and morphine in *Pericarpium papaveris*. The cation-exchanger function was based on sulfonic groups which not only affected the separation but also afforded for the EOF. It was shown that high concentration of organic modifier (90 % ACN) promoted hydrophilic interaction of the analytes with the stationary phase containing the diol groups that effected as hydrophilic interaction sites [103]. Using a similar monolithic CEC column, very fast separation of the major protoberberine alkaloids in extracts of *Coptis chinensis* Franch could be obtained. The mobile phase used was composed of phosphate buffer pH 7.4 in the presence of 60 % ACN as organic modifier [104]. CEC using hybrid silica monolith columns allowed a very fast analysis of methylxanthines in tea beverages. In the analyzed samples, theophylline was not detected whereas the caffeine content was found to be more than 10-fold lower compared to the tea infusions [105].

6 Capillary Electrophoresis–Mass Spectrometry (CE-MS)

Hyphenation of separation techniques with MS allows the direct sensitive detection of the resolved analytes regardless the presence in their molecule of chromophore/ fluorophore or electroactive groups. Most importantly, by MS hyphenation, structural information useful to the unambiguous identification of the analytes can be achieved, making this approach one of the method of choice in phytochemical studies [106]. Among the hyphenated techniques including the well-established gas chromatography–MS (GC-MS) and liquid chromatography–MS (LC-MS), CE-MS has been introduced more recently. The electrospray ionization source (ESI) is well suited for CE-MS interfacing, since it produces ions directly from liquid solutions at atmospheric pressure. Some of the limitations are related to buffer composition and upon the necessity for introduction of “makeup” liquid (sheath flow) to support the ionization. The advantages of CE-MS using ESI ionization source are mainly (a) the wide application range (from small ions to macromolecules, such as proteins) and (b) separation of charged species that can be easily transferred by the source from liquid to gas phase [60, 107].

Alkaloids that are separated in CZE as cationic species, on application of positive ESI mode, give rise to the corresponding pseudomolecular $[M + H]^+$ ions whose *m*/*z* values provide a useful confirmatory information for univocal identification. Often the CE separation is optimized for conventional UV detection; thus, some modifications have to be introduced for development of effective CE-MS methods. The specific parameters to be considered in hyphenation with ESI mode include electrospray voltage, nebulizing gas flow rate, drying gas flow rate, and temperature. In addition, the composition of the coaxial makeup liquid and its flow rate significantly affect the ionization efficiency and have to be appropriately selected for good coupling performances. The drying gas is used to accelerate buffer desolvation, and to increase sensitivity however is a critical factor to be optimized because the nebulizing gas has been reported to have an aspirating effect which influence the quality of the separation of the analytes because of a pressure-induced flow [108]. The most common buffers used for a conventional electrophoretic separations such as borate and phosphate are not suitable for CE coupling with ESI-MS because of their poor volatility and the risk of MS source contamination. More suitable buffers are based on formic and acetic acid, ammonium carbonate, and ammonium acetate. A fully aqueous BGE composed of ammonium acetate 20 mM pH 8.5 with sheath liquid constituted of 2-propanol/water (50/50, v/v) was used for CE-MS analysis of choline and atropine in hairy root cultures of *Cannabis sativa* L. [109]. Atropine and isomeric tropane alkaloids (Fig. 36.4) from extracts of *Atropa belladonna* L. and *Schizanthus grahamii* were also analyzed by CE-MS using mixed organic–aqueous BGEs [9, 66]. Highly sensitive and reproducible analysis of tobacco alkaloids was obtained using NACE-MS based on a BGE constituted of ACN:methanol (70:30, v/v) containing ammonium formate [110]. Anticancer alkaloids such as vinblastine and the monomeric precursor vindoline and catharanthine were determined by CE-MS in *Catharanthus roseus* obtaining LOD lower than 0.8 μg mL^{-1} [111].

In a series of studies, Sturm et al. showed the advantages of CE-MS in analysis of isoquinoline alkaloids in *Fumaria officinalis* herba and its phytotherapeutic tablets [75]. NACE-MS/MS conditions were optimized for the selective separation of structurally related compounds detected and identified by specific migration times and characteristic pseudomolecular $[M + H]^+$ ion. In Fig. 36.7a, the CE-MS electropherogram of a real sample of *F. officinalis* is reported. The peaks **7** and **8** corresponding to (−)-fumarophycine (*m*/*z* 398) and (−)-O-methylfumarophycine (*m*/*z* 412), respectively, were only partially resolved; however, the different Mw as determined by ESI-MS allowed for their selective qualitative and quantitative determination. In addition, the usefulness of MS/MS experiments in providing typical fragmentation pattern for the unambiguous identification of isobaric analytes was shown. As an example, compounds **4** and **5** corresponding to (+)-adlumine and (−)-α-hydrastine showed pseudomolecular ions with the same *m*/*z* at 384. In this case, the MS/MS experiments resulted in different fragmentation patterns; in particular, the signals at *m*/*z* 206.0 of compound **4** (Fig. 36.7b) were associated with the substitution of the isoquinoline moiety bearing to methoxy groups, whereas the signal at *m*/*z* 190 of compound **5** was assigned to the methylenedioxy group. The same authors applied NACE-MS conditions to analyze a set of 79 samples belonging to *Corydalis* species; 39 analytical peaks were characterized by the MS spectra, and a number of them were unambiguously identified as isoquinoline alkaloids [76].

Principal component analysis (PCA) performed using peak areas as the data basis allowed selection of eight compounds (including allocryptopine, protopine, corycavidine, bulbocapnine, and tetrahydropalmatine) as putative biomarkers for discrimination of the *Corydalis* species. The proposed method provided also the sensitive quantitation of palmatine, protopine, bulbocapnine, corydaline, and tetrahydropalmatine (LOD values in the range 0.7–8.3 μg mL^{-1}) [76]. A further study aimed to the characterization of *Stemona* alkaloids possessing a complex molecular structure defined as pyrido[1,2-*a*]azepine. NACE coupled with ESI-MS detection was developed to characterize the highest number of alkaloids in *S. curtisii*, *S. collinsae*, and *S. tuberosa*, and at least 40 and 50 alkaloids or charged *N*-bearing compounds were detected. Interestingly, notable differences in the alkaloids profiles of the three species were observed; in addition by the availability of references compounds, the unambiguous identification was achieved. CE-MS^n experiments were then undertaken to acquire information useful to elucidate the structure of additional *Stemona* alkaloids. Due to the structural complexity and number of reported *Stemona* alkaloids, reflected in the highly diverse fragmentation patterns, MS/MS experiments were not sufficient for a proper identification of unknowns but allowed unambiguous peak assignment of the single compounds in the various samples [77].

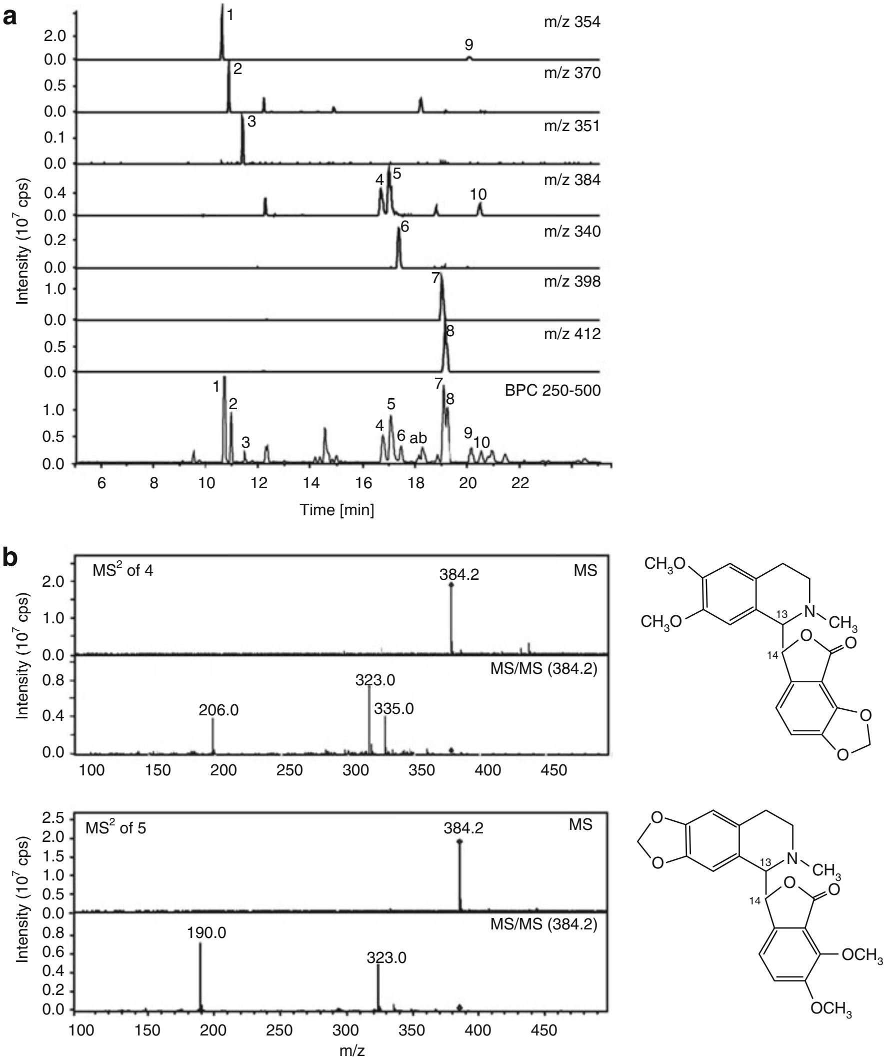

Fig. 36.7 (**a**) CE-MS electropherogram of the ten isoquinoline alkaloid fraction of F. officinalis (base peak chromatogram and selected ion traces). The peaks **7** and **8** are (−)-fumarophycine and (−)-O-methylfumarophycine, respectively. (**b**) CE-MS-derived mass spectra (MS and MS/MS) of alkaloids **4** and **5** (see related structure) identified as *13S/14S*, (+)-adlumine and *13R/14R*, (−)-α-hydrastine, respectively (Modified from ref. [75])

TOF-MS (time of flight MS), with its high mass-resolving power is capable of generating mass data of sufficient accuracy and precision to establish the elemental composition of an analyte. The high accuracy, fast response time, and ease of peak deconvolution capability make TOF-MS an ideal online detector to interface with high-resolution separation techniques. The hyphenation of TOF-MS with CE enhances further the capability of CE-MS for the unequivocal identification of components in complex samples. This approach was applied in characterization of *Atropa belladonna* L. compounds [9] and to the analysis of protoberberine alkaloids in *Rhizoma coptidis* [29]. In particular, the high accuracy of mass measurements allowed the unequivocal identification of eight protoberberine alkaloids in real extracts. Besides mass measurement, the isotopic mass distributions could be achieved, and this data provided information about the elemental composition of the species. The software in TOF-MS approach was capable of calculating all possible molecular formula satisfying the observed data. By means of this approach, the analytes could be unequivocally identified. As an example, in Fig. 36.8a, the CE-ESI-TOF-MS electropherograms of the eight alkaloids in *R. coptidis* extract are reported. The peaks **5** and **7** showed the same *m/z* 338 and also the same elemental composition ($C_{20}H_{20}NO_4$); thus, it was concluded that the two compounds were considered to be structural isomers. CE-MS experiments with a higher fragmentor voltage were performed providing extensive fragmentation in both compounds. The characteristic fragmentations were found to be closely similar (see Fig. 36.8b). Since also the exact masses of these fragmentation ions could be determined by TOF analysis, the determination of elemental compositions with mass errors under 5 ppm for each of the fragments was achieved. Finally, by comparing the observed MS data with the literature values, the analytes corresponding to peaks **5** and **7** could be identified as columbamine and jatrorrhizine, respectively [29].

7 Detection Sensitivity Improvement in Analysis of Alkaloids by CE

The small inner diameter of the separation capillary used in CE implies a short optical pathway, and the consequent poor concentration sensitivity when conventional UV detection is used. To overcome this drawback several techniques have been developed; some of them consist in application of general approaches that are not specifically addressed to CE analysis of alkaloids. One is the use of LIF detector for analysis of alkaloids with native fluorescence [68, 69] or after their off-line derivatization [64, 88, 112]. Sample pretreatment, a second major approach, is popularly employed in combination with sample extraction and can be conveniently applied in analysis of alkaloids because they can be easily retained in cationic-exchange sorbents in solid-phase extraction (SPE) mode [113, 114]. It may be interesting to focus on more specific aspects to detect very low levels of analytes using limited amounts of samples; to this regard chemiluminescence reactions and the use of online preconcentration methods will be considered.

7.1 Chemiluminescence Coupled to CE

Analytes separated by CE can be subjected to chemiluminescence reactions using specific reagents. The chemiluminescence emission can be measured using

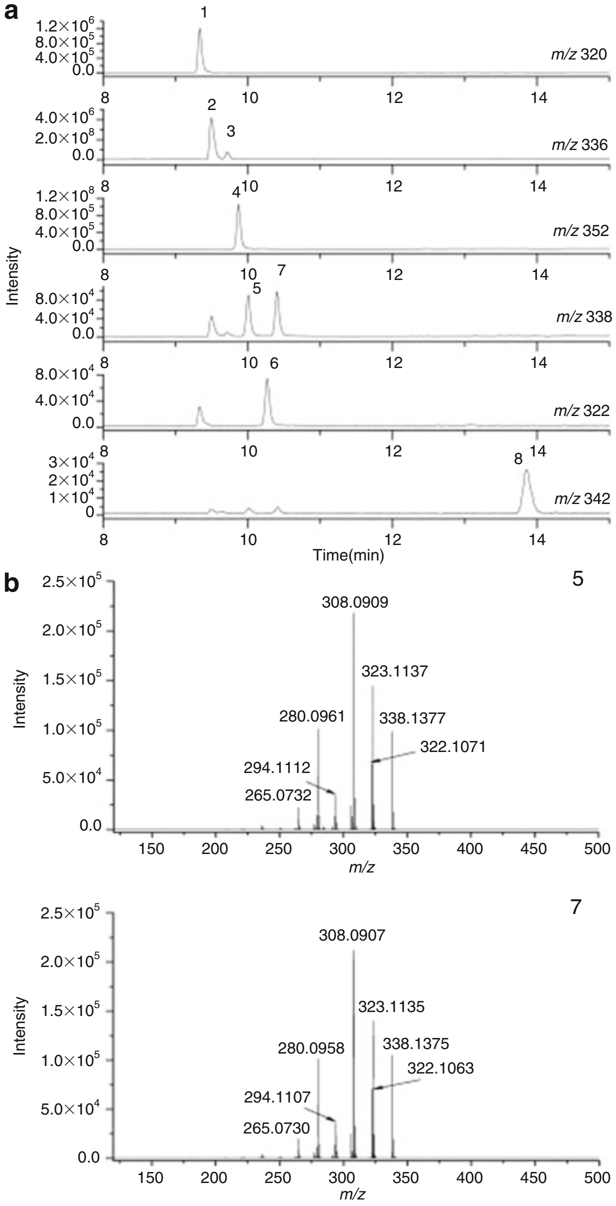

Fig 36.8 (**a**) CE-ESI-TOF-MS of eight alkaloids in *R. coptidis* extract. (*1*) Coptisine, (*2*) berberine, (*3*) epiberberine, (*4*) palmatine, (*5*) columbamine, (*6*) tetradehydroscoulerine/tetradehydrocheilanthifolinium, (*7*) jatrorrhizine, and (*8*) magnoflorine (**b**). Characteristic fragmentations of peaks 5 and 7 that were found to correspond to columbamine (*5*) and jatrorrhizine (*7*) (Modified from ref. [29])

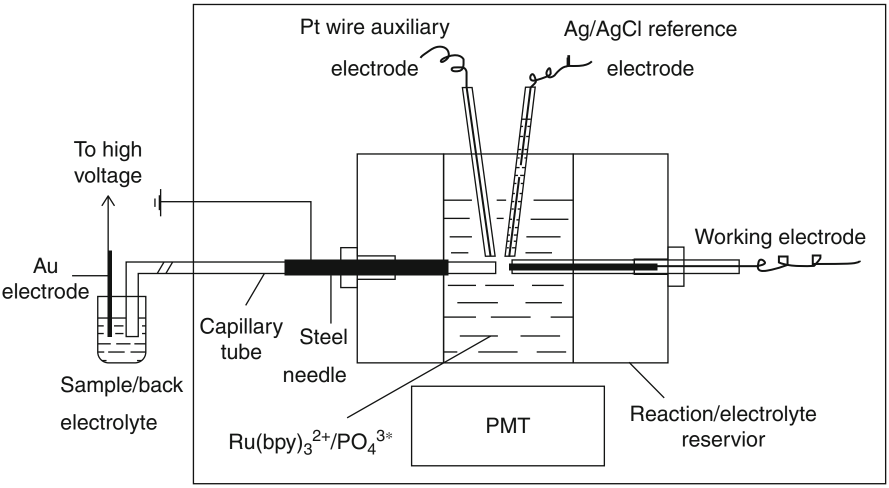

Fig. 36.9 Schematic diagram of the CE-ECL detection system (Modified from ref. [116])

a multichannel data collection analyzer based on a sensitive photomultiplier tube (PMT). Using the described configuration, no external light source is needed; the optical system is simple; and since strong background light levels are excluded, the noise signal is reduced with the result of an improved sensitivity [115]. Chemiluminescence reactions that involve species generated by electrochemical reactions give the so-called electrochemiluminescence (ECL) detection that has found several applications in sensitive and selective CE analysis of alkaloids. To this regard, the complex tris(2,2′-bipyridyl)ruthenium, Ru(bpy)$_3^{2+}$ is often selected as an ECL label. Solutions of this specie, typically in phosphate buffer (i.e., 75 mM, pH 8.0), are used to fill a reservoir of the detection cell as reported in the schematic diagram of Fig. 36.9 describing a system developed for the sensitive analysis of sinomenine, a morphinane alkaloid in *Sinomenium acutum* [116]. The complex undergoes oxidation on the working electrode surface, in general at the voltage of +1.3 V to generate the reactive specie Ru(bpy)$_3^{3+}$.

The alkyl amine groups of alkaloids eluting from the separating capillary react with the complex to form the excited state [Ru(bpy)$_3^{2+}$]* which will decay to the ground state emitting orange light at 610 nm. The detection potential and the distance of the separating capillary to the electrode are important instrumental aspects to be designed; other relevant parameters affecting the ECL response are the nature, concentration, and pH of the buffer in the detection reservoir and in the separating CE capillary [115, 116]. Importantly, tris(2,2′-bipyridyl)ruthenium is stable and soluble in aqueous media, and the high efficiency over a wide pH range makes it compatible with most buffers used in CE. The reactivity of the complex Ru (bpy)$_3^{3+}$ follows the order tertiary>secondary>and primary alkyl amines; thus, alkaloids are efficient co-reactants in the electrochemical process as highlighted by several applications in the field of plant analysis. A remarkable example is related to analysis of quinolizidine alkaloids from *Sophora* species (e.g., *S. tonkinensis* and *flavescens*) used in traditional Chinese medicine (TCM) for treating acute hepatitis and jaundice. Interestingly, at relatively high-dose levels, the compounds exhibit adverse effects, and the quality control of the quinolizidine-containing herbal drugs needs for accurate quantitation. Most of these alkaloids (i.e., sophoridine, matrine, and oxymatrine, Fig. 36.2) lack significant chromophores; thus, ECL detection can be considered as the method of choice for their sensitive analysis. Sophoridine and matrine, owing to the presence of tertiary amine function, react with $Ru(bpy)_3^{3+}$ to produce an intense ECL response, thus providing very low detection limits (1.0 nM). Unexpectedly, the ECL response was also observed for oxymatrine (quinolizidine *N*-oxide) that however showed higher detection limit (40 nM). Actually, in oxymatrine, the withdrawing oxygen attached to the nitrogen atom carries the charge from negative to positive, thus destabilizing the electrooxidation product. Under these detection conditions, the LOD values were 1,000-fold compared to those achieved by CE-UV at 200 nm [10, 12, 31, 117]. The highly sensitive detection of ECL using tris(2,2′-bipyridyl)ruthenium (II) was advantageously applied in CE analysis of aconitine alkaloids in TCM (caowu and chuanwu). The study was undertaken because of the necessity to provide efficient and sensitive analytical methods for the quality control and the safe use of *Aconitum* plants characterized by limited therapeutic index. Using ECL, detection limit of 10^{-8} M (in the order of ng mL^{-1}) was achieved [118]. Similar CZE (18 mM aqueous phosphate buffer, pH 9.0) and ECL detection conditions were applied to the analysis of galanthamine in *Bulbus Lycoridis Radiatae*, widely used in TCM. The ECL detection allowed LOD of 0.25 ng mL^{-1} to be achieved; however, the level of galanthamine found in the *Bulbus Lycoridis Radiatae* phytopreparations was 0.13 mg g^{-1} thus affordable also by UV detection [119]. The versatility of ECL detection mode is shown by several applications of CE performed in the presence of additive to the running buffer usually supplemented to tune the separation selectivity and using nonaqueous conditions. As an example, the analysis of anisodamine, atropine, and scopolamine in *Flos daturae*, a traditional Chinese crude herb, used as antitussive, antispasmodic, and analgesic agent, was performed in aqueous alkaline [8] or neutral conditions [43] and NACE [120]. In order to improve the separation of scopolamine and atropine, β-cyclodextrin (βCD) was supplemented at the concentration of 4 mM to the BGE showing to be compatible with the ECL detection. The reported detection limits were in the order of 0.3–13 ng mL^{-1} [43]. Ionic liquids may be used to prepare BGEs in CE with ECL detection [23, 38, 121]. In particular, a separation buffer constituted of 25 mM borate alkaline solution supplemented with the ionic liquid 1-ethyl-3-methylimidazolium tetrafluoroborate (1E-3MI-TFB) was used for separation of aconitum alkaloids in TCM products [121] and morphinane alkaloids in opium poppy [23]. In particular, borate buffer was useful in improving the separation selectivity by complexation of borate anions with the hydroxyl groups of the studied alkaloids. On the other hand, the ionic liquid, because of the higher conductivity compared to that of phosphate buffer present in the detection cell, provided a field-amplified effect, resulting in improved sensitivity (LOD of 1 × 10^{-6} to 1 × 10^{-9} M) [23]. Similar conditions, involving a running buffer constituted of alkaline phosphate solution in the presence of 40 mM 1B-3MI-TFB ionic liquid, were successfully used for separation of steroidal alkaloids verticine and verticinone. These compounds have been isolated from plants belonging to *Liliaceae* family, i.e., *Bulbus fritillariae*, used as a traditional herb remedy in Japanese, Turkish, Pakistani, and Southeast Asian folk medicines [122]. The *B. fritillariae*-based phytopreparations are used as antitussive, anti-asthmatics, and expectorants. Extracts from different *Fritillariae* species were obtained with chloroform in the presence of ammonia to afford the free base of the alkaloids that are characterized by cholestane carbon skeleton (isosteroid alkaloids) with a hexacyclic benzo[7,8]fluoreno[2,1-*b*]quinolizine nucleus devoid of significant chromophores. Under ECL detection conditions a very low LOD (about 50 pg mL^{-1}) was achieved. The analysis of real samples showed that verticine was found in any of the analyzed extracts (*Fritillariae hupehensis*, *F. walujewii*, *F. cirrhosa*, *F. thunbergii*, *F. ussuriensis*, *F. pallidiflora*, and *F. przewalski*) at amounts ranging within 5.40–12.35 mg L^{-1}, whereas verticinone was found at lower levels (5.66–8.07 mg L^{-1}) only in *F. hupehensis*, *F. cirrhosa, F. thunbergii*, and *F. ussuriensis* [38].

7.2 Online Sample Preconcentration

Online sample preconcentration is the term used to indicate CE techniques where a large volume of sample is injected into the separation capillary; successively, the analytes dissolved in the large sample zone are focused into a narrow zone before separation, in order to obtain improved concentration sensitivity [123]. One of the simplest online preconcentration technique is field-amplified sample stacking (FASS) that can be easily performed by injecting charged solutes in highly diluted buffer. When the injection voltage is applied, the charged analytes experience enhanced velocity in the lower-conductivity sample zone and are stacked into a narrow zone at the boundary sample/BGE because they experience the higher conductivity of the separation buffer. Yu et al. applied FASS in analysis of quinolizidine alkaloids (sophoridine and congeners) by using for separation, an MEEKC system based on the use of sodium cholate as surfactant [97]. The proposed system, that involved addition of magnesium salts to suppress the EOF, allowed LOD of 0.1 ng mL^{-1} using UV detection at 200 and 214 nm. Wang et al. applied FASS using a nonaqueous buffer (ACN/methanol, 30:70, v/v) in combination with ESI-MS detection in analysis of quinolizidine alkaloids in *Sophora flavescens* and *S. tonkinensis*, achieving LOD values of 0.02–0.04 ng mL^{-1} [124].

Sweeping is a phenomenon whereby analytes are injected in a CE system containing a pseudo-stationary phase (micelles or microemulsions) that by penetrating the sample zone allows for the accumulation of the solutes. Higher is the hydrophobicity of the analyte, and higher is the concentration efficiency. Although the sweeping technique proposed by Quirino and Terabe was originally used for

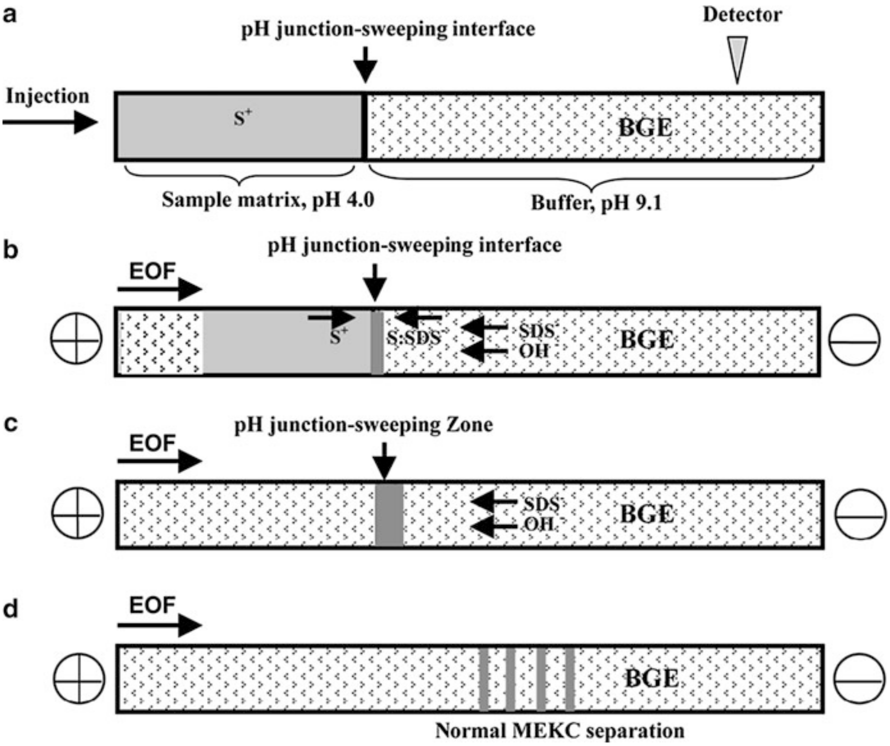
a
pH junction-sweeping interface
Detector
Injection
S+
BGE
Sample matrix, pH 4.0
Buffer, pH 9.1
b
pH junction-sweeping interface
EOF
S+
S:SDS
SDS
OH
BGE
c
pH junction-sweeping Zone
EOF
SDS
OH
BGE
d
EOF
BGE
Normal MEKC separation

Fig. 36.10 Schematic diagrams of the dynamic pH junction-sweeping strategy. Steps: (**a**) large-volume sample injection, (**b**) beginning of pH junction sweeping, (**c**) end of pH junction weeping, and (**d**) normal MEKC separation (Modified from ref. [131]). The analysis is performed on four pyrrolizidine alkaloids whose pKas were in the range 5.44–6.65

concentration of neutral molecules [125], it has shown to be applied equally well to ionic analytes as long as they have high affinity toward the pseudo-stationary phase [126]. A typical example is related to the analysis of berberine, coptisine, and palmatine (all containing a quaternary nitrogen) by means of an MEKC method. By working under strongly acidic conditions (pH 1.82), the EOF was significantly suppressed; the pseudo-stationary phase was obtained using SDS at 15 mM concentration, and in order to optimize the separation of the alkaloids, 10 % (v/v) THF was added. The sample solution was prepared at the same pH and electrolyte concentration as the BGE. After the introduction of a very long sample zone by pressure, a negative voltage is applied in order to drive the micelles of the BGE toward the sample zone. Under this condition, the cationic analytes move in the opposite direction so that the length of the sample zone becomes smaller and the solutes are incorporated into the micelles. With this sweeping method the LOD of the alkaloids was found to be 2.5 ng mL^{-1} which is 500-fold lower than that obtained using conventional injection [127]. Similarly, highly sensitive analysis of ephedra alkaloids in *Ephedra herba* samples [128], strychnine and brucine in *Strychnos nux-vomica* L. [129], and morphinane alkaloids in poppy straw samples [130] was carried out by sweeping methods optimized by means of a careful selection of separation buffer composition. The obtained sensitivity was 100-fold higher compared to that of conventional CE method.

In *dynamic pH junction* the analytes are dissolved in buffer whose pH provides either ionization or its suppression; then, they are injected in a CE system containing a BGE providing significant changes in ionization states of the analytes. The pH discontinuity is the key to focus the analytes at the boundary of sample plug and BGE. Yu and Li applied dynamic pH junction for sensitive detection in CE of pyrrolizidine alkaloids. In particular, senkirkine, senecionine, retrorsine, and seneciphylline with pKa ranging within 5.37–6.55 were dissolved in an acidic sample plug and injected in a CE filled with a BGE at pH 9.1. After application of the voltage, since the protonated alkaloids experience alkaline pH at the boundary with the BGE, their migration resulted to be stopped. The described dynamic pH junction could not be effective, in this specific situation, because the unprotonated alkaloids were only pushed out by the EOF. Thus, the dynamic pH junction was coupled to sweeping using as a BGE an SDS solution; the micelle by penetrating the neutral alkaloids provided an electrokinetic focusing of the large sample volume. After the compression of the sample plug, the MEKC led to separation. The schematic diagrams of the described method are reported in Fig. 36.10 [131].

8 Conclusion

Capillary electrophoresis can be considered a versatile separation technique useful in analysis of alkaloids for the quality control of herbal drugs and medicinal plants. The optimization of experimental parameters affecting separation, such as (a) nature, concentration, and pH values of background electrolyte in CZE; (b) choice of surfactant type and concentration in MEKC and MEEKC; (c) use of organic solvents; (d) use of additives which can form inclusion complexes such as CDs; and (e) opportunity to combine hybrid separation mechanisms (chromatographic and electrophoretic in CEC), represents valuable feature for the development of selective and fast analytical methods. The variety of separation mechanisms involved in EKC approaches offers the opportunity to achieve orthogonal selectivity compared to HPLC and GC, thus providing effective alternative analytical methods. The possibility to combine CE with LIF and ECL detectors, beside the online preconcentration systems, greatly enhances the sensitivity making CE useful in analysis of toxic alkaloids and for standardization of phytopharmaceuticals containing bioactive compounds with low therapeutic index. More advanced detection approaches, as CE-ESI-MS and CE-ESI-TOF-MS, are mainly addressed to the phytochemical characterization of new potentially bioactive alkaloids and plant metabolites.

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Part IV

Alkaloids: Pharmacology

Pharmacology of Macroalgae Alkaloids 37

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Contents

1 Introduction 1205
2 PEA Derivatives 1205
  2.1 Structure Activity Relationship of PEA Derivatives 1205
  2.2 PEA Derivatives as TA Family Members 1205
  2.3 PEA 1206
  2.4 TYR 1207
  2.5 *N*-ACPEA and *N*-ACTYR 1208
  2.6 HORD 1209
  2.7 DOP 1209
3 Indole Derivatives 1210
  3.1 CLP 1210
  3.2 FRG 1211
  3.3 MRT A 1212

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3.4 MRF A ... 1212
3.5 DTC ... 1212
4 Halogenated Indole Alkaloids ... 1212
4.1 BRI ... 1212
4.2 PHLI ... 1212
5 2, 7-Naphthyridine Derivatives ... 1212
5.1 LO A and LO B ... 1212
6 Conclusion ... 1213
References ... 1213

Abstract

Constituents of marine algae gained importance in the last four decades due to the growing interest in their potential for medicinal use. Till today, 44 alkaloids were isolated from macroalgae; some of which were reported to induce important pharmacological effects. These alkaloids can be classified under four groups as 2-phenylethylamine, indole, halogenated indole, and 2,7-naphthyridine derivatives, and each group displays a relatively distinguished activity profile. Overall, pharmacological effect spectrum of macroalga alkaloids is wide and includes neuromodulation, neurotransmission, growth regulation, cytotoxicity, angiogenesis, antioxidation as well as antibacterial, antifungal, and larvicidal activities.

Keywords

Pharmacology • alkaloids • macroalgae

Abbreviations

BRI	Bromoindole
CLP	Caulerpin
DOP	Dopamine
DTC	Denticin
FRG	Fragilamide
HORD	Hordenine
LO A	Lophocladine A
LO B	Lophocladine B
MRF A	Martefragin A
MRT A	Martensine A
N-ACPEA	*N*-Acetylphenylethylamine
N-ACTYR	*N*-Acetyltyramine
PEA	B-Phenylethylamine
PHLI	Polyhalogenated indoles
TA	Trace amine
TAAR	Trace amine-associated receptor
TYR	Tyramine

1 Introduction

Seaweeds were used in the era of folk medicine since old Chinese and Egyptian civilizations [1]. The importance and the need for increasing knowledge on seaweed use in pharmaceutical, cosmetic, food, and textile industries grew over years [2]. Advances in isolation methodologies yielded more information about the potential for medicinal use of marine algae and their constituents, especially in the last four decades [3]. Compounds isolated from marine algae were demonstrated to have anticoagulant, hypolipidemic, hypoglycemic, antitumor, and antiulcer activities [4]. Progressively increasing data reveal interesting pharmacological properties of macroalga alkaloids and may provide valuable avenues for further research.

Following isolation of the first alkaloid hordenine, many alkaloids were found in marine algae, which were presented in the recent reviews [5, 6]. TYR, DOP, and HORD are biological amines, and among these, only HORD is defined as an alkaloid. These amines were included in this chapter because all are derived from the parent compound PEA and have important pharmacological activities.

2 PEA Derivatives

2.1 Structure Activity Relationship of PEA Derivatives

PEA derivatives isolated from marine algae are PEA, *N*-ACPEA, TYR, *N*-ACTYR, HORD, and DOP. All possess sympathomimetic activity, i.e., their effects resemble the activity observed when sympathetic nervous system is stimulated.

%PEA can be considered as the parent compound and is composed of a benzene ring plus ethylamine moiety. A hydroxyl group is substituted at position 4 (C4) of the benzene ring in TYR, HORD, and DOP. DOP has an additional hydroxyl substitute at position 3 (C3) of the benzene ring. Activity of α- and β-receptors of sympathomimetic amines is determined by hydroxyl group substitution on positions 3 and 4. If the biogenic amine lacks either of these groups and if there is no aromatic substitution, sympathomimetic potency is reduced [7]. Positioning of hydroxyl group modifies activity, e.g., introduction of a β-hydroxyl group weakens, but phenolic-hydroxyl substitution strengthens sympathomimetic potency [8].

2.2 PEA Derivatives as TA Family Members

TAs were previously referred to as little more than metabolic by-products [9] or false transmitters, microamines, and noncatecholic amines [10]. Today, TAs are well-characterized neurotransmitters and neuromodulators, as well [9–12]. These endogenous monoamines are present at relatively lower concentrations, approximately a thousandfold, than classical monoamines, namely, DA, norepinephrine, and serotonin, although their synthesis rates are nearly equivalent. PEA and TYR are among traditional members of TA family. They are found as endogenous ligands in the central nervous system, but also produced in foods from the natural breakdown of the amino acids [9, 10].

Endogenous TAs act as agonists of a TA-associated receptor 1 (TAAR1) [12–14]. Thyronamines like 3-iodothyronamine, which are thyroid hormone derivatives, also show potent agonistic activity for TAAR1 [12]. TAAR1 mRNA is expressed in trace (in cerebellum, dorsal root ganglia, hippocampus, hypothalamus, liver, medulla, pancreas, pituitary, pontine reticular formation, prostate, skeletal muscle, and spleen), low (amygdala, kidney, lung, and small intestine), and moderate (stomach) amounts in human tissues [13].

TAAR1 functions as an important modulator of dopaminergic neurotransmission [15, 16]. In mouse brain slices, TAAR1 agonists inhibit firing frequency of dopaminergic and serotonergic neurons in the ventral tegmental area and dorsal raphe nucleus, respectively. Thus TAAR1 activation prevents hyperdopaminergic and hypoglutamatergic activity [16]. TAAR1 agonist 3-iodothyronamine induces anergia, hypothermia, bradicardia, hypoglycemia, and hyperinsulinemia [17]. In brief, accumulating evidence has rendered this receptor a promising therapeutic target for the treatment of affective disorders, psychosis, addiction, hyperactivity, and primary headache [12, 16].

Both PEA and TYR are agonists of TAAR1, but PEA is more potent than TYR [18]. On the other hand, TYR and PEA are partial allosteric antagonists at beta-adrenergic receptors 1 and 2, which are highly similar to TAAR 1 [18].

2.3 PEA

In 1990, PEA was proposed to act as a neuromodulator of catecholamine neurotransmission [19]. TAAR1 activation by PEA alters the function of dopamine transporter which is co-localized with TAAR1 in dopaminergic neurons of rhesus monkey and mouse brain substantia nigra [20]. DOP transporter mediates reuptake of DOP and thus terminates the action of synaptically released DOP [21].

Localization of TAARs particularly in amygdala may explain the role of PEA in affective disorders. 24-h mean urinary levels of phenylacetic acid (PAA), end catabolic product of PEA in healthy males, were measured following treadmill exercise. PAA level was increased by 77%. It was concluded that antidepressant effects of exercise may be related to increased PEA activity [22]. PEA has shown antidepressant effect in several preclinical and clinical studies, and its effect is nearly indistinguishable from those of classical antidepressants [9]. Urinary excretion of PEA is also elevated following profound stress, like a parachuting exercise [23]. Accordingly, in preclinical studies, stress was accompanied by elevated PEA levels, indicating its endogenous anxiogen role [11].

PEA is synthesized from phenylalanine by the enzyme aromatic L-amino acid decarboxylase (AADC). Accordingly, high phenylalanine levels in the brain as in the case of phenylketonuria disease result in increased PEA levels. Altered AADC activity, hence altered steady-state level of PEA, has been linked to the pathogenesis of schizophrenia, attention deficit and hyperactivity disorder, and addiction like nicotine dependence and alcoholism [11].

Phenylalanine is a precursor in both PEA and DOP synthesis in the dopaminergic neurons of the nigrostriatal system; therefore, both metabolic pathways are closely related. For this reason, decreased PEA level in body fluids may reflect nigrostriatal degeneration. Significantly lower PEA levels were reported in cerebrospinal fluid of patients with Parkinson's disease than controls and patients with peripheral neuropathy [24]. Similarly, lower plasma levels of PEA were found in patients with Parkinson's disease as compared to controls. Three years of follow-up of patients with Parkinson's disease, PEA levels showed a downward trend in the progressive group but remained unchanged in the nonprogressive group [25].

PEA produced a reversible hyperpolarization and inhibited spontaneous firing in mesencephalic dopaminergic neurons [26]. Modulation of dopaminergic transmission is actualized by reduction of both presynaptic and postsynaptic $GABA_B$ receptor-mediated responses. PEA, and also TYR, in the presence of specific DOP receptor antagonists, inhibited $GABA_B$ receptor-dependent presynaptic inhibition of GABAergic inputs to dopaminergic neurons of substantia nigra [27]. In a study conducted in rat midbrain dopaminergic neurons, PEA depressed the amplitude of electrically evoked $GABA_B$-mediated slow inhibitory postsynaptic potential, interfering with the inwardly rectifying potassium channels. These observations indicate a probable role of PEA in neural regulatory mechanisms related to emotion, reward, addiction development, and locomotion [28].

PEA induced seizures in a dose-dependent manner, and its convulsant effects could be prevented by increasing brain GABA levels [29]. Seizure inducing effect of PEA at high doses was dependent on inhibition of K^+ currents, as examined in the cell membranes of rat cerebral cortical neurons. PEA suppressed both time- and voltage-dependent K^+ current (M-current) and leakage K^+ current. Suppression of M-current weakens the control on excessive neural firing, and suppression of the leakage K current triggers neuronal excitation. As a result of hyperexcitation, generalized convulsions occur at high PEA doses [30].

PEA has a similar structure to amphetamine and is well known to increase locomotion and induce stereotyped behaviors like amphetamine at higher, supraphysiological doses [10, 11, 19]. It was shown that the psychomotor effect of PEA may involve cholinergic and glutamatergic pathways in the striatum. PEA increased acetylcholine release in the striatum. PEA-associated stimulation of acetylcholine release was mediated by AMPA-type glutamatergic receptors [31].

2.4 TYR

TYR is the decarboxylation product of tyrosine, which is the precursor of DOP and norepinephrine. TYR is an indirect-acting sympathomimetic and promotes norepinephrine release from the nerve terminals. Consumption of TYR-rich food like cheese, sour creams, chocolate while taking antidepressant monoamine oxidase inhibitors may interfere with the body's ability to metabolize endogenous and exogenous monoamines and result in hypertensive crisis, which is known as "cheese effect" [32]. Potency of TYR on blood pressure rise was found to be a 100-fold weaker than adrenalin [33]. TYR's effect on membrane potential and conductance was investigated in frog ventricular myocytes. TYR induced a concentration-dependent decrease in sodium currents and blocked sodium channels directly as shown by a whole-cell voltage clamp technique [34].

The effects of TYR in TAAR1 were investigated in knockout mice. Their aim was to dissociate dopamine transporter-related and receptor-mediated effects. In TAAR1 knockout mice, dopaminergic neurons had a higher spontaneous firing rate in the ventral tegmental area. TYR did not decrease spike frequency of these neurons in TAAR1 knockout mice in contrary to the wild type. This finding supports TAAR1-related neuromodulator role of TYR in dopaminergic neurotransmission [15].

TYR also modulates GABAergic transmission. TYR depressed $GABA_B$-mediated inhibitory postsynaptic potential in a concentration-dependent and reversible manner in dopaminergic neurons [28].

TYR predisposes susceptible patients to epileptic seizures. Oral TYR was reported to activate preexisting EEG abnormalities in epileptic patients, whereas chocolate (which is a PEA-rich food) and PEA had weaker effects [35].

TA directly mediates feeding threshold determination. In the blowfly *Phormia regina*, proboscis extension reflex was used as an indicator of behavioral sensitivity for taste. Acquired experiential effects of nonappetitive or appetitive odors were linked to TYR levels in the brain [36].

2.5 *N*-ACPEA and *N*-ACTYR

PEA and TYR are substrates for *N*-acetyltransferase (NAT)-mediated biotransformation process. NATs metabolize agents that contain an aromatic amine or hydrazine group. Addition of an acetyl group leads to a less soluble molecule, altering its pharmacokinetics [37]. Therefore, *N*-acetylation is used by cells as an inactivation process of excess amines. Substrate specificity of NAT for biogenic amines was investigated *in Fasciola Hepatica*. TYR and PEA were found to be the best substrates with a relative rate of 98–100 % as compared to serotonin, norepinephrine, and DOP [38].

N-ACTYR is the neutral metabolite of TYR. *N*-ACTYR is excreted after conjugation with glucuronic acid and sulfate, and less than 0.2% is detected as free unchanged compound [39]. In neuroblastoma, elevated levels of catecholamines or its metabolites can be found in the urine, and urinary *N*-ACTYR was detected in these patients [40].

N-acetyl derivatives are alike their parent drugs with immediate onset and short duration of action [33]. However, *N*-acetyl derivatives were shown to elicit weaker responses with regard to their parent compounds. *N*-ACTYR had a weaker sympathomimetic potency than TYR, as shown on a rat carotid artery model [33]. PEA and *N*-ACPEA were compared with respect to their effects on the rotational behavioral response in animals with a unilateral 6-hydroxydopamine lesion of the nigrostriatal dopamine system. Intravenous (i.v.) injections of both agents induced rotations ipsilateral to the side of the brain lesion, but activity of *N*-ACPEA was 90% less active than β-PEA [41].

N-ACPEA and *N*-ACTYR were compared for their inhibitory effects on sepiapterin reductase, a catalyzing enzyme for synthesis of tetrahydrobiopterin which is a required cofactor for phenylalanine, tyrosine, and tryptophan hydroxylases. In MOLT-4 T cell leukemia and MCF-7 breast cancer cell lines, both *N*-ACPEA and *N*-ACTYR (*N*-acetyl-p-tyramine) showed poor inhibitory effects on sepiapterin reductase, whereas *N*-acetyl-m-tyramine was a strong inhibitor [42].

2.6 HORD

Since HORD was used to feed animals, its toxicity was investigated in several animal species. In rats and dogs, HORD showed a positive inotropic effect, increased systolic and diastolic pressures, enhanced peripheral blood volume, and inhibited gut movement. All effects are short and only observable at high doses [43]. HORD administered at an oral dose of 2.0 mg/kg body weight to horses did not change heart rate, respiratory rate, basal body temperature, and behavior. But when HORD was injected by rapid i.v. route to horses, defecation within 60 s, substantial respiratory distress, approximately 250% increase in heart rate, and profuse sweating were observed [44]. These effects were transient and abolished within 30 min.

The vasopressor effect of HORD is not centrally induced but by its stimulatory action on the heart muscle [45]. In isolated vas deferens of guinea pig, relative maximal response of HORD ethiodide was found to be higher (0.22) than tyramine (0.09) as compared to norepinephrine (1.00), which served as the reference [46]. HORD isolated from *Phyllophora nervosa* increased blood pressure, contracted nictating membrane when injected by i.v. route to cats. This epinephrine-like effect was less potent but more durable than epinephrine [5].

HORD and its derivatives are central depressants [47]. At very high doses, the consequence could be fatal, causing respiratory arrest and death [45].

In the past, plants containing HORD have been used as a diuretic and also for the treatment of diarrhea and dysentery as folk remedies. HORD was shown to be a weak antiseptic and inhibit some soluble ferments [45].

2.7 DOP

DOP is a key neurotransmitter in the body. It's synthesized from phenylalanine and tyrosine. DOP binds to five distinct groups of receptors. D1 receptor is abundant in kidney, retina, cardiovascular system, and especially in CNS. DOP decreases blood pressure by vasodilatation and increasing urinary sodium excretion. DOP decreases prolactin secretion from the pituitary gland. DOP modulates many functions of CNS, like addictive behavior, emotions, cognition, learning, memory, and locomotor activity. DOP receptor agonists are indicated in the treatment of Parkinson's disease, hyperprolactinemia, and restless leg syndrome. DOP receptor antagonists are used in schizophrenia and other psychotic diseases, show promise in the treatment of addiction [21]

3 Indole Derivatives

Indole group alkaloids with reported pharmacological activity include CLP, CLS, FRG, MRT, MRF, and DTC.

3.1 CLP

Although *Caulerpa* material was reported to cause poisoning, one of its constituents, a red pigment, namely, CLP exhibited low toxicity [48, 49]. Acute toxicity study in mice followed up for 15 days revealed that LD50 of CLP is greater than 2 and 0.2 g/kg, following oral and i.v. administration, respectively [50].

CLP regulates plant growth and stimulates shoot and root growth [51–54]. Effect of CLP on cell growth was studied in a *Saccharomyces cerevisiae* tryptophan auxotroph with human indoleamine 2, 3-dioxygenase (IDO) expression. IDO is a degradation enzyme for tryptophan, and its overexpression when accompanied by low tryptophan levels restricts cell growth. In this yeast assay, CLP was able to restore growth [55].

Cytotoxic activity of CLP has been extensively investigated. CLP exhibited a moderate in vitro antitumor activity against crown-gall tumor [56]. The crude acetone extract of *Caulerpa racemosa* had a cytotoxic effect on C32 human amelanotic melanoma cells. C32 cells were treated for 72 h within 5.0–100 mcg/ml concentration range, and cytotoxic activity was measured by sulforhodamine (SRB) assay. The growth inhibitory effect of the crude extract at 50.0 and 100.0 mcg/ml concentrations continued for 3 days following exposure. However, CLP, purified from the crude extract, displayed no cytotoxicity in C32 cell line following 72 h of exposure. CLP had a high cytotoxic effect on normal fibroblast cell line FEK4 at 10.0 and 50.0 mcg/ml as compared to the control group [57]. Similarly, a cell line-dependent cytotoxic activity of CLP was demonstrated. CLP (1, 3, 10, and 30 mcM for 48 h) inhibited cell growth in breast tumor (T47D, MCF-7, and MDA-MB-231), prostate tumor (DU145 and PC-3), and primary human mammary epithelial cell (HMEC) lines as determined by SRB assay. Growth inhibitory effect varied between cell types. PC-3 was the most susceptible cell line, and viability was inhibited by 52% at 30 mcM [58]. CLP showed no cytotoxic activity in HL-60 leukemic and MCF-7 breast cancer cell lines [59].

Hypoxia increases intracellular concentration of a gene regulatory protein, hypoxia-inducible factor-1a (HIF-1), which stimulates vascular endothelial growth factor (VEGF). In T47D cells, CLP suppressed HIF-1 activation. This concentration-dependent effect of CLP is observed in hypoxia (1% O_2) as well as chemically induced hypoxia. In the same assay, CLP also suppressed hypoxia-induced secretion of vascular endothelial growth factor (VEGF), an important protein in tumor angiogenesis. Antimetastatic activity of CLP was further demonstrated in a MDA-MB-231-based wound healing model. In this wound healing process in cell culture, CLP inhibited migration of MDA-MB-231 cells in a dose-dependent manner [58]

Exposure of the marine sponge *Geodia cydonium* to nontoxic (non-apoptotic) concentrations of water pollutant tributyltin together with CLP (10 mcg/ml) led to apoptosis. Increased toxicity of tributyltin was at least partially attributed to inhibition of multixenobiotic resistance pump by CLP [60]. This important observation elucidates the potential effect of CLP in overcoming resistance against chemotherapeutic agents.

In addition to its effects on cell growth, CLP elicits antinociceptive and anti-inflammatory activities. Peripheral antinociceptive activity was investigated by acetic acid-induced writhing model. CLP (0.1–100 mcM/kg) inhibited acetic acid-induced abdominal constrictions dose dependently (IC50 = 0.0945). Central antinociceptive activity of CLP was demonstrated in the hot plate test. CLP (100 mcM/kg, p.o.) increased latency time 90, 120, and 150 min after treatment. Similarly, CLP caused an inhibition of nociception in formalin test. Anti-inflammatory activity of CLP was evaluated in capsaicin-induced ear edema and carrageenan-induced peripheral inflammation tests. Following oral administration at a dose of 100 mcM/kg, CLP inhibited capsaicin-induced ear edema by 55.8% and reduced leukocyte migration to the inflammatory sites by 48.3% [61].

CLP was shown to exhibit antifungal [51], antibacterial [62], and larvicidal [63] activities.

CLP also attracted attention in type II diabetes and obesity-related research. CLP inhibited human protein tyrosine phosphatase 1B (hPTP1B) which can hydrolyze phosphotyrosines on the insulin receptor and deactivates it [59].

3.2 FRG

ROS scavenging ability of FRG was tested using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) solution-based chemical assay and a 2,2,7,2-dichlorodihydrofluorescein diacetate (DCFH-DA) HL-60 cell line-based assay. The cell-based fluorescent DCFH assay enables detection of antioxidant molecules which can penetrate cell membranes and inhibit ROS production in living cells. The antioxidant activity was determined by TPA-stimulated control cells with and without FRG. FRG showed strong antioxidant activity in both systems [64].

3.3 MRT A

MRT A showed antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Mycobacterium smegmatis* [65].

3.4 MRF A

MRF A is an antioxidant and a potent inhibitor of lipid peroxidation [66–68]. MRF A showed inhibitory activity on NADPH-dependent lipid peroxidation (IC50 = 2.8 mcM) in rat liver microsomes, being 30 times and 70 times stronger than alpha-tocopherol (IC50 = 87 mcM) and ascorbic acid (IC50 = 200 mcM), respectively. Indole (IC50 = 330 mcM) and tryptamine (IC50 = 260 mcM), the partial structures of MRF A, had much weaker effects on lipid peroxidation [67]

3.5 DTC

DTC(s) have an anti-photo-oxidative activity [69].

4 Halogenated Indole Alkaloids

4.1 BRI

2,3,5, 6-Tetrabromo-1 *H*-indole showed antimicrobial activity and, at a level of 100 mcM per 12.7-mm disk, produced inhibition zones of 16 and 14 mm for *Bacillus subtilis* and *Saccharomyces cerevisiae*, respectively [70, 71].

4.2 PHLI

Strong antifungal activity detected in the crude extract of *Rodophyllis membranacea* Harvey was related to the presence of polyhalogenated indoles [72].

5 2, 7-Naphthyridine Derivatives

5.1 LO A and LO B

Although structurally similar, LO A and LO B possess distinct pharmacological properties. As measured by SRB assay, LO B had moderate cytotoxic activity in MDA-MB-435 breast cancer and NCI-H460 lung cancer cell lines with IC50 of 3.1 mcM and 64.6 mcM, respectively. However, LO A was inactive in these two cell lines. Both LO A and LO B did not display any cytotoxicity in neuro-2a neuroblastoma cell line [73].

LO B induced cell cycle arrest at G2/M phase in MDA-MB-435 cells. Cytotoxic effect was related to microtubule inhibition. LO B was shown to be a moderate tubulin depolymerizer in a rat aortic smooth muscle cell line, A-10 [73].

Affinity studies with LO A and LO B at 10 mcM were conducted in muscarinic, opiate, GABA, and histaminergic receptors. LO A was found to be antagonist of δ-opioid and NMDA receptors [73].

6 Conclusion

Available results highlight the potential of macroalga alkaloids for medicinal use. Further research in this field seems likely to yield promising results for the development of anticancer, anti-inflammatory, antioxidant, and antimicrobial agents as well as novel therapies against affective disorders, psychosis, addiction, hyperactivity, and locomotor diseases.

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Pharmacological Effects of Ephedrine 38

Renata Pereira Limberger, Ana Laura Bemvenuti Jacques, Gabriela Cristina Schmitt, and Marcelo Dutra Arbo

Contents

1 Introduction 1218
1.1 Occurrence 1220
1.2 Biosynthesis and Chemical Aspects 1221
1.3 Analytical Aspects 1224
2 Pharmacological Applications 1225
3 Mechanism of Action 1226
4 Toxicity 1227
5 Bioavailability and Metabolism 1230
6 Clinical Trials 1232
7 Conclusion 1233
References 1233

Abstract

Ephedrine is a sympathomimetic agent that has widespread use as an adrenergic stimulant. It comes from plants of the genus *Ephedra* sp., being an alkaloid mainly found in the specie *Ephedra sinica*. This amine can be obtained by industrial synthesis, as a product of biotransformation or by extraction of the plant. Other derivatives of ephedrine can also be found in *Ephedra*, such as

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pseudoephedrine that also has adrenergic activity. The purest form of ephedrine is used for therapeutic purpose, primarily as a bronchodilator and decongestant. Its pharmacological applications are related to its sympathomimetic properties, which in turn occur due to its stimulating action on α-, β1-, and β2-adrenergic receptors through direct and indirect effects. Nowadays, there is a concern about the indiscriminate use of ephedrine, since it started to be used to weight loss and enhance athletic performance. Associated with this tendency, a large number of adverse effects related with cardiovascular issues started to be reported. This chapter will discuss botanical, chemical, pharmacological, toxicological, and analytical aspects of ephedrine.

Keywords
Ephedra • Ephedraceae • Ephedrine • Phenylethylamine alkaloid • Sympathomimetic amine

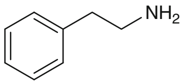
Abbreviations

AMP	Adenosine 3′,5′-monophosphate
CE	Capillary electrophoresis
CNS	Central nervous system
GC–FID	Gas chromatographic–flame ionization detector
GC–MS	Gas chromatographic–mass spectrometer
GC–NPD	Gas chromatographic–nitrogen–phosphorus detector
GCxGC	Gas chromatographic–gas chromatographic
HS	Headspace
LC–MS/MS	Liquid chromatographic–mass spectrometer mass spectrometer
LC–UV	Liquid chromatographic–ultraviolet
LLE	Liquid–liquid extraction
MAO	Monoamine oxidase
NE	Norepinephrine
PAL	L-phenylalanine ammonia-lyase
Phe	L-phenylalanine
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TLC	Thin-layer chromatography
VMT	Vesicular monoamine transporter

1 Introduction

Ephedrine (1*R*-2*S*-2-methylamino-1-phenylpropan-1-ol) is an adrenergic amine present in many kinds of pharmaceutical preparations, obtained by synthesis or from natural sources. Belonging to the genus *Ephedra* (Ephedraceae), the Chinese species *Ephedra sinica* and *Ephedra equisetina*, also known as ma huang, and the Indian and Pakistani species *E. gerardiana*, *E. intermedia*, and *E. major* are the primary source of ephedrine [1]. Besides *Ephedra* species, low amounts of ephedrine (less than 2 %) can also be found in the leaves of the Malvaceae *Sida cordifolia* (Linn) (known as country mallow) [2, 3] and in the Chinese herb *Pinellia* (known as ban xia), with around 0.003 % of ephedrine [4].

Ephedrine belongs to β-phenylethylamine core structure (**1**) and is kinetically and dynamically close related to amphetamine-type derivatives that include stimulants, psychedelics, and entactogens, as well as anorectics, bronchodilators, decongestants, and antidepressants [5]. Ephedrine (**2**) and its analogues pseudoephedrine, norephedrine (phenylpropanolamine) and norpseudoephedrine (cathine), as well as methylephedrine and methylpseudoephedrine (**3–7**) cannot be considered typical alkaloids, since they do not have the nitrogen as part of a heterocyclic ring system, being called protoalkaloids into the *sensu lato* alkaloid class [1].



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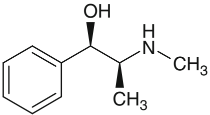

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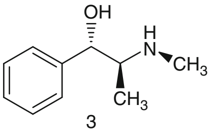

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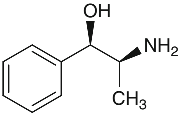

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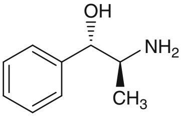

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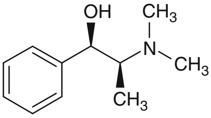

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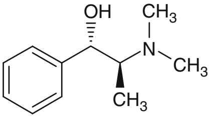

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The medicinal herb *Ephedrae* (ma huang or merely "ephedra") has been millenary used in traditional Chinese medicine to induce perspiration, reduce fever, and treat coughs and asthma. Recently, *Ephedra*-containing dietary supplements have been used as an aid in diets and as a stimulant to boost energy and athletic performance. The activity of ephedra is attributed to the presence of three pairs of diastereoisomeric alkaloids: (1*R*,2*S*)-(−)-ephedrine and (1*S*,2*S*)-(+)-pseudoephedrine, (1*R*,2*S*)-(−)-norephedrine and (1*S*,2*S*)-(+)-norpseudoephedrine, and (1*R*,2*S*)-(−)-*N*-methylephedrine and (1*S*,2*S*)-(+)-*N*-methylpseudoephedrine (**2–7**), being ephedrine the major constituent of it. More than 50 species of *Ephedra* are known and at least 18 of them contain these alkaloids. The amounts of ephedrines vary according to the species, time of harvest, geographical location, and growing conditions [6].

The long history of *Ephedra*'s use has begun in oriental medicine, especially for the treatment of asthma, colds, cough, fever, headache, and nasal congestion [7, 8]. Some applications still remain in modern medicine, but much has changed after the beginning of ephedrine's use for weight loss and muscle gain [9]. The record of cardiovascular adverse effects attributed to the use of ephedrine usually associated with others substances such as caffeine, salicin, and/or p-synephrine led to a higher concern about the use of this amine. Thus, after several attempts, the FDA banned the sale of ephedrine-containing products in the United States [10]. However, the pharmacological and toxicological properties strongly depend on the concentration of ephedrine, interactions with other drugs, individual susceptibility, and the presence of contaminants [8].

Based on these considerations, this chapter presents a literature review over several aspects of ephedrine, focusing on its botany, chemical composition, general uses, pharmacological properties, toxicity, and analytical aspects.

1.1 Occurrence

Plants of the genus *Ephedra* sp. (Ephedraceae, Gnetales) are the main natural source of ephedrine and related alkaloids. The aerial parts of different *Ephedra* species contain at least six optically active constituents that are structurally related to ephedrine. Among these, ephedrine and pseudoephedrine are the main psychoactive constituents, although others as optical isomers and methylated derivatives are also important [11].

A major source of *Ephedra* is near to the seacoast in southern China and this local formerly supplies most of the American market [12]. These popular herbs, also known as ma huang in traditional Chinese medicine, can be found in subtropical zones in Asia, Europe, and America [8, 13]. The name ma huang has Chinese origins, in which "ma" means astringent and "huang" means yellow, probably referring to the taste and color of the plant or tops of *Ephedra sinica* Stapf [12].

The *Ephedraceae* family consists of branched shrubs, rarely being small trees, with opposite leaves, scales and very small size [14]. The shrubs can reach 1 m in height and can grow in semiarid and desert conditions, being the six continents suitable for the growth of that genus [15]. Studies indicate that there are between 50 and 65 species of *Ephedra* in the world [16]. The plants are small bushes, dioecious, and with minute leaves, giving the appearance of leafless shrub. The shrub grows 60–90 cm high and has slender aerial stems, which are green, erect, small ribbed, and channeled. It is 1.5 mm in diameter and usually terminates in a sharp point. At the nodes, which are 4–6 cm apart, the leaves appear as whitish, triangular, scarious sheaths. Small blossoms appear in the summer [12, 17]. The concentration of ephedrine alkaloids can vary from 0.02 % to 3.40 % according to species and the isomer (−)-ephedrine represents from 30 % to 90 % of the total content of alkaloids [17, 18].

The different proportions of the alkaloids vary between plants of the same species and between different species of the genus *Ephedra* [19]. The collection aspects are also important. The plant should be collected in the autumn, since the amount of alkaloid shows considerable variation at different seasons [1].

Ephedrine and pseudoephedrine are the main active constituents of the *Ephedra*. Described in pharmacopoeias of China, Germany, and Japan, the dried young branches of *Ephedra sinica, E. equisetina*, *and E. gerardiana* contain not less than 1.25 % of alkaloids [20]. Japanese pharmacopoeia specifies not less than 0.6 % [21].

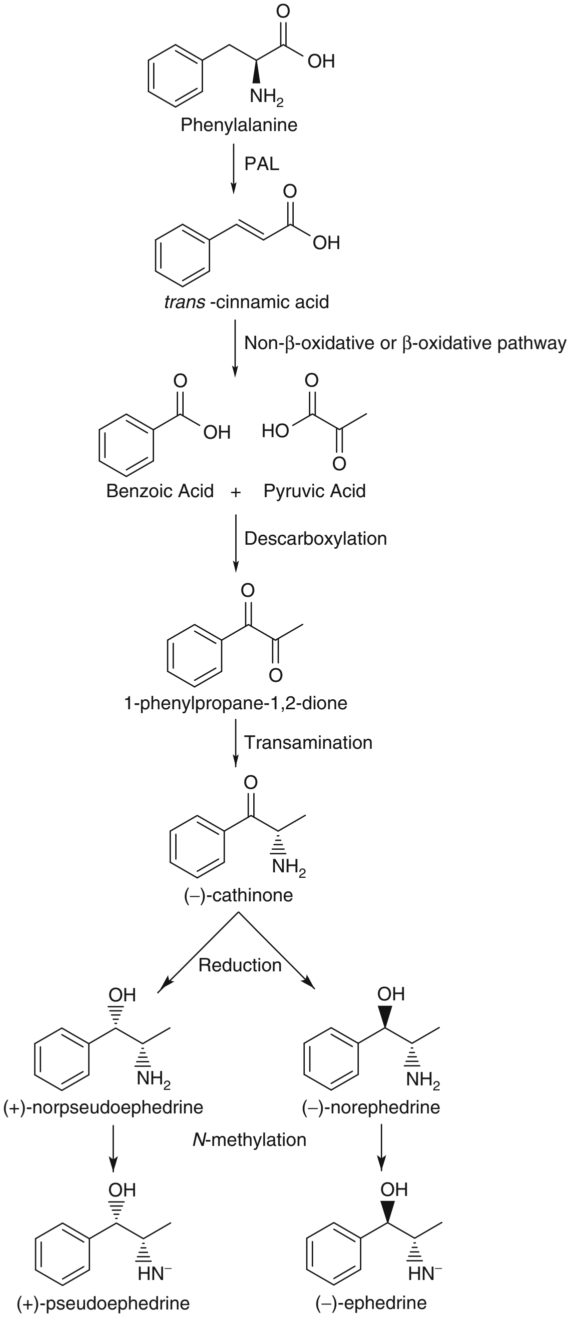
Ephedra sinica is the primary source of ephedrine alkaloids, although other species of this genus may contain the active constituents as *E. equisetina, E. intermedia*, *E. gerardiana, E. alata*, *E. distachya, E. botschantzevii*, *E. fragilis, E. major*, *E. minuta, E. monosperma*, *E. pachyclada, E. likiangensis*, *E. saxatilis, E. lomatolepis*, *E. lepidosperma, E. przewalskii*, and *E. regeliana* [22]. Eurasian species are characterized by the presence of alkaloids, while American species are considered devoid of such metabolites [7, 22–24]. Approximately 40 species of *Ephedra* are grown for commercial purposes; among them are *Ephedra sinica* Stapf and *E. equisetina* Bunge, from southern China, and *E. intermedia* Schrenk and *E. gerardiana* Wall, from India and Pakistan [17, 25]. The less-known alkaloid ephedroxane was found in *Ephedra intermedia* and at least in six more species [26].

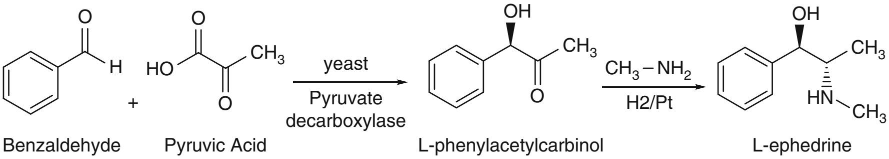
Besides *Ephedra* species, low amounts of ephedrine can also be found in the leaves of the Malvaceae *Sida cordifolia* (Linn) (known as country mallow), with less than 2 % of content [2, 3] and in the Chinese herb *Pinellia* (known as ban xia), with around 0.003 % of ephedrine [4, 27]. Due to the low content of ephedrine, no stimulatory effects were observed in pharmacological tests with *Pinellia*, showing the opposite effect of sedation [28]. In addition to other species previously mentioned, other monoamine alkaloids can be found in khat (*Catha edulis* Forsk.) such as norpseudoephedrine and norephedrine [29, 30].

1.2 Biosynthesis and Chemical Aspects

L-phenylalanine (Phe) is the precursor of the biosynthesis of ephedrine in plants, unlike the majority of alkaloids [31]. Although the backbone of this amino acid has clear resemblance with ephedrine, phenylalanine provides only the aromatic ring and the first carbon of the side chain (C1–C6) of this amine. Supporting this finding, it was shown that cinnamic acid, benzoic acid, and benzaldehyde are easily incorporated into ephedrine [32], being the remaining two carbons (C2–C3) derived from pyruvate and the nitrogen introduced by reaction of transamination at the end of biosynthesis, as can be seen in Scheme 38.1 [33, 34]. The biosynthetic route from phenylalanine occurs with the metabolism of L-phenylalanine through cinnamic acid to benzoic acid. The conversion of phenylalanine into *trans*-cinnamic acid is well characterized by the enzyme L-phenylalanine ammonia-lyase (PAL) [35]. At least two possible courses of Phe side-chain shortening is currently accepted in benzoic acid biosynthesis: the β-oxidative and non-β-oxidative pathway [36]. Besides PAL, no other enzymes involved in biosynthesis of amphetamine analogues were isolated [31]. Benzoic acid may undergo esterification with coenzyme

Scheme 38.1 Biosynthesis of ephedrine and related alkaloids


Phenylalanine
PAL
trans -cinnamic acid
Non-β-oxidative or β-oxidative pathway
Benzoic Acid + Pyruvic Acid
Descarboxylation
1-phenylpropane-1,2-dione
Transamination
(–)-cathinone
Reduction
(+)-norpseudoephedrine
(–)-norephedrine
N-methylation
(+)-pseudoephedrine
(–)-ephedrine



Scheme 38.2 Synthesis of ephedrine by fermentation reaction on benzaldehyde, followed by reductive condensation with methylamine

A following by acetylation with pyruvate, which in turn is decarboxylated to diketone. A transamination reaction occurs with diketone giving cathinone. Reduction of the carbonyl group of cathinone provides norephedrine or norpseudoephedrine which in turn suffer *N*-methylation generating ephedrine or norpseudoephedrine [37].

The ephedrine and pseudoephedrine commercially available and used for medicinal purposes are obtained either synthetically or by the extraction of plant material. Three possible methodologies can be used for the commercial production of ephedrine. The first one is the extraction from plants, especially from those of the genus *Ephedra*, and it is still used for herbal formulations, although being an expensive and prolonged method. The extraction method consists basically in the treatment of the alkaloids with alkali followed by addition of an organic solvent to extract the substance [31]. The commercial production can also occur by a chemical synthesis that has been developed to obtain racemic mixtures of ephedrine [38]. The third method is the most common and consists of a combination of fermentation and chemical synthesis, being a semisynthetic process. [31]. It involves the biotransformation of benzaldehyde to L-phenylacetylcarbinol, catalyzed by the enzyme pyruvate decarboxylase. The chemical procedure of fermentation usually uses brewer’s yeast (*Saccharomyces* sp.) or others species of yeast. The chemical counterpart of the method is based on a reductive condensation with methylamine in combination with hydrogen and metals as catalysts, as shown in Scheme 38.2. The growth phase is followed by biotransformation and added sugars, seeking to maximize L-phenylacetylcarbinol production. This process yields (−)-ephedrine with very high enantioselectivity, [12, 17], being (1S,2R)-pseudoephedrine produced from its diastereomer via Welsh rearrangement [31].

Considering the chemical characteristics, ephedrine has two chiral centers, and therefore four isomers can be identified: (±)-ephedrine and (±)-pseudoephedrine [39]. (1R,2S)-(−)-ephedrine is the major isomer found in Ephedra sp., and pharmacological studies have shown it as responsible for the pharmacological activities of ephedra. Not only (−)-ephedrine has the widespread use but also (+)-pseudoephedrine is added in over-the-counter decongestant preparations [40]. The four isomers of ephedrine may be naturally present in *Ephedra* species, and they are usually used as a hydrochloride form, being the classical purification method for ephedrine hydrochloride a combination of conventional infusion and organic solvent extraction or adsorption [41]. Other alkaloids are also present in smaller amounts, and the minor ephedrine alkaloids include (+)-pseudoephedrine and the demethyl analogues (−)-norephedrine and (+)-norpseudoephedrine [40].

Ephedrine occurs as white, rosette, needle, and crystals. It is soluble in water, alcohol, chloroform, ether, and in liquid petrolatum [12] and has a melting point of 187 °C–188 °C. It is marketed as ephedrine hydrochloride and may be found some traces of benzoic acid as a by-product of the process [42].

1.3 Analytical Aspects

A great number of publications on ephedrine-containing products analysis are proposed in the literature [43]. Extraction of ephedrines in body fluids and pharmaceuticals using liquid–liquid extraction procedures (LLE), solid-phase extraction (SPE), solid-phase microextraction (SPME), and headspace (HS) have been reported [44]. Gas chromatographic (GC) and liquid chromatographic (LC) procedures with different detectors (e.g., mass spectrometer, MS; nitrogen–phosphorus detector, NPD; flame ionization detector, FID; and ultraviolet, UV) have been used as analytical strategies, in addition to immunoassays, thin-layer chromatography (TLC), and capillary electrophoresis (CE). The employ of them depends on the purpose of analysis and the kind and amount of matrix used. Ephedrine-related compounds may be quantified in blood, plasma, or urine to monitor possible abusers, such as athletes, or to confirm a diagnosis of poisoning and to assist a medicolegal death investigation.

The choice of the method depends on the sensitivity and the resolution required for the analysis, the complexity of the sample matrix, and the time needed for sample pretreatment. Considering that extracts of the plants are a complex matrix, the analysis need to be preceded by preparative methodologies in order to clean up the samples. Nowadays, solid-phase extraction (SPE) is considered the gold standard for pretreatment of samples. This methodology allows the passage of large volumes of sample through the extractor column, which will selectively retain the analytes. The interfering compounds can then be discarded and the previously retained analytes are eluted with small amounts of organic solvent in a concentration range suitable for instrumental analysis. SPE also pre-concentrate the analyte, improving the sensitivity of the analysis [24] consisting in a better approach when compared with liquid–liquid extraction.

Analytical strategies including GC–NPD [45], GC–FID [46], GCxGC [47], GC–MS [48–50], LC–UV [6, 51], and LC–MS/MS [6, 52–55] have been employed in the routine laboratories to normal and chiral analysis. The use of mass spectrometry detection is mandatory to the confirmatory steps of the analyses. Methods by CE have been successfully used to separate chiral isomers of ephedrine and related compounds [56, 57]. Chromatographic techniques can easily distinguish (−)-ephedrine from other phenylethylamine derivatives and diastereoisomers.

LC–MS/MS procedures have the advantage of decrease work-up time due to the ability to analyze underivatized compounds. These techniques have also shown the ability to separate structural isomers such as norpseudoephedrine and norephedrine with relatively short analysis times, having a reported instrument cycle time of 8 min per sample [53, 58]. Currently, GC–MS is still the preferred method for forensic confirmation and has the advantage of low equipment costs. However, a previous derivatization step should be employed in order to improve the accuracy and the chromatographic profile of the analysis. An attractive analytical approach for routine confirmations can be created by combination of extractive–derivatization techniques with fast GC–MS, considering that the analytes of interest are separated by chromatography and accurately quantified [59], through a well-validated method.

2 Pharmacological Applications

Ma huang is one of the oldest known drugs and has been used as a medicine in China for more than 5,000 years [17]. Its use in modern medicine began in 1923 with the discovery of the valuable properties of ephedrine [12] which in turn was discovered in 1887 [37].

Ephedrine is a sympathomimetic amine with similar effects to those of adrenaline, so it is taken most commonly as a decongestant or for weight loss and energy enhancement as a dietary supplement [17, 60]. The purest form of ephedrine is currently used as a bronchodilator, decongestant, and vasopressor due to its sympathomimetic effects. The bronchodilator activity promotes relief in asthma, relaxing the bronchial smooth muscle in asthmatic patients. The vasoconstrictor action on mucous membranes makes it a superior nasal decongestant [17], being that the main clinical use for ephedrine. It may be used alone or in combination with other agents for the relief of cold symptoms being used topically and orally [20].

Other presentations of ephedrine have the same or very similar clinical uses. Ephedrine sulfate, for example, is used to combat hypotensive states, nasal congestion, and allergic disorders, such as bronchial asthma. Ephedrine hydrochloride is used similarly to ephedrine as a sympathomimetic agent [12]. Ephedrine hydrochloride or sulfate has been used as a bronchodilator, but the more selective β2-sympathomimetic bronchodilating agents, such as salbutamol, are now preferred. The salts of ephedrine have been given parentally to combat a fall in blood pressure during spinal anesthesia, since ephedrine is no longer generally advocated for orthostatic hypotension due to risk of shock, circulatory collapse, or hemorrhage. They are sometimes used in motion sickness, usually associated with hyoscine or an antihistaminic. Other uses include diabetic neuropathic edema and nocturnal enuresis, although other treatments are usually preferred [20]. Ephedrine derivatives are also largely employed, being pseudoephedrine widely used for the treatment of cough and cold and as a decongestant [17].

Besides the features already mentioned, ephedrine has also the capacity to stimulate thermogenesis in human subjects [61]. Probably because of its ability to stimulate the CNS and thermogenic properties, ephedrine may be employed to lose weight and enhance performance in endurance training and body building [62]. Thus, this pharmacological property triggered an unbridled use of it as a weight-loss compound, as well as an enhancer of athletic performance. So, ephedrine became quickly one of the main active components used in dietary supplements, gaining market share. The marketing of ephedrine for this purpose was restrained only after serious adverse effects reports arising from its use. Thus, the toxicity of this alkaloid began to be studied in order to determine the toxicological profile of this amine either alone or in combinations with stimulants such as caffeine. Ephedrine is considered nowadays one of the substances most widely used in sport, together with amphetamines, cocaine, caffeine, and anabolic steroids [63].

Owing to its sympathomimetic activity, ephedrine may be contraindicated for individuals with hypertension or other cardiovascular diseases, glaucoma, diabetes, and hyperthyroidism [64].

It is currently being traded as “herbal ecstasy” due to its CNS stimulation that in overdoses can lead to hallucinations, paranoids, and psychosis [17]. However, the large number of reports of serious adverse effects related to the use of this substance increased the attention to its use.

3 Mechanism of Action

The adrenergic effects caused by ephedrine occur mainly due to its capacity to be a sympathomimetic agonist at both α- and β-adrenergic receptors. As consequence of these stimulations, an increase in cardiac rate and contractility, peripheral vasoconstriction, bronchodilation, and CNS stimulation can be observed as general effects caused by the use of this amine [62, 65].

Ephedrine stimulates α-, β1-, and β2-adrenergic receptors through direct and indirect effects on adrenergic receptors [20]. The β-adrenergic effects are believed to be pronounced by the activation of adenylcyclase which increases the productions of cyclic AMP, while α-adrenergic effects result from inhibition of adenylcyclase [12].

Considering the chemical structure of ephedrine, it is interesting to observe the lack of the phenolic group, characteristic of catecholamines. However, ephedrine remains capable to stimulate α- and β-receptor directly and displace norepinephrine (NE) from storage vesicles, releasing these catecholamines at synaptic areas in the brain and in the heart. These released substances act on receptors promoting the adrenergic effect [17, 66].

Due to this mechanism of releasing catecholamines, ephedrine is also classified as indirect-acting sympathomimetic amine. The chemical similarity between ephedrine and epinephrine allows the transport of ephedrine into the nerve terminal by NE transporter. Inside the terminal nerve, an exchange of NE by ephedrine occurs by the vesicular monoamine transporter (VMT). Thus, NE accumulates in cytosol, being part of it degraded by MAO and partly released in the synaptic cleft by NE transporter. This causes an increase in the amount of NE in the synaptic cleft and consequently increases the effect on noradrenergic postsynaptic receptors. Although the indirect action of ephedrine is well established, it is known that there are other adrenergic mechanisms, such as direct stimulation of adrenergic receptors, inhibition of NE transporter (NE remains longer in the synaptic cleft), and partial inhibition of MAO (the metabolism of NE is reduced) [63].

As expected, there are many drugs that can strongly interact with ephedrine, increasing or decreasing its effect. Classically, these drugs are those capable of modifying the noradrenergic transmission. So, MAO inhibitors potentiate the effect by preventing the inactivation of NE previously displaced from the vesicle. Drugs that inhibit NE transport prevent the catch of ephedrine by the nerve endings, decreasing the ephedrine effect [63].

By the mechanisms mentioned above, it is possible to correlate the clinical effects observed with the use of ephedrine, as well as to establish the possible effects before the consumption. The use of ephedrine in therapeutic doses raises the blood pressure by increasing cardiac output and also by inducing peripheral vasoconstriction. It may cause tachycardia. Its action on β- adrenergic receptors in the heart produces a positive ionotropic effect. Ephedrine has a stimulant action on the respiratory center. It also causes bronchodilatation, reduces intestinal tone and motility, and usually reduces the activity of the uterus. Its effects on α-adrenergic receptors results in vasoconstriction in the skin and mucous membranes [12], leading to hypertension, which may produce cerebral hemorrhage and pulmonary edema.

Sympathomimetic agents are capable to produce a wide range of adverse effects, most of which are due to excessive stimulation of the sympathetic nervous system. Therefore, these effects depend on the relative agonist activity of the drug at a given dose on different types of receptor. General adverse effects related to the use of sympathomimetic agents are fear, anxiety, restlessness, tremor, insomnia, confusion, irritability, and psychotic effects. Cardiovascular issues such as tachycardia and cardiac arrhythmias, angina pain, palpitations, and cardiac arrest may be produced by stimulation of β1-adrenergic receptors of the heart. On the other hand, vasodilatation caused by β2-stimulation may lead to hypotension with dizziness and fainting and flushing. These kinds of stimulants have important effects on the CNS. The development of tolerance and dependence and the significant abuse of these drugs are consequences related to the CNS effects of ephedrine [20].

Considering the foregoing, it is reasonable to recommend patients at risk of stroke, myocardial infarction, uncontrolled blood pressure, seizures, and general anxiety disorder to avoid ephedrine [67].

4 Toxicity

If used for therapeutic purposes with dose control, ephedrine has no potential toxicity. However, the toxicity of this amine may be expressed in overdosages, which can lead to paranoid psychosis, delusions, and hallucinations [20]. Large doses of ephedrine may cause hypertension, headache, dizziness, palpitations, vomiting, nervousness, and insomnia [12].

Nowadays, the use of ephedrine with nontherapeutic purpose, being marketed as an herbal or a dietary supplement containing *Ephedra* species, is common. The whole issue with *ephedra* alkaloids is most of the time a paradox: depending on the relevant legislation of each country, the whole extract is considered a supplement, while the purified or synthesized alkaloids are considered pharmaceuticals being marketed as drugs. This usually means that the manufacturers of the alkaloid are compelled to prove both safety and efficacy of their products through clinical trials before being allowed to bring them to market, while the supplements have less demanding legislation [68]. Since ephedrine has thermogenic properties and is capable to stimulate lipolysis, the use of this amine had turned the attention to promote weight loss and improve athletic performance. Then a variety of compounds containing ephedrine, mainly dietary supplements, appeared on the market, being marketed with the appeal of easily promoting weight loss. Besides, the increasingly widespread use has been accompanied by the misconception of safety of ephedrine, mainly due to its natural origin [60]. In these cases, the use of ephedrine does not obey doses and leads to an uncontrolled consumption pattern often related to overdoses. Furthermore, there is no reliable quality control of these products due to a lack of effective monitoring on it, becoming difficult to ensure that the levels of ephedrine are consistent with that expressed in the label.

Scientific papers report that the pharmacological and toxicological properties of *Ephedra*-containing products depend strongly on the content of ephedrine and other associated substances. Clinical and preclinical studies and the long history of use of *ephedra* and its alkaloids indicate that its use can be safe if administrated according to the recommendations of the official codes. The serious adverse effects observed are related mainly to its overuse in dietary supplement formulations, to the associations and interactions with other drugs, individual susceptibility, and presence of contaminants, among other factors. Thus, the knowledge of the chemical composition of species of *Ephedra* and the correct orientation are essential to prevent accidents [8].

The study of the toxicity of commonly used associations became important when considering that it may cause an increase in cardiovascular risks when ephedrine is associated with other substances. Among the possible associations, the combination of ephedrine with *p*-synephrine (*Citrus aurantium* extract), salicin, and caffeine is one of the most usually found in the market, and it is used as an athletic performance enhancer and as a weight-loss compound. Recent studies have elucidated the acute and subchronic toxicity of this association. In the acute toxicity test in mice for both sexes, there were observed signs of toxicity such as ptosis, piloerection, and alterations in locomotor activity in all doses. A curious difference between genders not previously related in the literature was found. Signs were more intense in males possibly due to hormonal or metabolic differences. Other findings were alterations in locomotor activity, motor coordination, and body temperature, besides death by cardiopulmonary hemorrhage, corroborating with clinical reports that indicated the association of *p*-synephrine, ephedrine, and caffeine as potentially toxic [69]. These data indicate that the mechanism of action and toxicity of the mixtures is more complex than it was taught, involving alterations in the pharmacokinetic and pharmacodynamic properties when the substances are administered together, promoting unexpected adverse responses [69]. The results of subchronic test in rats helped to establish the toxicological profile of the association. The repeated dose given orally showed no clinical signs of toxicity, neither weight alterations nor deaths occurred as well as any significantly alterations in hematological parameters. There were observed lipid peroxidation, hepatic and renal damages, and a reduction in glutathione levels in male rats. In females, there were no alterations observed, showing again the different toxicity profile displayed by male and female probably due to hormonal influence. Although results showed the capacity of the association to change the oxidative status and promote renal and hepatic damages, it is still necessary that more studies be conducted to elucidate the role of hormones on toxicological effects presented by the weight-loss association [70].

The toxicity of ephedrine is closely related to adverse cardiovascular events, since the clinical presentation of toxicity reflects the sympathomimetic activity of these agents. The adrenergic effects can shorten cardiac refractory periods, permitting the development of reentrant cardiac arrhythmias. The worst complication related to the use of ephedrine is thrombotic stroke, presumably resulting from vasoconstriction of large cerebral arteries that in turn leads to local thrombosis [71]. Other adverse effects include hypertension, diaphoresis, hypothermia, and agitation. The best treatment in an overdose is the rapid identification of the symptoms followed by supportive management.

Arbo and coworkers [72] performed a study to evaluate the female reproductive toxicity of *E. sinica*, *C. aurantium*, ephedrine, and *p*-synephrine. The uterotrophic assay was used, which is commonly applied to detect (anti)estrogenic activity of chemical substances or mixtures. Ephedrine at the dose of 0.5 mg/kg/day showed a reduction of the uterus relative mass, indicating an antiestrogenic effect of this amine. However, the authors emphasized the need of more studies to completely characterize the mechanism of action of ephedrine in the endocrine system, since the uterotrophic assay is only a screening assay. Another interesting result obtained in this study was a reduction in the adrenals relative mass in the groups which received ephedrine, *p*-synephrine, and both doses of the extracts. The authors attribute this finding to the α1-adrenoceptor agonist activity of these substances through the vasoconstriction and reduction of the liquid in the organ [72].

In 2004, the Food and Drug Administration banned the sale of ephedra-containing over-the-counter dietary supplements due to a several number of reports involving adverse effects related to its use [73]. However, these products remain available to the public through illicit channels. Anticipating the possible ban of ephedrine sales, the industry of supplements quickly reformulated and developed “Ephedra-free” products, intending to replace those which contain ephedrine. The strategy used was the replacement of ephedrine by *p*-synephrine, a structural analogue of ephedrine primarily found in *Citrus* sp. extracts. There are two chemical differences between ephedrine and *p*-synephrine: the presence of a hydroxyl group replacing a hydrogen atom and the absent of a side-chain methyl group replaced by hydrogen, both in *p*-synephrine. It is believed that the pharmacological effects of *p*-synephrine do not differ from ephedrine, providing the same stimulatory and potentially toxic effects on the CNS and cardiovascular system as ephedra [74]. The lipolytic effect of *p*-synephrine is alleged to occur by specific stimulating of β3-adrenergic receptor, and consequent thermogenesis [75, 76] is questionable.

In spite of its popularity, there is no strong evidence confirming the effectiveness of *p*-synephrine as a thermogenic agent. The use of *p*-synephrine in dietary supplements has been accompanied by human toxicity reports, which lead to the idea of high toxicity of this amine. The most common adverse effects were the cardiovascular ones, as well as those that happened with ephedrine. However, the complex combination of different compounds must be considered, since *Citrus* extracts contain other compounds than *p*-synephrine. The presence of contaminants or other stimulants may influence the pharmacological and toxicological profiles of the products, being difficult to attribute the occurrence of adverse events only to *p*-synephrine [77].

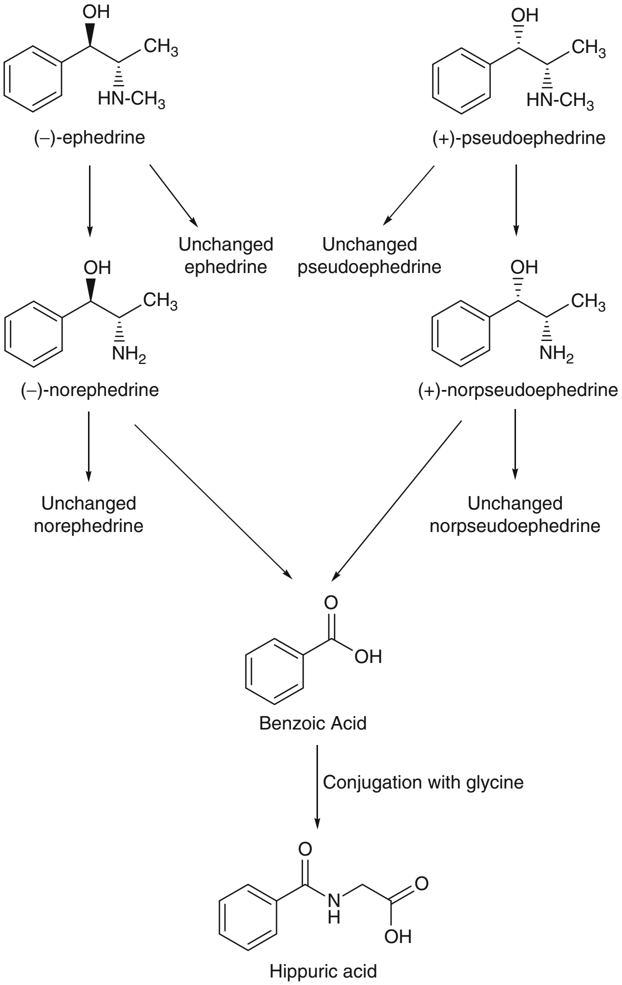
Considering that, studies have been developed to establish the toxicological profile of *p*-synephrine. The acute administration of *C. aurantium* extract (2.5 % *p*-synephrine) and *p*-synephrine produced reduction in locomotor activity, gasping, exophthalmia, piloerection, and salivation, corroborating to the hypothesis that *p*-synephrine acts not only in β3-adrenoreceptors but also in other adrenergic receptors. However, all the effects were reversible and persisted for 3–4 h. The adrenergic stimulation alerts for the same possible side effects of *p*-synephrine and *C. aurantiu*m [78]. The subchronic toxicity of *Citrus aurantium* extract and *p*-synephrine were also determined and indicated a low subchronic toxicity in mice but a possible alteration in the oxidative metabolism [79].

5 Bioavailability and Metabolism

The administration of ephedrine may be given orally or topically as nasal drops or sprays [20].

Ephedrine is orally active, being readily and completely (about 100 %) well absorbed from the gastrointestinal tract after oral administration. It has longer duration though less potent action than epinephrine [17, 20, 80]. The absorption is better with the oral administration of the pure alkaloid than with herbal alkaloid extracts, which the presence of many others substances may retard the rate of absorption. The heat stress may increase the absorption rate of ephedrine [81], as well as it may elevate blood pressure significantly greater under these stress conditions. Considering that *Ephedra* extracts are often taken prior to exercise in a warm environment, these stress conditions may enhance the cardiovascular effects experienced during physical activity [68].

Ephedrine has a large volume of distribution and is not bound to plasma proteins, remaining in the free form with high tissue affinity and capacity to reach high concentrations in the CNS. High doses of sympathomimetic drugs may cross the blood–brain barrier due to lipophilic properties of the molecule and therefore exercise adrenergic stimulation on the CNS, especially when orally administrated [82]. This amine is reported to have a plasma half-life ranging from 3 to 6 h, but its effects last about 1 h. The plasma half-life is influenced by the elimination process, which in turn depends on the urinary pH, since in acid urine, the elimination is enhanced, being the half-life in these cases accordingly shorter [20, 80].


(–)-ephedrine
(+)-pseudoephedrine
Unchanged ephedrine
Unchanged pseudoephedrine
(–)-norephedrine
(+)-norpseudoephedrine
Unchanged norephedrine
Unchanged norpseudoephedrine
Benzoic Acid
Conjugation with glycine
Hippuric acid

Scheme 38.3 Biotransformation and excretion of ephedrine

Ephedrine undergoes hepatic biotransformation being excreted mostly unchanged in urine [83]. From 8 % to 20 % of ephedrine is excreted after demethylation and delamination according to its metabolism showed in Scheme 38.3.

Excretion is dependent on several factors including urine volume, urinary pH, and individual variability. Ephedrine and amphetamines are weak organic bases, and therefore, they are easily ionized in acidic medium because of an ionizable amino group on the molecule. The acidification prevents reabsorption promoting excretion because of the positively charged amino group. Alkaline urine has the opposite effect [84, 85]. The excretion of ephedrine also occurs in breast milk and crosses the placenta, which poses concerns for women who may be taking ephedrine during pregnancy or breast-feeding. It is excreted primarily unchanged and with small amounts of metabolites in the urine over 24 h [20]. It is resistant to metabolism by monoamine oxidase, increasing its activity by oral ingestion.

6 Clinical Trials

Ephedrine is the most commonly used drug among the vasopressors to prevent hypotension during spinal anesthesia. A study randomized 42 women undergoing elective Cesarean section under spinal anesthesia into two groups (n = 21 per group), a control and an ephedrine-treated group. Shortly after the spinal injection, ephedrine 0.5 mg/kg in the ephedrine group or saline in the control group was injected intravenous for 60 seconds. The mean of highest and lowest heart rate in the ephedrine group was higher than those of control group. Also, there were significant lower incidences of hypotension and nausea and vomiting in the ephedrine group compared with the control group. The findings suggest the prophylactic bolus dose of 0.5 mg/kg intravenous ephedrine given at the time of intrathecal block after a crystalloid fluid preload, plus rescue boluses reduce the incidence of hypotension [86]. A similar randomized, double-blinded, controlled trial was conducted using propofol as anesthetic inducer (n = 156). Authors concluded that adding 30 mg of ephedrine to 20 ml of 1 % propofol is as effective as adding lidocaine in preventing injection pain, and it results in a more stable hemodynamic profile in the patients [87].

Ephedrine was first reported to be effective in the treatment of myasthenia gravis in the 1930s but was subsequently superseded by anticholinesterases and corticosteroids. The mechanism by which it may affect neuromuscular transmission at pharmacologic doses is unclear. Anecdotally, patients with congenital myasthenic syndrome (CMS) have reported benefit from ephedrine, often tried because of a failed response to other therapies. A study was conducted following 10 patients with Dok-7 mutation CMS. Dok-7 is an adaptor protein that is a key component of the muscle-specific tyrosine kinase (MuSK) signaling pathway and is essential for postsynaptic specialization of the neuromuscular junction. In this rare condition, the clinical phenotype is typically characterized by a definite onset of weakness in early childhood, although in retrospect symptoms consistent with a CMS may have been present at birth, sparing of the external ocular muscles in most cases, and a predominant limb–girdle distribution of weakness. A surprising feature of this form of CMS is the lack of response or worsening of weakness with anticholinesterase treatment and a variable response to 3,4-diaminopyridine (3,4-DAP), the conventional CMS treatments. This study provided Class IV evidence that ephedrine given at doses between 15 and 90 mg/day improves muscle strength and mobility in Dok-7 CMS [88].

A randomized, controlled, double-blind, clinical trial was conducted to assess the efficacy and safety of herbal extracts of *E. sinica* in Korean premenopausal women (n = 125). Subjects were administered ephedra extract in capsules (pseudoephedrine 31.52 mg) or placebo capsules as well as participating in a low-calorie diet for 8 weeks. Ephedra combined with a low-calorie diet was effective in reducing body mass index [89].

7 Conclusion

Ephedrine is a classical sympathomimetic agent used for centuries in medicine. In spite of its natural origin, most of the commercial ephedrine comes from synthetic or semisynthetic processes. Nowadays, it is commonly used as a nasal decongestant and for weight loss and for recreational purposes. Furthermore, promising uses have been investigated, such as for the reduction of the incidence of hypotension during spinal anesthesia and for the treatment of some cases of myasthenic syndrome. In spite of the reports of serious adverse cardiovascular side effects attributed to ephedrine, it is a safe drug if correctly used. However, some attention has been given to the toxicity of the association of ephedrine with other stimulant compounds for weight-loss purposes.

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Lycopodium Alkaloids: Pharmacology 39

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Contents

1 Introduction 1241
2 Lycopodium Alkaloids 1242
3 Pharmacological Applications 1243
3.1 Club Mosses and Chinese Traditional Medicine 1243
3.2 Alzheimer's Disease and AD Drugs 1244
3.3 Huperzine A and B 1244
3.4 Products on the Market 1245
3.5 Methods to Supply HupA and Other Lycopodium Alkaloids 1245
4 Mechanism of Action 1246
4.1 Classical Cholinergic Effects 1246
4.2 NMDA Receptor Antagonism 1249
4.3 Neuroprotective Effects 1249
5 Toxicity 1249
6 Bioavailability and Metabolism 1251
7 Other Biological Activities 1252
8 Clinical Trials 1253
9 Conclusion 1257
References 1257

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Abstract

The aim of this work is to review the current knowledge on the lycopodium alkaloids with an emphasis on their pharmacology and potential medical application. Lycopodium alkaloids are produced by club mosses, a vulnerable group of slow-growing lower plants. (−)-Huperzine A (hupA) isolated from *Huperzia serrata* is known for its potent and reversible acetylcholinesterase (AChE) inhibiting activity and is used as a drug for Alzheimer's disease (AD) in China. In addition, hupA has been shown to have neuroprotective effects in preclinical studies. It is by far the most intensively studied lycopodium alkaloid and clinical trials do indicate positive effects on AD symptoms with minimum adverse effects. At present, the greatest hurdle for research and application of lycopodium alkaloids is the lack of sustainable methods to supply these compounds. Lycopodium alkaloids which resemble hupA in having favorable bioavailability, pharmacokinetics, and toxicological profiles, in addition to interesting biological activities, are likely to be included in the search for new drug leads in the future, for example, in the development of multi-target and multidrug therapies for AD and other neurodegenerative diseases.

Keywords

Acetylcholinesterase • Alzheimer's disease • biological activity • clinical trials • club mosses • huperzine A • Lycopodiaceae • lycopodium alkaloids • pharmacology

Abbreviations

Aβ	Beta-amyloid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AChR	Acetylcholine receptor
AD	Alzheimer's disease
ADAS-Cog	Alzheimer's disease assessment scale – cognitive section
ADL	Activities of daily living
BBB	Blood brain barrier
b.i.d.	Twice a day
BuChE	Butyrylcholinesterase
BuChEI	Butyrylcholinesterase inhibitor
ChE	Cholinesterase
CNS	Central nervous system
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
FDA	Food and drug administration
hupA	(−)-huperzine A
hupB	(−)-huperzine B
i.m.	Intramuscular
i.p.	Intraperitoneal

i.v.	Intravenous
MCI	Mild cognitive impairment
MMSE	Mini Mental State Examination
nAChR	Nicotinic acetylcholine receptor
NGF	Nerve growth factor
NIH	National Institutes of Health
NMDA	*N*-methyl *D*-aspartate
NOAEL	No-observed-adverse-effect-levels
OP	Organophosphate
p.o.	Per os (oral)
s.c.	Subcutaneous
*Tc*AChE	*Torpedo californica* acetylcholinesterase

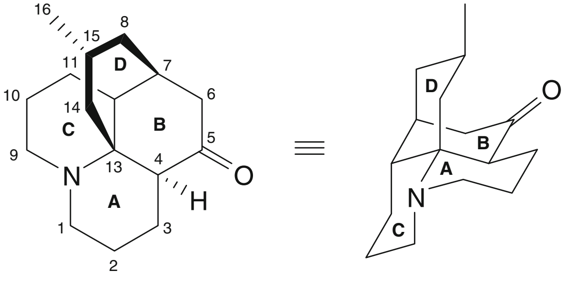
1 Introduction

Club mosses comprise an ancient group of spore-forming, slow-growing vascular plants that have survived since the late Silurian geological period or about 400 million years [1]. The classification of the order of club mosses, that is, Lycopodiales, is still a matter of debate and some taxonomists consider them to consist of one family of Lycopodiaceae, subdivided into four genera: *Lycopodium*, *Diphasiastrum*, *Lycopodiella*, and *Huperzia* [2], while others have suggested seven or even more genera and placed *Huperzia* in a separate family Huperziaceae [3, 4]. More than 500 club moss species grow worldwide [5, 6] and the alkaloid content has been studied for about 10 % of the species [7]. They produce alkaloids of condensed polycyclic structures commonly known as the lycopodium alkaloids [6–9].

Huperzia serrata is a club moss species that has been used in traditional Chinese medicine for centuries for various ailments including myasthenia gravis, an autoimmune neuromuscular disease, caused by circulating antibodies that block acetylcholine (ACh) receptors at the postsynaptic neuromuscular junction, consequently inhibiting the stimulative effect of the neurotransmitter [10]. This traditional use of *H. serrata* motivated Chinese scientists in the 1980s to search for compounds that might be effective in treatments of diseases that included cholinergic dysfunction such as AD [3, 11, 12]. This led to the isolation of the potent AChE inhibitor hupA [13, 14]. This alkaloid was previously isolated from Canadian *H. selago* and named selagine; however, the structure was incorrect and was later corrected and shown to be identical to hupA [15, 16]. The discovery of the potent inhibition of AChE by hupA evoked a renewed interest among scientists for the lycopodium alkaloids. HupA, in particular, has been the subject of intense research in relation to its cholinergic as well as non-cholinergic activities, and to its beneficial effects on AD patients in clinical trials [17]. Thus, the overwhelming majority of pharmacological studies on club moss alkaloids have been performed on hupA and the following chapter will reflect this fact; however, other lycopodium alkaloids will be included whenever appropriate.

2 Lycopodium Alkaloids

The first lycopodium alkaloid was isolated from the club moss *Lycopodium complanatum* by Bödeker as early as 1881 [18]; much later its structure was shown to be lycopodine [19]. Since the first comprehensive review on lycopodium alkaloids was published in 1968 [20], new structures have been isolated and the chemistry and biology of this class of compounds have been reviewed several times [6–9, 21]. According to Ayer [6], the lycopodium alkaloids are generally considered to comprise of four major classes. Compounds **1–4** are representatives of each group: lycopodine **1** (class I), lycodine **2** (class II), fawcettimine **3** (class III), and phlegmarine **4** (miscellaneous class IV).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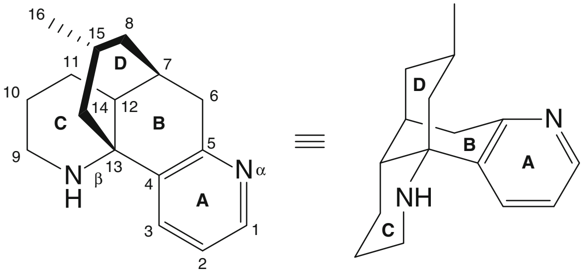
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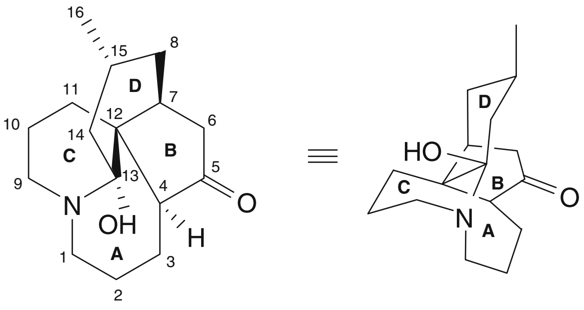
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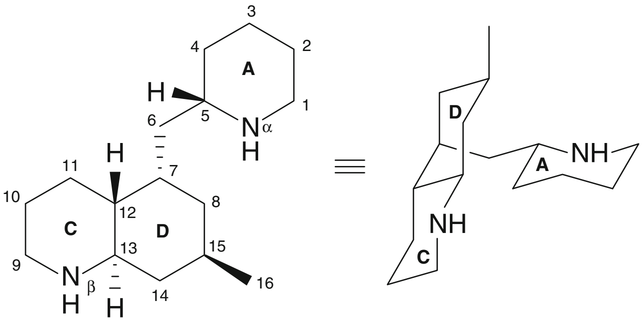

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For class I compounds represented by lycopodine **1**, the A, B, and C rings are stable and the structural variations are mainly in the D ring. For class II or lycodane-type alkaloids, the B, C, and D rings are as in lycopodine **1**; however, the A ring is opened and rearranged. HupA and hupB belong to this class. Class III comprises of compounds that result from a C4-C13 to C4-C12 bound migration from lycopodane-type precursors. The miscellaneous group represented by phlegmarine **4** includes all compounds that do not belong to the other three classes [6, 8]. The reported structures of lycopodium alkaloids are now close to 250 [7]. They are all structurally related, usually having 16-carbon skeletons although they can have up to 32 carbons (dimers) and down to 15 as in hupA [8]. Further discussion on the chemistry and structures of the lycopodium alkaloids in general is beyond the scope of this chapter and will be restricted to relevant structures in connection with the pharmacological activities discussed.

3 Pharmacological Applications

3.1 Club Mosses and Chinese Traditional Medicine

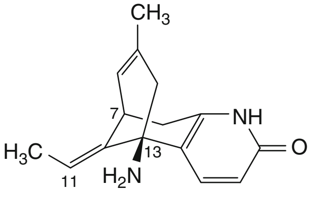
Qian Ceng Ta, a Chinese medicinal herb *H. serrata* (Thunb) Trev., as well as some other species of club mosses, have a long history of use for the treatment of a number of indications including bruises, strains, swellings, and, more recently, schizophrenia, myasthenia gravis, and organophosphate (OP) poisoning [3, 8]. This herb is described in old Chinese pharmacopeias sometimes under the name Shi Song, which is somewhat confusing as this name was used to describe several different medicinal herbs. However, all of the Shi Song herbs were species of Lycopodiaceae, e.g., *L. japonicum*, *L. annotinum*, *L. obscurum*, *D. complanatum*, besides *H. serrata* [3]. When Chinese scientists discovered that *H. serrata* contained lycopodium alkaloids with potent and reversible AChE-inhibiting activity, this herbal medicine gained worldwide attention based on scientific evidence that indicated positive effects on learning and memory which might benefit patients suffering from AD [3, 21].

3.2 Alzheimer's Disease and AD Drugs

AD is an age-related degenerative brain disorder that affects elderly people and the prevalence is increasing with rising age of the population. AD destroys neurons and neuronal connections in the cerebral cortex, leading to loss of brain mass and brain function. The symptoms are progressive cognitive impairment that leads to death usually within 3–10 years. The pathophysiology of AD is complex and far from fully understood. However, it is known that level of ACh in the brain is lowered in AD and after years of studies in both animals and humans, it is clear that cholinergic pathways in the brain contribute considerably to the modulation of several important aspects of cognition [22, 23]. In addition, the postmortem brains of AD patients show several distinct neuropathological features such as beta-amyloid (Aβ) peptide-containing senile plaques and intracellular neurofibrillary tangles which cause neurodegeneration [17, 24, 25]. Other non-cholinergic neurodegenerative factors are also believed to play a role, e.g., overstimulation of *N*-methyl *D*-aspartate (NMDA) receptors by glutamate and oxidative stress [26]. There is no cure for AD but the currently approved symptomatic drug treatments in Western countries consists of the AChEIs donepezil, rivastigmine, and galanthamine as well as memantine which is a NMDA receptor antagonist. These treatments have their limitations as they are not effective for all patients, and a number of cholinergic side effects such as nausea, vomiting, diarrhea, and tremors can also limit their use [27]. It is evident that the need for new and better treatments for AD is immense. "Mild cognitive impairment" or MCI is a condition defined for people suffering from less severe but abnormal cognitive impairment and a high risk for developing AD [27, 28]. This group could undoubtedly benefit from new medicines, especially neuroprotective and disease-modifying drugs that could prevent the MCI from developing into AD [29].

3.3 Huperzine A and B

The lycopodium alkaloids, hupA **5** and hupB **6**, were isolated from *H. serrata* and shown to be potent AChEI. Their natural configurations are 7*R*,13*R*,11*E* and 7*S*,13*R*,12*R*, respectively, or the (−)-form [13, 14]. They have the rigid lycodane-type polycyclic structures **5** and **6**.



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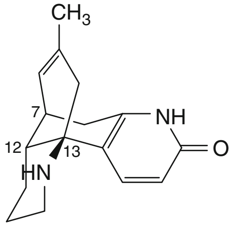

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The (+)-enantiomeric form of huperzine A has been synthesized and tested for AChE inhibition in vitro and the activity compared to the natural (−)-enantiomer. The (−)-enantiomer of huperzine A was shown to be the more active form, as well as being the most potent of all lycopodium alkaloids currently reported [13, 30].

3.4 Products on the Market

The discovery of AChEIs among the lycopodium alkaloids and the growing evidence supporting the cholinergic hypothesis of AD promoted the development of commercial products. Shuangyiping, a tablet form of hupA, was developed from *H. serrata* extracts and was marketed in China in 1996, for symptomatic treatment of AD [31]. Powdered *H. serrata* plant material containing hupA is sold in USA as a dietary supplement in tablets or capsules [3] and marketed as a memory enhancer. Although hupA is currently not approved by the Food and Drug Administration (FDA) as an AD drug, these products are commercially promoted as a treatment for AD [32].

3.5 Methods to Supply HupA and Other Lycopodium Alkaloids

The club mosses are the only natural sources of lycopodium alkaloids and they are vulnerable slow-growing plants which are very difficult to cultivate; in addition, their alkaloid content is low, e.g., about 0.005–0.025 % in *H. serrata* for the much demanded hupA [17]. At present, the market is supplied with *H. serrata* plant material and isolated hupA primarily from the wild growing plants of *H. serrata* and other *Huperzia* species in China, which are subsequently becoming endangered species [3, 8]. Accordingly, it is important to find sustainable methods to produce these compounds. The synthesis of hupA has been studied since 1989 and different approaches have been reported in the literature but none of them seem to be industrially feasible [17]. However, a new eight-step enantioselective synthetic route to hupA in 35–45 % yield was recently published by Tun et al., and the authors conclude that the method will provide a reliable supply of this compound for future applications [33].

Besides synthesis, other alternative methods to supply hupA have been sought. An in vitro propagation in *Phlegmariurus squarrosus* was described by Ma and Gang; prothalli and sporocytes were produced from in vitro cultures and grown to plants which produced higher yields of hupA than the naturally occurring plants [34]. Another approach has been to culture hupA-producing endophytic fungi from *H. serrata*. Wang et al. [35] recovered over 120 endophytic fungi from *H. serrata* and nine of them, belonging to seven different fungal genera, were found to produce hupA. The most potent AChE inhibition effect was found in a *Shiraia* sp. and the yield of (−)-hupA produced in a potato dextrose broth was 327.8 μg l^{-1}. Interestingly, two extracts of endophytic fungi sp. which did not contain hupA, showed potent AChE inhibition, indicating the presence of unknown AChEIs [35].

4 Mechanism of Action

The generally recognized mechanism of action for the beneficial effect of hupA and other AChEI in the symptomatic treatment of AD is the inhibition of AChE which results in elevation of the abnormally low ACh level in the brains of AD patients [27]. In addition, preclinical studies show that hupA is a NMDA receptor antagonist and it has been suggested that hupA, and possibly other lycopodium alkaloids might have neuroprotective effects through several mechanisms that altogether, could contribute to a disease-modifying action on AD [26].

4.1 Classical Cholinergic Effects

AChE is an enzyme that terminates transmission at cholinergic synapses and neuromuscular junctions by rapid hydrolysis of ACh [36, 37]. In an animal study, the cortical ACh levels in rats were shown to increase in a dose-dependent manner after p.o. administration of hupA, and to be higher than for equimolar doses of the AD drugs donepezil and rivastigmine, indicating more potency and longer lasting effect of hupA [38].

The interactions of hupA and hupB with AChEs have been studied extensively by molecular modeling and docking studies, revealing that the catalytic site resides inside a narrow gorge lined with hydrophobic aromatic amino acid residues. Comparison of X-ray structures of native AChE from *Torpedo californica* (*Tc*AChE) with and without bound hupA or hupB showed surprisingly few interactions between the protein and the ligand and a significant change of the main chain conformation of the protein [30, 39]. Figure 39.1 shows hupA docked in the active site of *Tc*AChE and the main interactions between hupA and the amino acids lining the active gorge of the enzyme.

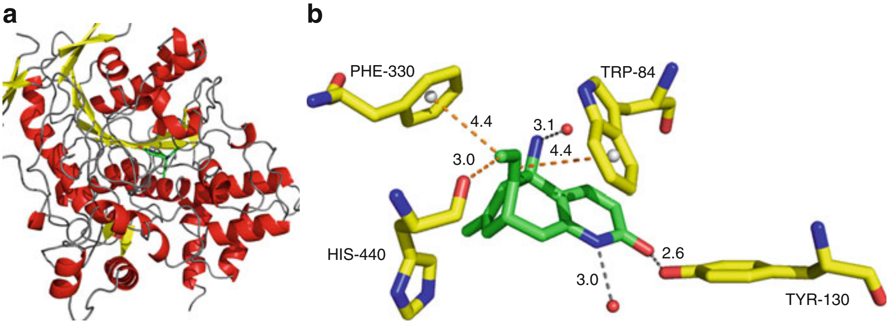
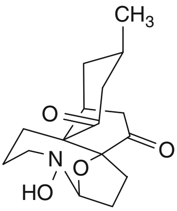


Fig. 39.1 Stereoview of hupA **5** within the active sited gorge of *Tc*AChE (PDB code 1VOT). (**a**) HupA (*green*) in complex with the enzyme (*cartoon*). (**b**) Selected protein residues (*yellow*) and water molecules (*red spheres*) showing important interactions with hupA **5** (*green*) within the binding site. Hydrogen bondings are labeled as *gray dashed lines*, other interactions as shown as *orange dashed lines*

On the one hand, the structure-based design of AChEIs is challenged by the large-amplitude fluctuations of the gorge structure and the fact that it contains more buried water molecules than a typical protein [40–42]; on the other hand, this renders opportunities which could involve lycopodium alkaloids and their analogues as possible ligands for this enzyme.

The ChE inhibition of hupA has been evaluated in vitro and in vivo using the colorimetric method of Ellman et al. [43]. It is a potent and reversible inhibitor of ChE with a high selectivity toward AChE over BuChE which has been linked to hupA's lower incidence of side effects in humans compared to registered AD drugs [8]. Another factor that has been suggested to contribute to hupA lower peripheral side effect profile is its preference toward the tetrameric G4 form of the enzyme which resides predominantly in the brain [44]. The AChE inhibition has been published for several lycopodium alkaloids isolated from different species of club mosses. The most active ones **5**–**11**, having IC50 $<$ 20 μM, are listed in Table 39.1 together with **12**–**15** which have low or no activity. However, it should be noted that these results are not directly comparable since they are from different studies and use different sources for the ChEs.



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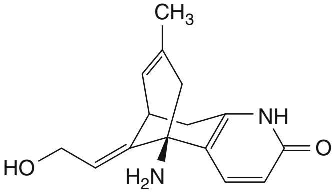

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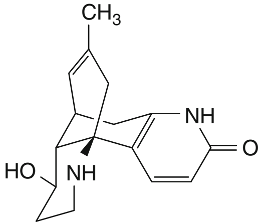

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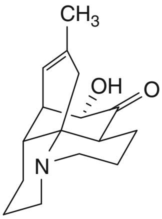

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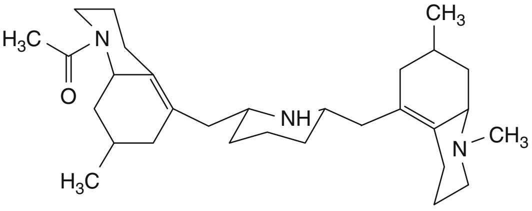

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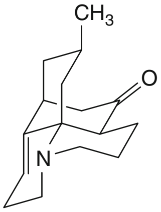

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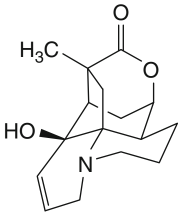

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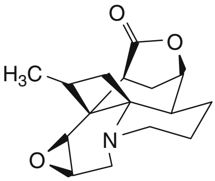

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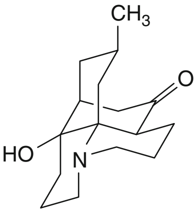

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Table 39.1 AChE inhibition of lycopodium alkaloids (**5**–**11**) with IC50 < 20 μM, and (**12**–**15**) with low or no activity. Note that the data are from different studies and different sources of ChEs are used

Lycopodium alkaloid	Source Plant species	AChEI IC50 μM	BuChEI IC50 μM	References
(−)-Huperzine A **5**	*H. serrata*	0.072[a]	70.2[b]	[45]
(−)-Huperzine B **6**	*H. serrata*	19.3[a]	227[b]	[45]
Sieboldine A **7**	*H. sieboldii*[g]	2.0[c]	–	[46]
Carinatumin A **8**	*H. carinata*[g]	4.6[d]	–	[47]
Carinatumin B **9**	*H. carinata*[g]	7.0[d]	–	[47]
Lycoposerramine H **10**	*H. serrata*[g]	16.7[e]	–	[48]
Cryptadine B **11**	*L. cryptomerinum*	18.5[d]	–	[49]
Anhydrolycodoline **12**	*L. annotinum*	191[c]	>2,000[f]	[50]
Annotine **13**	*L. annotinum*	860[c]	2,000[f]	[50]
Annotinine **14**	*L. annotinum*	>2,000[c]	>2,000[f]	[50]
Lycodoline **15**	*L. annotinum*	>2,000[c]	>2000[f]	[50]

Source of ChE used:
[a] Rat cortex
[b] Rat serum
[c] Electric eel
[d] Bovine erythrocyte
[e] Not indicated
[f] Equine serum
[g] *L. serratum* = *H. serrata*, *L. carinatum* = *H. carinata*, *L. sieboldii* = *H. sieboldii* [51]

Two of the lycopodium alkaloids with IC50 < 20 μM, i.e., carinatumin A **8** and carinatumin B **9**, have structures similar to hupA **5** and hupB **6** except for an additional hydroxy group. Sieboldine A **7** with two carbonyl groups and an N-hydroxy group and the phlegmarane-type cryptadine **11**, are somewhat different tetracyclic and pentacyclic structures, respectively. Lycoposerramine H **10** resembles the lycopodane-type skeleton although it has a C8-C15 double bond similar to hupA **5** and hupB **6**. Lycopodane-type alkaloids tested by Halldorsdottir et al., e.g., anhydrolycodoline **12**, annotine **13**, annotinine **14**, and lycodoline **15**, all have low or no activity. Molecular modeling studies have indicated weaker interaction of these lycopodane-type compounds **12–15** with the active site of the enzyme compared with the lycodane-type hupB **6**. However, novel binding orientation of anhydrolycodoline **12**, which has a C11-C12 double bond, found in these docking studies suggests the possibility of designing analogues with increased potency [50].

Chronic inflammation is considered to be a contributing factor in AD. Besides the low ACh levels in the brains of AD patients, decreased number of nicotinic (nAChR) and muscarinic acetylcholine receptors (mAChR) has been detected in AD patients brains as well as in experimental AD animals [24, 27]. In a recent publication, hupA has been shown to reduce both acute and chronic cerebral inflammation in rats, by a mechanism that is believed to be mediated through the nAChR [11].

4.2 NMDA Receptor Antagonism

Glutamate overstimulation of NMDA receptors is considered to contribute to the neurodegeneration seen in AD and other neurodegenerative diseases. HupA was shown to protect cultured rat brain cells from glutamate-induced cell death [52, 53], and both hupA and hupB have been shown to exert an antagonistic effect on NMDA receptors in rat cerebral cortex [54, 55] and could therefore reduce neuronal cell death caused by glutamate. In contrast to the different activities of the two enantiomeric forms of huperzine A toward the AChE enzyme, their potency is similar toward the NMDA receptor, reflecting a non-stereoselective interaction with the receptor [30, 56].

4.3 Neuroprotective Effects

Several preclinical studies have suggested that hupA might also be neuroprotective through non-cholinergic mechanisms, e.g., modification of Aβ peptide processing, reduction of oxidative stress, neuronal protection against apoptosis, and regulation of the expression and secretion of nerve growth factor (NGF) and NGF signaling [3, 26, 57]. A simplified overview of several connected factors suggested to contribute to an overall neuroprotective effect of hupA in AD that is presented in Fig. 39.2. More detailed description can be found in reviews by Zhang and coworkers [26, 57].

These effects of hupA are considered to protect the cells from toxic and apoptotic effects caused by Aβ. In contrast to the AChE-inhibiting effect, the neuroprotective effects in rats have been shown to be non-stereoselective toward the two enantiomeric forms of huperzine A [58]. In summary, the therapeutic effect of hupA observed in the treatment of AD is probably based on a multi-target mechanism [26, 57], where neuroprotective effects could be contributing factors [26].

5 Toxicity

In general, the lycopodium alkaloids are considered to be "…moderately toxic, but owing to the inedible nature of the club mosses, no fatalities appear to have been recorded in either humans or farm animals…" [59]. Studies describing the toxicity of lycopodium alkaloids besides hupA are scarce; however, a study from 1947 describes moderate toxicity of lycopodine and annotinine in frogs, mice, and rabbits and lethal doses were accompanied by symptoms of muscle cramps [60].

Toxicological studies of hupA have been conducted after acute and chronic administration in several animal species including mice, rats, rabbits, and dogs [61–63]. Cholinergic symptoms characterize its side effects and toxicity, similar to

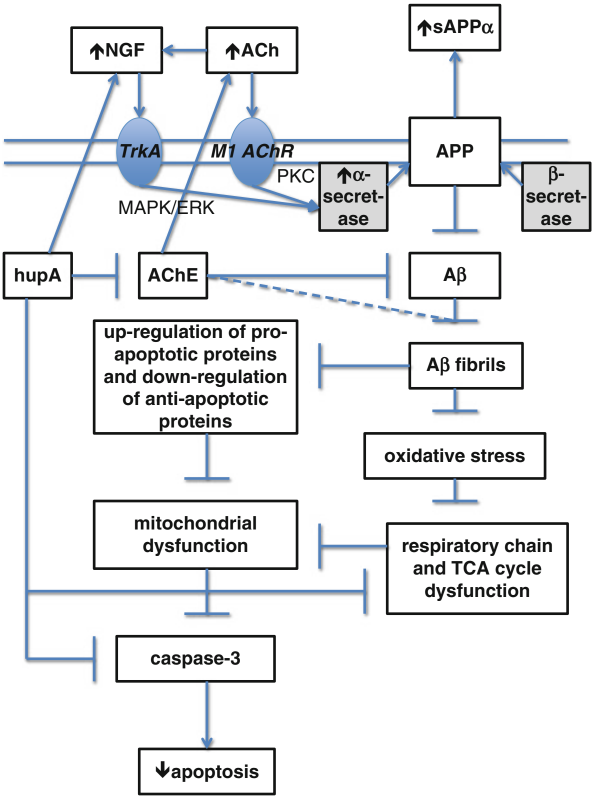
↑NGF
↑ACh
↑sAPPα
TrkA
M1 AChR
APP
PKC
↑α-secret-ase
β-secret-ase
MAPK/ERK
hupA
AChE
Aβ
up-regulation of pro-apoptotic proteins and down-regulation of anti-apoptotic proteins
Aβ fibrils
oxidative stress
mitochondrial dysfunction
respiratory chain and TCA cycle dysfunction
caspase-3
↓apoptosis

Fig. 39.2 Suggested effects leading to neuroprotective activity of hupA in AD. HupA is considered to modulate the APP processing by inducing the activity of α-secretase. The increased activity of α-secretase causes enhancement of nutritional APP (sAPPα) and consequently inhibition the Aβ pathway. These APP-modulating effects are mediated through M1 muscarinic receptor–mediated PKC-dependent cascade and Trk receptor–mediated ERK/MAPK-dependent cascade and caused by increased levels of ACh and NGF in the synaptic junction. Increased release of sAPPα and decreased Aβ release from APP followed by inhibition of Aβ fibril formations affects the regulation of the expression of apoptotic proteins, attenuates oxidative stress, and allows the mitochondria, respiratory chain, and TCA cycle to function normally. *APP* amyloid precursor protein, *PKC* protein kinase C. *ERK* extracellular signal-regulating kinase, *MAPK* mitogen-activated protein kinase, *TCA* tricarboxylic acid cycle (citric acid cycle)

that of other ChE inhibitors, with the earliest sign of muscular tremor, followed by drooling, tears, increased bronchial secretions, and incontinence. The experimental data for the toxicity of hupA were included in the investigational new drug (IND) submission to the FDA (IND#63,997). In rats, the LD50 administered as a single oral dose is 2–4 mg kg^{-1} of body weight for females, and >4 mg kg^{-1} for males. The chronic toxicity of hupA studied in rats for 180 days indicated tolerability at 1.5 mg kg^{-1}day^{-1}. In a similar study in rabbits and dogs, deaths started to occur at an i.m. dose of 0.6 mg kg^{-1}day^{-1} for the rabbits, while the dogs showed transient muscle twitching at this dose. Pathological examination of the treated rabbits and dogs showed abnormalities in heart and brain [62].

The no-observed-adverse-effect-levels (NOAEL) for hupA have been determined after chronic 30-day oral administration. The NOAEL was found to be 1 and 3 mg kg^{-1}day^{-1} for female and male rats, respectively, and the only drug-related effect detected was a transient decrease in food consumption. The NOAEL for dogs was 0.1 mg kg^{-1}day^{-1}, and after a single dose of 10 mg kg^{-1} the dogs showed signs of acute cholinergic toxicity such as convulsions, salivation, lacrimation, tremors, impaired limb function, emesis, and death [62].

The maximum recommended human daily dose of hupA in China is 0.45 mg [64]. Studies to evaluate the toxicity of this natural product have also been carried out in China and support the seemingly favorable therapeutic index of hupA. The LD50 per kg in mice was shown to be 4.6 mg (p.o.), 3.0 mg (s.c.), 1.8 mg (i.p.), and 0.63 mg (i.v.). Histopathological examination in dogs (0.6 mg kg^{-1} i.m.) and in rats (1.5 mg kg^{-1} p.o.) performed to evaluate subacute toxicity showed no changes in liver, kidney, heart, lung, or brain after administration of hupA for 180 days. No mutagenicity was detected in rats, and no teratogenic effect in mice or rabbits [63]. Atropine exerted a significant antagonistic effect on the toxicity induced by hupA [65].

6 Bioavailability and Metabolism

The pharmacokinetic profile of hupA has been studied in healthy volunteers with oral as well as transdermal administration [66, 67]. In a recent study, hupA was administrated p.o. to 12 healthy, young volunteers as a single therapeutic dose of 0.4 mg and was found to be well tolerated with no adverse events reported. HupA was absorbed rapidly, and occurred in the plasma 5–10 min after administration and reached $C_{\max}$ 2.59 ng ml^{-1} at a $T_{\max}$ of 58.33 min. It was shown to distribute widely in the body at a moderate rate and to cross the blood–brain barrier (BBB) easily. The results conformed to a two-compartment model with the elimination half-life of α-phase and β-phase to be approximately 21 and 716 min, respectively [66]. Another study performed in China in 1995 which included six volunteers receiving a single 0.99 mg p.o. dose of hupA showed somewhat different results with shorter absorption and elimination half-lives, i.e., 13 and 288 min, respectively, compared to the previous study. However, both studies revealed rapid absorption and wide distribution, followed by a slower elimination rate [17, 66].

Since most oral AChEIs exhibit a dose-dependent relationship with undesirable cholinergic adverse effects, researchers have in recent years been focusing on slow-release administration to minimize the fluctuations in plasma concentration [67, 68]. Ye and coworkers have developed transdermal patches containing 4 mg of hupA with the average daily dose of 0.456 mg absorbed [68]. A recent clinical study on 30 healthy volunteers revealed that the transdermal administration provided continuous drug delivery over 120 h and the pharmacokinetic behavior in vivo presented linear dynamic characteristics with prolonged $T_{\max}$, lowered $C_{\max}$, and relatively constant plasma concentrations. This indicated similar efficacy of the patches to orally administrated hupA and a more favorable tolerability profile [67]. In addition, comparison of a single and multiple dose pharmacokinetics between patches and conventional tablets of hupA in six beagle dogs showed that transdermal administration exhibits good controlled-release properties with prolonged $T_{\max}$ (24 h vs. 3 h), lowered $C_{\max}$ (3.4 ng ml^{-1} vs. 9.8 ng ml^{-1}), and reduced fluctuations in serum concentrations [68]. Furthermore, pharmacokinetic studies of hupA in rat plasma and cerebrospinal fluid (CSF) after intranasal administration [69, 70] were compared to i.v. infusion. Approximately 20 % of the concentration in plasma reached the CSF after both routes of administration, indicating no direct transport of hupA from nasal cavity to CSF in rats [69].

An autoradiographic study in mice after i.v. injection of a dose of 183 μg kg^{-1} showed the presence of [^{3} H]-hupA in all regions of the brain 60 min after injection. Radioactivity was practically absent in all parts of the body after 12 h [71]. In an additional study on mice receiving [^{3} H]-hupA p.o., the highest radioactivity was observed in the kidneys and liver [65].

An in vitro study on rat liver microsomes was performed to identify which cytochrome P450 (CYP) isoenzymes are involved in the major metabolic pathway of hupA. The results revealed that the metabolism was primarily inhibited by a CYP1A2 antibody (76.2 %), with a probable secondary contribution of CYP3A1/2 antibody (17.8 %), while inhibitory effects produced by CYP2C11 and 2E1 antibodies were minor [72]. CYP1A2 is about 13 % of total CYP isoenzymes in the liver [73] with both pharmacological and toxicological significance [74]. In addition, hupA showed little effects on the activity and expression of CYP in rats that demonstrate: The isoenzymes were not affected at pharmacological doses, but may elicit a slight inductive response in CYP1A2 at a toxicological dose [75].

ZT-1, a semisynthetic prodrug of hupA, is transformed nonenzymatically into hupA. It has been administered by i.v., oral, immediate-release and by sustained-release formulations in the form of implants [76]. In vitro studies show that ZT-1 inhibits AChE similar to hupA, but a weaker BuChE inhibition was observed. Pharmacokinetic studies suggest that ZT-1 has similar properties to hupA regarding oral bioavailability, the ability to cross the BBB, and longevity of action [8].

7 Other Biological Activities

Prophylaxis and treatment of OP poisoning: *O*-Pinacolyl methylphosphonofluoridate or soman is an extremely toxic OP poison that has been used in chemical warfare and terrorism. However, most OP compounds are used in industry and in veterinary and human medicine as insecticides [77, 78]. OPs are irreversible inhibitors of AChE, resulting in accumulation of ACh at the synaptic junctions, which causes severe cholinergic symptoms, e.g., tremors and seizure, progressing to impairment of respiratory drive and coma [77]. HupA has been tested as a pretreatment of OP poisoning in animals. The advantages of hupA over current treatment (pyridostigmine) are that it crosses the BBB and acts in a slow and reversible manner, partially protecting the peripheral and central AChEs from binding with OP [3, 79]. Animal studies have been promising, showing hupA to be active against soman-induced seizures and mortality; however, this pretreatment has not been authorized for human use [79–81].

Insecticidal activity: HupA was shown to be insecticidal against the Australian carpet beetle, *Anthrenocerus australis* (LD50 110 ppm), the Australian sheep blowfly *Lucilia cuprina* (LD50 2,380 ppm), and the webbing clothes moth, *Tineola bisselliella* (LD50 630 ppm) and reduced the feeding by *A. australis* by 97 % at 63 ppm [82].

Cytotoxicity against cancer cells: Several lycopodium alkaloids have been shown to possess cytotoxic effects on cancer cells. Cermizine A, B, and D, cernuine N-oxide, and lycocernuine N-oxide isolated from *L. cernuum*, senopodine G and H isolated from *L. chinense* [83], lycopladine A isolated from *L. complanatum* [84], and the dimeric lycopodium alkaloid complandine D isolated from *L. complanatum* [85] exhibited cytotoxicity against murine lymphoma L1210 cells in vitro, with IC50 ranging from 4.9 to 8.2 μg ml^{-1}. Furthermore, nankakurine A isolated from *L. hamiltonii* was shown to be cytotoxic to human epidermoid carcinoma KB cells (IC50 3.1 μg ml^{-1}) in vitro [86].

Antifungal activity: Lycovatine A isolated from *L. clavatum* var. *robustum* and complanadine C and D were shown to have antifungal effect on *Cryptococcus neoformans* (MIC 0.52, 0.52, and 0.26 μg ml^{-1}, respectively) and *Aspergillus niger* (MIC 2.06, 2.05, and 4.16 μg ml^{-1}, respectively) [85].

8 Clinical Trials

Several clinical trials on the efficacy of hupA for the treatment of AD have been conducted in China, and recently, a phase II trial was completed in the USA. Clinical tests, e.g., MMSE (Mini Mental State Examination) and ADL (Activities of daily living), as well as other memory tests, were used to evaluate the effects of the treatment. Table 39.2 reviews the setup, trial conditions, and results from the clinical trials. Note that for the trials conducted in China, only the four studies selected by a meta-analysis performed by Wang et al. in 2009 are included in Table 39.2 [64, 87–90].

Wang et al. evaluated 11 clinical studies conducted in China on efficacy and safety of hupA in the treatment of AD, and four placebo-controlled, randomized trials (Table 39.2) using MMSE and ADL as outcome measures were included in their meta-analysis. A total of 474 AD patients were included, 235 received 0.3–0.5 mg of hupA p.o. daily for 8–24 weeks, and 239 in the control group. The results presented a significant improvement in both MMSE and ADL and a meta-regression showed that the estimated effect size of MMSE and ADL was increased over the treatment time. Adverse events were mild-to-moderate peripheral

Table 39.2 Clinical trials on hupA as a treatment for AD

Location publication year	Number of patients (*n*) (HupA/Control)	Study design	Treatment	Memory test and results	Adverse effects	References
China 1995	103 (50/53)	DB, M, PC, RC	0.4 mg/day (0.2 mg b.i.d.), 8 weeks	MMSE: HupA, +3.0; placebo, +1.0	No significant difference found between groups	[60, 86]
				HDS: HupA, +4.0; placebo, −1.0		
				ADL: HupA, −4.0; placebo, +0.1		
				MQ: HupA, +12.0; placebo, +4.0		
China 2002	202 (100/102)	DB, M, PC, RC, DE	Up to 0.4 mg/day (0.2 mg b.i.d.), 12 weeks[a]	MMSE: HupA, +2.7; placebo, +0.19	Mild adverse events observed for 3 % of patients in the treatment group	[60, 87]
				ADAS – Cog: HupA, 56.1 %; placebo, 10.1 %		
				CIBIC-plus: HupA, 59.2 %; placebo, 40.6 %		
				ADAS – non-Cog: HupA, 70.0 %; placebo, 36.3 %		
				ADL (p = 0.001): HupA, −2.4; placebo, −0.5		
China 2003	65 (35/30)	SB, PC, RC	0.3 mg/day (0.15 mg b.i.d.), 16 weeks	MMSE: HupA, +4.3; placebo, +0.1	No serious adverse events, only mild adverse effects	[60, 88]
				ADL: HupA, −6.6; placebo, 0.0		
China 2006	120 (60/60)	SB, PC, RC	0.5 mg/day, 24 weeks[b]	MMSE: HupA, +5.0; placebo, +0.0	Gastrointestinal effects, ALT increase	[60, 89]
				ADL: HupA, −5.0; placebo, +2.0		
USA 2008	15 (12/3)	PC, RC, DE Phase Ia	Up to 0.4 mg/day (0.2 mg b.i.d.), 4 weeks	No clinically significant abnormalities were detected in the studies	No serious adverse events	[62]

USA 2008	15 (12/3)	PC, RC, DE Phase Ib	Up to 0.8 mg/day (0.4 mg b.i.d.), 4 weeks	No clinically significant abnormalities were detected in the studies	No serious adverse events, only mild cholinergic events	[62]
USA 2011	210 (70/70/70)	DB, M, PC, RC, DE Phase II	0.2 mg/day or 0.4 mg/day, 16 weeks	MMSE: HupA 0.4 mg/day, +0.65; HupA 0.8 mg/day, +1.10; placebo, −0.40 ADAS – Cog: HupA 0.4 mg/day, −0.32; HupA 0.8 mg/day, −1.92; placebo, −0.34 ADCS-ADL: HupA 0.4 mg/day, −1.18; HupA 0.8 mg/d, −0.85; placebo, −2.64 ADL and NPI – not significant in either dose	No serious adverse events	[90]

SB single-blind, *DB* double-blind, *M* multicenter, *PC* placebo-controlled, *RC* randomized-controlled, *DE* dose-escalation trial, *MMSE* Mini Mental State Examination, *ADAS-Cog* Alzheimer's Disease Assessment Scale – Cognitive Section, *ADAS* – non-Cog, Alzheimer's Disease Assessment Scale – non-Cognitive Section, *ADL* Activities of daily living, *NPI* Neuropsychiatric Inventory, *ADCS* – *ADL* Alzheimer's Disease Cooperative Study – Activities on Daily Living, *CIBIC-plus* Clinical Interview-Based Impression, *HDS* Hasegawa Dementia Scale, *MQ* Memory Quotient

[a] Both groups got Vitamin E: 200 mg/day (100 mg b.i.d.)

[b] Control group received *Salvia miltiorrhiza* tablets

cholinergic side effects, including nausea, vomiting and diarrhea and no serious adverse events occurred [64].

In the USA, powdered *H. serrata* is available as a dietary supplement, and the recommended dose contains 50 μg b.i.d. of hupA [3]. Two US phase I studies were conducted to determine the safety and tolerability of hupA. Phase Ia and phase Ib (Table 39.2) were identical in design except for the dose which was up to 0.2 mg and 0.4 mg b.i.d in phase Ia and phase Ib, respectively. No serious adverse events occurred in these studies, although mild-to-moderate side effects were observed in the phase Ib study with the higher dose [62].

Subsequently, a multicenter phase II study was organized by NIH in over 30 sites in the USA (Table 39.2). The results showed that hupA was generally well tolerated at doses up to 0.4 mg b.i.d. for 24 weeks, even though many of the patients participating had been unable to tolerate at least one previous trial of treatment with a marketed ChE inhibitor. The cognitive benefits as indicated by the change in the Alzheimer's disease Assessment Scale – Cognitive Section (ADAS – Cog) score after 16 weeks, for the 0.2 mg b.i.d. dose on the one hand, did not indicate efficacy, although it had favorable influence on the MMSE score. On the other hand, the 0.4 mg b.i.d. dose showed evidence of cognitive enhancement in both ADAS – Cog and MMSE. The effect on ADL was not significant in either dose. The authors conclude that the results suggest a possible benefit of hupA which requires confirmation in additional studies [91].

It is evident from Table 39.2 that the scores for MMSE, ADL, and ADAS – Cog cognitive tests are considerably higher in the Chinese studies than in the US study for comparable doses of hupA. However, studies in both countries indicate dose-dependent improvements of symptoms, although they do not agree on the effective therapeutic dose [62, 64, 91]. It has been implied that results are not directly comparable across trials which are conducted in different countries, as different inclusion/exclusion criteria may have been used, and other factors in the implementation may differ as well [62, 64]. According to the US phase II study, the recommended doses for the marketed products for AD treatment in both USA and China are too low to be effective. All the studies in Table 39.2 agree on the good tolerability of hupA up to a dose of 0.8 mg/day in the phase II US study. However, if hupA is to become an established AD drug first, the maximum tolerable dose needs to be determined and secondly, large, well-designed, multinational phase III clinical trials are needed.

ZT-1, a semi-synthesized prodrug of hupA, has been tested for the treatment of AD symptoms. An open label phase II study in patients with mild-to-moderate AD with once-daily oral formulation of ZT-1 was reported to have good efficacy and tolerability comparable to donepezil [76]. A phase I study for a sustained-release ZT-1 implant dosage form concluded that the implants were safe up to a dose of 15 mg month^{-1} and continuously released the active component over a period of 28 days [76]. Subsequently, a phase II study was designed for this implant and the investigators argued that a continuous slow-release form of ZT-1 could reduce the side effects and increase the compliance for AD treatment compared to donepezil [76]; however, to our knowledge, the results of these trials have not yet been published.

9 Conclusion

The traditional use of club mosses in Chinese herbal medicine promoted the interest for lycopodium alkaloids as possible drug leads for dementia. HupA, the most potent AChEI of the natural lycopodium alkaloids tested so far, binds selectively and reversibly to the enzyme with prolonged biological half-life. In addition, preclinical studies indicate an overall neuroprotective effect of hupA which implies, although not clinically confirmed, a disease-modifying potential in AD. HupA has many other advantageous characteristics favoring a CNS drug lead, e.g., to cross the BBB smoothly and having good bioavailability and pharmacokinetic properties, low toxicity, and mild adverse effects in humans.

The lycopodium alkaloids, in general, are interesting molecules to explore as drug candidates. They often have rigid and stable structures which can interfere with numerous targets both outside and within the CNS, thus representing feasible model molecules for multi-target or multidrug development aimed at AD and other neurodegenerative diseases. Noteworthy, the dose-limiting adverse effects of hupA and other AChEIs used for AD are indeed cholinergic in nature, suggesting that instead of focusing on more potent AChEIs, future drug development should aim at multi-target approach including some of the many targets of neuroprotection. The numerous structurally diverse lycopodium alkaloids already discovered are likely to play a role in this future quest for more effective drugs. An important challenge in that respect is to develop sustainable methods, e.g., synthesis and bioprocessing of lycopodium alkaloids, to supply these interesting natural products for future studies and application.

One of the major barriers in the current clinical evaluation of potential drug candidates for AD is the definition of the therapeutic effect in AD clinical trials, including the lack of tests evaluating the progression of the disease [29] and to clarify symptomatic versus disease-modifying potential. More accurate methods of evaluation of AD symptoms together with better understanding of the complex pathophysiology behind the disease would definitely benefit the development of more efficient drug treatment for AD.

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Scope of Alkaloids in Antileishmanial Drug Discovery and Development

40

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Contents

1 Introduction 1264
2 Taxonomy of *Leishmania* spp. 1266
3 Parasite Biology 1266
4 Drug Targets 1268
4.1 Sterol Biosynthetic Pathway 1268
4.2 Glycolytic Pathway 1268
4.3 Nucleoside Transporters 1269
4.4 Purine Salvage Enzymes 1269
4.5 GPI Biosynthetic Pathway 1269
4.6 Proteinases as Drug Targets 1270
4.7 Folate Biosynthesis 1270
4.8 Glyoxalase System 1271
4.9 Topoisomerases 1271
4.10 Hypusine Pathway 1272
5 Bioavailability and Metabolism 1272
6 Current Chemotherapy of Leishmaniasis 1273
6.1 Pentavalent Antimonials 1273
6.2 Pentamidine 1274
6.3 Amphotericin B 1274
6.4 Miltefosine 1275
6.5 Paromomycin 1275
7 Other Drugs 1276
8 Natural Products as Folk Medicines Against Leishmaniasis 1278

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9 Alkaloids 1282
9.1 Benzo[*c*]phenanthridine Alkaloid 1282
9.2 Quinoline Alkaloids 1283
9.3 Furoquinoline Alkaloids 1284
9.4 Isoquinoline Alkaloids 1284
9.5 Naphthylisoquinoline Alkaloids 1286
9.6 Bisbenzylisoquinolinic Alkaloids 1287
9.7 Indole Alkaloids 1288
9.8 Steroidal Alkaloids 1289
9.9 Benzoquinolizidine Alkaloids 1289
9.10 Diterpene Alkaloids 1290
9.11 Pyrrolidinium Alkaloids 1290
9.12 Acridone Alkaloids 1290
9.13 *β*-Carboline Alkaloids 1291
9.14 Alkaloids from Marine Sources 1291
10 Future Perspectives 1294
References 1295

Abstract

Leishmaniasis, a parasitic disease resulting from infection of macrophages by obligate intracellular parasites of genus *Leishmania*, is prevalent in tropical and subtropical regions of 88 countries, affecting about 12 million people in the world. Leishmaniasis displays a spectrum of clinical manifestations, ranging from mucocutaneous and cutaneous lesions to visceral leishmaniasis, which is usually fatal for untreated patients. Moreover, significant increases in the rate of *Leishmania*-HIV coinfections across the world and cases of resistant parasites are aggravating this problem. Chemotherapy is the only effective treatment for leishmaniasis. However, the growing incidence of resistance for most of the antileishmanial drugs in endemic and nonendemic regions has seriously hampered their use in these regions. The present chapter briefly illustrates leishmaniasis epidemiology, occurrence, parasite biology, drug targets, bioavailability and metabolism, and treatment around the world and also critically discusses the key points in alkaloids-based drug discovery protocols.

Keywords

Alkaloids • leishmaniasis • natural products • parasite biology • therapeutic options

1 Introduction

The vector-borne parasitic disease leishmaniasis continues to be one of the six entities on the World Health Organization tropical disease list [1]. The disease threatens about 350 million men, women, and children in 88 countries around the world covering tropical and temperate regions of world ranging from rainforests in

Central and South America to deserts in West Asia and the Middle East. As many as 12 million people are believed to be currently infected, with about 1–2 million estimated new cases occurring every year [2].

The protozoan parasites of family Trypanosomatidae and order Kinetoplastida are responsible for three main clinical syndromes of leishmaniasis: cutaneous (caused by *Leishmania tropica* and *L. mexicana*), mucocutaneous (caused by *L. braziliensis*), and visceral (caused by *L. donovani*). The *Leishmania* species, *L. tropica* and *L. donovani* found in Africa, Europe, and Asia, are often termed as the "Old World," while the species *L. mexicana* and *L. braziliensis* are restricted to the America and commonly referred to as the "New World" [3]. The visceral leishmaniasis (VL) has an estimated incidence of 500,000 new cases and 60,000 deaths each year with more than 90% of cases are centralized to India, Bangladesh, Nepal, Sudan, and Brazil [4].

Leishmaniasis is caused by 20 species of *Leishmania* and transmitted by 30 species of sand fly. Females of *Phlebotomus* (in the Old World) or *Luztomiya* (in the New World) sand flies are the proven vector of the disease [5]. These sand flies are about one-third the size of typical mosquitoes, thus are too small to see and get realized by the people. Sand flies become infected by biting an infected animal (e.g., rodents or dogs) or human and spread the disease to other healthy vertebrates [6]. Besides, leishmaniasis can also be spread by blood transfusions or contaminated needles. The disease rarely spreads from a pregnant woman to her baby. Usually, the disease is more common in rural than urban areas, and people of all ages if living or travel in disease prevailing region are at high risk [7].

The symptoms for leishmaniasis include fever, weight loss, enlarged spleen, swollen glands, skin sores, splenomegaly, lymphadenopathy, hepatomegaly, pancytopenia, progressive anemia, and hypergammaglobulinemia with hypoalbuminemia. People suffering from cutaneous leishmaniasis (CL) usually develop one or more skin sores (either painful or painless, with or without a scab) within few weeks to months after bitten by an infected female sand fly. Some people may have swollen glands near the sores. VL patients may have low blood counts (including a low RBC, WBC, and platelet count); progressive emaciation; enlargement in visceral organs (liver and spleen); hyperpigmented skin of the forehead, abdomen, hands, and feet in light-skinned persons; and occasional bleeding. Leishmaniasis is always fatal when left untreated, and sometimes patients, i.e., 50% in Sudan and 1–3% in India, develop post-*kala-azar* dermal leishmaniasis (PKDL) [8].

There are a growing number of reports of *Leishmania*-human immunodeficiency virus (HIV) coinfections across the world. *Leishmania*-HIV coinfection has been globally controlled in southern Europe since 1997 by highly active antiretroviral therapy (HAART), but it appears to be an increasing problem in other countries such as Ethiopia, Sudan, Brazil, or India where both infections are becoming more and more prevalent [9]. The situation is particularly alarming in southern Europe, where 50–75% of adult VL cases are HIV positive. Among the 45 million people infected by HIV worldwide, an estimated one-third lives in the zones of endemic *Leishmania* infections [10]. Over 850 *Leishmania*-HIV coinfection cases recorded worldwide, the majority are from Europe, where 7–17% of HIV-positive individuals with fever have amastigotes, suggesting that *Leishmania*-infected individuals without symptoms will express symptoms of leishmaniasis if they become immunosuppressed. However, there are indirect reasons and statistical data demonstrating that intravenous drug addiction plays a specific role in transmission *Leishmania* species. To date, the greatest prevalence of *Leishmania*-HIV coinfection has been recorded in the Mediterranean basin, where among more than 2,000 cases notified to the WHO, 90% of them belong to Spain, Italy, France, and Portugal [11]. Still no comparative clinical studies about the leishmaniasis in HIV-infected and non-HIV-infected patients have been reported, and aspects of its epidemiology, clinical features, and management remain unknown.

The present chapter briefly illustrates the current status of leishmaniasis, occurrence, morphology and life cycle, drug targets, bioavailability and metabolism, and treatment around the world and also critically discusses the key points in natural product-based drug discovery protocols. Finally, a comprehensive coverage of alkaloids with significant activity against *Leishmania* species has been given substantially. In order to highlight any possible structure-activity relationships, the chapter has been organized according to chemical structural class.

2 Taxonomy of *Leishmania* spp.

Earlier various classifications have been successively applied to heteroxenous protozoan *Leishmania*; however, the simplest one can be summarized from Fig. 40.1 [1, 4].

3 Parasite Biology

The obligate intracellular *Leishmania* parasites exist in two morphologic forms: promastigotes and amastigotes. The long spindle-shaped flagellated (15–28 μm) promastigotes measuring about 114.3–20 μm in length and 1.5 to 1.8 μm at their widest part [12] are found in digestive tract of sand fly [13]. The small, round to oval bodies called amastigotes (2–3 μm in length) are the noninfective *Leishmania* parasites occurring in monocytes, polymorphonuclear leucocytes, or endothelial cells of vertebrate hosts.

The *Leishmania* promastigotes are transmitted by sand fly to vertebrate hosts, e.g., canines, marsupials, edentates, and rodents (Fig. 40.2). Once inside the bloodstream of reservoirs for the disease, promastigotes are phagocytosed by the mononuclear phagocytic cells and are transformed to amastigotes which multiply by means of binary fission. On lyse of host cell, the free parasites spread to new cells and tissues of different organs including the spleen, liver, and bone marrow. Amastigotes in the blood as well as in the monocytes are ingested during a blood

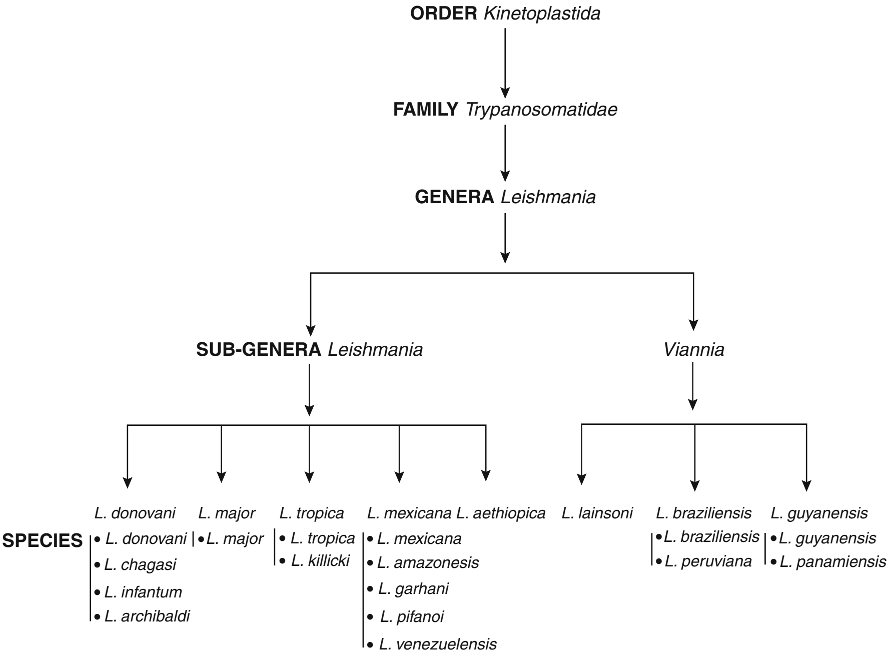

ORDER Kinetoplastida
FAMILY Trypanosomatidae
GENERA Leishmania
SUB-GENERA Leishmania
Viannia
L. donovani
L. major
L. tropica
L. mexicana
L. aethiopica
L. lainsoni
L. braziliensis
L. guyanensis
SPECIES
• L. donovani
• L. chagasi
• L. infantum
• L. archibaldi
• L. major
• L. tropica
• L. killicki
• L. mexicana
• L. amazonesis
• L. garhani
• L. pifanoi
• L. venezuelensis
• L. braziliensis
• L. peruviana
• L. guyanensis
• L. panamiensis

Fig. 40.1 Classification of *Leishmania* parasite

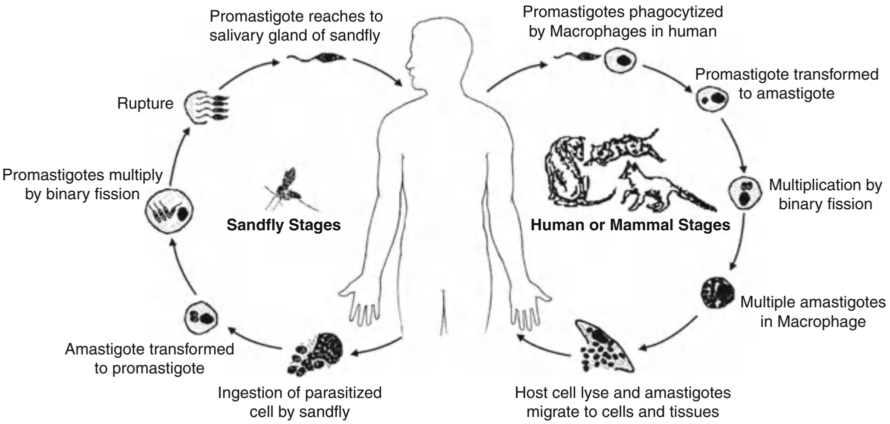

Promastigote reaches to salivary gland of sandfly
Promastigotes phagocytized by Macrophages in human
Promastigote transformed to amastigote
Rupture
Promastigotes multiply by binary fission
Sandfly Stages
Human or Mammal Stages
Multiplication by binary fission
Multiple amastigotes in Macrophage
Amastigote transformed to promastigote
Ingestion of parasitized cell by sandfly
Host cell lyse and amastigotes migrate to cells and tissues

Fig. 40.2 Life cycle of *Leishmania* parasite

meal by female sand fly. Once ingested, the amastigotes migrate to the midgut of the sand fly and transform into the promastigotes. Infected sand fly during the second blood meal regurgitates the infectious promastigotes from its pharynx into the bloodstream of the vertebrate host, and the life cycle is repeated [1, 14].

4 Drug Targets

Identification and characterization of novel cellular targets and answering the problem of drug resistance in *Leishmania* has always been the main thrust of protozoal research worldwide. However, putative target in a pathogen can be identified and validated successfully only if it is either absent in the host or substantially different from the host homolog. The cell organization in *Leishmania* species is significantly different from the mammalian cells, and thus, it is possible to find targets that are unique to these pathogens. Secondly, the target selected should be absolutely necessary for the survival of the pathogen [15]. Some of the crucial targets significant in drug discovery and development against leishmaniasis have been discussed below.

4.1 Sterol Biosynthetic Pathway

Sterols are important components of the cell membrane that are vital to various cellular functions and maintenance of cell structure. The major sterol in promastigotes of *Leishmania tropica*, *L. donovani*, and 3 subspecies of *L. mexicana* is ergosta-5,7,24(28)-trien-3*β*-ol. Ergosta-7,24(28)-dien-3*β*-ol is the major constituent of *L. major*. 24-Methylcholesta-5,7,22-trien-3*β*-ol and 24-ethylcholesta-5,7,22-trien-3*β*-ol are the minor constituents, while traces of ergosta-5,7,22,24(28)-tetraen-3*β*-ol and a C27-diene have also been detected in some species. Lanosterol and 4,4-dimethylcholesta-8,24-dien-3*β*-ol have been also identified in these species, while squalene is known to occur in *L. tropica*. The enzyme D24,25-sterol methyltransferase (SMT) is a putative target in ergosterol biosynthesis. The occurrence of this enzyme is limited to trypanosomatids only (absent in human host), thus would be exploited effectively as a potential drug target [16].

4.2 Glycolytic Pathway

Glycosomes are peroxisome-related organelles containing glycolytic enzymes that are found only in kinetoplastids. The unique compartmentalization of glycolytic enzymes in glycosomes of *Leishmania* and their large phylogenetic distance with the mammalian hosts can be exploited by designing specific inhibitors that can bind to parasitic enzymes with the high affinity. In this context, the specific inhibitors have been designed for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Studies have shown that the adenosine being a poor inhibitor of the enzyme shows enhanced inhibition on substitutions at the 20-position of ribose and *N*6-position of adenosine (disubstituted analogs). Likewise, *N*-(1-naphthalenemethyl)-2′-(3-methoxybenzamido) adenosine inhibits the growth of *L. mexicana* with IC_{50} of 0.28 μM [17]. Thus, with the design and development of specific inhibitors of enzymes frequently involved in glycolytic pathway, the energy production in parasite can be blocked effectively. Since glycolysis is the only source of energy for these parasites, it can serve as an excellent drug target.

4.3 Nucleoside Transporters

In *Leishmania*, the LdNT1 and LdNT2 are the two specific transporters that transport the nucleosides across the membrane. LdNT1 occurs in promastigotes as well as amastigotes and transports adenosine and pyrimidine nucleosides, while LdNT2, occurring in amastigotes only, is responsible for transportation of purine nucleosides, i.e., inosine and guanosine [18]. Similarly, among the two purine nucleobase transporters, namely, LmaNT3 and LmaNT4 identified from *L. major*, the LmaNT3 is known to transport hypoxanthine, xanthine, adenosine, and guanine bases only but none of nucleosides, while LmaNT4 at neutral pH takes up adenine only but also takes up hypoxanthine, guanine, and xanthine at acidic pH [19]. The parasitic transporters are different from the mammalian transporters in terms of their higher specificity toward the substrate. There are so many pathways for the uptake of purines; thus, it is very difficult to target them with selective inhibitors. But, they will remain pharmacologically important as these transporters also uptake toxic nucleoside analogs which are inhibitory to the cell growth.

4.4 Purine Salvage Enzymes

Salvage pathway, a pathway in which nucleotides (purine and pyrimidine) are synthesized from intermediates in the degradative pathway for nucleotides, is used to recover bases and nucleosides that are formed during degradation of RNA and DNA. Since *Leishmania* species cannot synthesize purine nucleotides de novo, they rely predominantly upon therapeutically germane phosphoribosyltransferase (PRT) enzymes. There are three PRTs identified in *Leishmania* for acquisition of purine from the host: hypoxanthine-guanine PRT (HGPRT), adenine PRT (APRT), and xanthine PRT (XPRT) [20]. Among these enzymes, the HGPRT is particularly important due to its difference in substrate specificity with the host enzyme. HGPRT converts hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate. Allopurinol is a most common HGPRT inhibitor used which on phosphorylation by HGPRT gets incorporated into nucleic acid and cause selective death of the parasite [21]. However, it has been established that PRTs are not essential for survival of *Leishmania* because there may be various alternative purine salvage pathways present in the parasite. Therefore, in antileishmanial chemotherapy it is necessary to target more than one enzyme at a time.

4.5 GPI Biosynthetic Pathway

In promastigotes of *Leishmania* parasites, the cell surface is coated by glycocalyx consisting of glycosylphosphatidylinositol (GPI)-anchored glycoproteins, GPI-anchored lipophosphoglycan (LPG), and a family of free GPIs, called as glycoinositolphospholipids (GIPLs) [22, 23]. GPI anchors are synthesized in endoplasmic reticulum membrane and after attachment to the nascent proteins, are transferred to the plasma membrane via the secretory pathway. GPI biosynthesis in case of parasites involves mannosylation of GlcN-PI which is followed by inositol acylation, whereas in yeast and human, inositol acylation is a prerequisite for mannosylation. The trimannosyl core of GPI anchors is added stepwise and involves three distinct mannosyltransferases (MTs) in which the structure of mannosyltransferase III (MTIII) in trypanosomes has been found to be significantly different from mammalian MTIII. Thus, species-specific inhibition can be achieved by synthesizing small molecule inhibitors [24].

On completion of GPI anchor synthesis, lipid remodeling comprising the exchange of fatty acid components with other fatty acid of the lipid moiety or the whole lipid component takes place. The mature GPI precursor is transferred to the protein through transamidation reaction by a multimeric protein complex, where -COOH terminal signal peptide is replaced by the formation of amide linkage to amino group of ethanolamine phosphate linked to the third mannose of GPI anchor. The protein components differ in different species, suggesting that these differences between the parasite and mammalian enzymes can be exploited for drug designing [24].

4.6 Proteinases as Drug Targets

Proteinases are of four main types – cysteine, serine, aspartate, and metalloenzymes. The name is given on the basis of the residue present in the active site. In case of parasitic protozoa, the most identified and characterized are the cysteine proteinases (CPs), which are homologous to mammalian cathepsins. CPs play an important role in the infection, replication, development, and metabolism of protozoan parasites [25], and their activity is necessary for the survival of *L. mexicana* within the macrophages, in vitro [26]. Knockout studies in *L. mexicana* have shown that cysteine proteases not only are virulence factors but also act as modulators of host immune responses [27]. Thus, CPs have become a potential target for chemotherapy and a candidate for vaccine development. Initial studies have confirmed the efficacy of CP inhibitors in treatment of *L. major* [28]. Immunization with the hybrid protein vaccine, consisting of *L. major* cysteine proteases CPB and CPA, is partially protected against leishmaniasis [29]. So far, functionally well-characterized CPs are from the New World species of *Leishmania* causing the cutaneous forms of leishmaniasis. The members of *L. donovani* complex also possess multiple classes of CPs, which are developmentally regulated and are not functionally well characterized. Therefore, there is a need to study the function of these proteases and their role in VL.

4.7 Folate Biosynthesis

A biochemical pathway that has been exploited earlier in the treatment of infectious diseases is the folic acid biosynthetic pathway. Folic acid consists of three building blocks: a pterin, *p*-aminobenzoic acid (*p*ABA), and glutamic acid. Analogs of folic acid that can inhibit cellular growth are called antifolates, while folates are important cofactors essential for cellular growth. The enzymes that are involved in their synthesis have been of great interest as drug targets, particularly, thymidylate synthase (TS) and dihydrofolate reductase (DHFR) that converts dihydrofolate to tetrahydrofolate, an important cofactor in the synthesis of thymine. Both the enzymes exist on a single polypeptide in case of trypanosomatids, with DHFR domain on amino terminus and TS domain on carboxy terminus. The protozoan parasite *Leishmania* is a folate and pterin auxotroph. The main biopterin transporter (BT1) and pterin reductase (PTR1) have already been characterized in *Leishmania*. A number of compounds have been screened against PTR1 in *L. major* [30]. Several compounds were found to be inhibiting both DHFRTS and PTR1. However, only few of them inhibited both the enzymes and the growth of the parasite potently. Thus, design and development of suitable inhibitors to target both the enzymes simultaneously or a combination of two compounds that can be used specifically for inhibiting both the enzymes may be crucial for antileishmanial drug discovery.

4.8 Glyoxalase System

The glyoxalase system is responsible for detoxification of the cell through removal of toxic and mutagenic intermediates (i.e., methylglyoxal) formed as a by-product of glycolysis. The glyoxalase system functions by activity of two enzymes, namely, glyoxalase I (lactoyl glutathione lyase) and glyoxalase II (hydroxyacyl glutathione hydrolase), and uses glutathione as a cofactor. Glyoxalase I has been characterized from *L. donovani* [31] and *L. major* [32], while glyoxalase II has been characterized from *L. donovani* [33]. Glyoxalase I is highly substrate specific and lacks the basic residues in its active site which are conserved in human homolog. These residues are important for binding to glutathione. The substrate for *L. donovani* glyoxalase II is thioester of trypanothione, which is positively charged, therefore, cannot be accommodated in the active site of the human enzyme. This observation strongly suggests that the parasitic enzymes are highly substrate specific. Therefore, trypanothione-dependent glyoxalase pathway has drawn attention for additional biochemical and genetic investigations as a possible target for rational drug design.

4.9 Topoisomerases

In kinetoplastids, the DNA topoisomerases play a key role in cellular processes affecting the topology and organization of intracellular DNA and play an important role in DNA replication, transcription, chromosomal condensation, segregation, and many other vital cellular processes. They are broadly classified as type I and type II topoisomerases. Type I and II topoisomerase have been characterized from *L. donovani* [34]. Interestingly, the topoisomerase II from *L. donovani* was found to contain both ATP dependent and independent activities. Many of the topoisomerase II inhibitors have shown strong activity against *Leishmania* species [35, 36]. The structural analysis of these enzymes will give us an insight into their catalytic mechanisms and will also enable us to design specific inhibitors against *Leishmania*.

4.10 Hypusine Pathway

Hypusine, an unusual amino acid derived from the polyamine spermidine, occurs in all the eukaryotes. The name is given on the basis of two structural components, hydroxyputrescine and lysine [37]. It is synthesized as a result of posttranslational modification occurring exclusively on one cellular protein, eukaryotic initiation factor 5A (eIF5A). Recent researches have shown that hypusine biosynthesis also occurs in *L. donovani*. Moreover, *Leishmania* has two genes containing DHS domains, namely, DHS-like gene (DHSL20) and DHS34 gene, of which only DHS34 protein was found to contain functional activity in vitro and is essential for the survival of parasite [38]. Structural modeling of DHS34 protein has shown that one of the NAD^+ binding domains lies in a 225-amino acid long insertion present in DHS34 protein, as compared to the human homolog. The recombinant DHS34 remained uninhibited by most potent inhibitors of the human enzyme like GC7 and other spermidine analogs, indicating that there is a difference between the spermidine binding sites of both the enzymes which could be exploited while designing new inhibitors.

5 Bioavailability and Metabolism

A little is known about the energy metabolism exhibited by *Leishmania* parasite during its life cycle. Studies have shown that *Leishmania* and trypanosomes share many features due to development from a common ancestor, and many of the genes in different trypanosomatids are proven orthologues. In spite of many common features, some substantial biological differences between *Leishmania* and trypanosomatids can be figured out by comparing their life history strategies [39]. The biology (e.g., their food source) of trypanosome vectors is significantly differing from sand flies. In mammalian host, *Leishmania* thrives inside a parasitophorous vacuole within mononuclear cells. Amastigotes are fully adapted for reaching this niche, and their appropriate metabolic capabilities have been evolved to ensure the intracellular existence. *Leishmania* exists as promastigote forms adapted to survive in different parts of the intestinal tract in sand fly excluding salivary glands or hindgut (except braziliensis complex of *Leishmania*).

The metabolism of in vitro grown promastigotes in nutrient-rich medium compared to those occurring naturally in sand flies is still not fully understood. The biochemical researches involving mixed populations of promastigotes (e.g., metacyclic promastigotes in addition to multiplicative forms) have indicated for the metabolic similarities with the vector stages of *T. cruzi* and *T. brucei*. The amino acids (notably proline) and sugars (including glucose) are the probable energy sources. The energy is supposed to be generated by both glycolysis (and glycosomes) and mitochondrial metabolism through an active electron transport chain. Experimental evidences have indicated for the presence of enzymes of tricarboxylic acid (TCA) cycle; however, cycle is not active [40]. Researches have shown for reversible metabolic arrest in promastigotes on inhibition of the electron transport chain, which might be an adaptation for survival during life in the sand fly [41].

Earlier, the presence of a glyoxylate cycle was reported [42] but was found to be unlike on the basis of genome analysis. The pathways that produce acetate and succinate are supposed to be responsible for CO_2 production. The succinate, acetate, pyruvate, and D-lactate have been identified as end products. Alanine, ammonia, and urea are also released. Due to less availability of *Leishmania* amastigotes for metabolic studies in compared to promastigotes, the knowledge of the energy metabolism in amastigotes is considerably more fragmentary. Studies using amastigotes isolated from in vivo lesions of *L. mexicana* have evidenced for an increased β-oxidation of fatty acids and a reduced need for proline and glucose consumption in compared to promastigotes [43]. The amastigotes contain considerably fewer glycosomes but greater number of enzymes, i.e., phosphoenolpyruvate carboxykinase and malate dehydrogenase than promastigotes [44–46].

The importance of some of the detected differences in gene content between trypanosomatids can be readily hypothesized. Moreover, some of the known metabolic differences between trypanosomatids are not fully explained by the presence or absence of genes that encode metabolic enzymes. Clearly, the situation will be affected by the control of gene expression and by enzyme stability at different stages of the life cycle. The current “best guess” on the metabolism that occurs in *Leishmania* amastigotes and promastigotes now requires detailed studies, and gene mining has indicated some sensible approaches to pursue and experiments that should be done [39].

6 Current Chemotherapy of Leishmaniasis

6.1 Pentavalent Antimonials

Pentavalent antimonials, sodium stibogluconate (Pentostam®), meglumine antimoniate (Glucantime®), etc., are being used for treatment of leishmaniasis over more than five decades. The pentavalent antimoniate (Sb^V) is a prodrug of trivalent antimonite (Sb^{III}), an active form of the drug which is generated by reduction from Sb^V either in macrophages or in the parasite [1]. Aquaporin-1 transporters in the plasma membrane of amastigotes are able to transport trivalent metalloids into the cell, though reduction into the trivalent form occurs only in a minor amount in phagolysosomes of the macrophage while the major amount is supposed to be converted within the parasite. These compounds are supposed to exert antileishmanial activity by inhibiting enzymes involved in the glycolysis and oxidation of fatty acids.

Instead of costly branded sodium stibogluconate (approximately 200 USD per patient), a generic sodium antimony gluconate (SAG, Albert David Ltd., India, 13 USD per patient) was used to treat patients satisfactorily without any significant difference in final cure [4]. Antimonials are administered intravenously or intramuscularly, and side effects like chemical pancreatitis, elevations in serum aminotransferases, and electrocardiographic abnormalities are usually reversible. One major drawback of therapy is the drastic emergence of resistance against antimonials. A prominent example is Bihar State in India, where the cure rate of antimony therapy against VL has declined to merely 35%, making conventional treatment no longer useful [47].

6.2 Pentamidine

Pentamidine is an aromatic diamine used to cure leishmaniasis as a second-line drug. Its isothionate and methanesulfonate salts are mainly used for the treatment of VL. The drug gets accumulated in mitochondria and enhances efficacy of mitochondrial respiratory chain complex II inhibitors suggesting its leishmanicidal activities due to decreased mitochondrial membrane potential. Pentamidine was the first drug used for the treatment of patient refractory to Sb^V in India [48]. Biophysical analysis, foot-printing studies, and the crystal structure have proved that the charged amidinium groups of pentamidine establish hydrogen bonding with O_2 of thymine or N_3 of adenine and form complexes with the minor groove of DNA. However, declining efficacy (cures only 70% of patients) and high resistance risk have led to its closure in India.

Pentamidine is highly toxic; causes myalgias, pain at the injection site, nausea, and headache; and less frequently results in a metallic taste, a burning sensation, numbness, and hypotension. Reversible hypoglycemia occurs in about 2% of cases [49].

6.3 Amphotericin B

Amphotericin B (AmB) is a pollen antibiotic that was recommended as first-line drug in India by National Expert Committee for Sb^V refractory regions of VL. At doses of 0.75–1.0 mg/kg for 15 infusions on alternate days, it cured more than 97% of patients. AmB shows high affinity for ergosterol, the predominant sterol of fungal and leishmanial cell membrane [50]. Despite high efficiency, AmB is toxic and treatment with it can cause serious adverse reactions including fever with rigor and chills, thrombophlebitis, and occasional serious toxicities like myocarditis, severe hypokalemia, renal dysfunction, and even death [51].

Currently, toxic effects of AmB have been largely ameliorated with the advent of lipid formulations. In these formulations, deoxycholate has been replaced by other lipids that mask AmB from susceptible tissues, thus reducing toxicity and facilitating its preferential uptake by reticuloendothelial cells. Thus, this drug delivery results in increasing efficacy and reduced toxicity. Three lipid-associated formulations of amphotericin are commercially available: liposomal amphotericin B (AmBisome), amphotericin B lipid complex (Abelcet), and amphotericin B colloidal dispersion (Amphocil). These compounds have been considered between the most striking advances in *Leishmania* therapy [52].

The antileishmanial activity of AmB and its lipid formulation is due to its interaction from both sterols, i.e., ergosterol of *Leishmania* and cholesterol of host macrophages. Since cholesterol is complexed by AmB, it markedly inhibits the binding of *L. donovani* promastigotes to macrophage. Further, at higher concentration (<0.1 M), it induces the formation of aqueous pores in leishmanial promastigotes cell membrane that result in osmotic changes leading to the cell lysis.

6.4 Miltefosine

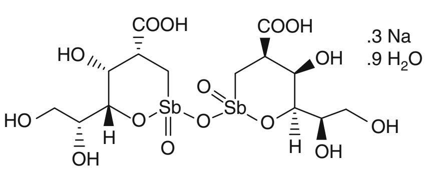
Miltefosine, an alkylphosphocholine (hexadecylphosphocholine) developed originally as an antitumor agent, was approved in India at 50–100 mg (~2.5 mg/kg) doses for 4 weeks against VL patients including children. The activity of miltefosine is due to intracellular accumulation of drug, which is regulated by two transporters, LdMT and its *β*-subunit LdRos3, a P-type ATPase, belonging to aminophospholipid translocase family. Miltefosine mediates apoptotic-like cell death in *L. donovani*, and, moreover, inhibition of alkyl-specific-acyl-CoA acyltransferase has also been reported [53]. Miltefosine blocks *Leishmania* proliferation, alters phospholipid and sterol composition, and activates cellular immunity. However, due to high cost and serious side effects, i.e., teratogenic and abortifacient nature, medical advisors generally avoid the drug in their prescriptions [54].

Miltefosine has been hailed as a novel oral drug for treatment of VL. Success in immunocompetent and immunocompromised patients is perhaps the most significant recent advance [55]. However, some studies have demonstrated the insensibility of *Leishmania* species from the New World including *L. braziliensis*, *L. guyanensis*, and *L. mexicana*. These results are disappointing as miltefosine was believed to provide a better alternative to drug therapy in America. The efficacy of miltefosine against *L. infantum* infection has been conducted in animals and needs a validation in clinical trials [56].

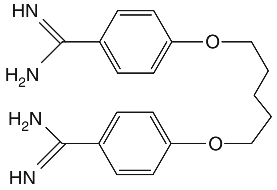
6.5 Paromomycin

Paromomycin, an amino glycoside antibiotic originally identified as an antileishmanial drug in the 1960s, acts synergistically with antimonials in vitro and was demonstrated significant (93% cure rate) at a dose of 16 mg/kg when given intramuscularly for 21 days to VL patients in India. Recent study has shown that cationic paromomycin binds to the negatively charged leishmanial glycocalyx suggesting mitochondria as a primary target. Like other amino glycosides, the drug acts by impairing the macromolecular synthesis and alters the membrane properties of *Leishmania* [57]. It binds to the 30 S ribosomal subunit, interfering with initiation of protein synthesis by fixing the 30 S–50 S ribosomal complex at the start codon of mRNA, leading to accumulation of abnormal initiation complex [58]. In parallel, experimental evidences have shown that paromomycin promoted ribosomal subunit association of both cytoplasmic and mitochondrial forms, following low Mg^{2+} concentration; induced dissociation; and also caused dysfunction in respiratory systems [59].

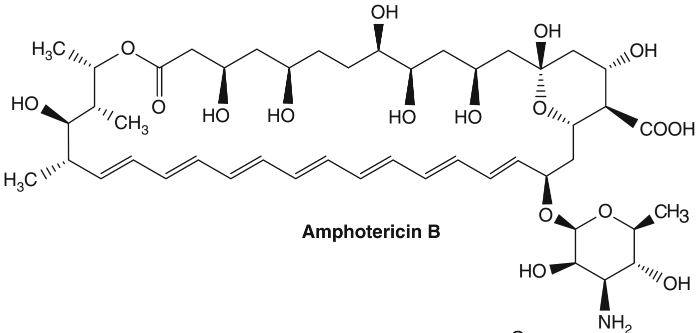
Three preparations of paromomycin ointments have been used for CL: paromomycin 15% plus methylbenzethonium chloride 12%, paromomycin 15% with urea 10%, and paromomycin plus gentamicin 0.5%. These formulations have shown variable results according to the species of *Leishmania* involved and the epidemiologic situation [4]. The most common side effect associated with the paromomycin is ototoxicity as well as problems in liver function [60]. In patients treated with the ointment formulation, skin rashes, local pruritus, and burs have been the side effects encountered.



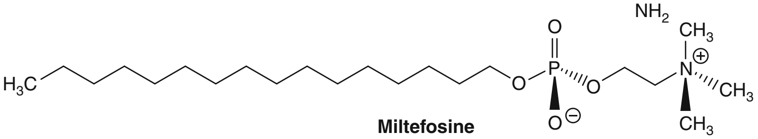
Sodium stibugluconate



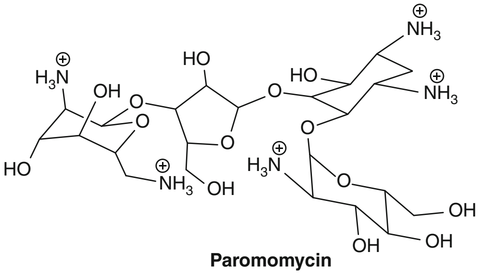
Pentamidine



Amphotericin B



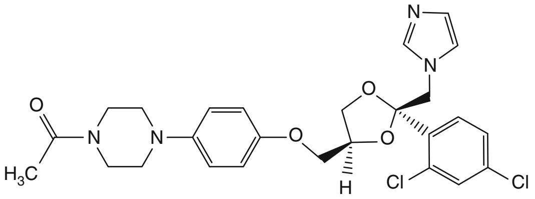
Miltefosine



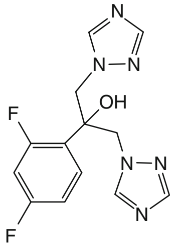
Paromomycin

7 Other Drugs

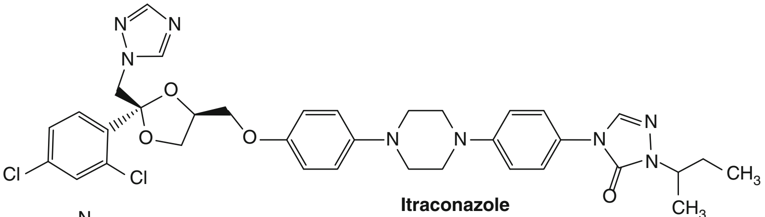
Some of imidazoles and triazoles are the potential inhibitors of 14-demethylase, a key enzyme in the sterol biosynthesis pathway, thus directly interfere with cell wall biosynthesis in *Leishmania*. Fluconazole, a triazole antifungal drug used in the treatment and prevention of superficial and systemic fungal infections, has been used for the treatment of *L. major* in Old World. Similarly, ketoconazole has been used in the New World against *L. panamensis* and *L. mexicana*. Itraconazole has been used in Old and New World but suffers from low efficacy. Itraconazole at a concentration of 0.75 μM/L causes complete cell lyses after 72 h against *L. major* promastigotes. Likewise, posaconazole shows activity against experimental *L. amazonensis* infection but still has not been evaluated in clinical trials [61].



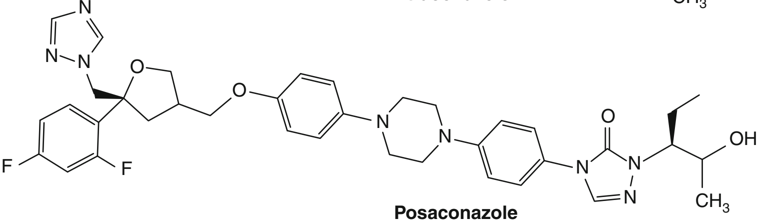
Ketoconazole



Fluconazole



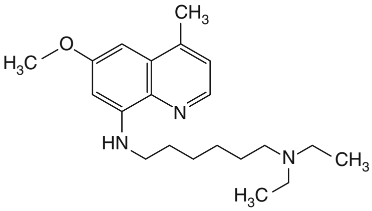
Itraconazole



Posaconazole

Sitamaquine, an orally active analog of 8-aminoquinoline, is in clinical development by the Walter Reed Army Institute in collaboration with GlaxoSmithKline (formerly SmithKline Beecham) to use for the treatment of VL. Sitamaquine at high concentration affects parasite motility, morphology, and growth. The drug involves electrostatic interaction between phospholipid anionic polar head groups and positively charged sitamaquine and then with phospholipid acyl chains leading to drug insertion within biological membranes. After binding to the membrane, sitamaquine accumulates in *Leishmania* cytosolic acidic compartments, acidocalcisome. However, correlation between its action and accumulation is not yet understood.

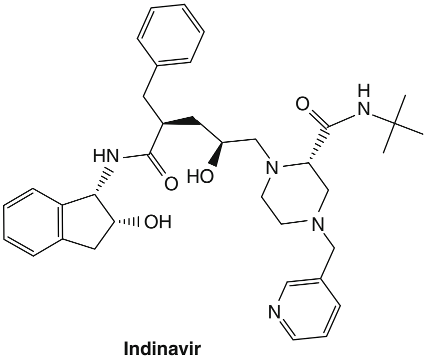
In a randomized, open label, and multicenter phase II trial in India and Kenya, the drug was found efficacious and well tolerated at various dose levels [62]. Despite efficacy, few side effects like vomiting, dyspepsia, cyanosis, nephritic syndrome, and glomerulonephritis were also observed in Indian phase II trial. However, the Kenyan phase II trial showed somewhat equal efficacy but observed side effects were abdominal pain, headache, and kidney dysfunction. As on March 2002, the drug is currently in phase III trials for the treatment of VL.



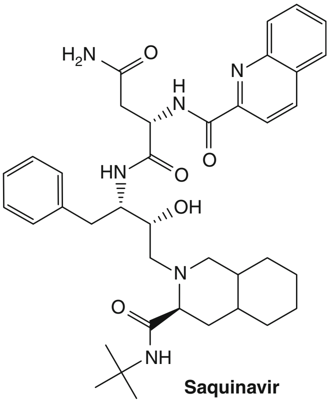
Sitamaquine

Leishmania-HIV coinfection is regarded as an “emerging” infectious disease, for in certain countries up to 70% of adult cases of leishmaniasis are related to HIV infection-AIDS. To date, the greatest prevalence of *Leishmania*-HIV coinfection has been in the Mediterranean basin. Among more than 2000 cases notified to the WHO, 90% of them belong to Spain, Italy, France, and Portugal. The most common species involved in *Leishmania*-HIV coinfection is *L. infantum*. Currently, no treatment has been completely effective, and the mortality rate is high (approximately 25%) during the first month after diagnosis [1, 4].

Indinavir and saquinavir, the two antiretroviral drugs capable of inhibiting the protease enzymes, have shown significant pharmacological activity against *L. major* and *L. infantum*. These results add new insights into the wide-spectrum efficacy of protease inhibitors and suggest for the study of their action on amastigote forms of *Leishmania* in order to validate their potential contribution against opportunistic infections in treated seropositive patients [63].



Indinavir



Saquinavir

8 Natural Products as Folk Medicines Against Leishmaniasis

Drugs derived from plants have been empirically used in the treatment of various human disorders for thousands of years in the form of the traditional Ayurvedic and Chinese medicine. A number of plants, i.e., *Cinchona calisaya* (bark), *Strychnos pseudoquina* (bark), *Deianira erubescens* (roots and leaves), and *Remijia ferruginea* (bark), were historically used against wide range of diseases. Drugs derived from microbial fermentations have played an equally seminal role in modern discovery and have revolutionized medicine, saving both human and animal lives. A number of plant-based natural products, e.g., aspirin, morphine, quinine, etc., are still in use today.

Ancient records have established the effectiveness of natural products as potentially rich sources of new and selective agents for the treatment of important tropical diseases caused by protozoans and other parasites. For example, in traditional Chinese medicine, the potential of plant extracts for the treatment of infectious diseases was recognized more than 2,000 years ago. Today, several compounds derived from plants or medical plants are applied in standard therapies, e.g., artemisinin, an important antimalarial drug was identified in the 1970s from traditional Chinese medicine *Artemisia annua*, and since then, many artemisinin derivatives have been prepared and evaluated in various preclinical and clinical trials to use in the treatment of malaria. In the year 2000, artemisinin (Artemotil®) was approved for the treatment of chloroquine-resistant *Plasmodium falciparum* malaria and cerebral malaria. However, the WHO has restricted the use of Artemotil® as a monotherapy since malarial parasites are developing resistance to the drug. However, combination therapies that include Artemotil® are the preferred treatment for malaria and are both effective and well tolerated in patients. Artemotil® is currently used only as a second-line drug in severe cases of malaria and is also increasingly being used against *P. vivax malaria*. As of May 2009, arterolane (RBx11160, OZ-277), a trioxolane modeled on artemisinin pharmacophore, is under phase III clinical development for the treatment of malaria by Ranbaxy in combination with piperaquine.

Plants are valuable sources for the screening of bioactive secondary metabolites, but also bacteria, fungi, and terrestrial or marine invertebrates produce pharmaceutically useful compounds. For example, paromomycin (Humatin™, King Pharmaceuticals), obtained from *Streptomyces krestomuceticus*, is an orphan drug that was approved by Drug-Controller General of India in September 2006 against VL. Paromomycin was originally developed by the Institute for OneWorld Health and is an off-patent antibiotic marketed in the USA to treat intestinal parasites also.

Natural products literature provides a growing research on several plant-derived compounds with a wide range of pharmacological activities, thus may be of potential utility in drug development and biomedical researches. Many plant-derived substances of diverse structural classes have been described in the literature showing antileishmanial properties, but none of them have been clinically evaluated in studies or projected to reach the clinical applications in near future. In this chapter, we have provided a brief overview of *Leishmania* occurrence, morphology and life cycle, bioavailability and metabolism, drug targets, *Leishmania* chemotherapy, and the active principles of established drugs. Furthermore, we have focused to cover the entire formal and constant researches on antileishmanial alkaloids up to July 2011 from the mid-1980 with special attention on structure-activity relationship (SAR)-based activity and mechanism of action (Table 40.1). Finally, a comprehensive coverage of plant-derived alkaloids with significant activity against *Leishmania* species has been given in detail. In order to highlight any possible structure-activity relationships, the chapter has been organized according to chemical structural classes.

Table 40.1 Alkaloids with leishmanicidal activity

Benzo[c]phenanthridine alkaloid
Dihydrochelerythrine (**1**)[b]
6-Acetonyldihydrochelerythrine (**2**)[b]
Lucidamine-A (**3**)[b]
Lucidamine-B (**4**)[b]
Quinoline alkaloids
2-n-Propylquinoline **(5)**[a,c]
Chimanine-D **(6)**[a,c]
Chimanine-B **(7)**[a]
Dictylomide-A **(8)**[a]
Dictylomide-B **(9)**[a]
Furoquinoline alkaloids
γ-Fagarine **(10)**[a,c]
Maculine **(11)**[a,c]
Isoquinoline alkaloids
O-methylmoschatoline **(12)**[a]
Liriodenine **(13)**[a]
Berberine **(14)**[a,c]
Isoguattouregidine **(15)**[a]
Anonaine **(16)**[a]
(+)-Neolitsine **(17)**[a]
Cryptodorine **(18)**[a]
Xylopine **(19)**[a]
Unonopsine **(20)**[a]
Duguetine **(21)**[a]
Duguetine *β*-*N*-oxide **(22)**[a]
Dicentrinone **(23)**[a]
N-methyltetrahydropalmatine **(24)**[a]
N -methylglaucine **(25)**[a]
Naphthylisoquinoline alkaloids
Ancistroealaine **-** A **(26)**[a]
Ancistrocladinium A **(27)**[a]
Ancistrocladinium B **(28)**[a]
Ancistrocladidine **(29)**[a]
Ancistrotanzanine-B **(30)**[a]
Ancistrotanazanine-A **(31)**[a]
Korupensamine A **(32)**[a]
Ancistrocongolines B **(33)**[a]
Ancistrocongolines C **(34)**[a]
Ancistrocongolines D **(35)**[a]
Bisbenzylisoquinolinic alkaloids
Daphanandrine **(36)**[a]
Obaberine **(37)**[a,c]
Gyrocarpine **(38)**[a,c]
Limacine **(39)**[a]

(*continued*)

Table 40.1 (continued)

Indole alkaloids
Dihydrocorynantheine **(40)**[a]
Corynantheine **(41)**[a]
Corynantheidine **(42)**[a]
Pleiocarpine **(43)**[a]
Gabunine **(44)**[a,b,c]
Ramiflorines-A **(45)**[a]
Ramiflorines-B **(46)**[a]
Steroidal alkaloids
Holamine **(47)**[a]
15- α -Hydroxyholamine **(48)**[a]
Holacurtine **(49)**[a]
N -desmethylholacurtine **(50)**[a]
Benzoquinolizidine alkaloids
Klugine **(51)**[a]
Cephaeline **(52)**[a]
Isocephaeline **(53)**[a]
Emetine **(54)**[a]
Diterpene alkaloids
15,22- *O* -diacetyl-19-oxo-dihydroatisine **(55)**[a]
Azitine **(56)**[a]
Isoazitine **(57)**[a]
Pyrrolidinium alkaloids
(2 *S* ,4 *R*)-2-Carboxy-4-(*E*)- *p-* coumaroyloxy-1,1-dimethylpyrrolidinium inner salt **(58)**[b]
Acridone alkaloids
Rhodesiacridone **(59)**[a,b]
Gravacridonediol **(60)**[a,b]
β - *Carboline alkaloids*
Harmaline **(61)**[a,b]
Harmine **(62)**[c]
Canthin-6-one **(63)**[a,c]
5-Methoxycanthin-6-one **(64)**[a,c]
N -hydroxyannomontine **(65)**[a]
Annomontine **(66)**[a]
Alkaloids from marine sources
Renieramycin A **(67)**[a]
Araguspongin C **(68)**[a,b,c]
Ciliatamides A **(69)**[a]
Ciliatamides B **(70)**[a]
Ciliatamides C **(71)**[a]
Almiramides A **(72)**[a]
Almiramides B **(73)**[a]
Almiramides C **(74)**[a]
Dragonamide A **(75)**[a]
Dragonamide E **(76)**[a]

(*continued*)

Table 40.1 (continued)

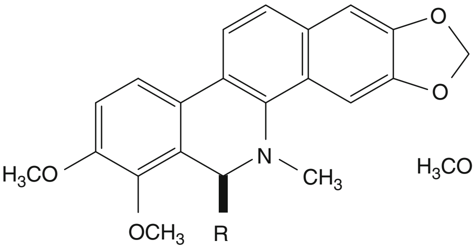
Herbamide B (**77**)[a]
Viridamide A (**78**)[a]
Venturamides A (**79**)[a]
Venturamides B (**80**)[a]
Valinomycin (**81**)[a]
Manzamine J (**82**)[a]
Manzamines A (**83**)[a]
Manzamine A *N* -oxide (**84**)[a]
(+)-8-Hydroxymanzamine A (**85**)[a]
Manzamine E (**86**)[a]
6-Hydroxymanzamine E (**87**)[a]
Manzamine F (**88**)[a]
Ircinol A (**89**)[a]

[a] In vitro activity against promastigotes
[b] In vitro activity against amastigotes
[c] In vivo activity

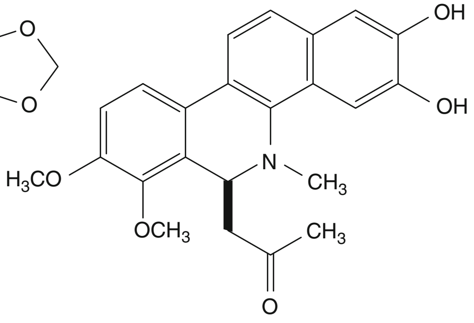
9 Alkaloids

9.1 Benzo[*c*]phenanthridine Alkaloid

The alkaloids constitute an important class of natural products exhibiting significant antileishmanial activities. The benzo[*c*]phenanthridine alkaloids, dihydrochelerythrine (1), 6-acetonyldihydrochelerythrine (2), and their semisynthetic derivatives lucidamine-A (3) and -B (4), isolated from the stem bark of *Garcinia lucida* (Clusiaceae), exhibit attractive antiprotozoal activity against *L. donovani*, with little toxicity to Vero cells and the host cells. Among these, the compound 1 with IC_{50} value of 2.0 μM shows promising antileishmanial activity, while compounds 2, 3, and 4 with IC_{50} values of 6.6, 10.8, and 6.8 μM, respectively, exhibit comparatively lower activity against *L. donovani* axenic amastigotes [64].



1 R = H
2 R = CH_2COCH_3



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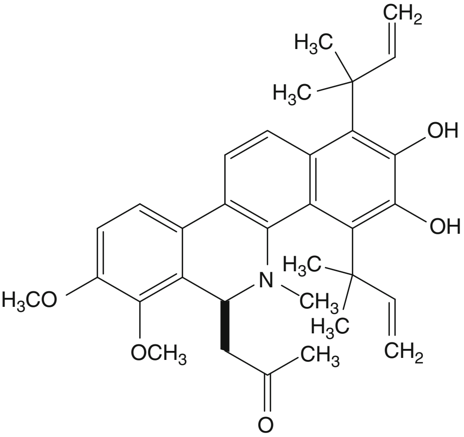

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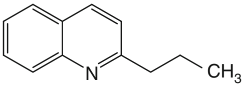
9.2 Quinoline Alkaloids

The quinoline alkaloids, 2-n-propylquinoline (5), chimanine-D (6), and chimanine-B (7), isolated from *Galipea longiflora* (Rutaceae), exhibit antileishmanial activity against promastigotes of *L. braziliensis* with an IC_{90} values of 50, 25, and 25 μg/mL, respectively. Oral in vivo studies using 5 in BALB/c mice demonstrate 99.9% suppression of liver parasites, while subcutaneous treatment with 6 causes 86.6% parasite suppression when given for 10 days at 0.54 mmol/kg [65]. However, oral treatment with 6 for 5 days results in 72.9% parasite suppression only. Likewise, dictylomide-A (8) and B (9), isolated from the bark of *Dictyoloma peruviana* (Rutaceae), causes total lyses of *L. amazonensis* promastigotes at 100 μg/mL concentration [66].



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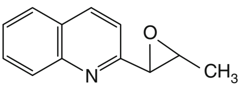

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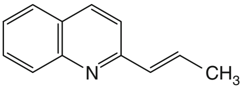

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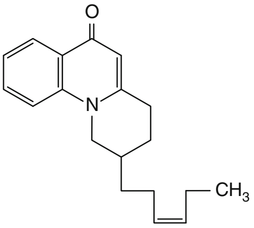

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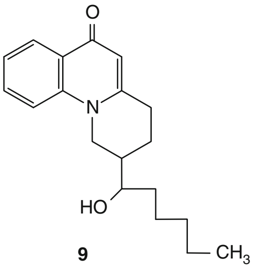
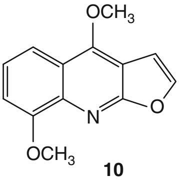


9

9.3 Furoquinoline Alkaloids

The furoquinoline alkaloids are biogenetically derived from the 2-substituted oxygenated 4-quinolones after a prenylation at C-3. The furoquinolines, γ-fagarine (10), and maculine (11), isolated from the stem bark of *Helietta apiculata* (Rutaceae), exhibit significant in vitro antileishmanial activities (IC_{50} between 17 and 30 mg/mL) against promastigote forms of *L. braziliensis*, *L. amazonensis*, and *L. donovani*. In vivo study reviles that the treatment with reference drug or compound 10 produces a significant reduction of the lesion weight by 66.9% (po0.01) and 90.5% (po0.001), respectively, and a drastic reduction of the lesional parasites by 95.2% (po0.01) and 97.4% (po0.005) [67].



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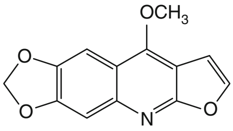

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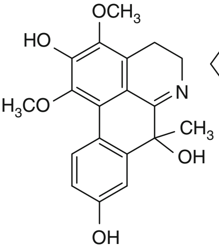
9.4 Isoquinoline Alkaloids

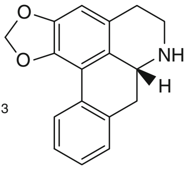
O-methylmoschatoline (12) and liriodenine (13), isolated from *Annona foetida* (Annonaceae), display in vitro activity against promastigote forms of *L. braziliensis* with an $IC_{50} < 60$ μM [68]. The SAR study among these oxoaporphine alkaloids reveals that 13 bearing methylenedioxy moiety is eight times more active against *L. braziliensis* and *L. guyanensis* than the 12. Berberine (14), occurring in many plant species of Annonaceae, Menispermaceae, and Berberidaceae, exhibits in vivo leishmanicidal activity with an IC_{50} value of 10 μg/mL against *L. major*. Isoguattouregidine (15) isolated from *Guatteria foliosa* (Annonaceae) shows activity at a concentration of 100 μg/mL against *L. donovani* and *L. amazonensis*. Anonaine (16) isolated from *Annona spinescens* (Annonaceae) exhibits activity against promastigotes of *L. braziliensis* and *L. donovani* [69].

The alkaloids (+)-neolitsine (17) and cryptodorine (18), isolated from *Guatteria dumetorum* (Annonaceae), display significant activity against promastigotes of *L. mexicana* at 15 and 3 μM concentrations, respectively. Xylopine (19), an aporphine alkaloid isolated from *Guatteria amplifolia* (Annonaceae), shows activity against promastigotes of *L. mexicana* (IC_{50} value 3 μM) and *L. panamensis* (IC_{50} value 6 μM) [70]. Unonopsine (20), a dimeric aporphine alkaloid isolated from the *Unonopsis buchtienii* (Annonaceae), displays antileishmanial activity (IC_{100} value 25 μg/mL) against *L. donovani* promastigotes [71].

The alkaloids, duguetine (21), duguetine *β*-*N*-oxide (22), dicentrinone (23), *N*-methyltetrahydropalmatine (24), and *N*-methylglaucine (25), isolated from subterranean stem bark of *Duguetia furfuracea* (Annonaceae), exhibit good to moderate antileishmanial activity. Among these, dicentrinone (23) with an IC_{50} value of 0.01 μM shows strongest leishmanicidal activity, however, at the same time also shows weak cytotoxicity. Likewise, metabolite 22 with an IC_{50} value of 0.11 μM shows promising leishmanicidal effect. The isoquinoline alkaloids 21, 25, and 24 with IC_{50} values of 4.32, 4.88, and 17.03 μM, respectively, display good to moderate activity against *Leishmania* parasites [72].


OCH3
HO
H3CO
N
CH3
OH
OH

12



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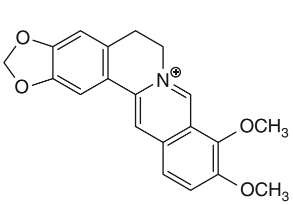
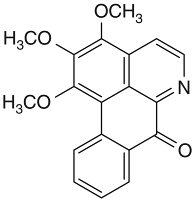
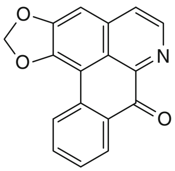

O
O
N⊕
OCH3
OCH3

14


OCH3
H3CO
H3CO
N
O

15



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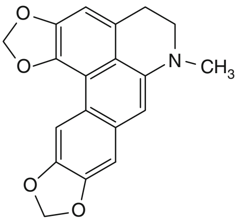

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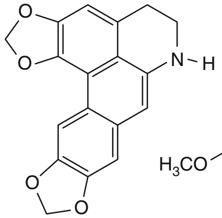

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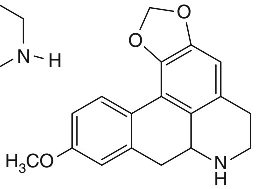

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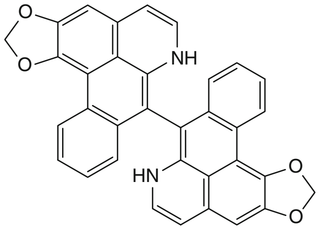

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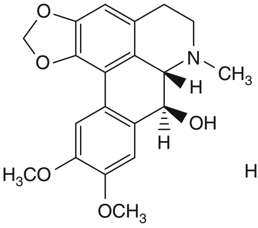
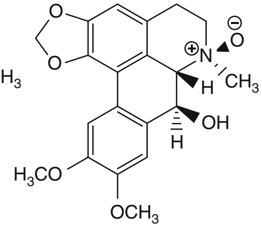
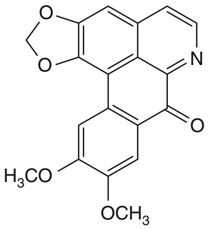

O
O
N
CH3
H
OH
H
H3CO
OCH3

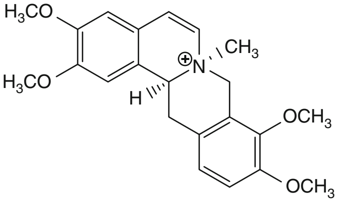
21


O
O
⊖
O
⊕N
H
CH3
OH
H
H3CO
OCH3

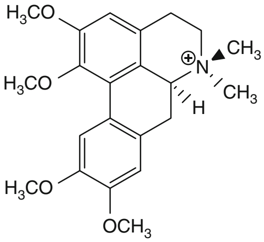
22


O
O
N
O
H3CO
OCH3

23


H3CO
H3CO
⊕N
CH3
H
OCH3
OCH3

24

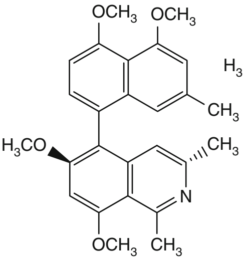

H3CO
H3CO
⊕N
CH3
CH3
H
H3CO
OCH3

25

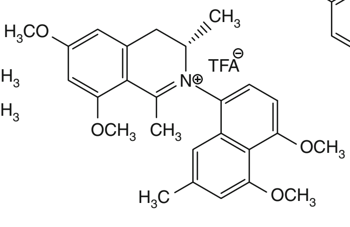
9.5 Naphthylisoquinoline Alkaloids

Among the naphthylisoquinoline alkaloids, ancistroealaine-A (26) isolated from *Ancistrocladus ealaensis* (Ancistrocladaceae) exhibits activity against *L. donovani* promastigotes with an IC_{50} value 4.10 μg/mL. Ancistrocladinium A (27) and B (28) isolated from yet an undescribed Congolese Ancistrocladaceae species require 2.61 and 1.52 μg/mL concentrations, respectively, to reach the IC_{50} toward *L. major* promastigotes. An apoptosis like death pathway is the possible mode of action for compounds 27 and 28 [73, 74]. Ancistrocladidine (29), isolated from *Ancistrocladus tanzaniensis* (Ancistrocladaceae), shows relatively weak activity by a factor of 2 against *L. donovani* when compared to ancistrotanzanine-B (30) (IC_{50} = 1.6 μg/mL) while by a factor of 10 in comparison to miltefosin (positive control) [75, 76].

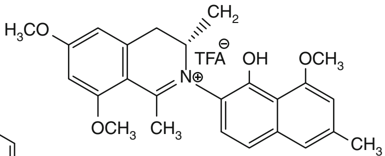
Likewise, ancistrotanazanine-A (31) exhibits activity against promastigotes of *L. donovani*. SAR-based studies among the alkaloids suggest that the compound-bearing *C,C*-biaryl axis connecting the naphthyl and isoquinoline moiety shows weak or no leishmanicidal activity. The korupensamine A (32) and ancistrocongolines B-D (33–35) isolated from *Ancistrocladus congolensis* exhibit weak to moderate antileishmanial activity. Among these, the ancistrocongolines 33, 34, and 35 show activity against *L. donovani* with IC_{50} value of 18.8, 19.3 and >30 μg/mL, respectively. The compound 32 with an IC_{50} value of 25.1 μg/mL demonstrates activity against *L. donovani* when compared to Pentostam as a standard drug (IC_{50} value of 47.2 μg/mL) [77].


OCH3
OCH3
CH3
H3CO
CH3
N
OCH3
CH3

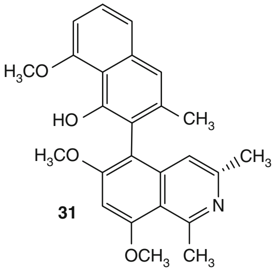
26


H3CO
CH3
TFA⊖
N⊕
OCH3
CH3
OCH3
H3C
OCH3

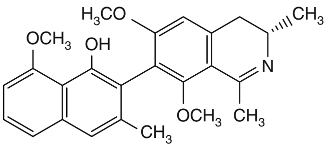
27


H3CO
CH2
TFA⊖
N⊕
OH
OCH3
OCH3
CH3
CH3

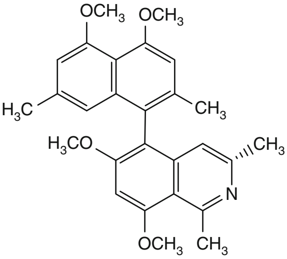
28


H3CO
HO
CH3
H3CO
CH3
N
OCH3
CH3

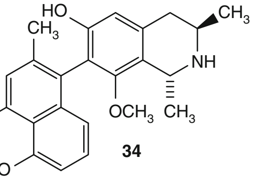
31


H3CO
CH3
OCH3
OH
N
OCH3
CH3
CH3

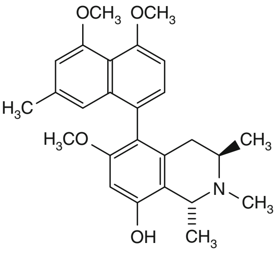
29


OCH3
OCH3
H3C
CH3
H3CO
CH3
N
OCH3
CH3

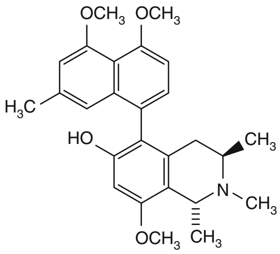
30


HO
CH3
CH3
NH
OCH3
CH3
H3CO
H3CO

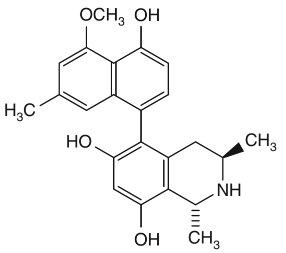
34


OCH3
OCH3
H3C
H3CO
CH3
N
CH3
OH
CH3

32


OCH3
OCH3
H3C
HO
CH3
N
CH3
OCH3
CH3

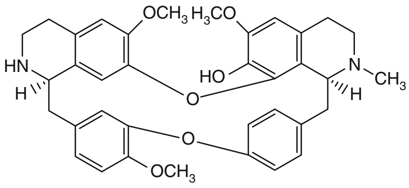
33


OCH3
OH
H3C
HO
CH3
NH
OH
CH3

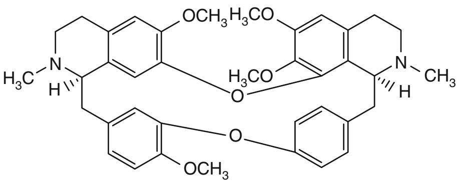
35

9.6 Bisbenzylisoquinolinic Alkaloids

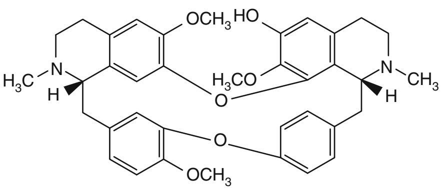
Daphanandrine (36) isolated from *Albertisia papuana* (Menispermaceae), obaberine (37) obtained from *Pseudoxandra sclerocarpa* (Annonaceae), gyrocarpine (38) produced by *Gyrocarpus americanus* (Hernandiaceae), and limacine (39) isolated from *Caryomene olivasans* (Menispermaceae), display activity against *L. donovani*, *L. braziliensis*, and *L. amazonensis* with an IC_{100} of ~50 μg/mL. SAR studies among these metabolites demonstrate that alkaloids with methylated nitrogen are more active than those with nonsubstituted or aromatic nitrogen, while quaternization of one or more nitrogen atoms results in the loss of antileishmanial activity [78].


OCH3
H3CO
HN
H
HO
N
CH3
H
O
O
OCH3

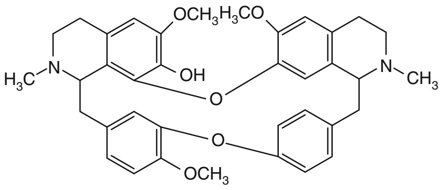
36


OCH3
H3CO
H3C
N
H
H3CO
N
CH3
H
O
O
OCH3

37


OCH3
HO
H3C
N
H
H3CO
N
CH3
H
O
O
OCH3

38

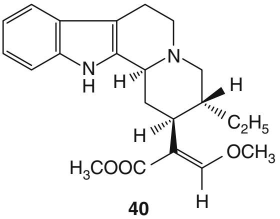

OCH3
H3CO
H3C
N
OH
N
CH3
O
O
OCH3

39

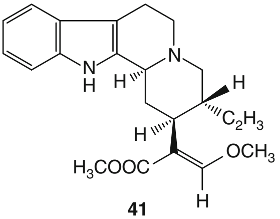
9.7 Indole Alkaloids

Dihydrocorynantheine (40), corynantheine (41), and corynantheidine (42), isolated from the bark of *Corynanthe pachyceras* (Rubiaceae), are the respiratory chain inhibitors exhibiting IC_{50} of 3 μM against *L. major*. Pleiocarpine (43) isolated from stem bark of *Kopsia griffithii* (Apocynaceae) shows in vitro antileishmanial activity with an $IC_{50} < 25$ μg/mL against *L. donovani* promastigotes. Gabunine (44), a bis-indole alkaloid obtained from stem bark of *Peschiera van heurkii* (Apocynaceae), exhibits in vitro activity with an IC_{50} 25 μg/mL against *L. amazonensis* amastigotes [79].

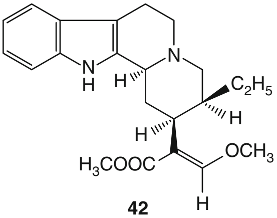
The indole alkaloids, ramiflorines-A (45) and -B (46), isolated from the stem bark of *Aspidosperma ramiflorum* (Apocynaceae), exhibit significant activity against *L. amazonen*sis with LD_{50} values of 16.3 ± 1.6 μg/mL and 4.9 ± 0.9 μg/mL, respectively, using pentamidine as standard drug (LD_{50} = 10.0 μg/mL) [80].


N
H
H
N
H
C2H5
H
H3COOC
OCH3
H

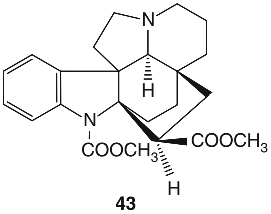
40


N
H
H
N
H
C2H3
H
H3COOC
OCH3
H

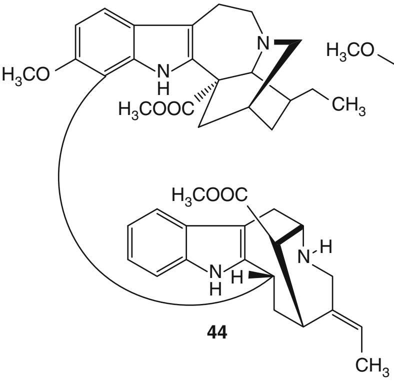
41


N
H
H
N
C2H5
H
H
H3COOC
OCH3
H

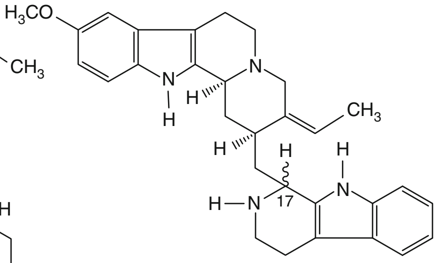
42


N
H
N
COOCH3
COOCH3
H

43


N
H3CO
N
H
H3COOC
CH3
H3COOC
N
H
H
N
H
CH3

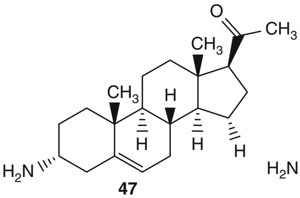
44


H3CO
N
H
N
H
CH3
H
H
H
H—N
17
N

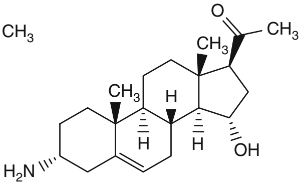
H-17- α 45
H-17- β 46

9.8 Steroidal Alkaloids

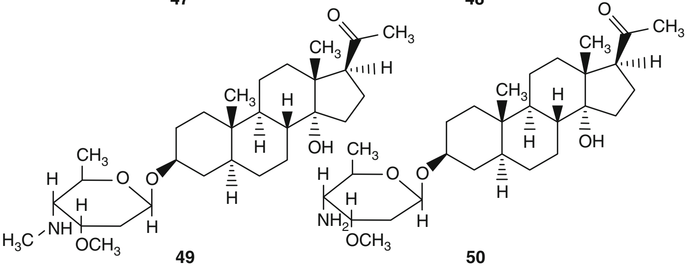
Among the alkaloids, holamine (47), 15-α-hydroxyholamine (48), holacurtine (49), and *N*-desmethylholacurtine (50), obtained from *Holarrhena curtisii* (Apocynaceae), the metabolite 47 exhibits strongest activity against *L. donovani* (1.56 > IC_{50} > 0.39 μg/mL) in compared to 48, 49, and 50 (6.25 > IC_{50} > 1.56 μg/mL) [81].



47



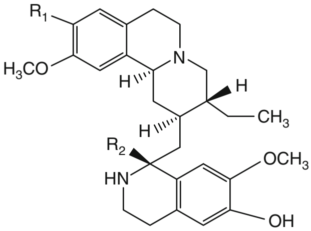
48



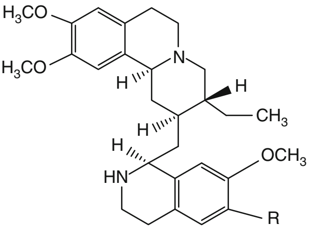
49 **50**

9.9 Benzoquinolizidine Alkaloids

Klugine (51), cephaeline (52), isocephaeline (53), and emetine (54), demonstrating significant leishmanicidal activity against *L. donovani*, have been isolated from *Psychotria klugii* (Rubiaceae). Among these metabolites, the compound 51 (IC_{50} of 0.40 μg/mL) and 53 (IC_{50} 0.45 μg/mL) exhibit <13- and <15-fold less potent activity in compared to 52, while compound 52 with IC_{50} of 0.03 μg/mL demonstrates >20- and >5-fold more in vitro activity against *L. donovani* when compared to pentamidine and amphotericin B, respectively. The alkaloid 54 exhibits activity against *L. donovani* with an IC_{50} value 0.03 μg/mL, however, produces toxicity in treatment of CL caused by *L. major* [82, 83].



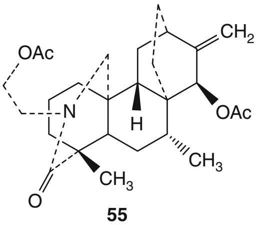
51 R_1 = OH; R_2 = OH
52 R_1 = OCH_3; R_2 = H



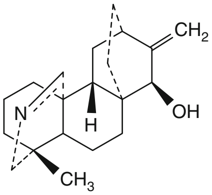
53 R = OCH_3
54 R = OH

9.10 Diterpene Alkaloids

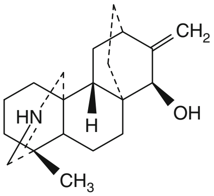
The alkaloids 15,22-*O*-diacetyl-19-oxo-dihydroatisine (55), azitine (56), and isoazitine (57), isolated from *Aconitum*, *Delphinium*, and *Consolida* species, show significant leishmanicidal activities. The metabolite 57 exhibits strongest activity against promastigotes of *L. infantum* with IC_{50} values 44.6, 32.3, and 24.6 μM at 24, 48, and 72 h of culture, respectively. The compound 56 and 55 with IC_{50} values of 33.7 and 27.9 μM at 72 h of culture, respectively, exhibit activity against promastigotes of *L. infantum* [84].



55



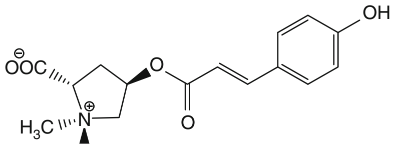
56



57

9.11 Pyrrolidinium Alkaloids

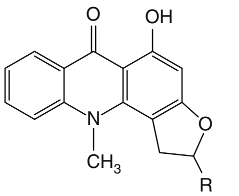
The pyrrolidinium alkaloid (2*S*,4*R*)-2-carboxy-4-(*E*)-*p*-coumaroyloxy-1,1-dimethylpyrrolidinium inner salt (58), isolated from *Phlomis brunneogaleata* (Lamiaceae), display activity with an IC_{50} of 9.1 μg/mL against axenic amastigotes of *L. donovani* [85].



58

9.12 Acridone Alkaloids

The rhodesiacridone (59) and gravacridonediol (60) isolated from *Thamnosma rhodesica* (Rutaceae) exhibit 69 % and 46 % inhibition at 10 μM concentration, respectively, against promastigotes of *L. major*. The compounds also display activity against *L. major* amastigotes and cause over 90 % and 50 % inhibition at 10 and 1 μM concentration, respectively [86].

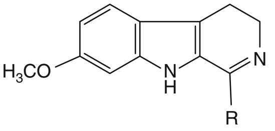


59 R = $C(OH)(CH_2OH)COCH_3$
60 R = $C(OH)(CH_3)CH_2OH$

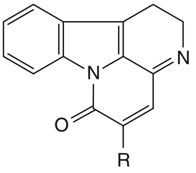
9.13 *β*-Carboline Alkaloids

The harmaline (61), isolated from *Peganum harmala* (Nitrariaceae), exhibits amastigote-specific activity (IC_{50} of 1.16 μM). Harmine (62) isolated from same plant species reduces spleen parasite load by approximately 40, 60, 70, and 80% in free, liposomal, niosomal, and nanoparticular forms, respectively, in mice model [87].

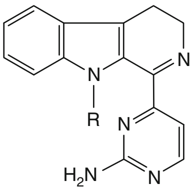
Canthin-6-one (63) and 5-methoxycanthin-6-one (64) occurring in plant species of Rutaceae and Simaroubaceae demonstrate in vivo activity against *L. amazonensis* in BALB/c mice model [88]. *N*-hydroxyannomontine (65) and annomontine (66) isolated from *Annona foetida* (Annonaceae) show efficient leishmanicidal potentials. The SAR studies suggest that the metabolite 66 (IC_{50} = 34.8 μM) displays 6 times more activity compared to 65 against *L. braziliensis* promastigotes. The compound 65 also exhibits activity against promastigotes of *L. guyanensis* while 66 remain inactive [68].



61 R = H
62 R = CH_3



63 R = H
64 R = OCH_3



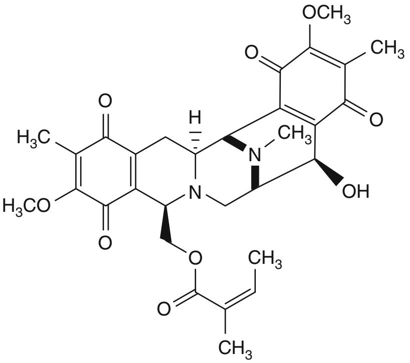
65 R = OH
66 R = H

9.14 Alkaloids from Marine Sources

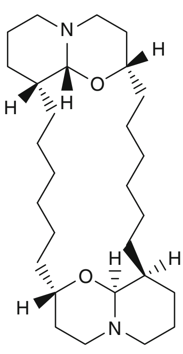
Many marine sponges, e.g., *Amphimedon viridis*, *Acanthostrongylophora* species, *Neopetrosia* species, *Plakortis angulospiculatus*, and *Pachymatisma johnstonii*, serve as rich sources of alkaloids with significant antileishmanial potentials [89]. Renieramycin A (67) isolated from *Neopetrosia* species is a La/egfp (expressing enhanced green fluorescent protein) inhibitor that shows efficient antileishmanial activity against *L. amazonensis* with IC_{50} 0.2 μg/mL [90].

Araguspongin C (68), isolated from a marine sponge *Haliclona exigua*, displays leishmanicidal activity against promastigotes as well as amastigotes at 100 µg/mL concentrations [91].

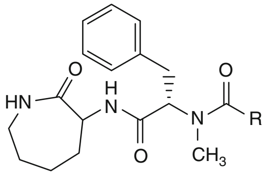
Among the ciliatamides A-C (69–71) isolated from *Aaptos ciliata*, the peptide 69 and 70 at 10.0 µg/mL concentrations inhibit 50 % growth of *L. major* promastigotes. The lipopeptides, almiramides A-C (72–74) isolated from cyanobacterium *Lyngbya majuscule*, exhibit significant in vitro antileishmanial activity against *L. donovani*. The SAR studies among these peptides suggest that 73 and 74 exhibit strong activity against *L. donovani* with EC_{50} values of 2.4 and 1.9 µM, respectively. The metabolites 73 and 74 also display weak cytotoxicity to mammalian Vero cells at 52.3 and 33.1 µM concentrations, respectively [92]. Dragonamide A (75), E (76) and herbamide B (77), isolated from same cyanobacterium strain, exhibit in vitro activity against *L. donovani* with EC_{50} values of 6.5, 5.1 and 5.9 µM, respectively [93].


OCH3
CH3
O
O
H3C
H
CH3
N
H3CO
N
OH
O
O
CH3
O
CH3

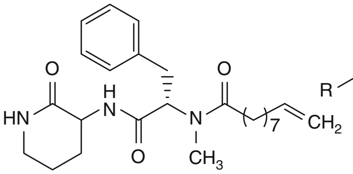
67


N
H
O
H
H
H
H
O
H
N

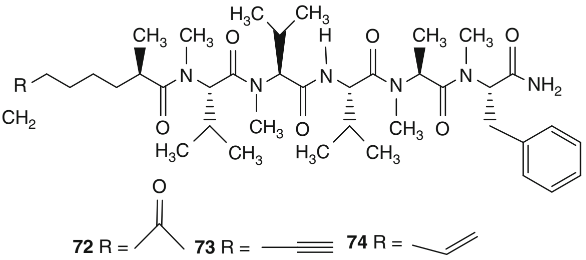
68

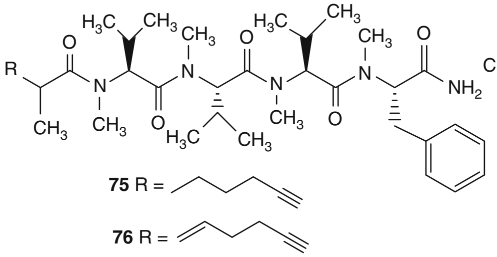

O
H
O
HN
N
N
R
O
CH3

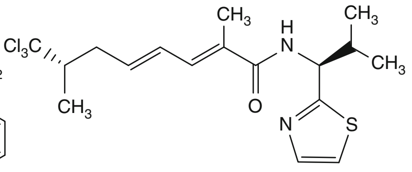
69 R = $(CH_2)_7CHCH_2$
70 R = $(CH_2)_6CH_3$


O
H
O
HN
N
N
7
CH2
O
CH3

71

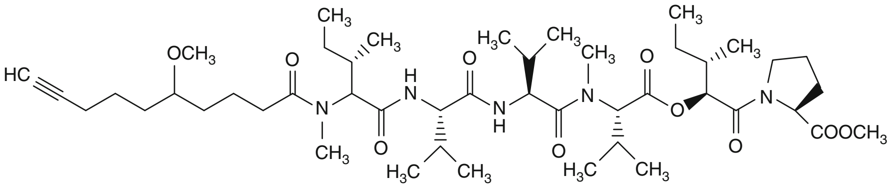

H3C
CH3
CH3
CH3
O
H
O
CH3
CH3
O
R
N
N
N
N
N
NH2
O
CH3
O
CH3
O
H3C
CH3
H3C
CH3
O
72 R =
73 R =
74 R =


H3C
CH3
CH3
H3C
CH3
CH3
O
O
CH3
O
R
N
N
N
N
NH2
CH3
CH3
O
H3C
CH3
CH3
O
75 R =
76 R =

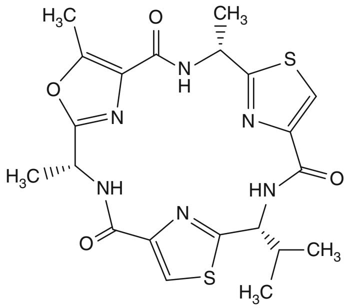

CH3
H
CH3
Cl3C
N
CH3
CH3
O
N
S

77

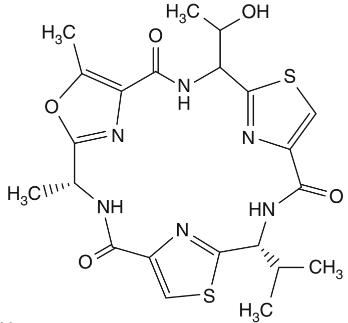
Viridamide A (78) isolated from *Oscillatoria nigroviridis* shows activity against *L. mexicana* with EC_{50} of 1.5 μM [94]. Venturamides A (79) and B (80) obtained from cyanobacterium *Oscillatoria* species exhibit activity against *L. donovani* with $EC_{50} > 19.0$ μM. Valinomycin (81), a dodecadepsipeptide isolated from *Streptomyces* strains, exhibits activity against promastigotes of *L. major* with $EC_{50} <$ 0.11 μM but at the same time shows cytotoxicity to 293 T kidney epithelial cells and J774.1 macrophages [95].



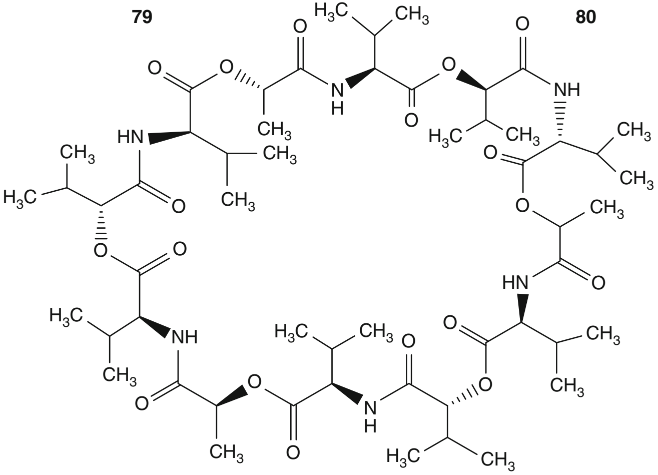
78



79

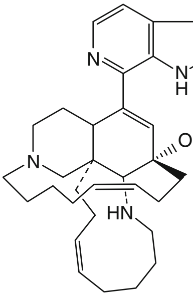


80

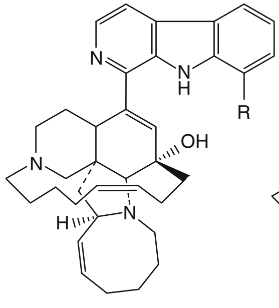


81

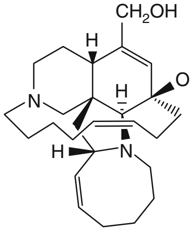
Manzamine-type alkaloids, namely, manzamine J (82), manzamines A (83), manzamine A *N*-oxide (84), (+)-8-hydroxymanzamine A (85), manzamine E (86), 6-hydroxymanzamine E (87), manzamine F (88), and ircinol A (89), isolated from a common Indonesian sponge of the genus *Acanthostrongylophora* show significant in vitro antileishmanial activity against *L. donovani* [96]. Comparison of antileishmanial activity among 83 and 82 suggested that the bond between N-27 and C-34 appears to be crucial for the leishmanicidal effect and provides valuable insight into the structural moieties required for activity against *Leishmania* parasites. The metabolites 83 and 89 with IC_{50} values of 0.9 µg/mL display excellent activity against parasites of *L. donovani* while 82 with IC_{50} value of 25 µg/mL exhibits lowest activity among all. Likewise, 84, 87, 86, 88, and 85 with IC_{50} values of 1.1, 2.5, 3.8, 4.2, 6.2, respectively, display good to moderate activity against *L. donovani* parasites [96].



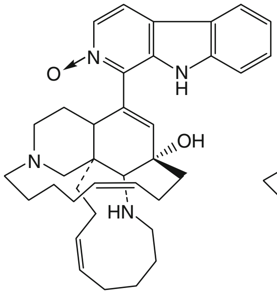
82



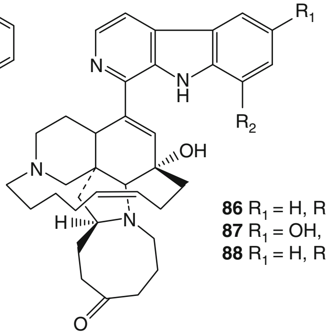
83 R = H
85 R = OH



89



84



86 R_1 = H, R_2 = H
87 R_1 = OH, R_2 = H
88 R_1 = H, R_2 = OH

10 Future Perspectives

Despite the advances in the parasitological and biochemical researches using various species of *Leishmania*, the treatment options available against leishmaniasis are far from satisfactory. In current situation, development of new drugs to combat leishmaniasis requires increased input from the disciplines of chemistry, pharmacology, toxicology, and pharmaceutics to complement the advances in molecular biology that have been made in the past 21 years. New potential drug targets should be identified with establishment of new methods for the rapid validation and characterization of targets. Instead of the conventional reductionist approach of finding a single drug that hits a single target, the systems biology approach should be followed using multiple compounds that hit multiple targets in different pathways to achieve the desired outcome. Mechanism of drug resistance and interspecies variation in drug susceptibility are also important areas to explore. Drug susceptibility in relation to genetic heterogeneity in *Leishmania* species and strains is another area of interest. Efforts should be made to develop drugs that target well-characterized genes essential for survival of the parasite selectively.

In determining the way forward to the development of plant-based drug candidates against leishmaniasis, the alkaloids as a source of antileishmanial drugs need to be discussed with respect to biochemical differences between protozoa and hosts. Ethnobotanic studies have identified many plants used by the local population in disease-endemic regions that would lead to the identification of new scaffolds for the future synthesis of second-generation compounds with optimized pharmacological profile for the treatment of leishmaniasis. The tremendous chemical diversity present in plants and the promising leads that have already been demonstrated significant against parasitic diseases are needed to be addressed also against *Leishmania* parasites. The development of antileishmanial natural products or their analogs in accordance to the considerations outlined above would have a dramatic positive impact on the treatment of leishmaniasis. A safer, shorter, and cheaper treatment; identification of the most cost-effective surveillance system and control strategies; and suitable vector control approach are some among the important aspects for the control and complete eradication of this deadly disease.

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Pharmacological/Biological Effects of Berberine

41

Eva M. Galvez, M. Perez, P. Domingo, D. Nuñez, V. L. Cebolla, M. Matt, and J. Pardo

Contents

1 Introduction 1303
2 Pharmacological Applications and Mechanism of Action 1303
2.1 Diabetes 1303
2.2 Hyperlipidemias 1305
2.3 Inflammatory Disorders 1305
2.4 Cardiovascular Disease 1306
2.5 Gastrointestinal Disorders 1307
2.6 Neurological Disorders 1307
2.7 Cerebral Ischemia 1308
2.8 Alzheimer's Disease 1308
2.9 Anxiety 1309
2.10 Schizophrenia 1309
2.11 Mental Depression 1309
2.12 Infectious Diseases 1310
2.13 Anticancer Properties 1311
2.14 Regulation of Cell Death and Proliferation 1313

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2.15 Solid Carcinomas 1314
2.16 Hematologic Malignancies 1315
2.17 Combined Therapies 1315
2.18 Inhibition of Tumor Invasiveness 1316
2.19 Other Applications 1317
3 Bioavailability and Metabolism 1317
4 Secondary Toxicity 1317
5 Clinical Trials 1319
6 Conclusions 1320
References 1320

Abstract

Berberine is a yellow alkaloid naturally produced by a diversity of plants. It is extracted from the roots, rhizome, and stem bark of several plants. This natural product has been used for centuries in the Chinese medicinal herb. Berberine presents a wide range of pharmacological applications such as antidiabetic, hypolipidemic, antihypertensive, anti-inflammatory, antioxidant, antidepressant, anticancer, antidiarrheal, hepatoprotective, and, above all, antimicrobial. It has been tested clinically in the treatment of oriental sore, diarrhea, trachoma, diabetes mellitus type 2, hypercholesterolemia, and congestive cardiac failure. This alkaloid is also used in the treatment of gastric and inflammatory diseases and more recently is being investigated for its application in neurology, cardiology, and oncology.

Keywords

Alkaloid • berberine • bioavailability • medicine

Abbreviations

AChE	Acetylcholinesterase enzyme
AML	Acute myeloid leukemia
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
AD	Alzheimer’s disease
Aβ peptide	Amyloid β-peptide
AR	Androgen receptor
ACE	Angiotensin-converting enzyme
ATM	Ataxia-telangiectasia mutated
Bcl-2	B cell lymphoma 2
COX2	Cyclooxygenase-2
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase1/2
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
iNOS	Inducible nitric oxide synthase
IC50	Inhibition concentration 50%

LD50	Lethal dose 50
LXR	Liver X receptor α
LDLR	Low-density lipoprotein receptor
MMP	Matrix metalloproteinase
MDR	Multidrug resistance
NF-κB	Nuclear factor-κB
PPAR	Peroxisome proliferator-activated receptor α
PI	Protease inhibitor
SREBPs	Sterol regulatory element-binding protein ()

1 Introduction

Berberine (Fig. 41.1) is an isoquinoline plant alkaloid with a bright yellow color that under ultraviolet light provides a strong yellow fluorescence. It belongs to the protoberberine structural class and represents one of the most natural alkaloids studied [1]. It is extracted from the plant *Coptidis rhizoma*, which has been used for centuries as a traditional Chinese medicinal herb where it was found to have curative properties. Berberine appears in the roots, flowers, shoots, and bark of a number of important medicinal plants like *Berberis* (Berberis aquifolium, Berberis vulgaris, Berberis aristata), *Hydrastis canadensis* (goldenseal), *Coptis chinensis*, *Tinospora cordifolia*, and *Eschscholzia californica* and has been medicinally used in many traditional medical cultures, including Ayurvedic herbal and Chinese herbal medicine. Berberine is an important traditional medicinal herb, which has been effectively used in the treatment of dysentery, diarrhea, stomatitis, throat infections, and hepatitis as folk medicine for a long time in China and other countries [2].

This compound has a wide range of pharmacologic effects, including protective effects on some gastric ulcer [3], treatment of inflammatory [3], cardiovascular or lipid, and glucose-related [4–11] diseases. In addition, berberine possesses antimicrobial activity against some bacterial [12, 13] or fungal infections [14]. Recent evidence has indicated that berberine has also anticancer properties and is currently being used to treat a variety of different diseases or medical disorders like diarrhea, high cholesterol levels, diabetes, and microbial infections such a fungal (*Candida*) or bacterial (*Salmonella*).

2 Pharmacological Applications and Mechanism of Action

2.1 Diabetes

The hypoglycemic effect of berberine was accidentally found in the 1980s in China when it was administered to diabetic patients with diarrhea [15].

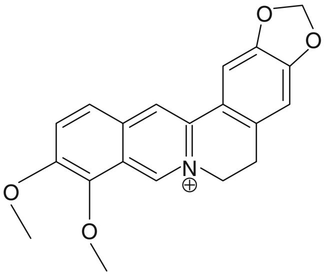

Fig. 41.1 Berberine cation

A large number of studies reported berberine to have antidiabetic properties in vivo in animal models as well as during human clinical trials [16–21], although its mechanism of action has not yet been completely elucidated. There are many studies that describe and propose different mechanisms of action of berberine related to the antidiabetic properties. Berberine is implicated in protection of β-cells (cells responsible for insulin secretion) in pancreas as well as contributing to their regeneration [22, 23]. In addition, it protects liver cells from ER stress, suggesting that berberine is able to exert a glucose-lowering effect in hepatocytes [24]. It regulates insulin secretion from β-cells acting as an agonist of the fatty acid receptor GPR40 [25]. Some works in vitro and in vivo [26, 27] show that berberine treatment results in increased AMPK activity in 3T3-L1 adipocytes and L6 myotubes. The consequence of this regulation is increased translocation of GLUT4 in L6 cells and reduced accumulation of lipid in 3T3-L1 adipocytes. These findings suggest that berberine displays beneficial effects in the treatment of diabetes and obesity at least in part via stimulation of AMPK activity.

On the other hand, berberine is also able to induce the expression of insulin receptors (at least in liver) in a dose-dependent manner, through upregulation of insulin receptor mRNA [28, 29]. This effect was dependent on the presence of insulin. Berberine has also been linked to improving post-receptor signal transduction [24].

The "lipotoxicity" hypothesis indicates that fat-induced hepatic insulin resistance (FIHIR) may play a major role in the pathogenesis of type 2 diabetes, the most common form of diabetes mellitus. It has been suggested that berberine exerts a therapeutic effect on FIHIR in a model of type 2 diabetes in hamsters by modulating the hepatic SREBPs, LXRα, and PPARα transcriptional programs [30].

One study has analyzed the hypoglycemic activity of berberine in comparison with current hypoglycemic drugs like metformin [5]. This study indicated that berberine might be beneficial for the recovery of β-cell function during diabetes or prediabetes.

Some of the studies mentioned above were found in in vitro models and should be interpreted with caution. As described later, berberine has low bioavailability and shows poor absorption, supporting the hypothesis that berberine may exert its antihyperglycemic effect in the intestinal tract before absorption before reaching other organs like pancreas or liver [2]. Recent evidences suggest that the gut microbiota composition is associated with obesity and type 2 diabetes [31], which are closely associated with a low-grade inflammatory state. This study hypothesizes that gut microbiota modulation may be one mechanism of the antidiabetic effect of berberine although the mechanism has not been yet explained.

2.2 Hyperlipidemias

Berberine presents promising lipid-lowering capabilities. It was found to reduce cholesterol in serum of human volunteers by a mechanism different to that of statins [4]. They showed in a rat model that berberine upregulated the LDLR in the liver [4, 32]. Recently Abidi et al. have shown that the root extract of goldenseal, an herbal supplement that contains the alkaloids hydrastine, berberine, berberastine, hydrastinine, tetrahydroberberastine, canadine, and canalidine, had a similar effect in a hamster model [11]. In both studies, the effect of this increase in LDLR was the reduction of LDL-associated cholesterol in blood.

Although there is striking evidence that berberine lowers cholesterol through mechanisms independent of statins, its ability to regulate cholesterol biosynthesis has yet to be considered.

A few studies have demonstrated that berberine increases bile formation and secretion [33–35]. It has also been shown that berberine is able to interact with micelles through hydrophilic and hydrophobic binding sites to form alkaloid–bile salt agglomerates [36, 37]. This process is thought to decrease the capacity of micelles to solubilize cholesterol and thus affects cholesterol absorption. However, the effect of berberine on cholesterol absorption has not been directly studied.

The hypothesis that berberine lowered plasma cholesterol and non-HDL cholesterol levels through a combination of inhibition of cholesterol absorption and stimulation of bile acid synthesis was recently analyzed. This study shows that berberine used in combination with other natural products, plant stanols, which lower cholesterol through a different mechanism, improved the cholesterol-lowering efficacy through a synergistic inhibition on cholesterol absorption.

Although the in vitro and in vivo evidences indicate that berberine possesses cholesterol-lowering potential, a recent work suggested that promotion of foam cell formation by berberine could counterbalance its beneficial effects [38]. By using the model of atherosclerosis in apolipoprotein E-deficient mice, they showed that berberine induced in vivo foam cell formation and promoted atherosclerosis development. Thus, this effect could counterbalance the beneficial effect of this cholesterol-lowering compound on atherosclerosis.

2.3 Inflammatory Disorders

The anti-inflammatory properties of berberine have been used worldwide for the treatment of several inflammatory diseases including rheumatism and lumbago [39, 40]. In some traditional medicines, the use of berberine to reduce fever is also described [39, 41]. Although many studies have shown the anti-inflammatory activity of berberine, the exact mechanism is unknown. However, it seems to be related to the ability of berberine to inhibit prostaglandin biosynthesis in vivo [2, 42]. This effect seems to be mediated by the inhibition of COX2 expression and activity [40, 42]. COX2 activity was not directly inhibited by berberine but through the inhibition of COX2 regulatory proteins.

It has also been suggested that berberine reduces inflammation by inhibiting TNF-α secretion from differentiating adipocytes [40]. Since cyclooxygenases have been implicated in inflammation and carcinogenesis, those in vivo observations may help to explain the anti-inflammatory and anticancer (see below) activity of berberine.

The beneficial effects of berberine as anti-inflammatory agent have also been shown in vivo during endotoxin-induced sepsis. Berberine treatment reduces mortality [43] and prevents heart and lung injury after endotoxin administration in mice [43–45]. The mechanism by which berberine exerts this function seems to be related to the inhibition of TNF-α production and phospholipase A2 activity [45].

2.4 Cardiovascular Disease

Berberine has been used in the treatment of several heart diseases including hypertension and arrhythmia in folk medicine [46, 47]. Several pharmacological studies have shown the beneficial effects of berberine on diverse cardiovascular disorders in animal and human, including prevention of ischemia-induced ventricular tachyarrhythmia, improvement of cardiac contractile function, and reduction of peripheral vascular resistance and blood pressure [48, 49]. Berberine has also positive inotropic, negative chronotropic, antiarrhythmic, vasodilator, and anti-atherosclerosis properties [50].

Data from in vivo studies in animals [51–53] and humans [49, 54] suggest berberine may be useful for patients with severe congestive heart failure.

Berberine is a known vasorelaxant in animals [46, 48] and has been used with success in humans [55], suggesting its possible clinical usefulness in the treatment of arrhythmias. Although the mechanism for berberine's antiarrhythmic effect is not clear, results from a rabbit model indicated it may be related to suppression of delayed afterdepolarization in the ventricular muscle [56]. Another work in rats suggested that, in addition to affecting several other parameters of cardiac performance, berberine has a vasodilatory/hypotensive effect attributable to its potentiation of acetylcholine [48]. The cardiovascular effects of berberine have been attributed to the blockade of delayed rectifier and ATP-sensitive potassium channels and stimulation of Na^+–Ca^{2+} exchanger. This alkaloid is also able to prolong the duration of ventricular action potential. Diverse cellular mechanisms regulated by berberine have been proposed to explain its vasodilator activity. It appears to work on both the endothelium itself and underlying smooth muscle [57]. Other mechanisms proposed involve the angiotensin-converting enzyme and the NO–cGMP system [58] or blockade of the α1-adrenoceptor [59, 60]

Berberine is also able to interfere with platelet function through diverse mechanisms like inhibition of thromboxane synthesis [61], enhancement of thrombolysis (breaking of clots) [2], interaction with α2-adrenoceptor on platelets [62], and inhibition of calcium influx [63].

Finally, berberine has been shown to protect myocytes in a model of ischemia reperfusion [64].

2.5 Gastrointestinal Disorders

In the case of gastric ulcers, berberine has also been shown to have beneficial effects [3]. It was shown that berberine could significantly protect gastric mucosa from damage by ethanol in rats. This effect may be due to reduced expression of iNOS. Thus, berberine may affect nitric oxide production and associated damage by inhibiting iNOS expression in damaged gastric tissue and, ultimately, improve in the healing of ulcers.

Several in vitro works conclude that berberine can ameliorate intestinal epithelial tight junction damage induced by the release of proinflammatory cytokines [65, 66]. This effect has been attributed to the downregulation of the NF-κB and myosin light chain kinase pathway. These findings suggest that this compound may help to restore barrier function in injured intestinal mucosa [65, 66].

Berberine is currently being used to treat gastrointestinal-related medical disorders due to infections including bacterial diarrhea caused by *Vibrio cholerae* and *Escherichia coli* and intestinal parasites. The mechanism of the antidiarrheal activity of berberine has not been described yet. According to the study performed by Eaker et al., berberine significantly inhibits myoelectric activity and transit of the small intestine that appears to be partially mediated by opioid and α-adrenergic receptors, concluding that the antidiarrheal properties of berberine may be mediated, at least in part, by its ability to delay small intestinal transit [67].

2.6 Neurological Disorders

Berberine seems to have antidepressant and neuroprotective effects in several neurodegenerative and neuropsychiatric disorders. It has been reported to modulate neurotransmitters and their receptor systems in the brain. Moreover, some works indicate a beneficial effect of berberine in the treatment of central nervous system-related disorders including Alzheimer's disease, cerebral ischemia, schizophrenia, anxiety, and mental depression [68–71]. Supporting the observed effect of berberine on the central nervous system, Wang et al. showed that berberine can cross the blood–brain barrier and could be transported into the neurons in a concentration- and time-dependent manner [72].

2.7 Cerebral Ischemia

Stroke, known as a cerebral vascular accident (CVA), is the third leading cause of death in industrialized countries and the leading medical cause of acquired adult disability [73], but few effective therapeutic drugs have been used in clinical practice.

Recently, it was reported that berberine protects neurons from ischemic injury in gerbils and mice by reducing N-methyl-D-aspartate (NMDA) receptor 1 activity [69] and by suppressing apoptotic processes [74]. Studies in vitro suggested that this effect was due to inhibition of ROS production and subsequent blockade of the mitochondrial apoptotic pathway [74]. MMPs are responsible for maintaining and remodeling the extracellular matrix, and in addition to these physiological roles, abnormal MMP expression and activity may be involved in the pathophysiology of cerebral disease. However, it is not much known about the effect of berberine on MMPs, which are closely related to ischemic cerebral injury. By using a mouse model of brain ischemia, Hong et al. concluded that berberine may attenuate the delayed hippocampal neuronal cell injury [75] that normally follows transient global cerebral ischemia. Their results suggest that suppression of MMP-9 activity contributes to the protective effect of berberine.

2.8 Alzheimer's Disease

AD is the most common form of dementia in elderly people, becoming one of the most threatening diseases in this population. However, there is no efficient therapeutic agent to combat it. It is not easy to find a single target to treat AD, since its pathogenesis is very complex [76]. Diverse factors seem to be involved in the development of AD including oxidative stress, acetylcholinesterase enzyme (AChE), butyrylcholinesterase (BChE), monoamine oxidase (MAO), and Aβ peptide aggregation [77–82].

New experimental results suggest berberine may have a potential for inhibition and prevention of AD due to the multiple activities that berberine possesses including antioxidant activity, AChE and BChE inhibitory activity, MAO inhibitory activity, and its abilities to reduce Aβ peptide level and to lower cholesterol. Many studies have proved AChE-inhibiting property of berberine [83–85]. AChE is mainly present in the central nervous system and its major role is to catalyze the hydrolysis of the neurotransmitter acetylcholine to choline. This process can return an activated cholinergic neuron to its resting state. The pathogenesis of AD is linked to acetylcholine deficiency in the brain.

The effect of berberine in vivo has been studied in a rat model of AD concluding that intraperitoneal administration of berberine increased the spatial memory of animals by increasing IL1β and iNOS expression in hippocampus [68].

It has also been shown in vitro that berberine reduces the secretion of Aβ peptide by human neuroglioma cells [86]. Since accumulation and aggregation of Aβ peptide seems to be the crucial event in the pathogenesis of AD [87, 88], inhibition of Aβ peptide generation is a promising therapeutic strategy to treat

AD. According to Zu et al., berberine decreases the production of Aβ peptide by inhibiting the expression of BACE via activation of the ERK1/2 pathway. However, the effect of berberine on Aβ peptide accumulation was not shown in vivo until recently. By using a well-established model of AD, the TgCRND8 transgenic mouse, Durairajan et al. showed that berberine ameliorated neuropathology and cognitive impairment in these mice [89]. The mechanism responsible for this effect was studied in vitro and seems to be dependent of the modulation of the PKB/glycogen synthase kinase 3 (gsk3) signaling pathway.

2.9 Anxiety

Peng et al. showed using two different models of experimental anxiety that high doses of berberine (100, 500 mg/kg) have anxiolytic effect, which was similar to that observed with 1 mg/kg diazepam and 2 mg/kg buspirone. They concluded that this effect might be related to the enhancement of turnover rates of monoamines in the brain stem and the reduction of serotonergic system activity. Moreover, berberine was able to reduce serotonergic system activity by regulating the activity of somatodendritic 5-HT1A autoreceptors and postsynaptic 5-HT1A and 5-HT2 receptors [71].

2.10 Schizophrenia

Prolyl oligopeptidase (POP) is a cytosolic serine peptidase that is known to hydrolyze proline-containing peptides at the carboxy terminus [90]. This enzyme has been associated with schizophrenia, bipolar affective disorder, memory loss, depression, and related neuropsychiatric disorders and therefore may be a relevant therapeutic target to treat these disorders. It is known that the activity of POP enzyme is decreased in depression and increased in psychotic conditions, such as mania and schizophrenia. However, current antipsychotic drugs do not affect the function of this enzyme [91]. According to Tarrago et al., berberine inhibits POP in a dose-dependent manner. As berberine is a natural compound that has been safely administered to humans, it is hypothesized that berberine might be used in the treatment of schizophrenia, opening up new perspectives for the treatment of neuropsychiatric diseases. Moreover, berberine also has D2 dopamine receptor antagonistic and D1 dopamine receptor agonistic activity [92], which may also play a role in its action.

2.11 Mental Depression

Depression is a common mental disorder. In vivo experimental evidences suggest that berberine possesses central nervous system activities, particularly the ability to inhibit monoamine oxidase-A, an enzyme involved in the degradation of norepinephrine and serotonin (5-HT) [70]. In this study, the effect of berberine was attributed to the nitric oxide pathway and/or sigma receptors.

During a mouse in vivo study, berberine was found to enhance the inhibitory neural pathway and decrease morphine dependence [93]. In addition, it modulated locomotor activity and induced hypnosis.

2.12 Infectious Diseases

Berberine possesses antimicrobial activity against bacterial [13, 94–96], fungal [97], protozoan [98, 99], and viral [100–102] infections. In vitro studies have shown that berberine is effective against *Entamoeba histolytica*, *Giardia lamblia*, *Trichomonas vaginalis* [98, 103], *Candida* spp. [97, 104], *Plasmodium falciparum* [99], *Staphylococcus aureus* [95, 105, 106], influenza virus [100], human immunodeficiency virus (HIV) [107], human cytomegalovirus [101], herpes simplex virus, [102] *Chlamydia trachomatis* [105], *Helicobacter pylori* [96], and *Leishmania donovani* [99].

However, few studies have analyzed the in vivo relevance of these findings. Overall, most of the data suggest that berberine is a weak antimicrobial per se, but its activity is strongly synergized by other drugs.

One of the major problems in treating bacterial and fungal infections is the acquisition of the so-called MDR. The weak antimicrobial activity of berberine is mostly due to the MDR pump present in the membrane of the microorganisms, and combination with MDR inhibitors highly enhances the antimicrobial activity of berberine. Ball et al. showed that covalently linking berberine to INF-55, an inhibitor of major facilitator MDRs, results in a highly effective antimicrobial compound that readily accumulates in bacteria [108]. In addition, it has been shown that its activity is strongly synergized by 5′-methoxyhydnocarpin, an inhibitor of the bacterial multidrug resistance pump (MDR). Since gram-positive bacteria are the main targets of plant-derived MDR inhibitors, this may explain why so few of these bacteria are plant pathogens. Plants produce compounds structurally similar to berberine that intercalate into the DNA helix. Since berberine also binds to DNA irrespective of its sequence [109–113], this compound fulfills the requirements for a highly effective antibacterial agent since it is unaffected by mutations.

There are several other examples in which berberine is combined with other substances to potentiate its antibacterial activity. Flavones, chrysosplenol-D, and chrysoplenetin from *Artemisia annua L. (Asteraceae)*, which possess very weak antibacterial action by themselves, produce potent combinations with berberine resulting in very effective inhibition of *Staphylococcus aureus* growth. The combination of amphotericin B and berberine can reduce by approximately 75 % the amphotericin B dose in the treatment of candidiasis in mice, implying that berberine indeed has synergy with amphotericin B against *C. albicans* [104]. Another example is methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria, which are responsible for substantial morbidity and mortality in hospitals. According to Yu et al., berberine is able to restore the effectiveness of β-lactam antibiotics against MDR strains of *Staphylococcus aureus* (MRSA) and inhibit the MRSA adhesion and intracellular invasion in HGFs (human gingival fibroblasts) [95]. However, the in vivo relevance of this finding is not clear. In chloroquine-resistant malaria, the combination of pyrimethamine and berberine gives the best results in clearing the parasite and is more effective than both tetracycline and co-trimoxazole [114]. Berberine also inhibits *T. vaginalis* and its effect is comparable to metronidazole as regards potency [103]. In the mechanistic aspect, one study has shown that berberine has potent inhibitory activity against sortase A (SrtA) and sortase B. The inhibition of sortase enzymes results in a marked reduction in the virulence and infection potential of *S. aureus*, so it may be an important mechanism in the antibacterial activity of berberine [106].

A clinical study also showed the beneficial effects of the administration of aqueous berberine for the treatment of *Chlamydia trachomatis* infection [115]. It was found that the conjunctival scrapings of patients receiving the berberine chloride eye drops were negative for *C. trachomatis* and there were no relapses, even 1 year after treatment. The results suggested that berberine chloride had no direct antichlamydial properties, but it seemed to cure the infection by stimulating some protective mechanism in the host.

Several studies have analyzed the efficacy of berberine analogs and/or derivatives as antimicrobial agents [116–118]. These studies suggest that the presence of lipophilic substituents with moderate sizes contribute to the optimal antimicrobial activity [116]. In addition, the replacement of methoxyl groups at different positions of the ring by a methylenedioxy group results in increased antibacterial activity [117]. Finally, it was found that alkyl-substituted analogs of berberine are more active than berberine against two strains of *Staphylococcus aureus* and fungal infections [118].

There are some studies where the ability of berberine to inhibit the HIV-I has been evaluated [107, 119]. Zha et al. identified a key cellular mechanism underlying the protective effect of berberine on HIV PI-induced inflammatory response in macrophages. Modulation of the endoplasmic reticulum (ER) stress response represents a potential therapeutic target for various inflammatory diseases and metabolic syndromes, including HIV PI-associated atherosclerosis. However, in vivo and clinical studies are needed to further clarify the putative application of berberine in anti-HIV therapy.

2.13 Anticancer Properties

Different chemotherapeutic drugs currently used to treat cancer patients are alkaloids like vincristine and vinblastine or taxanes paclitaxel and docetaxel. Although berberine is still not used in a clinical setting, it also possesses antitumoral activity against a number of tumor types. Indeed, a study using 60 tumor cell lines showed that the IC50 value for berberine was similar to that of other alkaloids used in chemotherapy like paclitaxel or vinblastine [120]. Moreover, in contrast to other antitumoral drugs, the secondary toxicity of berberine is very low as explained in

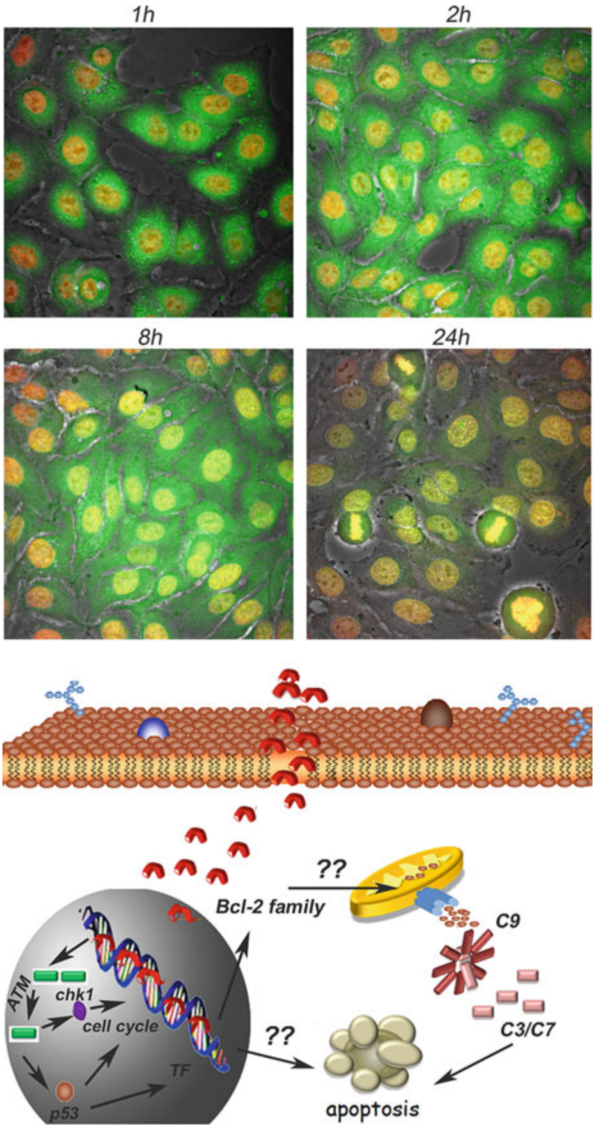

Fig. 41.2 Cellular location and schematic representation of the apoptotic pathways activated by Berberine in tumor cells. *Upper panels*, *berberine accumulates in the nucleus of A549 lung carcinoma cell line*. Cells were incubated with berberine (50 μM) for the indicated times and, subsequently, nuclei were stained with the fluorescent dye DRAQ5. Images were taken in a Leica TCS SP2 confocal microscope. Pictures correspond to the central section in a confocal z-stack acquired sequentially. Green: berberine; Red: DRAQ5; Grey: DIC image. *Lower panel*, *berberine activates in vitro the intrinsic cell death pathway*. Berberine-induced apoptosis is initiated after DNA damage by cell cycle arrest in G0/G1 mediated by the ATM/p53 pathway. In addition, berberine is able to block cell cycle in G2M by activation the ATM/Chk1 p53-independent pathway in some tumor cell types. Activation of these pathways leads to transcription (TF) of

next sections. Cancer cells sensitive to berberine include hematological malignancies or solid carcinomas as explained below in more detail [121]. Most of the antitumor activity is based on the in vitro evidence on the ability of berberine to affect several steps of tumor development including cell proliferation, cell death, and invasiveness [122, 123]. However, there are increasing evidences suggesting that berberine is also able to inhibit tumor growth in vivo [122]. In addition, berberine enhances the efficiency of chemotherapy or radiotherapy effects. During the next sections, we will summarize the ability of berberine alone or in combination with other treatments to induce tumor cell death or to inhibit tumor cell invasiveness.

2.14 Regulation of Cell Death and Proliferation

Cell death induced by berberine in vitro mostly exhibits a typical apoptotic phenotype, including the activation of a family of proteases, the caspases, and cytochrome c release from mitochondria. Berberine has been shown to activate the two major proapoptotic mechanisms known as extrinsic and intrinsic pathways. Intrinsic (also known as mitochondrial) pathway is regulated by the members of the Bcl-2 family. This family comprises proapoptotic (Bak, Bax, Bid, Bim, Puma, etc.) or antiapoptotic (Bcl-2, Bcl-XL, Mcl-1, A1, etc.) proteins [124]. Activation and/or inhibition of proapoptotic or antiapoptotic members, respectively, induces the permeabilization of the mitochondrial outer membrane and, subsequently, the release of apoptogenic factors like cytochrome c, serine protease Htr/Omi, endonuclease G, SMAC/Diablo, or apoptosis-inducing factor (AIF). Cytochrome c together with Apaf-1 and caspase 9 forms a complex known as apoptosome that activates caspase 3 by proteolysis. Active caspase 3 is the ultimate effector molecule, responsible for cell dismantling including chromatin condensation and DNA fragmentation. The intrinsic pathway is activated at different levels like cell cycle arrest, disruption of antiapoptotic/proapoptotic ratio of Bcl-2 family members, DNA damage, and subsequent activation of the p53 repairing factor or proteasome inhibition. The extrinsic (also known as death receptor) pathway is activated after a specific ligand (death ligand, i.e., FasL) binds to its receptor (death receptor, i.e., Fas) on the cell membrane [125]. This binding activates a proteolytic signaling pathway initiated by caspase-8 that induces the direct or mitochondrial-mediated caspase 3 activation and the subsequent apoptotic cell death.

Berberine activates in vitro the intrinsic cell death pathway in several mouse and human tumor cell lines. Most of the studies suggest that berberine-induced

Fig. 41.2 (continued) several pro-apoptotic genes, including members of the Bcl-2 family. Berberine promotes the release of cytochrome c from mitochondria to the cytosol and the formation of the apoptosome, inducing the activation of caspase-9 and, subsequently, of the effector caspase-3 and caspase-7. However, it is not clear the molecular mechanism that links nuclear damage with activation of the mitochondrial pathway apoptosis is initiated after DNA damage by cell cycle arrest in G0/G1 mediated by the ATM/p53 pathway [122, 126–129]. However, it has been recently showed that cell cycle arrest in G2M induced by berberine is mediated by the ATM/Chk1 p53-independent pathway in prostate cancer cells [130] (Fig. 41.2). Indeed, berberine is a cationic molecule able to interact with DNA in cell-free systems as well as in intact cells (Fig. 41.2) [110, 112–114]. Although apoptosis induced by berberine seems to be caspase dependent in most cell types [127, 131–133], the molecular mechanism/s that links cell death after DNA damage and cell cycle arrest with caspase activation, overall the role of the different members of the Bcl-2 family, is not known. Indeed, all evidences suggesting the involvement of Bcl-2 family members during cell death induced by berberine are indirect, and it has not been shown yet how overexpression or inhibition of those proteins affects this process. Thus, a formal proof of the implication of these proteins in cell death induced by berberine is still pending.

Two studies have proposed that berberine induces cell death by the extrinsic pathway in human colon carcinoma [134] and human hepatoma [131] cell lines; however, direct evidences of this mechanism were lacking. In neither of those studies, the role of Fas or FasL was tested if blocking the ligand or receptor could prevent cell death induction by berberine.

2.15 Solid Carcinomas

Berberine is active against diverse types of solid carcinomas when tested in vitro, including tongue, liver, lung, thyroid, prostate, breast, brain, skin, esophageal, oral, or colon carcinomas [133–143].

Most interestingly, berberine also prevents the development of solid carcinomas in vivo in both tumor xeno- or syngeneic transplants and carcinogen-induced tumorigenesis. Regarding models of tumor xenotransplants in immunocompromised mice, it has been shown that berberine inhibits the growth of different human lung [127] as well as human prostate cancer cell lines [144]. In both cases, p53-positive cell lines were more sensitive to berberine than p53-negative cell lines, suggesting a crucial role of this protein in the antitumoral effect of this alkaloid in vivo. Concerning human prostate cancer cells, it was later shown that berberine reduced the expression of the androgen receptor in tumor cells in vivo, indicating that it could interfere with pro-survival function of this receptor during the different stages of prostate cancer [145]. Finally, it was also showed that administration of berberine inhibited the growth of human tongue cancer SCC-4 cells, suggesting a beneficial effect of berberine-containing plants in the prevention of this type of neoplasm [135].

Strikingly, although berberine inhibited the growth of human Lewis lung carcinoma implanted subcutaneously in nude mice [127], it did not affect the growth of a mouse Lewis lung carcinoma after orthotopic implantation in lung parenchyma [141]. These apparent contradictory results could be due to the usage of different lung cell lines, the site of implantation, or the usage of xeno- versus syngeneic transplant models. Thus, the interpretation of results coming from human tumor xenotransplant models must be made with caution.

A beneficial effect of berberine has been also described for chemical-induced carcinomas. In a mouse model of two-stage chemical-induced carcinogenesis, it was found that berberine inhibited the enhancer effect of teleocidin in skin tumors induced by 7,12-dimethylbenz[a]anthracene (DMBA) [146]. This effect was attributed to the ability of berberine to inhibit 32Pi incorporation into phospholipids of cell membrane and hexose transport mediated by teleocidin. Supporting the efficacy of berberine against DMBA-induced tumorigenesis, using another tumor model, an independent group showed that berberine also prevented oral carcinogenesis induced by this chemical in mice [147]. Berberine was also active against methylcholanthrene- or N-nitrosodiethylamine-induced carcinogenesis in mice and rats [148].

2.16 Hematologic Malignancies

As well as in solid carcinomas, berberine is also able to kill diverse types of hematologic tumor cell lines in vitro such as acute myeloid leukemias, acute lymphocytic leukemias, and some types of non-Hodgkin lymphomas [111–114, 122, 129, 139, 149]. Despite that the first analyses about the antitumoral activity of berberine were conducted in leukemic cells [112, 114], at present there are much fewer studies about the efficacy of berberine against hematologic neoplasias than against solid carcinomas. Specifically, only one study has analyzed the activity of berberine against cells from patients with AML [150]. The results showed that berberine was able to inhibit migration of AML cells by reducing the levels of stromal-derived factor-1 secreted by the bone marrow stromal cells from AML patients. The same effect was observed against cancer stem cells isolated from those patients.

A number of studies have analyzed the efficacy of berberine in vivo against transplanted hematologic tumor cell lines as well as during virus-induced leukemia. Specifically, it has been showed that berberine has antitumoral potential in vivo against transplanted mouse acute lymphocytic leukemia P388 [112] and acute myeloid leukemia WEHI-3 [151]. In addition, this alkaloid was able to inhibit the progression of erythroleukemia induced by mouse retrovirus, Friend, in immunocompetent mice [152].

2.17 Combined Therapies

One of the main problems during cancer management is the presence and/or apparition of resistant tumor cells due to mutations in the molecule/pathway targeted by the selected therapy [153]. During the last years, the combination of two or more treatment modalities (i.e., combination of different drugs or combination of drugs with radiotherapy) has emerged as a promising tool to overcome tumor resistance. Berberine has also been shown to enhance the antitumoral effect of chemo- and radiotherapy [122, 154].

Esophageal cancer has a very bad prognosis due to the low efficacy of current treatments. It was recently found that berberine enhances the sensitivity of human esophageal squamous cell carcinoma lines to radiotherapy [140]. This effect was related to the downregulation of the repair protein RAD51. Although this protein was present in specimens from patients with esophageal carcinoma, its role in disease pathogenesis is not clear. Other tumor cell lines that show enhanced susceptibility to radiotherapy in the presence of berberine are lung carcinoma [155], hepatoma [156], and glioma cells [157].

Berberine also enhances the cytotoxicity of chemotherapeutic drugs against tumor cells, including cisplatin [158], evodiamine [159], arsenic trioxide [160], 5-fluorouracil [154], doxorubicin [154], TNF-a [154], TRAIL [161], or nitrosourea [143]. It has been suggested that this effect was due to the suppression of antiapoptotic gene products by inhibiting the activity of the transcription factor NF-kB [154]. This transcription factor that is constitutively expressed in several tumor cell types is regulated by the upstream IkB (inhibitory subunit of NF-kB) kinase (IKK). IKK phosphorylates IkB, promoting nuclear translocation of active NF-kB and transcription of target genes. Berberine was able to inhibit IKK and, thus, prevented IkB phosphorylation and NF-kB activation.

2.18 Inhibition of Tumor Invasiveness

Apart from its cytotoxic potential, berberine is able to inhibit tumor cell motility and invasiveness in vitro at noncytotoxic doses in a number of human tumor cell lines like lung, melanoma, liver, breast, gastric, tongue, or nasopharyngeal carcinoma cell lines [123, 162–167]. In most cell types, inhibition of cell invasiveness by berberine was dependent on the downregulation of matrix metalloproteinase expression by interfering with diverse signaling pathways like those regulated by ERK, PI3K, or Rho GTPases [163–167]. Moreover, the finding that berberine is able to directly inhibit the activity of the transcription factor NF-kB [154] suggests that this alkaloid may reduce the expression of matrix metalloproteinases by passing the above-mentioned upstream signaling pathways.

Concerning the ability of berberine to interfere with tumor cell invasiveness in vivo, it was shown that this alkaloid inhibited the formation of lung metastasis after inoculation of mouse melanoma B16 cells [167]. In addition, it was found that berberine inhibited the apparition of mediastinal lymph node metastasis after orthotopic implantation of mouse Lewis lung carcinoma cells in lung parenchyma [141]. At the berberine dose tested, it was not observed to have any effect on the growth of the tumor at the site of implantation. However, the combination of both camptothecin-11 and berberine was able to inhibit both tumor growth and metastasis. This promising result suggests the usage of berberine as antimetastatic drug in combination with other cytotoxic antitumoral agents.

2.19 Other Applications

Berberine has also been employed as an analgesic for the treatment of neurological conditions. Berberine has been shown to ameliorate tolerance inducement to some narcotics like morphine [168].

Berberine has been shown to suppress lipid synthesis from sebaceous glands, and thus, it has potential to be used as a topical agent in skin-related infections [169]. In addition, it has surfactant activity, enhancing skin permeability to polar drugs [170]. However, it should be noted that when used on skin, berberine has the potential to form free radicals induced by sunlight [171].

3 Bioavailability and Metabolism

It is widely accepted in the pharmaceutical industry that the distribution, metabolism, and efficacy of many drugs can be modulated depending on their affinity to serum albumin [172]. Hu et al. [173] investigated the association of human serum albumin (HSA) with berberine. It was found that an interaction occurred at the IIA domain of HSA. Since other alkaloid drugs may share with berberine binding sites in HSA, this finding may help to design therapeutic drugs with reduced binding constant to HSA and, thus, more efficient than current ones.

As described above, berberine has a wide spectrum of pharmacological actions, but the poor bioavailability may limit its clinical use. According to Pan et al., berberine is hardly absorbed in the intestine (less than 5 % in 2.5 h) [174]. Some studies [175, 176] suggest that the poor intestinal absorption is due to the negative effect of intestinal P-glycoprotein on this process. If true, P-glycoprotein inhibitors could be of therapeutic value by improving berberine bioavailability. Some works have been directed to improve berberine bioavailability showing that the use of a water-soluble form of vitamin E, called “D-α-tocopheryl polyethylene glycol 1,000 succinate” (TPGS), enhances the bioavailability of berberine [177–179]. For example, 2.5 % TPGS content of the solution enhanced berberine absorption by 1.9-fold [178]. This effect was attributed to the inhibition of P-glycoprotein efflux [179]. Another study concludes that sodium caprate could significantly promote the absorption of berberine in intestine and enhance its antidiabetic effect without any serious mucosal damage [180].

4 Secondary Toxicity

The most promising finding concerning the use of berberine as a therapeutic agent, especially in cancer, is its low secondary toxicity. One of the major problems of drugs used in chemotherapy is the secondary toxic effect against healthy tissues

Table 41.1 Clinical trial

Study	Status	Condition	Interventions
Polycystic Ovary Syndrome (PCOS): Effect Of Letrozole and Berberine	Recruiting	Polycystic ovary syndrome	Drug: letrozole–berberine Drug: letrozole Drug: berberine
Role of Pioglitazone and Berberine in Treatment of Non-Alcoholic Fatty Liver Disease	Completed	Nonalcoholic fatty liver disease	Lifestyle intervention Drug: pioglitazone Drug: berberine
Effect of Berberine on Hormonal and Metabolic Features in Obese Women With Polycystic Ovary Syndrome (PCOS)	Active, not recruiting	Polycystic ovary syndrome Obesity	Drug: berberine Drug: placebo
Efficacy and Safety of Berberine in the Treatment of Diabetes With Dyslipidemia	Completed	Diabetes mellitus, type 2 Metabolic syndrome	Drug: berberine
Therapeutic Effects of Berberine in Patients With Type 2 Diabetes	Completed	Type 2 diabetes	Drug: berberine Drug: metformin
Armolipid Plus and Metabolic Syndrome	Completed	Metabolic syndrome	Dietary supplement: Armolipid Plus
The Role of a Combination of Nutraceuticals in the Control of Cardiovascular Risk	Active, not recruiting	Metabolic syndrome	Drug: Armolipid Prev
Combined Effects of Bioactive Compounds in Lipid Profile	Recruiting	Hyperlipidemia low-density-lipoprotein-type elevated triglycerides	Dietary supplement: Armolipid Plus Dietary supplement: placebo
Effects of Armolipid Plus on Cholesterol Levels and Endothelial Function	Completed	Hyperlipidemia endothelial dysfunction	Dietary supplement: mixture of berberine, policosanol, red yeast, placebo
Ezetimibe Versus Nutraceuticals in Statin-intolerant Patients	Not yet recruiting	Coronary artery disease	Drug: ezetimibe Drug: nutraceuticals

and/or cells. This is due in part to the low number of specific targets in transformed cells. Indeed, most of the drugs target processes in cancer cells, which are still ongoing in normal cells and tissues.

In vitro and in vivo evidences suggest that berberine is more targeted than other chemotherapeutic drugs. It has been reported that naive or activated T cells are resistant to berberine [181]. Furthermore, it has been shown that berberine is able to protect thymocytes (precursors of T cells) from cell death induced by dexamethasone, etoposide, or camptothecin [182]. Finally, it has been reported that macrophages are not only resistant to berberine-induced cell death but also able to get activated and produce immune-stimulatory cytokines like IFNgamma and IL12 in vitro [183] and in vivo [184].

Concerning other organs and/or cell types, recently it was reported that berberine killed hepatoma cells without affecting the viability of normal hepatocytes. In addition, berberine shows much higher specificity for oral-derived tumor cells than for normal oral tissue [185]. Finally, it has been showed that berberine inhibits pathogenic forms of the androgen receptor in prostate cancer cells without affecting its function in normal cells and the morphology of normal prostates [186].

Apart from the low toxicity of berberine against normal tissues, it has also been showed that this molecule exerts a protective effect on lung tissue during irradiation [187].

Several groups have studied the pharmacokinetics and toxicity of berberine in mouse and rat models in vivo. Berberine toxicity is low in rat and mouse models when tested by histopathological criteria and with relatively high LD50 (dose at which 50 % of animals die) values [123]. The results show that the lethal dose 50 (LD50, amount of a compound required to kill 50 % of the treated animals) in rats is 205 mg/kg when given intraperitoneally and that it was well tolerated in doses of 100 mg/kg when given orally to rats [188]. An independent study found that the LD50 of berberine in mice after intravenous or intraperitoneal administration was 9 mg/kg and 57 mg/kg, respectively [189]. This group did not find any LD50 for berberine after oral intragastric administration. However, as indicated in the previous section, low amounts of berberine enter the circulation by this way. Of note, during a 3-month trial with patients suffering from type 2 diabetes, it was found that therapeutic doses of berberine did not affect liver or kidney and only produced transient gastrointestinal effects on around 35 % of the patients [190]. Janhke et al. published in 2006 for first time the developmental toxicity evaluation of berberine in rats and mice and no fetal adverse effects were noted [191].

Although berberine has not been reported to be mutagenic, this compound is able to intercalate into the DNA double helix [192, 193]. Thus, it is possible that this binding may lead to mutations in the genome of exposed cells. Some studies have shown than non-transformed cells take up much lower levels of berberine than do tumor cells [194]. However, at present the basis of this phenomenon is not understood and needs to be investigated.

The most common side effects include constipation, laxative, anaphylaxis, and other skin allergies such as dermatitis and rashes, and high dosages may cause respiratory and circulatory system problems [123]. Furthermore, berberine could displace bilirubin from serum-binding proteins, causing jaundice, kernicterus, and brain damage in infants [195–197]. Therefore, exposure of pregnant women to berberine is not recommended because it may lead to jaundice and kernicterus in the fetus [196].

5 Clinical Trials

Clinical trials have been conducted using berberine suggesting that this compound may be beneficial in the treatment of trachomas (eye infections), bacterial diarrhea, and leishmaniasis (parasitic disease). Berberine has been shown to be safe in the majority of clinical trials. However, there is a potential for interaction between berberine and many prescription medications, and berberine should not be used by pregnant or breastfeeding women, due to potential for adverse effects (Table 41.1).

6 Conclusions

Berberine, a natural alkaloid used for centuries in folk medicine, shows proven as well as promising therapeutic effects against a number of disorders including infection, cancer, inflammation, diabetes, or cardiovascular diseases. The versatility of this alkaloid to treat this great variety of apparently nonrelated affections may be due to the ability of berberine to target key molecular pathways including NF-kB, ROS, iNOS, COX, or AMPK. The low toxicity of berberine as well as its synergistic and protective effects in combination with other drugs becomes a promising molecule for the treatment of diseases with serious drug-associated sequelae like cancer. A weakness of berberine is its low bioavailability during in vivo administration. However, several efforts have been developed during last years in order to solve these problems and ensure a targeted therapeutic activity of berberine.

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Alkaloids and Drug Discovery for Neurodegenerative Diseases

42

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Contents

1 Introduction 1333
2 Cholinesterase (ChE) Inhibitor Alkaloids and Derivatives 1335
2.1 Galantamine 1335
2.2 Huperzines 1338
2.3 Physostigmine 1340
3 Alkaloids and Derivatives as Ligands at Cholinergic Receptors 1343
3.1 Muscarinic Receptor Ligands 1343
3.2 Nicotinic Receptor Ligands 1345
4 Alkaloids and Derivatives that Modulate Dopaminergic Function 1348
5 Monoamine Oxidase Inhibitory Alkaloids and Derivatives 1352
6 *Vinca* Alkaloids and Derivatives 1353
7 *Coffea arabica* L. Alkaloids and Derivatives 1353
8 Conclusion 1355
References 1355

Abstract

Neurodegenerative diseases include a range of disorders that involve dysfunction of the central nervous system, due to degeneration of neurons and associated pathological processes. These diseases include Lewy body dementia, vascular dementia, Huntington's, Parkinson's (PD), and Alzheimer's diseases (AD). Treatment strategies are limited and frequently only provide symptomatic relief through the use of drugs that modulate neurotransmitter disturbances. Of the five drugs developed to treat symptoms of AD, the most common form of dementia, two are derived from plant alkaloids: galantamine, originally from *Galanthus*

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woronowii, and rivastigmine, which is based on the chemical structure of physostigmine from *Physostigma venenosum*. These drugs inhibit acetylcholinesterase to improve cholinergic neuronal dysfunction and the associated cognitive symptoms that occur in AD. Many other alkaloids and their derivatives have been investigated for their ability to modulate cholinergic functions in AD and other dementias. Other alkaloids have been explored as potential treatments for the motor symptoms that occur in PD, via modulation of dopaminergic neurotransmission. Lead compounds for drug discovery include ergot alkaloids from *Claviceps purpurea*, which provided templates for the development of synthetic drugs such as bromocriptine, used to alleviate PD symptoms. Numerous other alkaloids and their derivatives have been investigated for their ability to alleviate symptoms in neurodegenerative diseases, with some also emerging as disease-modifying agents. Other alkaloids, such as nicotine and caffeine, have been suggested to provide protective effects against the development of some neurodegenerative diseases and are discussed from an epidemiological perspective, with consideration of their mechanistic effects.

Keywords

Acetylcholinesterase inhibitors • Alzheimer’s disease • caffeine • cholinergic • dopaminergic • monoamine oxidase • muscarinic receptor • nicotinic receptor • Parkinson’s disease • *Vinca*

Abbreviations

Aβ	β-Amyloid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer’s disease
APP	Amyloid precursor protein
BACE1	β-Secretase
BBB	Blood-brain barrier
BChE	Butyrylcholinesterase
BDNF	Brain-derived neurotrophic factor
BPSD	Behavioral and psychological symptoms of dementia
ChE	Cholinesterase
ChEI	Cholinesterase inhibitor
CNS	Central nervous system
DA	Dopamine
GI	Gastrointestinal
H_2O_2	Hydrogen peroxide
LBD	Lewy body dementia
mAChR	Muscarinic acetylcholine receptor
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor

MCI	Mild cognitive impairment
MMSE	Mini-Mental State Examination
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
nAChR	Nicotinic acetylcholine receptor
NGF	Nerve growth factor
NMDA	*N*-Methyl-D-aspartate
6-OHDA	6-Hydroxydopamine
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PKC	Protein kinase C
RCT	Randomized controlled trial
TGF-β_1	Transforming growth factor β_1
VaD	Vascular dementia

1 Introduction

Neurodegenerative diseases encompass a range of disorders including the dementia pathologies such as Lewy body (LBD) and vascular (VaD) dementias and Huntington's, Pick's, Parkinson's (PD), and Alzheimer's (AD) diseases; the latter is the most common form of dementia, with PD as the second most common neurodegenerative disorder [1, 2]. Symptoms of dementia are associated with multiple cognitive impairments, particularly memory loss, with deficits in attention, language, and social skills. These symptoms can occur in addition to movement disorders associated with PD (Parkinson's disease dementia [PDD]) and LBD, and cerebrovascular events in VaD [3]. In addition, behavioral and psychological symptoms of dementia (BPSD) can include psychosis, agitation, anxiety, sleep disorders, and depression [3].

Treatments for neurodegenerative diseases are limited and frequently only provide symptomatic relief. This appears to reflect a relative lack of knowledge of the etiology and pathology of dementias such as AD. The discovery that deficits in the cholinergic neuronal system occur in the central nervous system (CNS) in senile dementia [4, 5] provided a breakthrough in the understanding of the pathology of dementia, particularly AD. This pioneering work led to the hypothesis that drugs that could prolong or mimic the action of the neurotransmitter acetylcholine (ACh) at cholinergic (muscarinic [mAChR] and nicotinic [nAChR]) receptors might restore cholinergic function and thus reduce symptoms in AD. In AD, the $\alpha_4\beta_2$ nAChR subtypes in particular are reduced [6]; thus, nicotinic agonists including the alkaloid nicotine have been explored for potential modulation of cholinergic function in dementia. Agonists at M_1 mAChRs can improve cognitive functions in animals and in AD patients, and antagonists at central presynaptic M_2 mAChRs can increase ACh release to improve cognition [7]; thus, ligands for these mAChRs have also been sought as dementia treatment strategies. In addition to the search for compounds that interact directly with cholinergic receptors, compounds that inhibit acetylcholinesterase (AChE) to reduce the hydrolysis of ACh by this enzyme have also been investigated to prolong the action of ACh at cholinergic receptors. Also emerging as a potential therapeutic target in AD is inhibition of butyrylcholinesterase (BChE). BChE is predominantly localized in glial cells, and although its function has not been fully elucidated, it is now known to metabolize ACh, and it is suggested that some neuronal pathways may function via BChE [8].

Other neurotransmitter abnormalities occur in dementia pathologies, including glutamatergic and serotonergic, and in LBD/PD, dopaminergic neuronal degeneration occurs, particularly in the substantia nigra [2, 3]. Thus, compounds that modulate these neurotransmitter systems are also relevant to alleviate symptoms. There are limited treatment options for neurodegenerative diseases such as Huntington's disease and the prion diseases. Those natural products, which include some alkaloids, that have been explored for use in these and some other neurodegenerative diseases have been recently reviewed [9] and will therefore not be the focus of this chapter. Other treatment strategies for neurodegenerative diseases have focused on the discovery of disease-modifying agents. For neurodegenerative diseases, inhibition of protein aggregation pathologies via prevention of β-amyloidosis and abnormal tau, for AD in particular, and reduction of α-synuclein deposition in LBD/PD are treatment strategies being investigated [10]. Compounds displaying more than one mode of action relevant to the multiple pathologies that are associated with neurodegenerative diseases may provide therapeutic advantages over drugs targeting just one mode of action.

Five drugs (four AChE inhibitors [AChEIs] and one glutamate receptor antagonist) have now been developed to treat symptoms of dementia (primarily in AD but for some drugs also PDD). Three of these drugs are synthetic (donepezil, tacrine, and memantine), but two (galantamine and rivastigmine) are AChEIs derived from plant alkaloids. There are numerous other alkaloids of natural origin, or their derivatives (semisynthetic and synthetic), that are being explored for their ability to modulate cholinergic functions, or to target other mechanisms, as our understanding of neurodegenerative disease pathology is evolving. There are many alkaloids and their derivatives that are cholinesterase (ChE) inhibitors (ChEIs) [11–13], but their inhibitory potency and therapeutic potential for use in neurodegenerative diseases vary considerably. This chapter summarizes some of the most therapeutically promising ChEIs derived from alkaloids; for other ChEI alkaloids of natural origin, readers are directed to reviews by Houghton et al. [11], Howes and Houghton [12], and Williams et al. [13]. In summary, this chapter presents examples of alkaloids that have been investigated in drug discovery studies for their therapeutic relevance to neurodegenerative diseases, with an emphasis on alkaloid drug leads for two of the most common forms of neurodegenerative disease, AD and PD.

2 Cholinesterase (ChE) Inhibitor Alkaloids and Derivatives

2.1 Galantamine

The isoquinoline alkaloid galantamine (**1**) was first isolated in the early 1950s from *Galanthus woronowii* Losinsk. (Amaryllidaceae), the snowdrop, and it was reported to have AChE inhibiting properties [14]. It was then isolated from *G. nivalis* L., species of *Narcissus* and *Leucojum aestivum* L. (summer snowflake), in the late 1950s when its ChE inhibiting action was confirmed [14]. Although galantamine (also referred to as galanthamine) was initially used therapeutically for several indications including in ophthalmology, gastroenterology, cardiology, and physiotherapy, it was not until the 1980s that it began to be explored for its potential to alleviate symptoms of dementia [14], prompted by the discovery in the 1970s of the cholinergic deficits that occur in dementia [4, 5]. Following the licensing of synthetic ChEIs for symptomatic treatment of AD, galantamine was the first naturally derived anti-ChE drug licensed to treat symptoms of mild to moderate dementia in AD.

Cochrane reviews assessing controlled trials for the effects of galantamine in AD concluded it could improve cognitive functions and activities of daily living, in addition to behavioral symptoms, in AD patients [15, 16]. Limited clinical data are available for the effects of galantamine in other forms of dementia, although some cognitive improvements have been observed with this drug in LBD [17], PDD [18], and VaD [19], with some improvements in BPSD also observed in VaD [20]. Studies in patients with mild cognitive impairment (MCI) showed galantamine to provide only marginal clinical benefit with increased death rates; thus, use in MCI is not recommended [16].

With regard to cholinergic function, galantamine is also an allosteric modulator of nAChRs particularly the $\alpha_4\beta_2$ and α_7 subtypes, an action that was considered to provide additional cognitive enhancement in AD. More recently, however, improved cognitive function by galantamine in early stage AD was not correlated with $\alpha_4\beta_2$ nAChR modulation [21]. However, the potentiation of presynaptic nAChRs by galantamine was associated with increased glutamatergic neurotransmission to increase cognitive performance in AD patients [22].

Although selective and competitive AChE inhibition is considered the primary mode of action of galantamine, other mechanistic effects relevant to treating neurodegenerative diseases have since been discovered (summarized in Table 42.1). However, many of these activities are reported in vitro only, with further studies needed to investigate if any of these effects occur with therapeutic relevance in humans.

Numerous other Amaryllidaceae alkaloids have been isolated and, in addition to many synthetic and semisynthetic derivatives, have been investigated for their ChE inhibitory effects and potential to improve cognitive functions, although few appear to have reached more advanced stages of clinical development. One prodrug of

Table 42.1 Mechanistic effects of acetylcholinesterase inhibitors

Mechanistic effect	Galantamine	Huperzines A and B, and derivatives	Physostigmine and derivatives
Neuroprotective/ anti-apoptosis	• Neuroprotective against Aβ via inhibition of calpain-calcineurin signaling [23] and against glutamate toxicity in vitro [24] • Prevents Aβ-induced oxidative damage via antioxidant effects [25] • Prevents Aβ-induced apoptosis in vitro [24]; apoptosis prevention may be via increased Bcl-2 expression [26] • Protects against oxygen-glucose deprivation-induced damage in rat hippocampal slices [24] • Rat primary cortical cells are protected by pretreatment with galantamine [24] • Prevents mitochondrial dysfunction and endoplasmic reticulum stress [27]	*Huperzine A* • Neuroprotective against Aβ, oxygen-glucose deprivation, free-radicals, H_2O_2, and glutamate; also an NMDA receptor antagonist [24, 32, 33] • Protects isolated rat brain mitochondria from Aβ [34] • Attenuates apoptosis via modulation of gene expression [24] • Neuroprotection may be via inhibition of caspase 3 activation [35] *Huperzine B* • Neuroprotective in vitro via attenuation of H_2O_2- and oxygen-glucose deprivation-induced injury [1] *Synthetic derivatives* • A huperzine B dimer derivative is neuroprotective against Aβ [12, 36] • Enantiomers [(+)- and (−)-] of dimethylhuperzine are neuroprotective against glutamate [1] • Bis(12)-hupyridone prevents H_2O_2-induced neuronal apoptosis (independent of AChE inhibition) [37]	*Rivastigmine* • Neuroprotective involving the synapse; observed in rat primary cortical cultures [41] • Neuroprotective against Aβ in vitro [42] *Neostigmine and pyridostigmine* • Neuroprotective against glutamate toxicity in vitro [24] *Ganstigmine* • Neuroprotective against Aβ toxicity in vitro [24]
Anti-Aβ	• Inhibits Aβ release and BACE1 expression via modulation of α_7 nAChRs and other messengers (e.g., PKC) [28] • Inhibits aggregation and cytotoxicity of Aβ [29] • Enhances microglial phagocytosis and Aβ clearance from brains of rodent AD models [30]	*Huperzine A* • Regulates APP processing via PKC and mitogen-activated protein kinase pathways in vitro [38] *Huprine X* • In animal AD models, reduces insoluble Aβ in the hippocampus (3xTg-AD mice) but had no effect in APPswe mice [39]	*Physostigmine* • Lowers cortical concentrations of insoluble Aβ and decreases Aβ deposition [26] *Physostigmine and analogues* • (Include phenserine, cymserine, and tolserine) modulate soluble APPα and Aβ [26, 43]

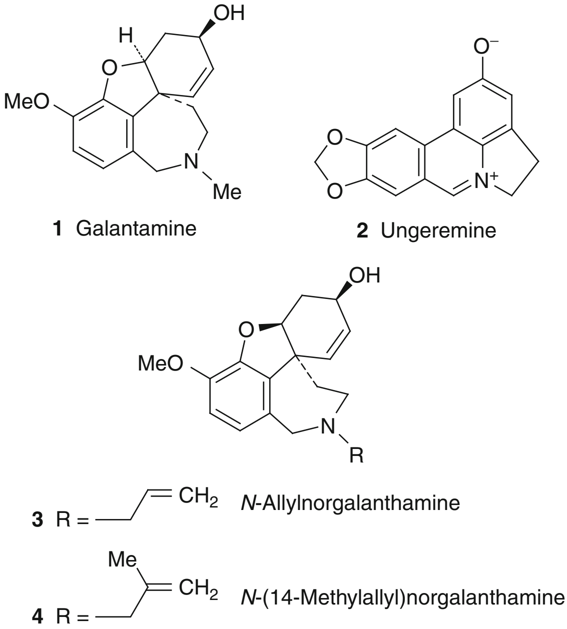
(*continued*)

Table 42.1 (continued)

Mechanistic effect	Galantamine	Huperzines A and B, and derivatives	Physostigmine and derivatives
			Cymserine analogues • Reduce Aβ levels in vitro and in a transgenic mouse AD model [44] *Phenserine* • Reduces Aβ levels by regulating APP translation in vitro [45]; (+)-enantiomer reduces APP in vivo [46] *Rivastigmine* • Lowers Aβ levels and increases soluble APPα levels in vitro [47]
Neurotrophic	• Limited evidence for neurotrophic effects, but galantamine increases ACh in anti-NGF transgenic rodents and reverses behavioral deficits and neurodegeneration [31]	*Huperzine A* • Increases neurite-bearing PC12 cells and upregulates NGF mRNA in vitro [33] • Enhances mRNA and protein levels of NGF, BDNF, and TGF-β_1 in the cerebral cortex and hippocampus in vivo [33] *Bis(12)-hupyridone* • Induces neurite outgrowth and promotes neuronal differentiation more potently than huperzine A (possibly via regulation of α_7 nAChRs) [40]	*(+)-Phenserine* • Increases neuronal differentiation of implanted human neural stem cells in mouse hippocampus and cortex [46] *Rivastigmine* • Potential synaptotrophic effects [41]

galantamine, Gln-1062 (Memogain$^{®}$), is being considered for drug development as an AChEI and nAChR modulator since it improved cognitive functions in an animal model of amnesia and had CNS bioavailability 15-fold that of galantamine, in addition to fewer gastrointestinal (GI) effects [10, 48]. Many other Amaryllidaceae ChE inhibitory alkaloids and their derivatives with potential for use in CNS disorders have been reported [11–13]. Those with more potent AChE inhibitory activity than galantamine include ungeremine (**2**), isolated from *Nerine bowdenii* W. Watson (also occurs in species of *Galanthus* and *Narcissus*) [13], and the *N*-alkylated galantamine derivatives *N*-allylnorgalanthamine (**3**), and *N*-(14-methylallyl)norgalanthamine (**4**), obtained from *Leucojum aestivum* L. leaves during the industrial production of galantamine [49] (Scheme 42.1).

Scheme 42.1 Amaryllidaceae derived alkaloids that inhibit cholinesterase



1 Galantamine

2 Ungeremine

3 *N*-Allylnorgalanthamine

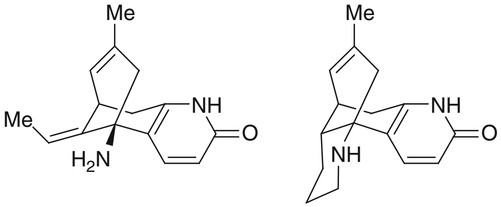
4 *N*-(14-Methylallyl)norgalanthamine

2.2 Huperzines

Huperzia serrata (Thunb.) Rothm. (Lycopodiaceae) is a traditional Chinese medicine that has been used to alleviate memory impairment [1] and was also marketed in the USA as a dietary supplement as powdered *H. serrata* for this purpose [50]. The quinolizidine-related alkaloids, huperzines A (**5**) and B (**6**), were isolated from *H. serrata* and identified as AChEIs [51, 52]. Both huperzines A and B improve cognitive functions in vivo [12, 32, 36]. Huperzine A (also occurs in other species including *H. selago* (L.) Bernh. ex Schrank and Mart. [53]) inhibits AChE more potently and selectively than huperzine B [3], with the naturally occurring isomer (−)-huperzine A, being more potent than the (+)-isomer [54]. Other biological activities that may have relevance for neurodegenerative diseases have been discovered for both huperzines A and B and their derivatives (summarized in Table 42.1), although AChE inhibition is considered the principal mode of action (Scheme 42.2).

Most studies to investigate the cognitive effects of these alkaloids have therefore focused on the more potent AChEI huperzine A, which has been evaluated in numerous controlled trials in dementia patients. Promising effects on cognitive functions are reported in elderly subjects and in those with AD and VaD [55–58], although evidence for efficacy in VaD is more limited compared to AD [58]. A meta-analysis of randomized controlled trials (RCTs) associated huperzine A with improved cognition (measured by Mini-Mental State Examination [MMSE]) and activities of daily living in AD patients [57]. Although huperzine

Scheme 42.2 *Huperzia serrata* alkaloids that inhibit cholinesterase



5 Huperzine A

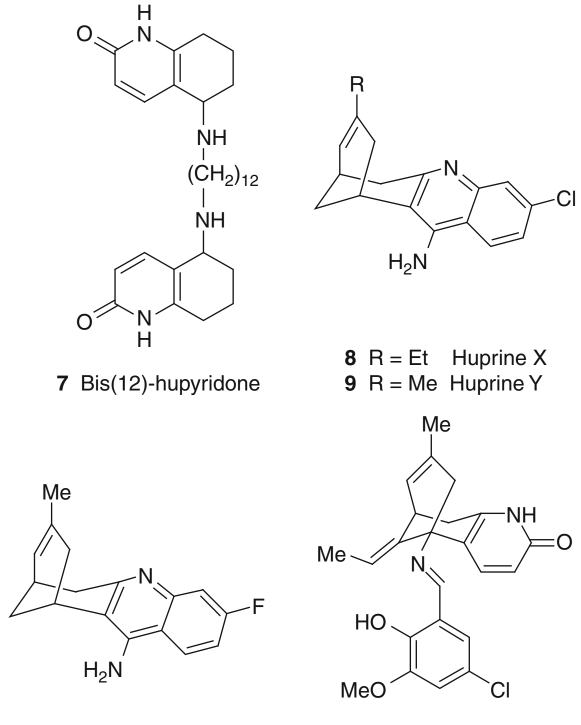
6 Huperzine B

A had reached the phase II trial stage, further trials on this oral formulation were suspended; a transdermal formulation has reached the phase I trial stage [10].

To optimize the AChE inhibitory potency and selectivity of the huperzines, an array of synthetic derivatives have been investigated. Dimerization of the inactive fragment of huperzine A produced dimers (hupyridone fragments linked with an 11 or 12 methylene tether) that were twice as potent at AChE inhibition compared to huperzine A [59]. Further studies with bis(12)-hupyridone (**7**) revealed that it inhibits AChE in the CNS in vivo, also indicating the ability to cross the blood-brain barrier (BBB) [60]. Other huperzine A analogues are in development in drug discovery programs as new AChEIs for neurodegenerative diseases [10]. To modify the binding to AChE, it has been rationalized that the H-bond between the C-14 methyl of huperzine A and the backbone carbonyl of His440 on AChE may be enhanced by replacing the C-14 methyl of huperzine A with fluorine. However, (±)-14-fluorohuperzine A was considerably less potent than huperzine A in the inhibition of AChE [61]. Other fluorinated huperzine A analogues have been developed but, disappointingly, were also less potent AChEIs than huperzine A [62, 63]. Substitution of the C-5 amino group in huperzine A with fluorine (hydroxyl or acetoxyl C-5 substituted and 5-desamino derivatives were also investigated) also produced less potent AChEIs compared to the natural alkaloid, presumably due to the importance of the C-5 amino group in the huperzine A structure for AChE inhibition, to imitate ACh by forming a quaternary ammonium under physiological conditions [64, 65].

Other compounds have been developed by combining structural features of both huperzine A and the synthetic AChEI tacrine [66, 67], to optimize AChE inhibitory activity. The hybrid compounds with structures based on these two AChEIs include the huprines X (**8**), Y (**9**), and Z (**10**). Huprine X has significantly greater affinity for AChE than huperzine A, tacrine, or the AChEI drug donepezil [68], in addition to reported affinity for M_1 and M_2 mAChRs and nAChRs [69], which may provide additional modulation of cholinergic function in the CNS. (±)-Huprines Z and Y are also potent AChEIs, with the latter (chlorine at position 3 in the structure, instead of fluorine) the more active [70]. Although the huprines are being pursued in drug development programs as AChEIs, M_1 mAChR, and nAChR agonists [10], there is a lack of available data on their toxicity. Hepatotoxicity is associated

Scheme 42.3 Synthetic derivatives of huperzine A



7 Bis(12)-hupyridone

8 R = Et Huprine X
9 R = Me Huprine Y

10 Huprine Z

11 ZT-1

with the AChEI tacrine [71], which limited its clinical use. Since the huprines consist of some structural features of tacrine, it is essential to consider their potential to cause hepatotoxicity. Other huperzine A hybrid structures have included the benzylpiperidine moiety of another synthetic AChEI drug, donepezil (E2020), but compounds developed were weaker AChEIs compared to donepezil [72]. Other huperzine derivatives in development include the prodrug Debio 9902 (ZT-1) (**11**), an AChEI and *N*-methyl-D-aspartate (NMDA) receptor antagonist, which improved cognitive functions in AD patients in a phase IIa trial [73, 74] (Scheme 42.3).

2.3 Physostigmine

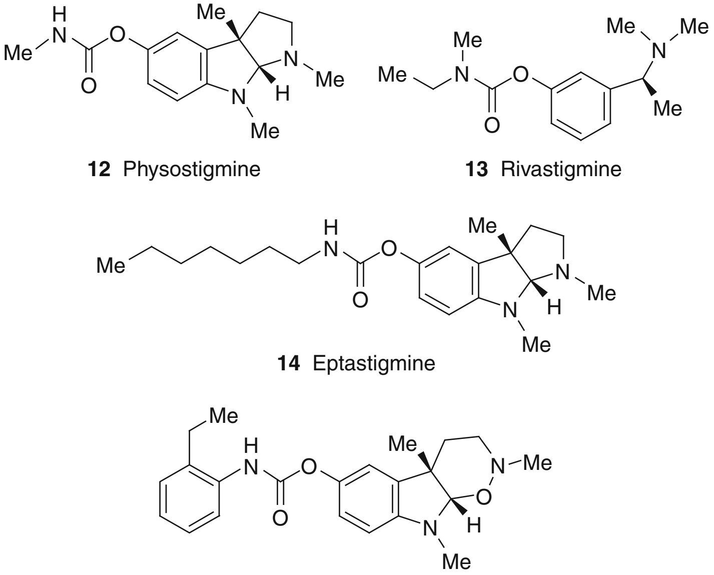
The calabar bean, seeds from *Physostigma venenosum* Balf. (Leguminosae), was used traditionally in Africa, particularly southeast Nigeria, for ritual deaths associated with the funeral of a chief and as an ordeal poison to determine the guilt or innocence of persons accused of a crime [1]. The pyrroloindole alkaloid physostigmine (**12**) (also referred to as eserine) was first isolated from the seeds of *P. venenosum* in the nineteenth century [75]. It is a short-acting and reversible inhibitor of AChE [12].

Physostigmine improves cognitive functions in vivo; it protects against oxygen deficit-induced cognitive impairment, improves learning in mice, and it antagonizes scopolamine-induced cognitive impairment in rats [1] and in zebrafish [76]. Although physostigmine improves cognitive functions in vivo and in AD patients [77], a Cochrane review in 2001 concluded there is limited evidence for efficacy in AD and adverse effects are common [78]. It should be noted, however, that although there is a lack of evidence to support the use of physostigmine for dementia, it is suggested to be of clinical value in severe cases of poisoning due to ingestion of *Datura* species (Solanaceae) [79].

Physostigmine has been used as a template for the development of new ChEIs, with the aim of improving efficacy and reducing adverse effects, which limited the development of physostigmine for clinical use. The only synthetic derivative to reach the phase IV clinical trial stage is rivastigmine (**13**), a reversible AChEI drug licensed to treat symptoms in mild to moderate AD and PD. Many rivastigmine analogues have been synthesized and investigated to produce optimized structures for ChE inhibition, with the aim of developing new pharmaceuticals [80]. Numerous other synthetic compounds based on the carbamate moiety of the physostigmine structure have also been developed for potential use in dementia; some examples of these are summarized below.

Eptastigmine (**14**), a more lipophilic heptyl derivative of physostigmine, inhibits AChE and BChE and improves cognition in AD, but hematologic effects hindered further investigation [81]. Ganstigmine (**15**) is a selective AChEI, and it attenuates scopolamine-induced amnesia in vivo [35]. Although investigated in phase I and II clinical trials, adverse events at higher doses have limited further clinical development [35] (Scheme 42.4).

Phenserine (**16**) is a phenylcarbamate derivative of physostigmine that inhibits both AChE and amyloid precursor protein (APP) synthesis [73]. Phenserine appears to be a more suitable drug candidate than physostigmine since it inhibits AChE more potently and selectively; it is more lipophilic, thus readily crosses the BBB, and it has a longer duration of action, reducing dosing frequency [82]. In a small trial, treatment of healthy elderly volunteers ($n = 32$) with phenserine tartrate was well tolerated, with dose-limiting adverse effects reported at doses $\geq$20 mg [83]. (−)-Phenserine, a more potent AChEI than the (+)-enantiomer [84], improved short-term memory in a placebo-controlled phase II study in 72 AD patients, when administered at 5 mg twice daily for 2 weeks, then 10 mg twice daily for 10 weeks [85]. It also improved cognitive functions in a small trial with 20 AD patients (30 mg/day) [86]. However, a phase III trial showed no difference in efficacy from placebo [87]. The (+)-enantiomer (Posiphen®) has reached the phase II trial stage [10]. The principal mode of action of (+)-phenserine is inhibition of APP synthesis, with associated reductions in β-amyloid (Aβ) and β-secretase levels [45, 88]. Various methyl analogues of phenserine have also been investigated for AChE and BChE inhibition and selectivity [89]. The C2′ methyl substitute of phenserine, tolserine (**17**), is a more selective inhibitor of AChE than BChE [87]. Tolserine is a potential drug candidate, as it is more hydrophobic than phenserine, augmenting transport across the BBB and is a potent and selective AChEI with



12 Physostigmine

13 Rivastigmine

14 Eptastigmine

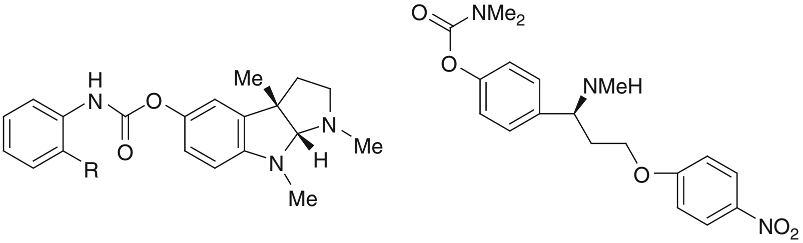
15 Ganstigmine

Scheme 42.4 Physostigmine and synthetic derivatives that inhibit cholinesterase

a long duration of action [90]. In addition to ChE inhibition, other modes of action are associated with physostigmine and derivatives and are summarized in Table 42.1.

Other compounds based on the structure of physostigmine that inhibit BChE (BChE inhibition is also considered a relevant AD treatment strategy [91]) more selectively than AChE include cymserine [92, 93], bisnorcymserine (*N*-demethylated cymserine) [94], dihydrobenzodioxepine cymserine [95], and tetrahydrofurobenzofuran cymserine [96]. Some cymserine analogues improved cognitive performance in aged rats [44]. Other synthetic carbamates are also under investigation as ChEI drug candidates [97].

To target both cognitive symptoms and BPSD, pharmacomodulation of carbamate derivatives has been attempted to produce compounds with dual modes of action. These include derivatives that incorporate structural features of physostigmine and the propargylamine pharmacophore of selegiline to produce compounds that inhibit both AChE and monoamine oxidase (MAO), although these compounds appear to lack therapeutic potential due to poor oral bioavailability and limited transport across the BBB [98]. Other compounds combine structural features of rivastigmine and fluoxetine to inhibit both AChE and the serotonin transporter, with one such compound, RS-1259 (BGC20-1259) (**18**), having completed phase I studies in healthy volunteers [99] (Scheme 42.5).



16 R = H Phenserine
17 R = Me Tolserine

18 RS-1259

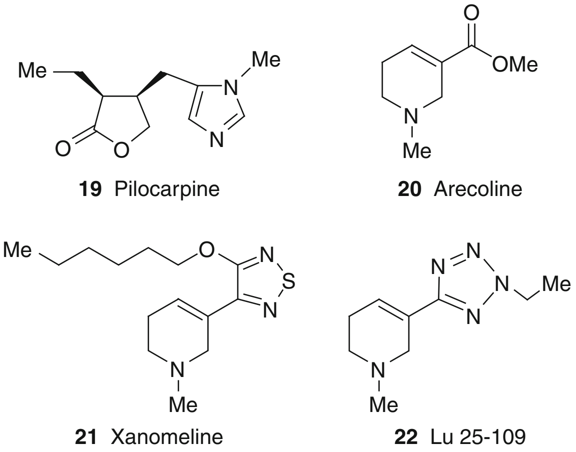
Scheme 42.5 Synthetic derivatives of physostigmine

3 Alkaloids and Derivatives as Ligands at Cholinergic Receptors

3.1 Muscarinic Receptor Ligands

The primary mode of action of mAChR ligands is to modulate cholinergic function, but some M_1 mAChR ligands may also reduce amyloidosis and decrease phosphorylated tau [100, 101]. Alkaloid ligands for the mAChR have been discovered from natural sources; although for many, toxicity appears to have limited their potential for clinical use. The alkaloid muscarine was first isolated from the fly agaric fungus *Amanita muscaria* (L.) Lam. (Amanitaceae) and also occurs in *Inocybe* (Inocybaceae) and *Clitocybe* (Tricholomataceae) species [102]. Although it is an agonist at mAChRs, along with the *Amanita* isoxazole alkaloids muscimol and ibotenic acid, it is considered to contribute to the psychotropic and other toxic effects of ingestion of *Amanita* species [102]; thus, *Amanita* alkaloids have not been pursued for clinical development in cognitive disorders. However, the chemical structures of other alkaloids that are mAChR ligands, such as pilocarpine (**19**) and arecoline (**20**), have provided templates for the development of semisynthetic/synthetic mAChR ligands aimed to modulate cholinergic function, with more appropriate pharmacokinetic and adverse effect profiles.

The use of *Pilocarpus* species (Rutaceae) as traditional medicines, particularly *P. microphyllus* Stapf ex Wardleworth and *P. jaborandi* Holmes, dates back to 1570 in Brazil, where *Pilocarpus* species were used to treat mouth ulcers, and around 100 years later, they were documented as remedies for various other purposes, including as an antidote to plant toxins [103]. The leaves of *Pilocarpus* species contain imidazole alkaloids including pilocarpine [104], an agonist at mAChRs, that has a chemical structure with features similar to those of ACh [105]. Although pilocarpine enhances cognitive functions in vivo [106–108], systemic use may cause adverse effects such as nausea, vomiting, sweating, and palpitations [109], and ocular administration may induce or exacerbate CNS symptoms in AD patients



Scheme 42.6 Muscarinic receptor ligand alkaloids and synthetic derivatives

[110, 111]. Among the synthetic pilocarpine derivatives, a thiolactone analogue, SDZ ENS 163 was suggested to be centrally active via agonism at M_1 and antagonism at M_2 mAChRs, with the advantage of no peripheral adverse effects [112]. However, this analogue was unsuccessful in reversing scopolamine-induced cognitive impairment in normal (non-AD) humans [113].

Arecoline is a reduced pyridine alkaloid derivative from *Areca catechu* L. (Arecaceae) (commonly known as betel nut) [104]. An agonist at mAChRs, arecoline may enhance cholinergic function [114, 115], explaining why it could improve scopolamine-induced cognitive impairments in vivo [116, 117] and cognitive functions and recognition skills in AD patients [118, 119]. However, arecoline is genotoxic and neurotoxic [120] and is associated with other therapeutic limitations. These include bioavailability issues, a short duration of action, and peripheral cholinergic effects [105]; consequently, numerous analogues have been synthesized. These include several morpholino and thiazolidinone arecoline derivatives, which bind to M_1 mAChRs and improve learning and memory in vivo [121, 122]. A synthetic analogue of arecoline is xanomeline (**21**), which contains a thiadiazole moiety in its structure instead of the labile ester moiety in arecoline. Although xanomeline improved cognition in clinical trials via M_1 mAChR agonism, in addition to alleviating BPSD (possibly via M_4 mAChR-mediated antidopaminergic activity), cholinergic adverse effects make this analogue unsuitable as a dementia treatment [7]. The fluorinated xanomeline derivative EUK1001 improved cognition in aged mice [123] and in an animal AD model (presenilin double knockout mice) [124]. Another arecoline derivative is Lu 25–109 (**22**), an M_1 mAChR partial agonist and M_2 mAChR antagonist, but this analogue failed to improve cognition in patients with mild to moderate AD and caused adverse effects such as nausea, diarrhea, and anorexia, when investigated in a 6-month double-blind, placebo-controlled trial [125] (Scheme 42.6). Numerous other functionally selective M_1 mAChR agonists, including those derived from alkaloids, have also been considered as potential treatments for cognitive disorders [7] but appear to be generally unsuccessful due to limited efficacy and unacceptable adverse effects.

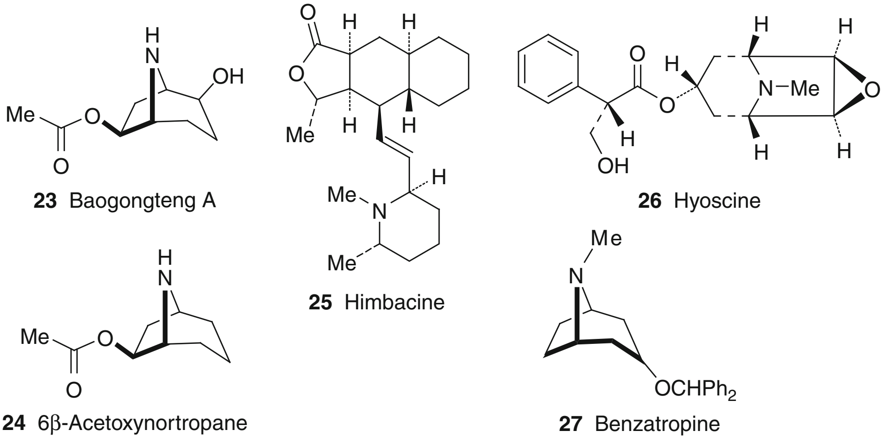
Although some tropane alkaloids (including atropine/hyoscyamine and hyoscine [scopolamine]) from *Atropa* and *Hyoscyamus* species, and other genera in the Solanaceae [104], are known mAChR antagonists [105], the nortropane alkaloid baogongteng A (**23**) from the stem of *Erycibe obtusifolia* Benth. (Convolvulaceae) is reported to be an mAChR agonist [126], although it does not appear to have been assessed for efficacy in cognitive dysfunction. The synthetic derivative 6β-acetoxynortropane (**24**) is considered a selective M_2 mAChR agonist, and it improved cognitive function in vivo [126]. Other derivatives of baogongteng A have been synthesized as mAChR agonists [127] but have yet to be further characterized pharmacologically. Other alkaloid derivatives under investigation as M_1 mAChR agonists for neurodegenerative disorders are the huprines, hybrid structures of the alkaloid huperzine A and tacrine [10] (see Sect. 2.2).

Compounds that antagonize M_2 mAChRs can increase ACh release and improve cognition, although may also cause tachycardia due to their effects on cardiac M_2 mAChRs [6]. *Galbulimima belgraveana* (F.Muell.) Sprague (synonym is *G. baccata* F.M.Bailey [Himantandraceae]) bark is a source of the pyridine alkaloid himbacine (**25**) [128], a selective antagonist at M_2 and M_4 mAChRs [105]. Most synthetic analogues of himbacine do not appear to show advantages over himbacine with regard to M_2 mAChR binding affinity [7], with limited data on analogues (e.g., 3-norhimbacine) that do show higher M_2 mAChR binding affinity [129].

With respect to PD, antimuscarinic compounds are used therapeutically to reduce the effects of the relative central cholinergic excess that occurs with dopamine (DA) deficiency, but since they are associated with cognitive impairment, they are generally not used for idiopathic PD [109] and may be unsuitable for use in those with PDD. Anticholinergic tropane alkaloids, such as hyoscine (**26**) and hyoscyamine, that occur in members of the Solanaceae (e.g., *Atropa*, *Hyoscyamus*, *Datura*, and *Duboisia* species) have been used to alleviate PD symptoms since they are antagonists at mAChRs but are not used routinely for this purpose [130], although synthetic derivatives such as benzatropine (**27**) are occasionally used to provide mild symptomatic control in PD [131] (Scheme 42.7).

3.2 Nicotinic Receptor Ligands

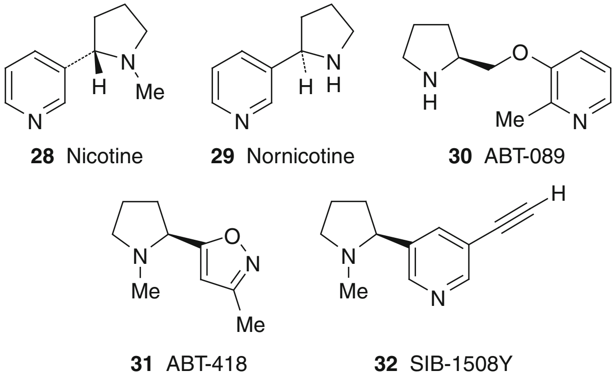
The pyridine and pyrrolidine alkaloid nicotine (**28**) (occurs in *Nicotiana* species [Solanaceae]) has shown numerous mechanistic effects relevant to neurodegenerative diseases. Nicotine can modulate cholinergic transmission via interaction with nAChRs, but may be more effective for attention deficits rather than memory, and more effective for MCI due to preservation of nAChRs compared to AD [3]. In addition to effects on cognitive functions in vivo, nicotine inhibits amyloidogenesis at different stages, is neuroprotective, upregulates nerve growth



Scheme 42.7 Muscarinic receptor ligand alkaloids and synthetic derivatives

factor (NGF) pathways, inhibits apoptosis, and reduces oxidative stress [1, 13, 50]. However, nicotine is reported to increase aggregation and phosphorylation of tau [13], which would be undesirable with respect to neurodegeneration. Early epidemiological data suggested smoking tobacco (*N. tabacum* L.), a source of nicotine, could protect against AD [50], which appeared to support the documented mechanistic effects of nicotine. Subsequent studies show smoking either has no effect or perhaps increases AD risk [3]. An inverse relationship between smoking and PD risk has been concluded; however, effects of tobacco smoking on PDD or LBD risk have not been determined, and the effects of smoking on risk of VaD are complicated, due to the association of smoking with cardiovascular disease [50]. With respect to PD, nicotine facilitates DA release by acting at somatodendritic and presynaptic nAChRs on mesolimbic and nigrostriatal neurons [132] and inhibits formation of α-synuclein fibrils in vitro [2]. The tobacco alkaloid nornicotine (**29**) is also an agonist at nAChRs, and it stimulates DA release from dopaminergic presynaptic nerve terminals [133]. Clinical trials investigating the effect of nicotine on cognitive performance in PD subjects have not consistently shown nicotine to improve cognition [134].

To optimize the therapeutic and pharmacokinetic profile, numerous nAChR agonists have been developed using nicotine as a template structure. Nicotine analogues that contain the pyrrolidine ring in their structure have been explored for their nAChR binding and cognitive effects, including ABT-089 (**30**) and ABT-418 (**31**). ABT-089, a pyridyl ether, is a partial agonist at $\alpha_4\beta_2$ and $\alpha_6\beta_2$ nAChRs, and it is neuroprotective; it reversed scopolamine-induced cognitive deficits in healthy volunteers but lacked efficacy in a phase II RCT in patients with mild to moderate AD [135, 136]. ABT-418, an $\alpha_4\beta_2$ nAChR agonist containing an isoxazole in place of the pyridine moiety of nicotine, reduced anxiety and enhanced cognition in animal models; the latter effect was also reported in an acute study with AD patients, but efficacy was not apparent in a larger placebo-controlled trial [137, 138]. SIB-1508Y (5′-ethynyl nicotine) (**32**) is an nAChR agonist that stimulates release of DA from rat



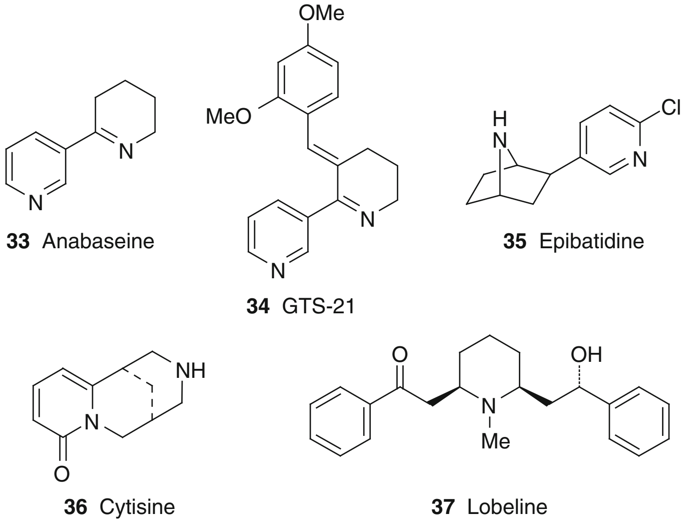
Scheme 42.8 Nicotinic receptor ligand alkaloids and synthetic derivatives

striatum, shows efficacy in animal models of PD, and has been investigated in clinical trials for PD [137, 139] (Scheme 42.8).

In addition to nicotine, minor alkaloids in tobacco modulate DA release from rat striatal slices and are suggested to be neuroprotective [140]. One of these alkaloids is anabaseine (**33**), which also occurs in some marine worms (Phylum *Nemertinea*) and aphaenogaster ants, but it is a relatively nonselective nAChR agonist [141–143]. A series of anabaseine derivatives have been associated with α_7 nAChR agonist and memory-related effects, with (*E*,*E*)-3-(2-methoxy-cinnamylidene)-anabaseine identified as the most potent in improving passive-avoidance behavior in rodents; (*E*,*E*)-3-(cinnamylidene)-anabaseine was also neuroprotective [144]. GTS-21 (3-(2,4-dimethoxybenzylidene)-anabaseine) (**34**) is a more hydrophobic derivative of anabaseine (thus favoring BBB permeability) that shows high affinity and partial antagonist activity for $\alpha_4\beta_2$ nAChRs and partial agonist activity at α_7 nAChRs [135, 143, 145]. In addition to neuroprotective activity in vitro, GTS-21 enhanced cognitive behaviors in vivo [135, 145]. GTS-21 improved cognitive functions in phase I clinical trials with healthy volunteers [145], and it has reached the phase II trial stage in AD patients [10], but outcomes are yet to be reported.

Alkaloids with affinity for nAChRs have been isolated from the frog, *Epipedobates tricolor* (Dendrobatidae). Most pharmacological studies have focused on one of these alkaloids, epibatidine (**35**), as it is antinociceptive and toxic, with selectivity for $\alpha_4\beta_2 > \alpha_3\beta_4 > \alpha_7$, $\alpha_1\beta_{1\gamma}\delta$ nAChRs; numerous synthetic analogues based on the structure of epibatidine are also reported [146]. Epibatidine increases DA output in the nigrostriatal pathway, and it is presumed to desensitize nAChRs, controlling the hyperactivity of this pathway that is associated with motor complications [147] (see Sect. 4), thus suggesting it may have some relevance as a lead compound for drug development in PD.

Numerous other alkaloids from natural origin are known ligands at nAChRs, such as the tropane alkaloid ferruginine (and several synthetic derivatives) [146, 148, 149], although there are limited data on their therapeutic potential in neurodegenerative diseases. Other alkaloids include the quinolizidine alkaloid cytisine (**36**) (occurs in



Scheme 42.9 Nicotinic receptor ligand alkaloids and the synthetic anabaseine derivative GTS-21

Sophora species [Leguminosae]) and the piperidine alkaloid lobeline (**37**) (from *Lobelia inflata* L. [Campanulaceae]), which have high affinity for $\alpha_4\beta_2$ nAChRs; lobeline also improves learning and is anxiolytic in vivo [137, 150, 151] (Scheme 42.9). Cytisine and derivatives may also be of therapeutic interest for PD (see Sect. 4). Huperzine A and tacrine hybrid structures (huprines) are also under investigation as nAChR agonists for AD, in addition to their documented ChE inhibitory and M_1 mAChR agonistic properties [10] (see Sect. 2.2).

4 Alkaloids and Derivatives that Modulate Dopaminergic Function

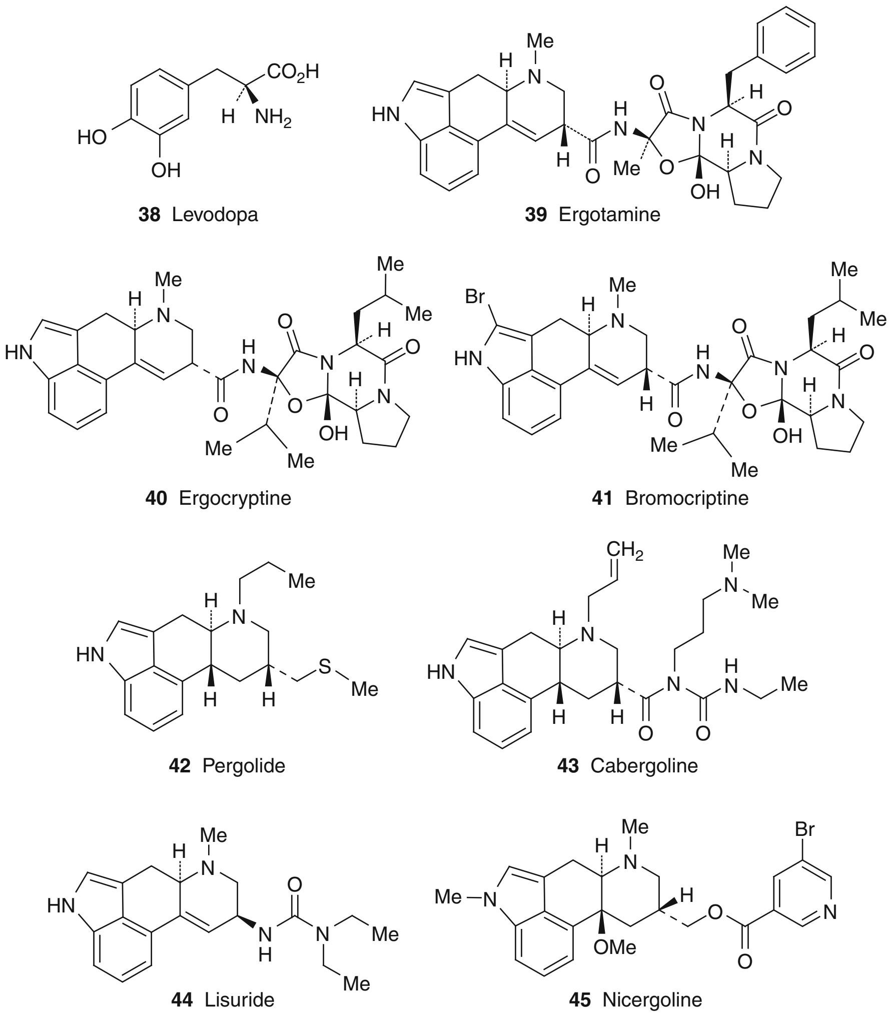
Dopamine (DA) agonists stimulate DA receptors directly to modulate dopaminergic function and reduce PD symptoms related to dopaminergic deficits. Levodopa (L-DOPA) (**38**) therapy aims to correct the striatal dopaminergic deficit in PD, and it is one of the principal drugs used to alleviate symptoms. Levodopa is also used for symptomatic treatment in Huntington’s disease [152]. It is a precursor of DA and is converted into DA via decarboxylation in the CNS; this conversion also occurs peripherally, thus extracerebral decarboxylase inhibitors are frequently prescribed in addition to levodopa to minimize peripheral adverse effects such as nausea and vomiting [109]. Levodopa has been detected in some members of the Leguminosae, including broad bean (*Vicia faba* L.) seedlings, and when extracts from *V. faba* were administered to rats, free DA was detected in the striatum, and urinary metabolites were consistent with those observed with levodopa administration [153].

V. faba beans have been suggested as an adjunctive therapy for PD, and short-term benefits have been observed in PD patients consuming *V. faba* beans [2], although evidence for efficacy is limited and their regular consumption has been associated with complications such as hemolytic anemia [154]. Levodopa also occurs in *Mucuna pruriens* (L.) DC., also known as the velvet bean [2]. An extract from beans of *M. pruriens* attenuated 6-hydroxydopamine (6-OHDA)-induced dopaminergic loss when administered to rats [2], although a later study showed an extract from *M. pruriens* beans could not significantly antagonize degeneration of dopaminergic neurons in a rat model of PD [155]. Levodopa as a treatment for PD is associated with motor complications [109], and other DA agonists have been developed to minimize adverse effects and to improve on the relatively short elimination half-life of levodopa [131].

The ergot fungus, *Claviceps purpurea* (Fr.) Tul. (Clavicipitaceae), can infect cereal crops such as rye (*Secale cereale* L. [Poaceae]), and it is a known poison [130]. The toxicity is due to the presence of indole alkaloids such as ergotamine (**39**) that cause vasoconstriction. The ergot alkaloids, such as ergocryptine (**40**), interact with DA receptors and may also increase neuronal DA release [156]. Synthetic derivatives of these alkaloids have shown dopaminergic activity, thus have been developed for clinical use in PD. These include the D_2 receptor agonists bromocriptine (**41**), pergolide (**42**), cabergoline (**43**), and lisuride (**44**) [130]. Neuroprotective properties have also been suggested for the DA receptor agonists, including bromocriptine and pergolide [131, 157]; bromocriptine protects against 6-OHDA and MPTP-induced cell loss and attenuates DA depletion in mouse striatum; pergolide upregulates superoxide dismutase in basal ganglia [158]. Bromocriptine also induces neurite outgrowth in vitro [159]. Although there is some evidence for their efficacy in PD, the use of these drugs has been limited by pulmonary, retroperitoneal, and pericardial fibrotic reactions [109]. Another synthetic ergot alkaloid derivative is nicergoline (**45**), and it enhances cognition in dementia; mechanistic effects include cholinergic activity and modulation of APP processing; it is also neuroprotective against Aβ [75] (Scheme 42.10).

The indole alkaloid ibogaine (**46**), from *Tabernanthe iboga* Baill. (Apocynaceae), enhances DA release in isolated striatal tissue and binds to NMDA receptors to prevent glutamate-induced cell death in hippocampal cultures [130, 160]. However, it has psychoactive and memory-altering effects, in addition to being tremorigenic at high doses [160], thus would be an unsuitable drug candidate for neurodegenerative diseases unless the alkaloid structure is modified to improve the adverse effect profile.

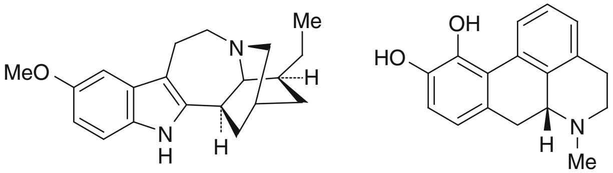
The opium poppy (*Papaver somniferum* L. [Papaveraceae]) latex contains benzylisoquinoline alkaloids and is a widely known source of the analgesic drugs morphine and codeine. The biosynthesis of benzylisoquinoline alkaloids begins with a condensation reaction catalyzed by norcoclaurine synthase of DA [161]. The structural features of benzylisoquinoline alkaloids derived from DA might provide some explanation for the documented affinity of some natural alkaloids of this class, and some synthetic derivatives, for DA receptors [162]. Indeed, the chemical structure of morphine has been used as a template for the development of the PD drug, apomorphine (**47**). Apomorphine includes a catecholaminergic moiety in its


38 Levodopa
39 Ergotamine
40 Ergocryptine
41 Bromocriptine
42 Pergolide
43 Cabergoline
44 Lisuride
45 Nicergoline

Scheme 42.10 Alkaloids and synthetic derivatives with affinity for dopamine receptors

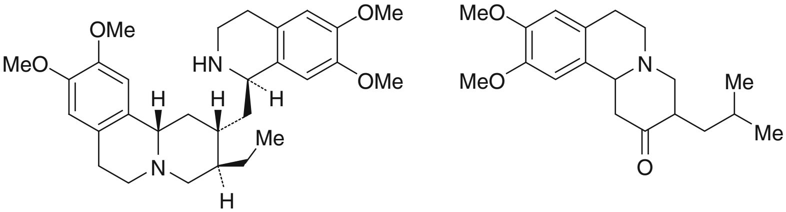
structure, similar to that in DA, and has D_1 and D_2 receptor agonist activity [130]. It is also suggested to be neuroprotective [157]; it protects neuronal cells against H_2O_2 and 6-OHDA in vitro via antioxidant effects [163]. It is effective at reducing symptoms in PD when administered subcutaneously, but its use may be limited by high first pass metabolism, so is unsuitable for oral administration, and by adverse effects such as nausea and vomiting [2]. Numerous synthetic analogues of apomorphine have been investigated [162], but none appear to have reached more advanced stages of drug development for PD (Scheme 42.11).

Cytisine (**36**) (see Sect. 3.2) and its derivative 5-bromocytisine significantly prevented the decrease of striatal DA loss induced by 6-OHDA in rats [164];



46 Ibogaine **47** Apomorphine

Scheme 42.11 The alkaloid ibogaine and the synthetic apomorphine, which modulate dopaminergic function



48 Emetine **49** Tetrabenazine

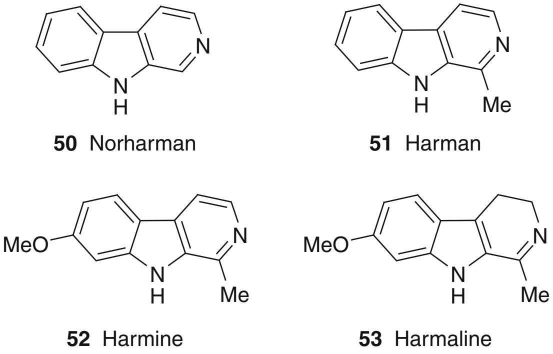
Scheme 42.12 Emetine and the synthetic derivative drug tetrabenazine

although 3-bromocytisine did not produce this effect [164], it increased motor activity in rats via effects on nAChRs in the striatum to modulate dopaminergic function [165]. These results suggest cytisine, or its bromo-derivatives may be relevant to explore as lead compounds for PD. Another nAChR ligand is epibatidine (**35**) (see Sect. 3.2), which enhances DA output in the terminal areas of the nigrostriatal and mesolimbic pathways, and it enhances locomotor activity [147].

Other alkaloids are reported to indirectly modulate dopaminergic function by neuroprotective effects and include *N*-fatty acyl derivatives of tryptamine that occur in some members of the Annonaceae (e.g., *Annona muricata* L.). When synthesized, these tryptamine-derived alkaloids (which occur at low levels in plant sources) were neuritogenic and neuroprotective in dopaminergic neurons in vitro [166].

Emetine (**48**) is an isoquinoline derived alkaloid from *Cephaelis* species (Rubiaceae) [104]. It was first isolated in 1817 [167] and was the lead compound for the development of the drug tetrabenazine (**49**). Tetrabenazine was originally developed as an antipsychotic drug in the 1960s, but in 2008 was approved by the US Food and Drug Administration for use in Huntington's disease chorea [168, 169]. The mode of action of tetrabenazine is reversible inhibition of the human vesicular monoamine transporter 2 to deplete monoamines, including DA, from nerve terminals [168]. Consequently, adverse effects can include extrapyramidal dysfunction and parkinsonism [109] (Scheme 42.12).

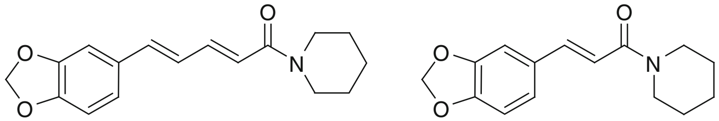
Scheme 42.13 Monoamine oxidase inhibitory alkaloids



5 Monoamine Oxidase Inhibitory Alkaloids and Derivatives

Inhibitors of the enzyme monoamine oxidase B (MAO-B) can increase the concentrations of both endogenous DA and exogenously administered levodopa by blocking the metabolism of DA in the CNS [131]. The indole β-carboline alkaloids, norharman (**50**) and harman (**51**), from natural products that include coffee (*Coffea arabica* L. [Rubiaceae]; for other *C. arabica* alkaloids, see Sect. 7), tobacco (*Nicotiana* species [Solanaceae]), and raisins (*Vitis vinifera* L. [Vitaceae]), inhibit MAO-A and -B, with the latter showing selectivity for MAO-A [9, 170, 171]; lack of selectivity for MAO-B may result in additional adverse effects due to MAO-A inhibition; thus, these alkaloids may not be suitable to pursue as drug leads for PD. There are reports of the roots from *Banisteriopsis caapi* (Spruce ex Griseb.) Morton (Malpighiaceae) being used to treat PD patients in Ecuador, which may be explained by the occurrence of β-carboline alkaloids (harmine (**52**) and harmaline (**53**)) in this species, which are MAO inhibitors (MAOIs) and they stimulate DA release from striatal cells [130]. However, harmine is selective for MAO-A [9] and not MAO-B, the target enzyme to inhibit in PD. Although harmine is also associated with causing convulsant effects and is hallucinogenic, it has been shown to increase glutamate transporter expression in vivo [172]. Thus, harmine may provide a lead chemical structure to develop compounds with MAOI activity that also modulate glutamatergic function in neurodegenerative diseases (Scheme 42.13).

Piperine (**54**) and antiepilepsirine (**55**), piperidine alkaloids that occur in *Piper* species (Piperaceae), are MAOIs with greater selectivity for MAO-B [173], yet they have not been studied extensively for therapeutic potential in PD. Some *Vinca* alkaloids, including vincristine, vinblastine, leurosine, and vindoline from *Catharanthus roseus* (L.) G.Don (Apocynaceae), are reported as selective MAO-B inhibitors, but cytotoxicity limits their development as potential drug candidates for PD [174, 175] (see Sect. 6) (Scheme 42.14).



54 Piperine **55** Antiepilepsirine

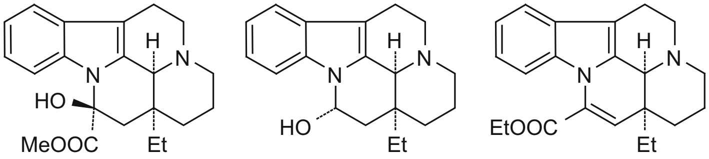
Scheme 42.14 Piperidine alkaloids that inhibit monoamine oxidase B

6 *Vinca* Alkaloids and Derivatives

Although some indole alkaloids from genera in the Apocynaceae are known to be cytotoxic, including the anticancer drugs vincristine and vinblastine from *Catharanthus roseus* (L.) G.Don (the Madagascan periwinkle; synonym is *Vinca rosea* L.) [104], and alkaloid fractions from *Vinca minor* L. (the lesser periwinkle) are cytotoxic [176]; some indole alkaloids derived from *V. minor* have shown potential for use in neurodegenerative diseases, particularly VaD. Mechanistically, *Vinca*-derived alkaloids are reported to be neuroprotective (including against hypoxia and ischemia), to modulate neurotransmitter (including ACh and DA) release and to stimulate cerebral metabolism, with vincamine (**56**), and the derivative vincanol (**57**) also reported to block voltage-gated Na^+ channels [177–179]. Vincamine and the synthetic derivative vinpocetine (**58**) also enhance cerebral blood flow, reduce cerebral insufficiency due to ischemia, and improve short-term memory [177, 180]. Vinpocetine and its metabolite, *cis*-apovincaminic acid, also protect against NMDA-induced neurotoxicity [181]. In addition, vinpocetine could improve learning and memory, possibly via cholinergic, neuroprotective, and antioxidant mechanisms, in an animal model of AD [182]. Studies in humans have focused on the effects of vinpocetine, which increased cerebral blood flow and improved cognitive functions in patients with MCI in a 12-week pilot study [179]. Double-blind placebo-controlled trials suggest vinpocetine is also efficacious in neurodegenerative dementia and VaD [177]. Although vinpocetine appears to show efficacy in dementia, particularly VaD, trial design flaws make promising data inconclusive [183, 184]; thus, it has been concluded there is insufficient evidence to support the use of vinpocetine in dementia [185] (Scheme 42.15).

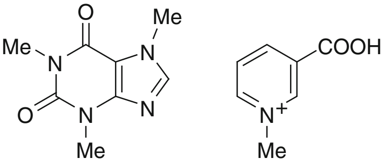
7 *Coffea arabica* L. Alkaloids and Derivatives

Epidemiological data suggest that dementia (AD in particular) risk is reduced in coffee (*Coffea arabica* L. [Rubiaceae])/caffeine (**59**) drinkers, compared with no or low coffee intake [186–188], although other studies do not support this conclusion since any protective effect of coffee or caffeine consumption against AD was not evident [189, 190]. Studies also suggest a high intake of coffee or caffeine is associated with a lower incidence of PD [2, 191], and an open-label pilot study



56 Vincamine **57** Vincanol **58** Vinpocetine

Scheme 42.15 *Vinca*-derived alkaloids



59 Caffeine **60** Trigonelline

Scheme 42.16 *Coffea arabica* alkaloids

with 25 PD subjects also indicated caffeine may improve some motor and nonmotor symptoms of PD [192].

Any effect of coffee on neurodegenerative disease risk therefore requires further evaluation, and the phytochemicals in coffee that might contribute to such an effect would also need to be assessed. Trigonelline (**60**) is a pyridine alkaloid that occurs in coffee, and it increases neurite outgrowth, inhibits AChE in vitro, and it improves memory retention in vivo [193, 194] (Scheme 42.16).

Most studies on coffee in relation to neurodegenerative diseases have focused on another alkaloid component, caffeine. This purine alkaloid produces several mechanistic effects that could be relevant to neurodegenerative diseases. Both coffee and caffeine are associated with neurodegenerative disease-modifying effects via reduction of Aβ [195], in addition to the CNS stimulant and cognitive enhancing effects [186, 188, 195]. Caffeine is also antagonistic at adenosine A_{2A} receptors, an effect that inhibits glutamate release and attenuates excitotoxicity, thus is neuroprotective [196]. Caffeine attenuates dopaminergic deficits and protects against neuronal degeneration in animal models of PD and AD, respectively [196]. In addition, caffeine protects against BBB disruptions in animal models of AD and PD, which may be protective against disease progression since BBB dysfunction has been implicated in the pathogenesis of neurodegenerative diseases [196]. Some synthetic caffeine analogues show dual modes of action relevant to PD. Analogues of (*E*)-8-(3-chlorostyryl) caffeine and of (*E*,*E*)-8-(4-phenylbutadien-1-yl) caffeine inhibit MAO-B and are antagonists at adenosine A_{2A} receptors [197, 198], thus have the potential to modulate dopaminergic neurotransmission and to provide neuroprotection in PD.

8 Conclusion

There are many examples of drugs that are derived from alkaloids, including those with CNS activities [199]. Of the drugs developed for neurodegenerative diseases, alkaloids have been a valuable source of lead compounds. The development of two ChEI drugs (galantamine and rivastigmine) from plant alkaloids to alleviate symptoms in AD or PD has set a precedent for drug discovery. Numerous other ChEI alkaloids of natural origin have been discovered [11–13], with many showing other mechanistic effects not related to ChE inhibition but relevant to neurodegenerative diseases (examples in Table 42.1). For PD, numerous alkaloids display activities relevant to modulating dopaminergic neurotransmission, with some ergot alkaloids providing template chemical structures for the development of the D_2 receptor agonist drugs such as bromocriptine; and for VaD, the *Vinca*-derived alkaloids such as the synthetic vinpocetine have shown promising although inconclusive results in controlled clinical trials.

Although alkaloids and their synthetic derivatives with mechanistic effects relevant to treating neurodegenerative diseases are widely reported, relatively few have reached the clinical trial stage of drug development. This may reflect the decline in natural product drug discovery by challenges such as reluctance from an industry perspective to invest in the costs of patenting, and technical issues such as isolation of a sufficient yield of a compound from a limited resource, or complex routes for total synthesis. However, since current treatment strategies for neurodegenerative diseases are limited, there is a need to discover new drugs that are more therapeutically successful with fewer adverse effects than those currently available, and natural sources continue to be a vast resource of alkaloids yet to be fully explored as new drug leads to treat neurodegenerative diseases.

From an epidemiological perspective, smoking tobacco that contains nicotine has been associated with a reduced risk of PD, and caffeine consumption in the diet has been suggested to reduce the incidence of AD and PD. Any protective effects that these or other alkaloids have against neurodegenerative diseases require further assessment, yet epidemiological data may be useful to determine the role of alkaloids in the treatment and prevention of neurodegenerative diseases.

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Opioids and Pain Treatment

43

Daniel Krashin, Andrea Trescot, and Natalia Murinova

Contents

1 Introduction 1368
2 Pharmacological Applications 1370
  2.1 Analgesia 1370
3 Mechanism of Action 1371
  3.1 Tolerance 1371
4 Toxicity 1373
  4.1 Physical Toxicity 1373
  4.2 Behavioral Toxicity 1375
5 Bioavailability and Metabolism 1375
  5.1 Morphine 1375
6 Other Biological Activities 1378
  6.1 Gastrointestinal 1378
  6.2 Antitussive 1378
  6.3 Antihypertensive 1378
7 Clinical Trials 1378
  7.1 Opioids for Acute Pain 1378
  7.2 Opioids for Chronic Pain 1379
  7.3 Opioids for Cancer Pain and Palliative Care 1379
8 Conclusion 1380
References 1380

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Abstract

Opiates are medications derived from the poppy plant that attach to opioid receptors in the brain. The extraction of morphine from opium marked the beginning of organic alkaloid chemistry and has led to the synthesis of a variety of semisynthetic and synthetic analgesics. Opium and its derivatives have been used for thousands of years not only for pain relief but also for the euphoria that they produce. It is this dual effect that allows them to be so useful but also addictive and therefore dangerous. Understanding their structure, action, use, and abuse reflects the close connection that we have with the plant world.

Keywords

Addiction • analgesia • codeine • laudanum • morphine • opiate • poppy • thebaine

Abbreviations

CGRP	Calcitonin gene-related peptide
CYP	Cytochrome P50
GABA	Gamma aminobutyric acid
NMDA	N-methyl-D-aspartate
NSAIDs	Nonsteroidal anti-inflammatory drugs
PCA	Patient-controlled analgesia
SNP	Single-nucleopeptide polymorphone

1 Introduction

Papaver alkaloids are compounds extracted from *Papaver somniferum*, the opium poppy. The historical record shows that the psychoactive properties of this plant have been known for millennia. Around 3400 BCE, the Sumerians described the poppy as *Hul Gil*, “the joy plant,” [1] and it was well known to the ancient Egyptians, Babylonians, Minoans, and Greeks [2]. In 1300 BCE, in Thebes, the Egyptians cultivated a poppy variety known as *opium thebaicum*, which they traded all over the known world. Hippocrates, the “father of medicine,” acknowledged opium’s usefulness as a pain reliever [3]. While the seeds and oil of the poppy plant are also consumed, the most important product is certainly opium, produced by scoring the unripe seed pods. The exuded latex “tears” of yellowish material, which darkens when dried, can be scraped off and collected. This bitter, granular material can be eaten, smoked, or made into a tincture with alcohol, known as laudanum. Laudanum was first described by the author Paracelsus (about 1522) [4], who recommended using it only sparingly, but laudanum later became a very popular remedy for numerous conditions in European medicine.

In 1805, a German pharmacist, Friedrich Sertürner, first reported that he had isolated an alkaloid substance from opium [5]. This isolation, produced by boiling the opium followed by immersing it in ammonia, yielded an insoluble substance. This

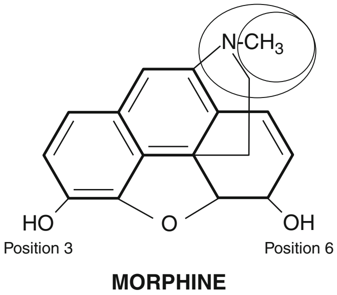


Fig. 43.1 Structure of morphine (Reprinted with permission from Trescot AM, Datta S, Lee M, Hansen H (2008) Opioid pharmacology. Pain Physician 11:S133–S53)

was the first example of the successful isolation of the "natural product," or active portion, of a plant-based medicine. He named this compound "morphium" in 1817, for Morpheus, the Greek god of sleep, after experimenting with its effects. The French chemist Joseph Guy-Lussac changed this to "morphine" upon translation of Sertürner's article into French, suggesting that "-ine" be used as a suffix to denote an organic base. The very existence of organic bases was widely disputed at the time, and this new substance fascinated many chemists of the era, though determination of the formula and synthesis were not accomplished until the mid-twentieth century.

The discovery of morphine marked the beginning of organic alkaloid chemistry, and the further analysis of papaver alkaloids proceeded quickly. There are over 50 papaver alkaloids in all, nitrogenous organic bases, but they can be divided into two major classes of papaver alkaloids – the isoquinolines and phenanthrenes. The phenanthrenes are the most medically significant, with morphine, codeine, and thebaine acting as important opioid agonists.

Morphine makes up about 10–20% of the mass of opium. Morphine (the archetypal opioid) consists of a benzene ring with a phenolic hydroxyl group at position 3 and an alcohol hydroxyl group at position 6 and at the nitrogen atom (Fig. 43.1). Both hydroxyl groups can be converted to ethers or esters. For example, codeine is morphine that is O-methylated at position 3, while heroin is morphine O-acetylated at position 3 and 6 (diacetyl morphine). The tertiary form of the nitrogen appears to be crucial to the analgesia of morphine; making the nitrogen quaternary greatly decreases the analgesia, since it cannot pass into the central nervous system. Changes to the methyl group on the nitrogen will decrease analgesia as well, creating opioid antagonists.

The second most common alkaloid in opium is codeine, at 0.5–1% of total mass. Codeine is a weak opioid, and is commonly used as an analgesic, sometimes in combination with acetaminophen. The third most common alkaloid is thebaine, at 0.3–1%. Thebaine has similar chemistry but very different biological effects than morphine or codeine, causing dysphoria and agitation in low doses and seizures in high doses. Thebaine is commonly used as a precursor for synthetic opioids. Papaverine is also about 1% of opium, and is a smooth muscle relaxant with little CNS effect. It should be noted that many specialty varieties of *P. somniferum* have

Table 43.1 Papaver alkaloids

Compound	% Opium	Action	Use
Morphine	10–20%	Analgesic	Pain relief
Codeine	0.5–1%	Weak analgesic	Pain relief, cough, diarrhea
Thebaine	0.3–1%	Dysphoria	Synthetic opioid precursor
Papaverine	1%	Smooth muscle relaxer	

been bred, some of which produce higher levels of one alkaloid or another, some that have been bred to minimize alkaloid production (Table 43.1).

2 Pharmacological Applications

The primary modern use of the papaver alkaloids is for analgesia. Morphine is considered the classic example of a high-potency opioid. *Opiates* are medications derived from the poppy; *opioids*, the more general term, refer to medications that work at the opioid receptors. All opiates are therefore opioids, but not all opioids are opiates.

2.1 Analgesia

2.1.1 Morphine

Morphine is still very widely used for analgesia. Indeed, it has been recommended by the World Health Organization as the standard opioid for cancer pain [6], since it is widely available and inexpensive. In the W.H.O. pain ladder, first published in 1986, the first step in pain management is described as adjuvant medications. The second step is weak opioids, such as codeine, while the third step is high-potency opioids such as morphine.

Morphine is available in a great variety of formulations, including short-acting and long-acting oral forms, as well as vaginal and rectal suppositories. There are several different morphine long-acting formulations, while the active ingredient in these remains identical, their duration of action and other characteristics may vary significantly. Some long-acting forms may be removed from their capsules to give in apple sauce or through a feeding tube, while the individual particles retain their timed release characteristics; on the other hand, time-released tablets should never be cut or crushed in order to prevent too much morphine becoming available all at one. Morphine is also available for injection or intravenous infusion in various strengths. Intravenous or subcutaneous infusion is the route of choice when patients cannot take oral medication. Morphine can also be given intrathecally, within the subarachnoid space surrounding the spinal cord. Recently, topical morphine has been used for skin ulcers and mucositis, and inhaled morphine has been used to treat pain and shortness of breath associated with cancer.

2.1.2 Codeine

Codeine is the prototypical weak opioid; when given orally, it is only about 25% as potent as oral morphine. Codeine as monotherapy is a less effective analgesic than NSAIDs or acetaminophen [7]. Therefore, it is commonly given in a combination with acetaminophen for improved pain relief.

3 Mechanism of Action

While opioids have been known to medicine since ancient times, the site of action in the brain was not clearly identified until the 1970s, when it was found that electrical stimulation of the periaqueductal gray in rats seemed to greatly reduce their avoidance of noxious stimuli [8]. Shortly thereafter, it was recognized that the brain was rich in opioid receptors in this area [9]. The nearly contemporaneous discovery of endogenous opioids [10] answered the question of why this elaborate system existed – the body makes use of opioid transmission to regulate pain perception and other bodily functions. Opioid receptors are found throughout the body, but primarily in the brain, spinal cord, and intestinal tract. These receptors are complex structures made up of seven amino acid chains, each of which bridges the membrane, forming a channel which can allow calcium ions to pass in or out of the neuron. When activated, an opioid receptor activates in turn a second messenger called a G-protein, which then detaches from the receptor complex and can have effects in distant parts of the cell (Fig. 43.2).

There are three families of opioid receptors: mu, delta, and kappa. Each type of receptor has a different affinity for specific opioids, and specific effects when activated. Each opioid receptor can cause analgesia; mu-agonists (such as morphine) also tend to cause more dependency, while kappa-agonists such as the endogenous opioid dynorphin cause more dysphoria.

Opioid-induced analgesia is produced through the action of opioid receptors on presynaptic terminals of the C-fibers and A-delta fibers. These fibers, which transmit nociceptive messages, are inhibited by the indirect effects of opioids, which in turn reduce the release of neurotransmitters such as substance P, CGRP, and glutamate. This effect occurs in the peripheral nervous system as well as at the primary afferent terminals in the spinal cord.

Another important site of opioid-mediated analgesia is the periaqueductal gray matter. This area of brain, as noted above, is rich in opioid receptors, and exercises descending anti-nociceptive influence on the dorsal horn of the spinal cord.

3.1 Tolerance

Opioid tolerance is a well-known phenomenon, which involves increasing dosage requirements with repeated opioid dosing in order to maintain the same analgesic effect. This effect is a common reason for dose escalation in pain patients. In humans, tolerance can be divided into *associative* and *nonassociative* forms.

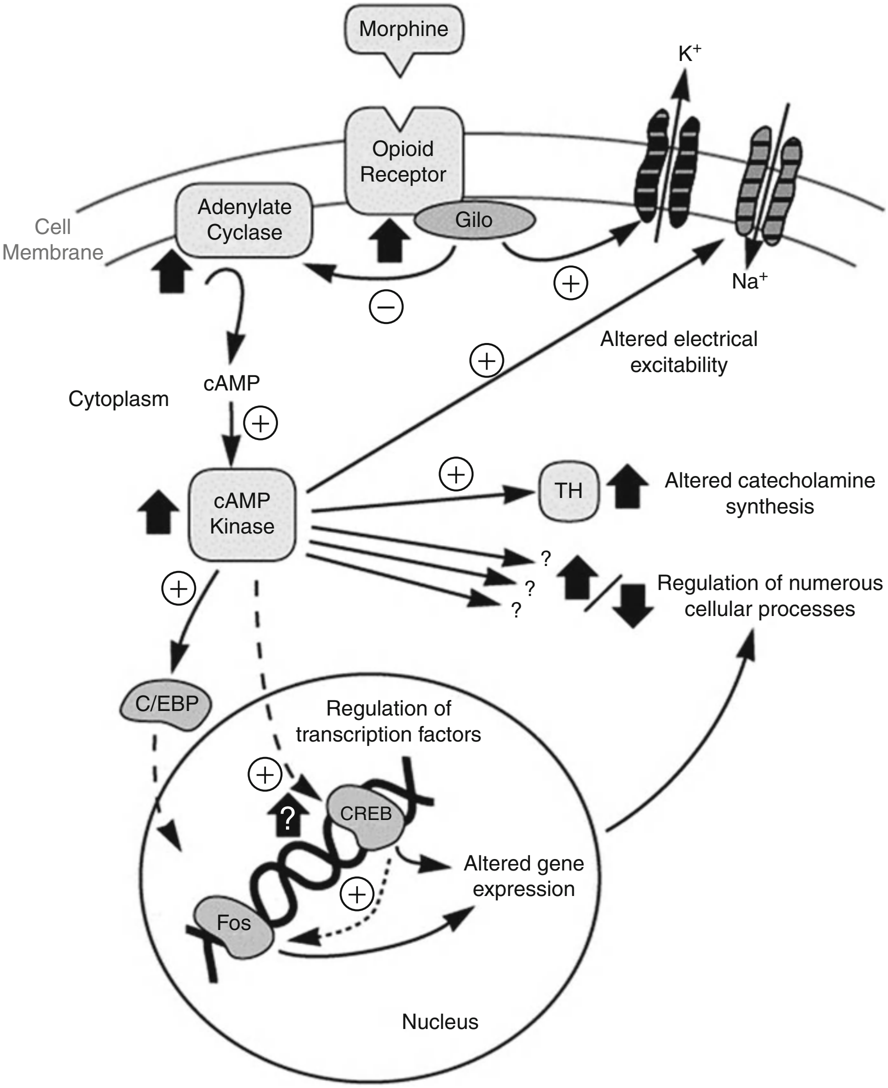

Morphine
K+
Opioid Receptor
Adenylate Cyclase
Gilo
Cell Membrane
Na+
⊖
⊕
Altered electrical excitability
Cytoplasm
cAMP
cAMP Kinase
TH
Altered catecholamine synthesis
?
Regulation of numerous cellular processes
C/EBP
Regulation of transcription factors
CREB
Altered gene expression
Fos
Nucleus

Fig. 43.2 Opioid action (Reprinted with permission from Trescot AM, Datta S, Lee M, Hansen H (2008) Opioid pharmacology. Pain Physician 11:S133–S53)

Associative, or learned tolerance is related to environmental cues and psychological factors. For example, opioid addicts may have greater tolerance for opioids used illicitly at home than an unfamiliar form given in the medical context of a hospital. Nonassociative, or adaptive tolerance, is a neurophysiological adaptation of the opioid receptor system, involving downregulation of the opioid receptors or decrease of opioid receptor sensitivity [11]. This process involves the NMDA receptor and increased expression of dynorphin.

4 Toxicity

4.1 Physical Toxicity

4.1.1 Respiratory Depression

The most significant and concerning side effect of opioids is respiratory depression. This effect of morphine was quickly recognized, with Sertürner himself reporting in 1827 the death of a dog he had injected with morphine. He and several human volunteers also suffered respiratory depression in the course of their experimentation [12].

Respiratory depression is mediated by the effect of mu-opioid receptors [13]. Opioid receptors in the brainstem at the nucleus accumbens have a dual effect, decreasing the respiratory drive as well as the response to decreasing oxygen levels and carbon dioxide levels in the bloodstream. Thus, patients on high doses of morphine feel less need to breathe, even when their respirations are becoming inadequate. This effect is the most serious and life-threatening toxicity associated with opioids. Recent research has shown that this risk is significantly greater when opioids are co-administered with sedatives such as benzodiazepines, or when patients have respiratory diseases or sleep apnea, as they may already have significant changes to their respiratory drive and be used to high partial pressures of carbon dioxide in the blood. The opioid antagonist naloxone can be used to quickly and safely reverse overdoses; it is memorable to see an unconscious, apneic patient suddenly "come to life" with the administration of naloxone – usually in a foul mood, as the naloxone also provokes acute withdrawal. With IV administration of morphine, the respiratory depression reaches full effect as quickly as 10 min after injection.

4.1.2 Other Neurological Symptoms

Opioids cause miosis (pinpoint pupils) in a dose-dependent manner. Sedation and hearing loss are also common at higher doses of opioids.

4.1.3 Hypotension

Especially when given intravenously, morphine can cause orthostatic hypotension and venous pooling.

4.1.4 Gastrointestinal

The decreased gut transit time associated with opioids, when not desired, can cause severe constipation and even bowel obstruction. Patients do not usually develop tolerance to these effects of opioids, even after long-term treatment. Morphine is also associated with spasms in the bile duct and sphincter of Oddi and the bladder. At higher doses, nausea and vomiting related to the chemoreceptor trigger zone in the fourth ventricle of the brain can be a limiting factor in opioid therapy.

4.1.5 Allergic Symptoms

Morphine triggers histamine release from mast cells in the skin. This reaction is not mediated by opioid receptors. This histamine can cause bronchospasm, hypertension, vasodilation, and facial flushing. True allergic reactions, including anaphylactic reactions, to opioids are rare but have been reported.

4.1.6 Hyperalgesia

Increased pain sensitivity is common in post-acute injury, nerve injury, and some forms of chronic pain. This sensitivity can take the form of hyperalgesia, or abnormally increased sensitivity to painful stimuli. For example, a patient with neuropathic pain may find a mild pinprick in the affected area unbearably painful. Allodynia is the perception of ordinary touch as painful, such as when a patient with neuropathic pain has a "hot coals" sensation when walking on a cool smooth floor. Considerable evidence suggests that chronic opioid use can contribute to hypersensitization of pain perception by affecting the responsiveness of nociceptive nerves in a manner analogous to chronic neuropathy [14]. This opioid-induced hyperalgesia has been described in patients taking opioids for pain as well as those taking opioids for addiction. In animal models of this phenomenon, changes take place in the neurons in the dorsal horn of the spinal cord to make them more reactive. These changes are long term and mediated by the NMDA receptor system, which is also involved in animal models of neuropathic pain and neurotoxic nerve damage. This sensitization occurs clinically in patients on long-term higher-dose opioids, who manifest both tolerance and sensitization.

4.1.7 Endocrine System

Chronic opioid use can affect the body's hormone balance, most notably in the adrenal hormones and gonadal hormones [15]. Chronic morphine use decreases blood levels of cortisol, a stress hormone. Opioids also change the balance of the pituitary hormones that regulate sex hormone production, increasing prolactin and decreasing luteinizing hormone, follicle-stimulating hormone, and the sex steroids of estrogen and testosterone. These changes in hormone levels cause effects in behavior, sexual function, and body physiology, including decreased libido, irregular menses or amenorrhea, galactorrhea, loss of muscle strength, and osteoporosis [16]. In patients who will receive long-term opioid treatment, sex steroid supplementation may be beneficial if there are no contraindications.

4.1.8 Immune System

Opioids affect the function and development of the immune system, both indirectly through its control system, and directly via the opioid receptors on immune cells. Bone marrow progenitor cells, macrophages, natural killer cells, immature thymocytes, B cells, and T cells, all express opioid-related receptors on their surfaces and have been shown to change their behavior in the presence of opioid agonists. Prolonged opioid exposure suppresses immune function in animal models, as

Table 43.2 Elements of aberrant behavior with opioid treatment

"Lost" prescriptions
Early refill requests
Abuse of other mind-altering substances or alcohol
Functional impairment
Doctor shopping
Diversion of medication
Failure to comply with treatment plans

does opioid withdrawal and untreated pain. Morphine may have greater immunosuppressive effects than some synthetic opioids. This is clinically relevant, particularly in AIDS patients, who are already suffering immune system difficulties and who may have higher viral loads when on high dose opioid regimens.

4.2 Behavioral Toxicity

4.2.1 Addiction

Opioids inhibit the release of GABA, an inhibitory neurotransmitter, in the ventral tegmentum of the brain. This facilitates the release of dopamine in the nucleus accumbens, the reward center of the brain, which is thought to be responsible for the habit-forming property of opioids. Despite enormous effort, little progress has been made in developing a compound that would possess the analgesic effect of morphine without morphine's addiction reinforcing effects.

4.2.2 Aberrant Behavior

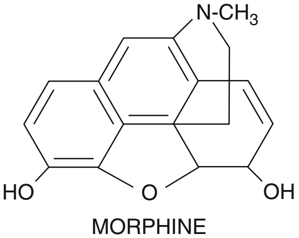
Aberrant behavior is a common complicating factor in opioid treatment, and sometimes leads to the failure of opioid treatment. Aberrancy has many potential causes, including miscommunication, and genuine under-treatment of pain, but should always be considered a red flag and potential indicator of such problematic situations as addiction and drug diversion. Many providers use a risk management approach with all patients who receive these medications, screening for risk factors associated with addiction and misuse and utilizing urine drug testing [17] (Table 43.2).

5 Bioavailability and Metabolism

5.1 Morphine

5.1.1 Chemical Properties

Morphine in the pure state is a white crystalline compound that is basic and insoluble in water. Since this has very poor bioavailability, morphine is available for pharmaceutical purposes as a salt, most commonly as morphine sulfate, but also sometimes as morphine acetate or morphine hydrochloride.



MORPHINE

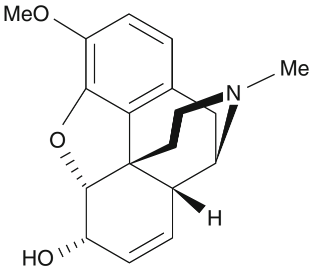
5.1.2 Pharmacodynamics of Morphine

Morphine, even in salt form, has poor solubility in lipids and therefore has trouble crossing the blood-brain barrier. It is primarily metabolized by conjugation with glucuronic acid, and is subsequently excreted renally. The majority of this is morphine-6-glucuronide (M-6-G), which also has an analgesic effect, but a small fraction is morphine-3-glucuronide (M-3-G), which is thought to contribute to opioid hyperalgesia. Morphine also has significant protein binding in the bloodstream. Therefore, less than half of an oral dose is available to the nervous system. Time to onset of analgesic effect of immediate-release oral morphine is about 30 min, and half-life is about 120 min. Therefore, immediate-release morphine is commonly given every 3–4 h. Long-acting morphine takes up to 90 min to take effect and can last from 8 to 24 h, depending on dosage and formulation.

Morphine is also hepatically metabolized to normorphine by the cytochrome P-450 enzymes CYP 3A4 and CYP2C8. Small amounts of codeine and hydromorphone are also produced, which is not clinically significant but can cause false positive urine drug tests for these substances. Morphine dosage should be reduced in patients with impaired renal function.

5.1.3 Codeine

Codeine is well absorbed after oral administration but has highly variable bioavailability and a plasma half-life of only 2.5 h.



CODEINE

Its metabolism is similar to that of morphine, with 80% being glucuronidated to codeine-6-glucuronide (C-6G) and smaller fractions being demethylated, creating morphine and norcodeine. It has been hypothesized that the small amount (roughly 10%) of morphine created through demethylation by the cytochrome P450 enzyme CYP2D6 is responsible for the majority of the analgesic effect, since people with the slow-acting variant of CYP2D6 get very little analgesic benefit from codeine [18]. Conversely, rapid CYP2D6 metabolizers may be at risk for morphine overdose, for themselves, or for breast-fed babies [19]. Codeine administration will produce both morphine and codeine in the urine. Codeine dosage should be reduced in patients with impaired renal function.

5.1.4 Pharmacogenetics of Opioids

There are multiple ways in which genetic variability can interact with opioid treatment in patients. Receptor polymorphisms can lead to a drug being more or less effective in an individual. Of the receptors that can be affected, OPRM1 (the mu-1 opioid receptor subtype) is the most clinically significant. A specific single-nucleotide polymorphism has been identified, (118A > G SNP) which causes a missense mutation that, through unclear mechanisms, greatly reduces the analgesic effect of opioids [20]. This is found in approximately 16% of Caucasians. However, a meta-analysis concluded that this polymorphism, though identifiable, was not clinically significant in pain treatment, and the authors did not recommend genotyping [21].

The other significant genetic effect on opioid treatment is the variability of the cytochrome P450 enzyme system, particularly CYP2D6. This enzyme is responsible for converting roughly 10% of the oral dose of codeine into morphine, which is primarily responsible for the analgesic effect of codeine. CYP2D6 effect is largely regulated at a single gene site, and, depending on which alleles are inherited, it can be rated as poor, intermediate, extensive, and ultrarapid [22]. Poor metabolizers (about 10% of Caucasians), those with two deficient alleles, will have almost no analgesic effect from normally therapeutic doses of codeine. These patients are also prone to developing toxicity due to elevated blood levels of numerous other drugs. Intermediate metabolizers are heterozygous for the deficient allele and will have some limited analgesic effect with codeine. Extensive metabolizers have the normally functioning alleles and respond to medications in the expected way. Ultrarapid metabolizers, who are only 1% of Caucasians but up to 30% of the North African population, may have greater than expected blood levels of morphine given standard codeine dosing. This has resulted in the death of breastfeeding babies when their mothers, who were ultrarapid metabolizers, were given codeine for postdelivery pain [19]. Some authorities have suggested that the genetically mediated variability of codeine effectiveness should lead to it being taken out of the first line of therapies for mild to moderate pain [18]. Others speculate that CYP2D6-deficient patients may be at less risk of addiction [23], perhaps because they do not get the euphoria from the morphine metabolite.

6 Other Biological Activities

6.1 Gastrointestinal

The gastrointestinal system is also liberally lined with opioid receptors. Mu-agonists, in particular, are known to cause decreases in gastric emptying, gut transit, and intestinal secretions. The constipating effects of codeine are not less than any other opioid, despite the weaker analgesia. Therefore, codeine may be used for diarrhea, although this use has now been largely supplanted by opioids that do not act centrally, such as diphenoxylate and loperamide.

6.2 Antitussive

The antitussive effects of opioids occur both centrally and peripherally. Mu-receptors [24] in the brainstem cough center and in the sensory arm of the vagus nerve, which controls airway reflexes, appear to mediate this effect. The weak analgesia associated with codeine makes it a good choice as an antitussive and it has frequently been used as a component of cough syrups. These cough syrups are sold over-the-counter in some areas and have been a source of opioid misuse and diversion. Codeine is not an active antitussive in young children and has potential for respiratory suppression in this patient population; for that reason, it is not recommended for children [25].

6.3 Antihypertensive

Morphine has hypotensive effects, increasing peripheral vascular capacity and decreasing blood pressure, which may be mediated through adenosine. For this reason, as well as its analgesic properties, intravenous morphine has been given for myocardial infarction, although it is not a part of the current standard of treatment.

7 Clinical Trials

7.1 Opioids for Acute Pain

Codeine has frequently been prescribed for mild acute pain, such as following dental procedures. The Cochrane Review database conducted large-scale meta-analysis of single-dose treatment for postoperative pain, which concluded that single-dose codeine 60 mg postoperatively was not an effective analgesic [7]. Codeine together with acetaminophen (paracetamol) yielded good relief in 50% of patients in the studies [26]. Codeine with acetaminophen is less effective that ibuprofen for pediatric arm fractures [27]. Codeine continues to be useful as a short-term medication for acute pain in adults when given short term.

Morphine is frequently given intravenously or orally for acute pain. Many patients prefer patient-controlled anesthesia (PCA) to intramuscular injections [28]. Morphine is also highly effective when given epidurally for postsurgical pain [29]. Comparisons of epidural and patient-controlled intravenous morphine suggest that both are equally effective for pain control, but epidural morphine may have slightly greater side effects [30]. The ability to combine epidural morphine with a long-acting local anesthetic such as ropivicaine or bupivacaine has allowed anesthesiologists to lower morphine dosages for epidural anesthesia [31].

7.2 Opioids for Chronic Pain

Codeine is effective for treatment of moderately severe chronic pain [32]. A Norwegian study indicated that while nearly 10% of the adult population was prescribed codeine at some point during the year 2005, only 1% had more frequent dosing, suggesting chronic administration [33]. An extensive systematic review of long-acting opioid preparations for chronic non-cancer pain has continued to show no difference between morphine and other long-acting opioids [34].

The subject of chronic opioid treatment for non-cancer pain has become significantly more contentious in the last decade, with studies suggesting that high-dose opioid use is concentrated in a small group of relatively high-risk patients [35]. Daily doses of opioids greater than 100 mg of morphine-equivalents/day have been shown to carry much higher risks of death [36]. And in the United States, an alarming trend toward high rates of opioid overdoses and overdose-related deaths has been seen in public health data since the year 1990 [37].

7.3 Opioids for Cancer Pain and Palliative Care

The World Health Organization guidelines for cancer pain recommend opioid treatment of moderate-to-severe cancer pain using oral medication whenever possible [6]. An extensive systematic review of opioids for cancer pain did not find clear evidence of superiority or inferiority of morphine over other opioids for cancer pain, and it remains a very commonly used treatment for cancer pain [38]. The variety of routes for morphine, including oral, rectal, intramuscular, intravenous, subcutaneous, and intrathecal, make it a highly versatile medication.

Many patients with moderate-to-severe cancer pain will be treated with a combination of a long-acting opioid as well as an immediate-release opioid for breakthrough pain. Immediate-release morphine can be given at 5–15% of the dose of long-acting morphine, and has been shown to work within 30–40 min [39]. Opioid treatment of cancer pain is sometimes limited by side effects such as sedation and nausea. In these cases, switching to intravenous administration can rapidly reestablish pain control [40]. Intravenous morphine for cancer pain must be closely monitored in patients with renal impairment, as the active metabolite morphine-6-glucuronide may accumulate [41].

In developed countries, cancer treatment centers should have access to pain specialists who can manage the complex issues involved in cancer pain, including changing medication requirements, changes in metabolism and pharmacokinetics of medications related to cancer treatments, and the tradeoffs between comfort and sedation frequently involved in end-of-life care. A large Italian study showed that access to palliative/supportive care teams was associated with a 31% lower chance of suffering severe pain during cancer care [42].

8 Conclusion

Poppy extracts have been the source of pain relief and euphoria for thousands of years. Opium derivatives, such as morphine and codeine, are the most effective analgesics available, and they have a wide variety of uses as well as potentially serious side effects. Recognition of the variability of receptor response and metabolism can help to explain the wide range of responses to the same dose of medications, and perhaps also explain why some people become addicted and others do not.

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Pyrrolizidine Alkaloid-Induced Toxicity to the Liver

44

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Contents

1 Introduction 1384
1.1 Traditional Herbal Medicines 1384
2 Pyrrolizidine Alkaloids 1385
3 Hepatocytotoxicity 1385
4 Review of Clinical and Pathological Features: Autopsy Findings and Histopathology 1386
5 Veno-Occlusive Liver Disease 1387
6 Use of Herbal Remedies and Extrahepatic Toxicities 1388
7 Sources of Poisoning 1389
8 Combining Herbal and Conventional Medicine 1390
9 Clinical Significance and Conclusions 1390
References 1391

Abstract

Background: Herbal-induced injury to the liver is a many-faceted phenomenon. The diverse aspects include the nature of the hepatotoxic agents, the character of the injury, the mechanism for the hepatotoxic effects, the circumstances of exposure, and the medical and social importance.

Purpose: The objectives are to encourage recognition and prevention of common morbidity encountered when using complementary and alternative medicine and 2) to review the toxic effect of herbal remedies containing pyrrolizidine alkaloids (PAs).

Design and Methods: A systematic literature search and review, and data interpretation are provided.2.

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Conclusions: Our results suggest that herbal remedies containing *PAs* induce veno-occlusive disease of the liver (VOD). Long-term use, monitoring, and avoiding overdose of such remedies may prevent the mitochondrial injury due to the glutathione depletion, which ultimately leads to the liver cell damage. Moreover, immediate "remedy" discontinuation will prevent further liver damage. The role of liver toxicity induced by "natural" herbal remedies and elucidating the herbal-drug interaction require further research.

Keywords

Complementary alternative medicine • herbal remedies • pyrrolizidine alkaloids • veno-occlusive disease of the liver • natural treatments or therapy

Abbreviations

PA	Pyrrolidizine alkaloids
VOD	Veno-occlusive disease

1 Introduction

1.1 Traditional Herbal Medicines

Herbal medicine – defined by the World Health Organization (WHO) as "herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, or plant materials, or combinations thereof" – is used to treat a multitude of ailments throughout the world. (http://whqlibdoc.who.int/hq/2002/WHO_EDM_TRM_2002.1.pdf).

We will review several herbal medicines used throughout the world, but we will focus on two genii *Senecio* spp. and *Callilepis laureola (Impila)* used in South Africa. It is currently estimated that 80 % of the South Africa population consult with traditional healers and use some form of traditional herbal medicine, usually in combinations [1–4]. Reports indicate it is used to treat cough [5], stomach problems [6], impotence [7, 8], to induce fertility [9], to facilitate labor [1, 10], or against "evil spirits" [11].

Acute poisoning due to the use of traditional medicines causes severe morbidity and mortality globally. The pyrrolizidine alkaloids are hazards posed by the environment that are ingested in ignorance of their toxicity or are taken as folk medicines. The toxicity of Senecio plants and related species has been a subject of interest for almost 100 years. At the turn of the century, they were suspected of causing disease in grazing domestic animals. The inappropriate use of medicinal herbs has resulted in numerous fatalities, especially in young children [11–20]. Within a few years, this suspicion was confirmed by a series of studies that have been reviewed in the excellent publication of McLean [20]. This chapter will review pyrrolizidine alkaloid characteristics, their role and pathological features of hepatotoxicity, VOD of liver, histopathological characteristics as well as extrahepatic toxicity characteristics, sources of PA, and description of preliminary efforts to combine conventional and traditional medicine.

2 Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids (PA) are complex molecules named for their inclusion of a pyrrolizidine nucleus: a pair of linked pyrrole rings. Each pyrrole can be drawn as a five-sided structure with four carbons and one nitrogen forming the ring [21]. Pyrroles are incorporated into the chlorophyll molecule; the biological role of PAs in plants remains unknown.

PAs are present in over 450 different species from 14 distinct plant families that grow worldwide [22–28]. The poisoning is usually accidental, by the ingestion of grain contaminated with pyrrolizidine-containing weeds, as has been reported in Tajikistan [29] and South Africa [30], expenditure of herbal or bush teas in the United States [23], or when taken as herbal infusions for medicinal purposes [31].

PAs are of special interest currently because several of them have been shown to cause clinical toxicity in humans. Comfrey, a well-known medicinal herb, contains PAs that are capable of causing liver damage [19].

Presently, there are over 300 PAs characterized with the highest numbers found in the genus *Senecio* (Asteraceae family), which contains senecionine with concentrations of up to 18 % of the dry weight of the plant [22], retrorsine [28], integerrimine, neosenkirkine, and florosenine [32].

Senecio spp. including golden ragwort proves to be a source of fatal poison that affects cattle [32]. Furthermore, the toxic effects are usually not detected until irreversible liver damage has occurred [33]. *S. latifolius* leaves are equipped as to treat wounds and burns, whereas decoctions are used to induce abortion or to cure infants [1, 5, 34–37].

3 Hepatocytotoxicity

The character of the injury produced by PAs includes several categories. Some agents lead to necrosis, steatosis, and cirrhosis. Others lead only to interference with bile secretion and to jaundice with little injury to the hepatic parenchyma. The possible relationship of the toxicity to glutathione binding leads to experimental in vitro exploration presenting similarity of the lesion to that of acetaminophen, and the recorded glutathione depletion [28]. Some PAs produce degenerative or vascular lesions. The entire range of known hepatic lesions can be produced by PAs. In the typical case of poisoning (with clinical and biochemical findings), ascites occurs in 96 % of patients, hepatomegaly in 85 %, and elevated liver enzymes in 92 % [33].

There is a growing concern over the use of herbal remedies containing PAs since pyrrolizidine-induced liver damage can be cumulative. The toxicity of PAs depends on the exposure time, dosage amount, and susceptibility of the organism. In humans, the dosage appears to fall within the range of 0.1–10 mg/kg per day [38]. Low levels of exposure to PAs likely results in lung lesions while high amounts of exposure often give rise to liver lesions [20, 39]. In a recent study looking at the five women (41–72 years old) that consumed for a long time PA-containing herbal remedies presented clinical ascites, jaundice, and hepatomegaly [40]. The imaging features were diffused, patchy hepatic enhancement, periportal edema, and ascites. Pathology ascertained that blood flow was obstructive at the site of sinusoid. PAs (senecionine and seneciphylline) were identified in all the three available herbal preparations ingested by the patients. Pyrrole-protein adducts were unequivocally found in all the five blood samples. Two patients recovered, two developed chronic illness, and one died due to liver failure and hepatic encephalopathy [40]. In other studies, mixed inflammatory cell infiltration, hepatocyte cytomegaly and karyomegaly, cytoplasmic vacuolization, centrilobular necrosis, bile duct hyperplasia, and hepatocellular adenomas are detected [41].

Enlargement of the liver and distension of the abdominal cavity were evident in cattle after ingestion of golden ragwort [42–44].

Watson and colleagues reported 50 toxicity cases at one hospital with fatal *Impila* poisonings in children between the ages of 0–12 [17].

There appear to be two clinical presentations of *Impila* poisoning. The most common is one in which severe hepatocellular failure predominates. In many of these cases, signs of renal failure accompany the hepatic necrosis [33]. The second and less common clinical presentation of *Impila* poisoning is where renal failure [7] is an early finding and precedes the development of hepatic failure [8].

Impila toxicity is very sudden in onset, and it is suspected that many patients, especially those residing in rural areas, do not reach the hospital alive [1, 7, 8]. Documented cases indicate that symptom duration prior to hospital admission is less than 1 day in 40 % of patients [3, 6]. In fatal cases, death occurs either within a few hours (<24) from hypoglycemic coma [33] or within a few days (<4) from acute hepatic failure [3, 17, 34]. In a few cases, patients have succumbed after several days from uremia due to acute renal tubular necrosis [3, 6]. The chance of survival after severe *Impila* intoxication is low. It is estimated that 63 % of patients die within 24 h and a further 28 % die within 4 days; thus, the total mortality from severe *Impila* poisoning is up to 91 % [3, 17].

4 Review of Clinical and Pathological Features: Autopsy Findings and Histopathology

Pathological features associated with *Impila* poisoning in humans are limited to only a few reports. In most of the cases, liver biopsies were not performed due to the severity of the symptoms [8].

The most detailed report of the pathological features associated with *Impila* poisoning in humans reviewed 235 cases of suspected *Impila*-related deaths (30 % of cases were children under the age of 5 years) [3]. Autopsy findings indicated good nutritional status, especially in children, with no evidence of other organic disease. In 50 % of cases, the liver was reduced in weight and appeared pale and yellow. In 70 % of cases, the renal weight was increased and the cortex was pale and swollen. Hemorrhages were often present in the lungs, skin, and intestine.

Histopathology of the liver showed centrilobular necrosis with a sharp demarcation. Death occurred rapidly, due to hypoglycemia. In these cases, centrilobular hepatocytes appeared swollen and showed karyolysis. In cases where death occurred only after a few days from liver failure, hepatocytes were necrotic. Fat vacuoles were seen in some hepatocytes, and the liver had minimal inflammatory infiltration. In cases of kidney failure, renal histology showed necrosis of the proximal convoluted tubules and loop of Henle. In these cases, the liver showed centrilobular collapse of the reticulum framework and cholestasis.

Watson and his colleagues examined 50 autopsy reports of children poisoned with *Impila* [17]. Upon presentation to hospital, 80 % of patients showed a disturbed level of consciousness (confusion, stupor, or coma), and in 52 %, this was associated with convulsions. Other common symptoms included abdominal pain, vomiting and diarrhea. Hypoglycemia (blood glucose <2.5 mmol/L) was found in 93 % of cases, metabolic acidosis (serum bicarbonate <19 mmol/L) in 87 %, uremia (elevated blood urea >6.64 mmol/L) in 60 %, hyperkalemia (serum potassium >5.3 mmol/L) in 63 %, hyponatremia (serum sodium <130 mmol/L) in 45 %, and leukocytosis (total white cell count $>11 \times 10^9$/L) in 80 %. In patients who survived more than 24 h, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactic dehydrogenase (LDH) were elevated up to 5–10 times the normal levels, and the prothrombin time was elevated up to 50 % of upper limit of normal. In all cases documented to date, concomitant jaundice has rarely been observed in the patients with hepatotoxicity, and the progression to death was very rapid.

Despite its reputed toxicity, *Impila* continues to be a commonly used traditional remedy in South Africa [1, 35–38]. The concentration of toxins found in plants has been shown to vary with season and soil and climate conditions [37, 39]. Also, the levels of toxin(s) in the poisonous plants may vary with the location [40, 41]. PAs are hepatotoxins, which have both acute and chronic effects in man and animals [3]. Depending on the dose, the observed toxicity is evident within days, weeks, or even months [3, 11–20]. A dose of 10 mg/kg per day produces acute toxicity in1–6 days. On the contrary, ingestion of 0.1 mg/kg/day poses chronic toxicity that presents clinically within months. A group of researchers from Brazil analyzed blood samples from 30 intoxicated animals that presented pathological liver lesions. Comparing the results with samples from 30 healthy animals, the investigators demonstrated that poisoning by *Senecio* spp. causes an increase in lipid peroxidation and consequently oxidative stress in bovine erythrocytes contributing to hemolysis [43].

5 Veno-Occlusive Liver Disease

Evidence of toxicity may not become apparent until some time after the alkaloid is ingested. The acute illness has been compared to the most common pro-thrombotic disorders that lead to Budd–Chiari syndrome, portal vein thrombosis, and sinusoidal obstruction syndrome, previously known as hepatic veno-occlusive disease of the liver. The term veno-occlusive disease of the liver refers to a form of toxic liver injury characterized clinically by the development of hepatomegaly, ascites, and jaundice. Histologically, there is a diffuse damage in the centrilobular zone of the liver. The cardinal histologic features of this injury are marked sinusoidal fibrosis, necrosis of pericentral hepatocytes, and narrowing and eventual fibrosis of central veins. The primary site of the toxic injury is suggested as the sinusoidal endothelial cells, followed by a series of biologic processes that lead to circulatory compromise of centrilobular hepatocytes, fibrosis, and obstruction of liver blood flow.

Other investigators have observed similar cases to the case we previously described with VOD, only the central veins of the liver lobule or sublobular veins are occluded. Toxic pyrrolizidines have acute and chronic effects on the liver, which frequently manifest as VOD [44]. Tomioka and his team report a case of a young woman developing VOD after taking cough remedies prepared using *S. tephrosioides* over a long period of time [45]. Additionally, VOD has been reported after a patient had taken commercial herbal preparations containing *S. vulgaris* [46]. Moreover, VOD has been associated with consumption of PA-containing dietary supplements [47]. Ridker and colleagues showed that as low as the 15 μg PA/kg body weight/day may cause VOD in humans [47].

Roulet and colleagues presented a. hepatic VOD in a newborn infant of a women drinking herbal tea [48]. Kumana's group described herbal tea induced hepatic VOD and was able to quantify the toxic alkaloid exposure in adults [49]. Investigators from Rio de Janeiro described a pediatric VOD case [50]. Hepatic VOD in Egyptian children has been documented [51].

In Peru, Ortiz and colleagues reported *a* VOD due to intake of *S. vulgaris* tea [52]. In Arizona, hepatic VOD due to pyrrolizidine (*Senecio*) poisoning was also described [53]. Tandon et al. reported "an epidemic of VOD in Afghanistan [54] and in Central India [55]. Moreover, ingestion *of* comfrey has been suggested to produce VOD [56, 57]. In the United States, the cases reported so far are four incidents related to comfrey ingestion in adults, two incidents of *Senecio* ingestion in infants and two incidents of Senecio ingestion in adults, four cases of *Heliotropium* ingestion in adults, two cases involving coltsfoot substitution by other PA-containing herbs in infants, and five other cases involving adults with unknown PA-containing herbs (three in China, one during a visit to Ecuador, and one using a tea product from South America). Although some cases of liver damage may have been due to something other than the herbs, in most instances, other sources of the intoxication were not evident or were deemed improbable.

6 Use of Herbal Remedies and Extrahepatic Toxicities

Some species have been reported to contain antimicrobial and therapeutic properties. *S. aureus* is used for injuries and serves as a diaphoretic and diuretic, although high dosages may cause abortion [1, 58]. Other species may cause other extrahepatic manifestations and teratogenesis [59].

S. bicolor is used to treat cataracts and conjunctivitis in the form of eye drops [60]. *S. scandens* possesses antipyretic activities [61]. In traditional Chinese folk medicine, *S. scandens* is known as "qianliguang" and classified in the 1977 edition of Chinese Pharmacopoeia as medication for bacterial diarrhea, enteritis, conjunctivitis, and respiratory tract infections [24]. Other Chinese herbals include *S. argunensis* and *S. integrifolius*, both of which are used in the treatment for febrile disease, inflammation, diarrhea, and cataracts. Both cause hepatotoxicity and extrahepatic manifestations [62]. In Perú, the decoction of the leaves of *S. culcitioides*, *S. tephrosioides*, and *S. canescens* are used to treat coughs, bronchitis, and asthma [63]. In addition, extracts of *S. canescens* have shown to exhibit antipyretic and barbiturate activities, consistent with its role as an analgesic in the treatment of breast pain [64]. *S. rhizomatosus* is used to treat wounds and increase biliary secretion. In Perú, it is used in the treatment of pneumonia [65].

People continue to be vulnerable to complementary and alternative medicine poisoning present in folk remedies, which are usually administered chronically. The concentration of PA in the plant extract differs since it depends on the area it was collected in, the season, and other climatic conditions. The standardization is not possible for several reasons: first, the content of the remedies are often poorly understood, thus making it difficult to discern toxicity level and almost impossible to place regulatory standards on which remedies are safe. Secondly, many patients are reluctant to disclose practice of folk remedies. This complicates the diagnosis process, which is already compromised due to poor analytical techniques [66, 67].

7 Sources of Poisoning

In addition to South Africa, there are many other countries which reported cases of deadly poisonings by herbal remedies in humans and animals: Afghanistan [54], Argentina [42], Brazil, Uruguay and Paraguay [32], Great Britain [56], Egypt [51], Greece [68], Hong Kong [49], India [55], Israel [41], Italy [69], Jamaica [70], Morocco [71], Scotland [72], and United States [23, 52].

In some countries, the larger population are exposed to low levels of alkaloids in commonly available foods, such as honey in Australia [73] and milk [74] and comfrey tea in Europe [19, 56].

S. *jacobaea* has been reported to contribute to honey production in Albania, Brazil, Italy, Switzerland, United Kingdom, United States, and Zimbabwe [75]. There is an alarming 3.9 μg of PAs per gram of honey from *S. jacobaea*, which translates to approximately 50–75 % of the plant when extraction efficiency is taken into consideration [75]. Although acute PA poisoning is still unlikely even when assuming maximum honey consumption of around 93 g per day for adults and 32 g per day for infants, the German Federal Health Bureau and the International Program on Chemical Safety both conclude that the level of PAs present in honey may contribute to chronic liver disease and liver tumors [76, 77].

S. longilobus is often confused with *Gordolobo yerba*, a popular Mexican herb obtained from *Gnaphalium macounii* [40]. This confusion often leads to erroneous preparation of the “yerba” herb, and resultant deaths include a 2-month-old boy and a 6-month-old girl after exposure to *S. longilobus* in *Gordolobo yerba* [40].

8 Combining Herbal and Conventional Medicine

In the last years, herbal medicines have frequently been used as an alternative or in addition to medical therapy for human immunodeficiency virus (HIV)-positive individuals and acquired immunodeficiency syndrome (AIDS) patients. A group of infectious disease specialists treated a pediatric population with antiviral and herbal remedies [78]. Others aimed to summarize research findings for herbal medicines, which are endowed with the ability to inhibit HIV. A Chinese herbal medicine, *Scutellaria baicalensis* and its identified components (i.e., baicalein and baicalin) have been shown to inhibit infectivity and replication of HIV [79]. There is insufficient evidence to support the use of herbal medicines in HIV-infected individuals and AIDS patients. Potential beneficial effects need to be confirmed in large, rigorous trials. Moreover, the possible toxicities due to the herbal remedies interactions with the antiviral therapy have to be considered.

9 Clinical Significance and Conclusions

Studying the facts involved in *herbal remedies*-induced toxicity has immense medical significance. Fatal or near-fatal poisoning cases that result from the use (or misuse) of traditional herbal medicines continue to be a common problem in many countries. Currently, there is no antidote or neutralization method for natural substances that induce veno-occlusive disease of the liver, *a*nd supportive care is the only therapy provided to these patients if time permits. This has been largely unsuccessful due to unavailability of medical support and/or fast deterioration of patient status due to long time lapse from ingestion or ingestion of high dose. As such, many of the cases often resulted in imminent death. The case and review presented here may contribute to the medical community awareness and thus finding a rational approach to limit or prevent VOD cases due to herbal remedies. Based on our clinical case of herbal remedies-induced VOD, this subject deserves further investigation and education. From a social point of view, the present knowledge on the pathophysiology of herbal remedies-induced VOD may assist in gaining a larger awareness of the problem, which will be required for the development of educational strategies aimed at physicians and the public about the potential dangers of these commonly used remedies. Herbal products are gaining widespread popularity across the globe. In the United States, it is estimated that up to 40 % of the adult population use herbal remedies [80], with similar trends occurring in Canada [81], Europe [72], and Australia [82]. The safety of these compounds, therefore, is not only a concern for third world countries, but should be made a priority in developed countries as well. While many medicinal plants offer significant therapeutic benefits, it is imperative that their potential risks are also recognized [83–85].

The common view that all “natural” compounds are safe compounds is a myth, and further studies on herbal-induced hepatotoxicity are needed to expose this dangerous misconception. Herbal remedies were used for centuries [86–88]. Diseases of domestic animals now known to be caused by the hepatotoxic PAs were a matter of concern to British farmers at the end of the eighteenth century until our days in Australia [89]. Moreover, in Germany, poisoning of horses and cattle by *Senecio jacobaea* is of renewed relevance for veterinary medicine. In addition, European and international authorities are concerned about possible residue levels in food of animal origin. The disease occurs almost entirely as a consequence of chronic poisoning and ends fatally due to formation of toxic metabolites of pyrrolizidine alkaloids in the liver, leading to cirrhosis. In addition, many pyrrolizidine alkaloids possess mutagenic and a few also carcinogenic properties [90].

Medicinal plants undoubtedly play a role in the treatment of disease, especially in developing countries. Although their toxicity has been known for a long time, PA-containing plants are still in use in many traditional medicines. Traditional healing systems have become of increasingly employed alone or as complementary medicine since many people believe that they can be used without any risk and side effects. This also applies to the traditional medicine of Madagascar and the Mascarene Islands, South Africa, Romania, and Israel [91, 92]. As with most therapeutic drugs, however, there is also a potential to cause toxicity, especially veno-occlusive disease of the liver [92]. It is important to understand the need of monitoring the use of herbal medicine with the aim of optimizing clinical complementary medicine use and maximizing the clinical and economic benefits, as well as, to enhance communication between scientists and physicians of all disciplines involved in complementary alternative medicine and clinical toxicology.

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Biological Activities of Pepper Alkaloids 45

Krishnapura Srinivasan

Contents

1 Piperine of Black Pepper (*Piper nigrum*) ... 1398
1.1 Introduction ... 1398
1.2 Biological Effects of Piperine ... 1399
1.3 Absorption and Metabolism of Piperine ... 1414
1.4 Conclusions ... 1415
2 Capsaicin of Red Pepper (*Capsicum annuum*) ... 1416
2.1 Introduction ... 1416
2.2 Biological Effects of Capsaicin ... 1417
2.3 Mechanism of Burning and Painful Sensation of Capsaicin ... 1427
2.4 Absorption and Metabolism of Capsaicin ... 1428
2.5 Conclusions ... 1429
References ... 1430

Abstract

The distinct biting quality of black pepper (*Piper nigrum*) widely used in human dietary is attributed to the alkaloid piperine. Black pepper is also used as a food preservative and as a vital component in traditional medicines in India and China. Several physiological effects of black pepper and its bioactive alkaloid piperine have been reported in recent decades. By stimulating the digestive enzymes of pancreas, piperine enhances the digestive capacity. Piperine has been documented to enhance the bioavailability of a number of therapeutic drugs as well as phytochemicals by its inhibitory influence on drug transformation reactions in liver and intestine. It strongly inhibits hepatic and intestinal aryl hydrocarbon hydroxylase and glucuronyl transferase. Piperine's bioavailability

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enhancing property is also partly attributed to increased absorption as a result of its effect on the ultrastructure of intestinal brush border. Piperine has been evidenced to have antidiarrheal property and an effect on intestinal motility and on the ultrastructure of intestinal microvilli improving absorbability of micronutrients. Piperine has been demonstrated in in vitro studies to protect against oxidative damage by inhibiting or quenching reactive oxygen species. Piperine treatment also lowers lipid peroxidation in vivo and beneficially influences antioxidant status in situations of oxidative stress. Piperine has been found to possess antimutagenic and antitumor influences.

Capsaicin, the pungent alkaloid of red pepper (*Capsicum annuum*), has been extensively studied for its biological effects which are of pharmacological relevance. These include cardioprotective influence, anti-lithogenic effect, anti-inflammatory and pain-relieving effect, thermogenic influence, and effects on gastrointestinal system. The involvement of neuropeptides, substance P, serotonin, and somatostatin in the pharmacological actions of capsaicin has been extensively investigated. Tropical application of capsaicin is proved to alleviate pain in arthritis, postoperative neuralgia, diabetic neuropathy, psoriasis, etc. Toxicological studies on capsaicin administered by different routes are documented. Capsaicin inhibits acid secretion and stimulates alkali and mucus secretion and particularly gastric mucosal blood flow which helps in prevention and healing of gastric ulcers. Antioxidant and anti-inflammatory properties of capsaicin are established in a number of studies. Chemopreventive potential of capsaicin is evidenced in cell line studies. The health beneficial hypocholesterolemic influence of capsaicin besides being cardioprotective has other implications, namely, prevention of cholesterol gallstones and protection of the structural integrity of erythrocytes under conditions of hypercholesterolemia. Beneficial influences of capsaicin on gastrointestinal system include digestive stimulant action and modulation of intestinal ultrastructure so as to enhance permeability to micronutrients.

Keywords

Alkaloids • biological activities • capsaicin • *capsicum annuum* • piperine • *piper nigrum*

1 Piperine of Black Pepper (*Piper nigrum*)

1.1 Introduction

Black pepper (*Piper nigrum*) – the dried berries of the plant belonging to the family Piperaceae – is one of the most widely used among spices, valued for its distinct biting quality. Black pepper has been used as a spice in India since prehistoric times; it is known to Indian cooking since at least 2000 BC [1]. Black pepper is produced from the green unripe berries of the pepper plant by briefly cooking in hot water [2]. White pepper which is commonly found in Western countries is produced

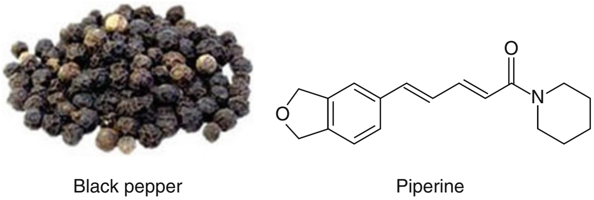


Fig. 45.1 Black pepper and piperine

by soaking ripe pepper berries in water for about a week, during which the flesh of the fruit softens and decomposes; rubbing off the skin would result in the naked seed which is then dried [2] (Fig. 45.1).

Black pepper is historically used also in traditional medicines and home remedies in India [3]. Black pepper figures in remedies in *Ayurveda*, *Siddha*, and *Unani* medicine in India for such illnesses as constipation, diarrhea, earache, gangrene, heart disease, hernia, hoarseness, indigestion, insect bites, insomnia, joint pain, liver problems, lung disease, oral abscesses, sunburn, tooth decay, and toothaches. Black pepper was relied upon to treat specific conditions such as diarrhea and fevers, but it appears that the extensive, generalized use was to enhance the effects of many herbal remedies [3].

Long pepper (*Piper longum*) and black pepper (*Piper nigrum*) (both of which are now known to contain piperine) have been prescribed in *Ayurvedic* system of medicine in India for thousands of years, a practice which may have enhanced pharmacological actions of other compounds in traditional herbal medicines. Black pepper is a component of *trikatu* (three acrids) along with long pepper and ginger (*Zingiber officinale*) in equal proportions; *trikatu* is widely used in combination with other *Ayurvedic* medications. Black pepper is specifically cited in *Ayurveda* to internally treat fevers, gastric and abdominal disorders, and urinary problems [4]. Medicinal external treatments with black pepper include treatments for rheumatism, neuralgia, and boils [4]. Possible uses of black pepper in Indian folk medicine include the treatment of respiratory diseases, dysentery, pyrexia, and insomnia [4]. Black pepper is part of a herbal, folk remedy relied to treat diarrhea [5]. Black pepper is also a constituent of traditional Chinese medicine for stimulation of digestion and relieving diarrhea [6]. Black pepper has been used in China as a folk remedy for epilepsy.

The spiciness of black pepper which is characterized by a distinct biting quality is due to the alkaloid compound piperine, which is found both in the outer fruit and in the seed [2]. Refined piperine is about 1 % as hot as the capsaicin of red chili pepper. The bioactive and pungent ingredient of black pepper was identified as piperine and isolated in 1820 by the Dutch chemist Hans Christian Oersted [2].

1.2 Biological Effects of Piperine

Many health beneficial physiological effects of black pepper or its bioactive alkaloid – piperine – have been reported in recent decades and been reviewed [7].

1.2.1 Inhibition of Drug-Metabolizing Enzyme System

In the context of piperine having been reported to enhance drug bioavailability, Atal et al. studied the interaction of piperine with hepatic drug biotransformation reactions in vitro and in vivo [8]. Piperine inhibited hydroxylation of aryl hydrocarbon, *N*-demethylation of ethylmorphine, *O*-deethylation of 7-ethoxy-coumarin, and glucuronidation of 3-hydroxybenzo (α-) pyrene (3-OH-BP) in rat liver in vitro in a dose-dependent manner. Piperine inhibited hepatic microsomal aryl hydrocarbon hydroxylase (AHH) noncompetitively from the untreated and 3-methylcholanthrene-treated rats with a K_i of 30 μM. Similarly, the kinetics of inhibition of ethylmorphine-*N*-demethylase from control rat liver exhibited noncompetitive inhibition with a K_m of 0.8 mM and K_i of 35 μM. These studies demonstrated that piperine is a nonspecific inhibitor of drug metabolism which shows little discrimination between different cytochrome P_{450} forms. Oral administration of piperine in rats strongly inhibited the hepatic AHH and UDP-glucuronyl transferase activities. Pretreatment with piperine prolonged hexobarbital sleeping time and zoxazolamine paralysis time in mice. These observations suggest that piperine is a potent inhibitor of drug metabolism. The mechanism of inhibition of glucuronidation by piperine has been explored by examining the rate of glucuronidation of 3-OH-BP and UDP-glucuronic acid (UDPGA) concentration in isolated epithelial cells of the guinea pig small intestine [9], and it was found that glucuronidation of 3-OH-BP was dependent on duration of incubation, cellular protein, and endogenous UDPGA concentration. Piperine caused a concentration-related decrease in UDPGA content and the rate of glucuronidation in these cells. Piperine noncompetitively inhibited hepatic UDP-glucuronyltransferase with Ki of 70 μM. The study demonstrated that piperine modifies the rate of glucuronidation by lowering the endogenous UDPGA concentration and also by inhibiting the transferase activity (Table 45.1).

Twenty-four hours following intragastric administration of piperine (800 mg/kg) in adult rats, a significant decrease in the hepatic levels of cytochrome P_{450}, benzphetamine *N*-demethylase, aminopyrine *N*-demethylase, and aniline hydroxylase has been observed [13]. An *i.p.* administration of rats with piperine (100 mg/kg) produced a significant decrease in hepatic cytochrome P_{450} and activities of benzphetamine *N*-demethylase, aminopyrine *N*-demethylase, and aniline hydroxylase 1 h after the treatment [15]. Twenty-four hours later, these parameters along with cytochrome b_5 and NADPH-cytochrome-C reductase remained depressed in piperine-treated rats. Piperine noncompetitively inhibited aromatic hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase activities in lung microsomes of rats and guinea pigs in vitro [10]. Piperine given at a dose of 25 mg/kg to rats caused a maximal inhibition of both the enzymes at 1 h. Similarly, upon daily treatment of piperine (15 mg/kg) to rats for 7 days, deethylase activity was consistently inhibited, while AHH showed faster recovery. Piperine thus appeared to cause differential inhibition of two forms of cytochrome P_{450} and thus would accordingly affect the steady-state level of those drugs metabolized by these pulmonary forms of cytochrome P_{450}.

Table 45.1 Inhibitory effects of piperine on drug-metabolizing enzyme system in vitro and in vivo

System	Observed effect	References
In vitro	(a) Inhibition of aryl hydroxylation, *N*-demethylation, *O*-deethylation, and glucuronidation in vitro by piperine	[8]
	(b) Decreased UDP-glucuronic acid concentration and rate of glucuronidation in isolated epithelial cells of guinea pig small intestine by piperine	[9]
	(c) Inhibition of aryl hydroxylase and *O*-deethylase activities by piperine in vitro in pulmonary microsomes	[10]
	(d) Suppression of aryl hydroxylation in cell culture is mediated by direct interaction of piperine with cytochrome P_{450} and not by downregulation of its gene expression	[11]
	(e) Piperine decreased the activities of liver microsomal aryl hydroxylase, *N*-demethylase and UDP-glucuronosyl transferase, and cytochrome P_{450}	[12]
Rats	(a) Lower aryl hydroxylase and UDP-glucuronyl transferase activities, prolonged hexobarbital sleeping time in piperine-treated rats	[8]
	(b) Inhibition of aryl hydroxylase and *O*-deethylase activities by piperine in vivo in pulmonary microsomes	[10]
	(c) Decreased activities of hepatic microsomal cytochrome P_{450}, *N*-demethylase, aryl hydroxylase by intragastric/intraperitoneal piperine	[13]
	(d) Inhibition of UDP-glucose dehydrogenase and UDP-glucuronyl transferase in liver and intestine by piperine	[14]
	(e) Lowered activity of *N*-demethylase, UDP-glucuronosyl transferase, and NADPH-cytochrome-C reductase as a result of piperine feeding	[12]
Guinea pig	(a) Inhibition of UDP-glucose dehydrogenase and UDP-glucuronyl transferase in liver and intestine by piperine	[14]

Piperine caused a concentration-related strong noncompetitive inhibition of UDP-glucose dehydrogenase (UDPGDH) reversibly and equipotently in rat and guinea pig liver and intestine [14]. However, the UDPGA contents were decreased less effectively by piperine in isolated rat hepatocytes compared with enterocytes of guinea pig small intestine. Data on UDPGA content and rate of glucuronidation suggested that piperine is a potent inhibitor of UDPGDH and it exerts stronger effects on intestinal glucuronidation than in rat liver. The effect of dietary piperine (0.02 %) on the activities of the liver drug-metabolizing enzyme system has been examined in rats [12]. Piperine significantly stimulated the activity of aryl hydroxylase, while the activities of *N*-demethylase, UDP-glucuronyl transferase, and NADPH-cytochrome-C reductase were lowered as a result of piperine feeding. Piperine also significantly decreased the activities of liver microsomal AHH, *N*-demethylase and UDP-glucuronosyl transferase, and cytochrome P_{450} in vitro when included at 1×10^{-6} mol/L.

A study of the modulation of benzo(α-)pyrene metabolism and regulation of cytochrome CYP1A1 gene expression by piperine in 5 L cells in culture revealed that piperine-mediated inhibition of AHH activity and consequent suppression of the procarcinogen activation results from direct interaction of piperine with cytochrome P_{450}1A1-protein and not because of downregulation of its gene expression [11]. Piperine was evaluated for beneficial effects in Alzheimer's disease by studying the potential for herb-drug interactions involving cytochrome P_{450}, UDP-glucuronosyl transferase, and sulfotransferase enzymes. Piperine was a relatively selective noncompetitive inhibitor of CYP3A with less effect on other enzymes evaluated [16]. Piperine inhibited recombinant CYP3A4 much more potently than CYP3A5.

1.2.2 Enhancing Effect on the Bioavailability of Drugs and Phytochemicals

Piperine is now established as a bioavailability enhancer of various structurally and therapeutically diverse drugs and other substances. Potential of piperine to increase the bioavailability of drugs in humans is of great clinical significance. Most of the clinical trials have shown that piperine increases levels of medications: phenytoin (used in epilepsy), propranolol (used for hypertension), rifampicin (used in tuberculosis), theophylline (lung medication), and coenzyme Q_{10}. This effect is due to the inhibitory interaction of piperine with cytochrome P_{450} enzymes of the liver and small intestine that are involved in drug metabolism, namely, CYP1A2, CYP1A1, CYP2D6, CYP3A4, and P-glycoprotein [17] (Table 45.2).

The scientific basis of the use of acrids (long pepper, black pepper, and ginger as constituents of *trikatu*) in a large number of medications in the indigenous *Ayurvedic* system of medicine in India has been evaluated by Atal et al. [18]. The observed >200 % increase in the blood levels of the test drug vasicine by *Piper longum* and of test drug sparteine by >100 % under the influence of piperine in a clinical study suggested that these acrids have the capacity to increase the bioavailability of certain drugs. These authors concluded that the *trikatu* group of drugs increases bioavailability of drugs either by promoting rapid absorption from the gastrointestinal tract, or by protecting the drug from being metabolized in its first passage through the liver after being absorbed, or by a combination of these two mechanisms. The effect of piperine on the bioavailability of propranolol and theophylline has been examined in a crossover study, wherein subjects received a single oral dose of propranolol (40 mg) or theophylline (150 mg) alone or in combination with piperine (20 mg/day for 7 days) [19]. An enhanced systemic availability of oral propranolol and theophylline was evidenced as a result of piperine treatment.

A pharmacokinetic study has examined the effect of piperine, a known inhibitor of hepatic and intestinal glucuronidation on the bioavailability of curcumin, the bioactive ingredient of the spice turmeric administered with piperine in healthy human volunteers [20]. After a dose of 2 g curcumin taken without piperine, serum levels were very low, while concomitant administration of piperine (20 mg) produced 2,000 % higher concentrations from 0.25 to 1 h post-drug. The study shows that in the dosages used, piperine enhances the serum concentration, extent of absorption, and bioavailability of curcumin in humans. When curcumin was given alone at 2 g/kg to rats, moderate serum concentrations were achieved over a period of 4 h [20]. Concomitant administration of piperine (20 mg/kg) increased

Table 45.2 Modulation of bioavailability of drugs, phytochemicals, and carcinogens by piperine

System	Remarks	References
Humans	(a) Increased bioavailability of vasicine and sparteine as a result of *Piper longum*/piperine treatment	[18]
	(b) Enhanced systemic availability of propranolol and theophylline as a result of piperine treatment	[19]
	(c) Increased serum concentration of curcumin by concomitant administration of piperine	[20]
	(d) Increased plasma levels of coenzyme Q_{10} by co-administration of piperine	[21]
	(e) Increased plasma concentration of phenytoin when co-administered along with piperine	[22]
	(f) Increased plasma concentration of antiretroviral agent nevirapine when co- administered along with piperine	[23]
Rats	(a) Decreased metabolic activation of fungal toxin aflatoxin B_1 and hence its increased accumulation in plasma	[24]
	(b) Enhanced bioavailability of β-lactam antibiotics – amoxicillin trihydrate and cefotaxime – by co-administration of piperine	[25]
	(c) Enhanced bioavailability of curcumin when administered concomitantly with piperine	[26]
Mice	(a) Delayed elimination of antiepileptic drug – phenytoin – by treatment of piperine	[27]
	(b) Increased plasma levels and delayed excretion of epigallocatechin-3-gallate from green tea as a result of intragastric co-treatment with piperine	[28]

the serum curcumin concentration for a short period of 1–2 h post-drug, and the bioavailability was increased by 154 %. Enhanced bioavailability of curcumin was evidenced when the same was orally administered concomitant with piperine in rats [26]. Intestinal absorption of curcumin was relatively higher when administered concomitantly with piperine, and it stayed significantly longer in the body tissues. This assumes importance in the context of diverse medicinal properties of curcumin.

Black pepper extract consisting of 98 % piperine has been shown to increase plasma levels of orally administered coenzyme Q_{10} in a clinical study [21]. The relative bioavailability of 90 and 120 mg of coenzyme Q_{10} administered in a single dose or for 14 and 21 days with placebo or with 5 mg of piperine was determined by comparing measured changes in plasma concentration. Supplementation of 120 mg coenzyme Q_{10} with piperine for 21 days produced a significant 30 % greater AUC than with coenzyme Q_{10} plus placebo. Piperine has been reported to enhance the oral bioavailability of phenytoin, an antiepileptic drug in human volunteers [22]. Piperine (20 mg administered along with phenytoin) increased significantly the mean plasma concentration of phenytoin in patients receiving either a 150 or 200 mg twice daily dose of phenytoin. A similar effect of piperine in altering the pharmacokinetics of phenytoin has been reported from a study on mice, where pretreatment of piperine significantly delayed the elimination of phenytoin [27]. There was a significant increase in AUC, C_{max}, and K_a. Enhanced bioavailability of nevirapine, a potent non-nucleoside inhibitor of HIV-1 reverse transcriptase indicated for use in combination with other antiretroviral agents for the treatment of HIV-1 infection, has been evidenced when administered along with piperine [23]. Mean maximum plasma concentration, area under the plasma concentration-time curve post-dose, was increased significantly when co-administered with piperine (20 mg) in healthy male subjects.

It has been observed that intragastric co-treatment with dietary piperine enhances the bioavailability of epigallocatechin-3-gallate (EGCG) from green tea in mice [28]. Co-administration of 164 μmol/kg EGCG and 70 μmol/kg piperine to mice increased the plasma C_{max} and AUC by 1.3-fold compared to mice treated with EGCG only. Piperine appeared to increase EGCG bioavailability by inhibiting glucuronidation and gastrointestinal transit. Co-administration of piperine enhanced the bioavailability of β-lactam antibiotics amoxicillin trihydrate and cefotaxime significantly in rats [25]. The improved bioavailability is reflected in various pharmacokinetic parameters, namely, t_{max}, C_{max}, half-life, and AUC of these antibiotics. The effect of piperine on the metabolic activation and distribution of aflatoxin B_1 (AFB_1) in rats has been studied [24]. Rats pretreated with piperine accumulated considerable AFB_1 in plasma and in the tissues examined as compared to the controls.

1.2.3 Beneficial Influences on the Gastrointestinal System

Digestive Stimulant Action

While studying the effect of spices on the secretion and composition of saliva in human subjects, it has been observed that black pepper enhances the secretion of saliva and the activity of salivary amylase [29]. The digestive stimulant action of spices is exerted through (1) a beneficial stimulation of the liver to produce and secrete bile rich in bile acids, which play a very important role in fat digestion and absorption, or (2) a beneficial stimulation of the activities of enzymes of pancreas and intestine that participate in digestion [30]. Black pepper and its bioactive alkaloid piperine, examined for their effect on bile secretion as a result of dietary intake for a period of time in experimental rats, did not show any beneficial stimulatory influence on bile acid production by the liver and its secretion into bile [31]. On the other hand, oral administration of piperine as a single dose significantly increased bile acid secretion. Influence of dietary intake of piperine on the pancreatic digestive enzymes and the terminal digestive enzymes of the small intestinal mucosa has been examined in experimental rats. Significantly increased activities of pancreatic lipase, amylase, chymotrypsin, and trypsin were observed as a result of dietary intake of piperine in these experimental rats [32]. Such beneficial influence of this spice on the activity of these enzymes was not evident when administered as a single oral dose. Piperine also significantly enhanced the activity of intestinal lipase and amylase in animals given single oral dose of piperine [33] (Table 45.3).

Dietary piperine (0.02 %) was examined for its influence on bile secretion, digestive enzymes of pancreas, and absorption of dietary fat in rats fed high fat (30 %) for 8 weeks [34]. Piperine enhanced the activity of pancreatic lipase and

Table 45.3 Beneficial influences of piperine on gastrointestinal system

System	Observed effect	References
Digestive stimulant action		
Rats	(a) Stimulation of digestive enzymes of pancreas by dietary piperine	[32]
	(b) Stimulation of digestive enzymes of intestine by dietary piperine	[33]
	(c) Oral administration of piperine increases biliary bile acid secretion	[31]
	(d) Enhanced activity of pancreatic lipase, higher secretion of bile acids in high-fat-fed rats which was associated with enhanced fat absorption	[34]
Influence on intestinal motility and food transit time		
Humans	(a) Increased orocecal transit time after black pepper consumption	[41]
Rats	(a) Gastrointestinal food transit time shortened by dietary piperine	[35]
	(b) Piperine inhibited gastric emptying of solids/liquids	[36]
Mice	(a) Piperine inhibited gastrointestinal transit	[36]
	(b) Piperine dose-dependently delayed gastrointestinal motility	[37]
Effect on gastric mucosa		
Humans	(a) Black pepper caused increases in gastric parietal and pepsin secretion and increased gastric cell exfoliation in humans	[38]
Rats	(a) Black pepper increased gastric acid secretion in anesthetized rats	[39]
	(b) Piperine increased gastric acid secretion	[40]
	(c) Piperine had protective action against stress-induced gastric ulcer	[41]
	(d) Dietary piperine showed protective effect on gastric and intestinal mucosa with respect to activities of antioxidant enzymes and gastric mucin content	[42]
	(e) Dietary piperine alleviated the diminished activities of antioxidant enzymes in gastric and intestinal mucosa during ethanol-induced oxidative stress	[42]
Mice	(a) Piperine had protective action against stress-induced gastric ulcer	[41]
Antidiarrheal property		
Mice	(a) Piperine inhibited diarrhea produced by castor oil, arachidonic acid, etc.	[36]
	(b) Piperine reduced castor oil-induced intestinal fluid accumulation	[43]
Influence on absorptive function		
Rats	(a) Piperine stimulated γ-glutamyl transpeptidase activity and enhanced uptake of amino acids in isolated epithelial cells of rat jejunum	[44]
	(b) Piperine modulated membrane dynamics and permeation characteristics, increasing absorptive surface and induction of synthesis of proteins associated with cytoskeletal function	[45]
	(c) Dietary piperine induced alteration in BBM fluidity and permeability property, associated with increased microvilli length, resulting in higher absorptive surface of the small intestine	[46]
	(d) Duodenum, jejunum, and ileum portions of small intestines isolated from rats pre-fed piperine showed higher uptake of iron, zinc, and calcium	[47]
	(e) Higher in vitro absorption of β-carotene in the intestines was evidenced in piperine-fed animals	[48]
	(f) Dietary piperine improved intestinal absorption of orally administered β-carotene	[49]

caused higher secretion of biliary bile acids in high-fat-fed rats which was associated with enhanced fat absorption. Stimulation of lipid mobilization from adipose tissue was suggested by the decrease in perirenal adipose tissue weight by dietary piperine. This was also accompanied by prevention of the accumulation of triglyceride in liver and serum in high-fat-fed rats. Activities of key lipogenic enzymes in liver were reduced which was accompanied by an increased activity of hormone-sensitive lipase. Thus, dietary piperine enhances fat digestion and absorption in high-fat-fed situation through enhanced secretion of bile salts and a stimulation of the activity pancreatic lipase. At the same time, the energy expenditure is facilitated to prevent the accumulation of absorbed fat.

Influence on Absorptive Function

Piperine (25–100 μM) has been shown to significantly stimulate γ-glutamyl transpeptidase (γ-GT) activity in vitro and enhance the uptake of amino acids in freshly isolated epithelial cells of rat jejunum [44]. The kinetic behavior of γ-GT was altered in the presence of piperine, suggesting that this alkaloid may interact with the lipid environment to produce effects leading to increased permeability of the intestinal cells. It is hypothesized that piperine's bioavailability-enhancing property may be partly attributed to increased absorption [45]. Piperine also caused an increase in intestinal brush border membrane fluidity and stimulated the activities of leucine aminopeptidase and glycyl-glycine dipeptidase due to the alteration in enzyme kinetics. This suggests that piperine modulates the membrane dynamics due to its apolar nature by interacting with surrounding lipids and hydrophobic portions in the protein vicinity, which may decrease the tendency of membrane lipids to act as stearic constrains to enzyme proteins and thus modify enzyme conformation. Ultrastructural studies with piperine showed an increase in microvilli length with a prominent increase in free ribosomes and ribosomes on the endoplasmic reticulum in enterocytes, suggesting that synthesis or turnover of cytoskeletal components or membrane proteins may be involved in the observed effect. Thus, piperine may induce alterations in membrane dynamics and permeation characteristics, along with induction of the synthesis of proteins associated with cytoskeletal function, resulting in an increase in the absorptive surface, thus assisting efficient permeation through the epithelial barrier.

Beneficial influence of black pepper and its bioactive alkaloid piperine on the small intestinal ultrastructure has been revalidated in a recent study [46]. Groups of rats were maintained on dietary black pepper (0.5 %) or piperine (0.02 %) for 8 weeks. Membrane fluidity study using an apolar fluorescent probe showed increased BBM fluidity in these spice-fed animals. This was corroborated by decreased cholesterol: phospholipid ratio in jejunal and ileal regions of the intestine. The dietary spice or its bioactive compound stimulated the activities of BBM enzymes – glycyl-glycine dipeptidase, leucine aminopeptidase, and γ-glutamyl transpeptidase – in jejunal mucosa, suggesting a modulation in membrane dynamics due to the apolar spice bioactive compounds interacting with surrounding lipids and hydrophobic portions in the protein vicinity and thus modify enzyme conformation. Scanning electronic microscopy of the intestinal villi in these spice treatments revealed alteration in the ultrastructure, especially an increase in microvilli length which would mean a beneficial increase in the absorptive surface of the small intestine, providing for an increased bioavailability of micronutrients. Thus, dietary black pepper and piperine were evidenced to induce alteration in BBM fluidity and passive permeability property, associated with the induction in the increased microvilli length, resulting in increased absorptive surface of the small intestine.

In view of dietary black pepper and piperine specifically altering the ultrastructure and permeability characteristics of small intestines, these dietary interventions have been examined for their possible influence on intestinal absorption of minerals [47]. Everted segments of duodenum, jejunum, and ileum portions of small intestines isolated from rats pre-fed this spice for 8 weeks and examined for the uptake of iron, zinc, and calcium from incubations containing digesta of finger millet showed higher absorption of these minerals. Dietary black pepper and piperine have also been studied for their possible influence on absorption of β-carotene by examining its uptake by the intestinal segments from rats pre-fed these spices [48]. Higher in vitro absorption of β-carotene in the intestines was evidenced in piperine-fed animals which was 47 % higher than control, while dietary black pepper produced 59 % increase. An animal study has also evaluated the influence of dietary piperine on the absorption of orally administered β-carotene and its conversion to vitamin A [49]. Concentration of β-carotene significantly increased in serum, liver, and intestine of piperine-fed rats 4 h after single oral administration of β-carotene suggesting improved intestinal absorption of β-carotene. Retinol concentration was not however changed in these animals suggesting that bioconversion of β-carotene to vitamin A was not similarly influenced. Activities of the two enzymes involved in the bioconversion of β-carotene to vitamin A, namely, β-carotene-15,15′-dioxygenase and retinal reductase in intestines and liver, remained unaffected by dietary piperine. Activities of these two enzymes involved in the bioconversion of β-carotene to retinal were inhibited by piperine in vitro, thus corroborating with in vivo observation.

Influence on Gastrointestinal Motility and Food Transit Time

An increase in orocecal transit time has been observed in human subjects after black pepper (1.5 g) consumption in a study of the effect on small intestinal peristalsis [50]. Piperine inhibited gastric emptying of solids/liquids in rats and gastrointestinal transit in mice in a dose- and time-dependent manner [36]. It significantly inhibited gastric emptying of solids and gastrointestinal transit at the doses extrapolated from humans (1 and 1.3 mg/kg *p.o.* in rats and mice, respectively). One week oral treatment with the same dose in rats and mice did not produce a significant change compared to single dose administration. Gastric emptying inhibitory activity of piperine is independent of gastric acid and pepsin secretion. Piperine which activates vanilloid receptors (0.5 – 20 mg/kg *i.p.*) dose-dependently delayed gastrointestinal motility in mice [37]. The inhibitory effect of piperine (10 mg/kg) was strongly attenuated in capsaicin (75 mg/kg)-treated mice. The study indicated that the vanilloid ligand piperine can reduce upper gastrointestinal motility. The effect of piperine involves capsaicin-sensitive neurons but not vanilloid receptors.

The gastrointestinal food transit time in experimental rats was significantly shortened by dietary piperine [35]. The reduction in food transit time produced by dietary piperine roughly correlated with its beneficial influence on digestive enzymes [30]. Thus, dietary piperine which has enhanced the activity of digestive enzymes also has markedly reduced the food transit time at the same level of consumption. This reduction in food transit time could probably be attributed to acceleration in the overall digestive process as a result of increased availability of digestive enzymes.

Antidiarrheal Property

Peppers are added in traditional antidiarrheal formulations of different herbs. In a study made in experimental mice, the antidiarrheal activity of piperine against diarrhea produced by castor oil, $MgSO_4$, and arachidonic acid has been evidenced at 8 and 32 mg/kg *p.o.* dose [51]. Inhibition of castor oil-induced entero pooling by piperine suggests its inhibitory effect on prostaglandins. Piperine (2.5–20 mg/kg, *i.p.*) dose-dependently reduced castor oil-induced intestinal fluid accumulation in experimental mice; piperine reduces castor oil-induced fluid secretion with a mechanism involving capsaicin-sensitive neurons [43].

Effect on Gastric Mucosa

Pungent spices have long been implicated to cause gastric mucosal injury. The effects of black pepper on the gastric mucosa were assessed using double-blind intragastric administration of the spice (1.5 g) to healthy human volunteers and found to be similar to aspirin [38]. Black pepper caused significant increases in parietal secretion, pepsin secretion, and potassium loss. Gastric cell exfoliation (as reflected in DNA loss in gastric contents) was increased after black pepper administration and mucosal micro bleeding was also observed.

On the other hand, protective action of piperine against experimental gastric ulcer has been evidenced in rats and mice wherein the gastric mucosa damage was induced by stress, indometacin, HCl, and pyloric ligation [41]. Piperine at 25 − 100 mg/kg *i.g.* protected animals from gastric ulceration in a dose-dependent manner as indicated by inhibition of the volume of gastric juice, gastric acidity, and pepsin activity. Black pepper has been reported to significantly increase gastric acid secretion in anesthetized rats [39]. Piperine has been shown to produce dose-dependent (20–142 mg/kg) increase in gastric acid secretion in rats [40]. Involvement of cholinergic receptors in the observed piperine-induced increase in gastric acid secretion is ruled out as the effect of piperine was significantly antagonized by cimetidine (1 mg/kg) but not by atropine (1 mg/kg). There is however an indication that increased acidity induced by piperine could be due to stimulation of histamine H_2 receptors by this pepper alkaloid.

In a recent study, the protective effect of dietary black pepper (0.5 %) and piperine (0.02 %) with respect to activities of antioxidant enzymes in gastric and intestinal mucosa was examined [42]. These dietary interventions significantly enhanced the activities of antioxidant enzymes − superoxide dismutase, catalase, glutathione reductase, and glutathione-*S*-transferase – in both gastric and intestinal mucosa, suggesting a gastrointestinal protective role for black pepper and piperine. In a separate study, these were found to alleviate the diminished activities of antioxidant enzymes in gastric and intestinal mucosa under conditions of ethanol-induced oxidative stress. The gastro protective effect was also reflected in their positive effect on mucosal glycoproteins, thereby lowering mucosal injury. The amelioration of the ethanol-induced decrease in the activities of antioxidant enzymes in gastric and intestinal mucosa by dietary spices suggests their beneficial gastrointestinal protective role.

1.2.4 Antioxidant Effects

Piperine has been demonstrated in in vitro experiments to protect against oxidative damage by quenching free radicals and reactive oxygen species and inhibiting lipid peroxidation [52]. Piperine is reported to have marginal inhibitory effects on ascorbate/Fe^{2+}-induced lipid peroxidation in rat liver microsomes even at high concentrations (600 μM) when compared to the beneficial inhibition of lipid peroxidation by antioxidants – vitamin E, *t*-butylhydroxy toluene and *t*-butylhydroxy anisole [53]. Both water and ethanol extract of black pepper exhibited strong total antioxidant activity and significant inhibition of peroxidation of linoleic acid emulsion [54]. Piperine is shown to be an effective antioxidant and offers protection against oxidation of human low-density lipoprotein (LDL) [55]. The aqueous extract of black pepper and piperine have been examined for their effect on human PMNL 5-lipoxygenase (5-LO), the key enzyme involved in biosynthesis of leukotrienes [56]. The formation of 5-LO product was significantly inhibited in a concentration-dependent manner. Thus, piperine might exert an antioxidant physiological role by modulating 5-LO pathway (Table 45.4).

Piperine treatment (10 mg/kg/day, *i.p.* for 14 days) has been assessed for protection against diabetes-induced oxidative stress in streptozotocin-induced diabetic rats [58]. Treatment with piperine reversed the diabetic effects on glutathione concentration in brain, on renal glutathione peroxidase and superoxide dismutase activities, and on cardiac glutathione reductase activity and lipid peroxidation. Thus, treatment with piperine for 14 days is only partially effective as an antioxidant in diabetes. The ability of piperine to reduce the oxidative changes induced by chemical carcinogens (7,12-dimethyl benzanthracene, dimethyl aminomethyl azobenzene, and 3-methyl cholanthrene) has been investigated in rat intestinal model [57]. A protective role of piperine against the oxidative alterations by these carcinogens was indicated by the observed inhibition of TBARS, a significant increase in the glutathione levels and restoration in γ-glutamyl transpeptidase and Na^+, K^+-ATPase activity in intestinal mucosa. Oral supplementation of piperine (50 mg/kg) effectively suppressed lung carcinogenesis by benzo (α)pyrene as revealed by a decrease in the extent of mitochondrial lipid peroxidation and concomitant increase in the activities of enzymatic antioxidants and nonenzymatic antioxidant levels when compared to lung carcinogenesis-bearing mice [61]. This suggests that piperine may extend its chemopreventive effect by modulating lipid peroxidation and augmenting antioxidant defense system.

The effect of supplementation of black pepper or piperine for a period of 10 weeks on tissue lipid peroxidation and enzymic and nonenzymic antioxidants has been examined in rats fed a high-fat diet (20 % coconut oil and 2 % cholesterol),

Table 45.4 Antioxidant effects of piperine

System	Observed effect	Reference
In vitro	(a) Inhibition/quenching of superoxides and hydroxyl radicals by piperine; inhibition of lipid peroxidation	[52]
	(b) Marginal inhibitory effect of piperine on ascorbate-Fe^{++}-induced lipid peroxidation in rat liver microsome	[53]
	(c) Water and ethanol extract of black pepper exhibited strong total antioxidant activity and inhibited peroxidation of linoleic acid emulsion	[54]
	(d) Piperine protects Cu^{++}-induced lipid peroxidation of human LDL	[55]
	(e) Black pepper aqueous extract and piperine inhibit human PMNL 5-lipoxygenase	[56]
Rats	Piperine treatment protected against oxidative stress induced in intestinal lumen by carcinogens	[57]
Streptozotocin-diabetic rats	*i.p.* administration of piperine for 2 weeks partially protected against diabetes-induced oxidative stress	[58]
High-fat-fed rats	Dietary black pepper/piperine reduces high-fat diet-induced oxidative stress by lowering lipid peroxidation, restoring activities of antioxidant enzymes and GSH	[59]
Mice	Piperine treatment decreased mitochondrial lipid peroxidation and augmented antioxidant defense system during benzo(α) pyrene- induced lung carcinogenesis	[60]

and it was observed that these can reduce high-fat diet-induced oxidative stress [59]. Simultaneous supplementation with black pepper or piperine lowered TBARS and conjugated diene levels and maintained antioxidant enzymes and glutathione levels in the liver, heart, kidney, intestine, and aorta near to those of control rats.

1.2.5 Antimutagenic and Tumor Inhibitory Effects

Black pepper has been shown to be effective in reducing the mutational events induced by the promutagen ethyl carbamate in *Drosophila* melanogaster [62]. Suppression of metabolic activation or interaction with the active groups of mutagens could be mechanism by which this spice exerts its antimutagenic action. While studying piperine for its immunomodulatory and antitumor activity, piperine was found to be cytotoxic toward Dalton's lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cells at 250 μg/mL [63]. Piperine was also found to be cytotoxic toward L929 cells in culture at a concentration of 50 μg/mL. Administration of piperine (1.14 mg/animal) could inhibit the solid tumor development in mice induced with DLA cells and increase the life span of mice-bearing Ehrlich ascites carcinoma tumor (Table 45.5).

The effect of piperine on the cytotoxicity and genotoxicity of aflatoxin B_1 (AFB_1) has been studied in rat hepatoma cells H4IIEC3/G-(H4IIE) using cellular growth and formation of micronuclei as endpoints [64]. Piperine markedly reduced the toxicity of aflatoxin. That is, AFB_1-induced formation of micronuclei in a concentration-dependent manner probably by suppressing cytochromes P_{450} mediated bioactivation

Table 45.5 Antimutagenic and cancer preventive effects of piperine

System	Observed effect	References
In vitro and cell lines	(a) Black pepper is effective in reducing mutational events induced by procarcinogen – ethylcarbamate in *Drosophila*	[62]
	(b) Piperine markedly reduced the AFB_1-induced formation of micronuclei in H4IIE cells in a concentration-dependent manner	[64]
	(c) Piperine counteracts $CYP_{450}2B1$-mediated toxicity of AFB_1 in Chinese hamster cells and therefore has chemopreventive effect against procarcinogens activated by $CYP_{450}2B1$	[65]
Rats	(a) Piperine administration effectively reduced cyclophosphamide-induced chromosomal aberrations in bone marrow cells	[66]
	(b) Dietary black pepper was evidenced to suppress colon carcinogenesis induced by the procarcinogen 1,2-dimethylhydrazine	[67]
Mice	(a) Tumor inhibitory activity of black pepper in mice implanted with Ehrlich ascites tumor	[68]
	(b) Piperine inhibited tumor development in mice induced with Dalton's lymphoma cells and increased the life span of afflicted mice	[63]
	(c) Anti-metastatic activity of piperine on lung metastasis induced by melanoma cells	[69]
	(d) Chemopreventive effect of piperine on benzo(α)pyrene induced experimental lung cancer	[61, 70, 71]

of the mycotoxin. The potential of piperine for inhibiting the activity of cytochrome $P_{450}2B1$ and protecting against AFB_1 has been investigated in Chinese hamster r2B1 cells engineered for the expression of rat $CYP_{450}2B1$ [65]. Piperine at 60 μM completely counteracted cytotoxicity and formation of micronuclei by 10 μM AFB_1 and reduced the toxic effects of 20 μM AFB_1 by > 50 %. The results suggest that (1) piperine is a potent inhibitor of rat $CYP_{450}2B1$ activity, (2) AFB_1 is activated by r2B1 cells to cytotoxic and genotoxic metabolites, and (3) piperine counteracts $CYP_{450}2B1$-mediated toxicity of AFB_1 in the cells and might, therefore, offer a potent chemopreventive effect against procarcinogens activated by $CYP_{450}2B1$.

The antimutagenic effect of piperine has been studied with respect to its influence on chromosomes in rat bone marrow cells [66]. Wistar rats orally administered piperine (100, 400, and 800 mg/kg) were challenged with cyclophosphamide (*i.p.* 50 mg/kg). Piperine (100 mg/kg) produced significant reduction in cyclophosphamide-induced chromosomal aberrations, suggesting that it may have antimutagenic potential. Black pepper extracts have been demonstrated to possess tumor inhibitory activity [72]. Tumor-reducing activity of orally administered extracts of black pepper was studied in mice transplanted *i.p.* with Ehrlich ascites tumor, wherein life span was increased in these mice by 65 % [68]. The antimetastatic activity of piperine has been demonstrated by the inhibition of lung metastasis induced by B16F-10 melanoma cells in C57BL/6 mice [69].

The cytoprotective effect of piperine on benzo(α-)pyrene-induced lung cancer has been investigated in mice and observed that piperine may extend its chemopreventive effect by modulating lipid peroxidation and augmenting antioxidant defense system [70]. Oral administration of piperine (100 mg/kg) effectively suppressed this experimental lung cancer. The protective role of piperine was examined during experimental lung carcinogenesis with reference to its effect on DNA damage and detoxification enzyme system [73]. The activities of detoxifying enzymes such as glutathione transferase, quinone reductase, and UDP-glucuronyl transferase were decreased while the hydrogen peroxide level was increased in the lung cancer-bearing animals. Supplementation of piperine (50 mg/kg) enhanced these detoxification enzymes and reduced DNA damage. These results explain the association between anti-peroxidative effect of piperine and ultimately the capability of piperine to prevent cancer. A significant suppression in the micronuclei formation induced by benzo(α-)pyrene and cyclophosphamide following oral administration of piperine at doses of 25, 50, and 75 mg/kg in mice has been reported [74].

Piperine has been evidenced to show chemopreventive effects when administered orally on lung cancer-bearing animals [61]. The beneficial effect of piperine is primarily exerted during initiation phase and post-initiation stage of benzo(α-) pyrene-induced lung carcinogenesis, via beneficial modulation of lipid peroxidation and membrane-bound ATPase enzymes. The ability of piperine to prevent lung carcinogenesis induced by benzo(α-)pyrene in mice and its effects on cell proliferation has been studied [60, 71]. Administration of piperine significantly decreased the levels of lipid peroxidation, protein carbonyls, nucleic acid, and polyamine synthesis that were found to be increased in lung cancer-bearing animals. Piperine could effectively inhibit benzo(α-)pyrene-induced lung carcinogenesis in albino mice by offering protection from protein damage and also by suppressing cell proliferation. Dietary (0.5 %) black pepper has been evidenced to suppress colon carcinogenesis induced by 1,2-dimethylhydrazine in rats [67].

1.2.6 Negative Influence on Reproductive System

Black pepper is used as contraceptive in folk medicine in parts of India. The reproductive toxicity of piperine has been studied in albino mice with respect to the effect on estrous cycle, toxicity to male germ cells, fertilization, implantation, and growth of pups [75]. Piperine (10 and 20 mg/kg) increased the period of the diestrous phase resulting in decreased mating performance and fertility. Postpartum litter growth was not affected by the piperine treatment, and sperm shape abnormalities were not induced at doses up to 75 mg/kg. Considerable anti-implantation activity was recorded after 5 days post-mating oral treatment with piperine. These results suggest that piperine interferes with several crucial reproductive events in a mammalian model. The effect of piperine on the fertilization of eggs with sperm has been investigated in female hamsters intragastrically treated with piperine at doses of 50 or 100 mg/kg from day 1 through day 4 of the estrous cycle [76]. During piperine treatment, these females were superovulated and artificially inseminated (AI) with spermatozoa from untreated male hamsters at 12 h after hCG injection. Administration of piperine to the superovulated animals markedly enhanced the percent fertilization at 9 h after AI (Table 45.6).

Table 45.6 Other biological effects of piperine

System	Observed effect	References
Effect on reproductive system		
In vitro	(a) Piperine decreased fertilizing ability of hamster sperms and degree of polyspermia in vitro	[77]
Rats	(b) Continued oral intake of piperine produced reduction in weights of testis, fall in sperm concentration, decrease in intra-testicular testosterone	[78]
Mice	(c) Oral intake of piperine decreased fertility due to interference with crucial reproductive events in albino mice	[75]
Anti-inflammatory activity		
Rats	Anti-inflammatory activity of piperine in experimental models: carrageenan- induced rat paw edema, cotton pellet granuloma, croton oil-induced granuloma pouch	[79]
Hepatoprotective activity		
Mice	Piperine exerted protection against *t*-butyl hydroperoxide and carbon tetrachloride in hepatotoxicity by reducing lipid peroxidation	[80]
Melanocyte stimulation		
In vitro	Growth stimulatory activity of black pepper extract in cultured melanocytes	[81]
Neuropharmacological activity		
Rats	(a) Piperine-administered animals possessed antidepression-like activity and cognitive-enhancing effect	[82]
	(b) Antidepressant-like effects of chronically administered piperine depend on the augmentation of the neurotransmitter synthesis	[83]
Anticonvulsant effects		
Human	Piperine treatment reduced the number of seizures in epileptic children	[84]

Piperine administration (10 mg/kg for 30 days) in mature male albino rats caused a significant reduction in the weights of testis and accessory sex organs [78]. Histological studies revealed that piperine caused severe damage to the seminiferous tubule, decrease in seminiferous tubular and Leydig cell nuclear diameter, and desquamation of spermatocytes and spermatids. The effect of piperine on the fertilizing ability of hamster sperm was investigated in vitro [77]. Addition of $0.18 - 1.05$ mM piperine reduced both the percentage of eggs fertilized and the degree of polyspermia in a dose-dependent manner. Rats orally administered piperine at doses of 1, 10, and 100 mg/kg for 30 consecutive days showed a decrease in the activity of antioxidant enzymes and sialic acid levels in the epididymis and thereby increased reactive oxygen species levels that could damage the epididymal environment and sperm function [85].

1.2.7 Other Physiological Effects

Anti-inflammatory activity of piperine has been reported in rats employing different experimental models like carrageenan-induced rat paw edema, cotton pellet granuloma, and croton oil-induced granuloma pouch [79]. Piperine acted significantly on early acute changes in inflammatory processes and chronic granulative changes. Pungent principles of dietary spices including piperine have been reported to induce a warming action via adrenal catecholamine secretion [86].

Piperine has been reported to exert a significant protection against *t*-butyl hydroperoxide and carbon tetrachloride-induced hepatotoxicity by reducing lipid peroxidation, by leakage of enzymes – alanine aminotransferase and alkaline phosphatase – and by preventing the depletion of glutathione and total thiols in the intoxicated mice [80]. The neuropharmacological activity of piperine-administered Wistar rats (5, 10, and 20 mg/kg once daily) was determined after single, 1, 2, 3, and 4 weeks of treatment [82]. Piperine showed antidepression-like activity and cognitive-enhancing effect at all treatment duration, suggesting its potential to improve brain function. Antidepressant-like effects of piperine have been demonstrated in two depressive models: forced swimming test and tail suspension test [83]. The results indicated that after 2 weeks of chronic administration, piperine (10 − 20 mg/kg) significantly reduced the duration of immobility in both the models. The study demonstrated that the antidepressant-like effects of piperine and antiepilepsirine might depend on the augmentation of the neurotransmitter synthesis or the reduction of the neurotransmitter reuptake.

Melanocyte stimulants are of interest as potential treatments for the depigmentary skin disorder vitiligo. Black pepper water extract and piperine promote melanocyte proliferation in vitro. Black pepper extract was found to possess growth-stimulatory activity in cultured melanocytes [81]. Its aqueous extract at 0.1 mg/mL was observed to cause nearly 300 % stimulation of the growth of a cultured mouse melanocyte line, in 8 days; hence, it is inferred that piperine is a potential repigmenting agent for the treatment of vitiligo.

A study of the in vitro effects of piperine on three bioenergetic reactions, namely, oxidative phosphorylation, ATPase activity, and calcium transport by isolated rat liver mitochondria, suggested that piperine inhibits mitochondrial oxidative phosphorylation at the level of respiratory chain [87]. Piperine did not inhibit the mitochondrial ATPase activity induced by dinitrophenol and was found to diminish calcium uptake. The influence of piperine on the enzymes and bioenergetic functions in isolated rat liver mitochondria and hepatocytes has been studied, and it was observed that piperine produces concentration-related site-specific effects on mitochondrial bioenergetics and enzymes of energy metabolism [88].

Piper longum and *Piper nigrum* are conventionally used as immuno-enhancers in Indian system of medicine. The underlying mechanism however remains unknown. Pepper has been used in China as a folk remedy for epilepsy. Piperine has been identified by researchers as having anticonvulsant effects in animal models, and antiepilepsirine, a derivative of piperine, has been used in China to treat epilepsy since 1975. A clinical trial on epileptic children-tested antiepilepsirine (10 mg/kg; two or three times a day) decreased the number of seizures in majority of subjects [84].

1.3 Absorption and Metabolism of Piperine

Tissue distribution and elimination of piperine has been examined following its oral intake in rats. Piperine administered to rats at a dose of 170 mg/kg by gavage or

85 mg/kg *i.p.* was absorbed to an extent of about 97 % [26, 89]. A maximum of 10.8 % of administered piperine was seen in tissues at 6 h. Only 3 % of the administered dose was excreted as piperine in the feces, while it was not detectable in urine. When rat intestinal segments were incubated with 100–1,000 μg of piperine, about 44–63 % of the added piperine disappeared from the mucosal side [89, 90]. Absorption of piperine in this in vitro system which was maximum at 800 μg per 10 mL was about 63 % [89]. The absorbed piperine could be traced in both the serosal fluid and in the intestinal tissue. When piperine was associated with mixed micelles, its in vitro intestinal absorption was relatively higher [90].

Highest concentration in stomach and small intestine was attained at about 6 h post-piperine dosage [91]. Only traces of piperine were detected in serum, kidney, and spleen from 30 min to 24 h. About $1 - 2.5$ % of the *i.p.*-administered piperine was detected in the liver during $0.5 - 6$ h after administration as contrasted with $0.1 - 0.25$ % of the orally administered dose. The increased excretion of conjugated uronic acids, conjugated sulfates, and phenols indicated that scission of the methylenedioxy group of piperine, glucuronidation, and sulfation appear to be the major steps in the disposition of piperine in the rat. After oral administration of piperine (170 mg/kg) to rats, the metabolites in urine were identified to be piperonylic acid, piperonyl alcohol, and piperonal and vanillic acid in the free form, whereas only piperic acid was detected in $0 - 6$ h bile [91]. Kidney appears to be the major excretion route for piperine metabolites in rats as no metabolite could be detected in feces. In a later investigation [92], a new major urinary metabolite 5-(3,4-methylenedioxy phenyl)-2,4-pentadienoic acid-*N*-(3-yl propionic acid)-amide was detected in rat urine and plasma using HPLC. This metabolite has a unique structure in that it retains methylenedioxy ring and conjugated double bonds while the piperidine ring is modified to form propionic acid group.

The absorption dynamics of piperine in intestine has been studied, and the data suggested that piperine is absorbed very fast across the intestinal barrier [93]. It may act as an apolar molecule and form apolar complex with drugs and solutes. It may modulate membrane dynamics due to its easy partitioning thus helping in efficient permeability across the barriers. Being essentially water insoluble, piperine is presumed to be assisted by serum albumin for its transport in blood after its intestinal absorption. The binding of piperine to serum albumin has confirmed by employing steady-state and time-resolved fluorescence techniques [94]. These observations are significant in understanding the transport of piperine in blood under physiological conditions.

1.4 Conclusions

Black pepper or its main bioactive alkaloid piperine, the ingredients used in a number of ancient and folk medicines, is now demonstrated to possess diverse health beneficial physiological effects. The most far-reaching attribute of piperine has been its inhibitory influence on hepatic and intestinal drug-metabolizing system. It strongly inhibits a particular cytochrome P_{450} and hence phase-I reactions

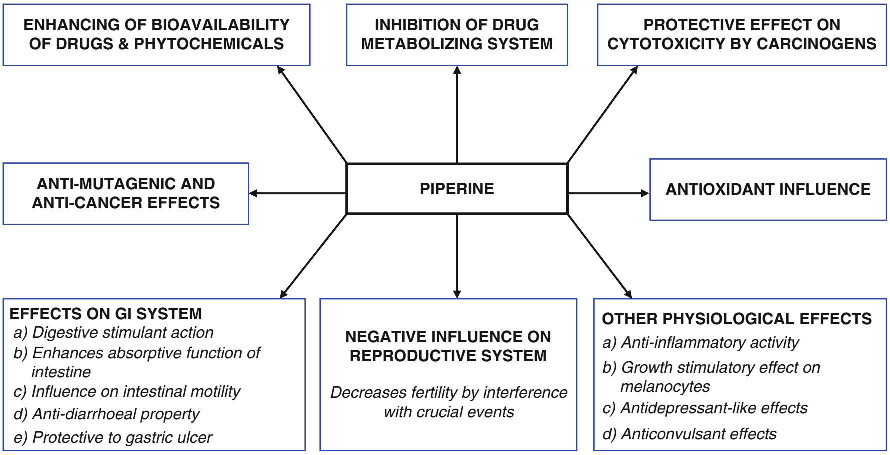

ENHANCING OF BIOAVAILABILITY OF DRUGS & PHYTOCHEMICALS
INHIBITION OF DRUG METABOLIZING SYSTEM
PROTECTIVE EFFECT ON CYTOTOXICITY BY CARCINOGENS
ANTI-MUTAGENIC AND ANTI-CANCER EFFECTS
PIPERINE
ANTIOXIDANT INFLUENCE
EFFECTS ON GI SYSTEM
a) Digestive stimulant action
b) Enhances absorptive function of intestine
c) Influence on intestinal motility
d) Anti-diarrhoeal property
e) Protective to gastric ulcer
NEGATIVE INFLUENCE ON REPRODUCTIVE SYSTEM
Decreases fertility by interference with crucial events
OTHER PHYSIOLOGICAL EFFECTS
a) Anti-inflammatory activity
b) Growth stimulatory effect on melanocytes
c) Antidepressant-like effects
d) Anticonvulsant effects

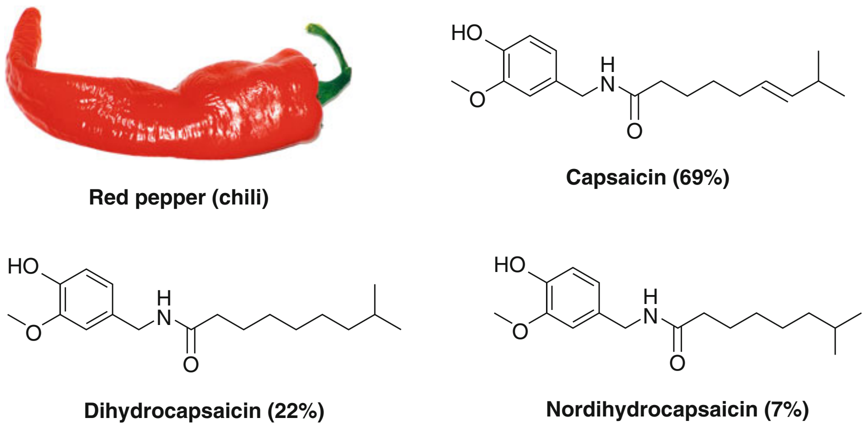
Fig. 45.2 Summary of the diverse physiological effects of piperine

mediated by the same, especially aromatic hydroxylation. It also strongly retards glucuronidation reactions of phase-II. As a result of interference with crucial drug-metabolizing reactions in the liver, piperine enhances the bioavailability of therapeutic drugs, i.e., increases their plasma half-life and delays their excretion. This particular inhibitory effect of piperine on drug metabolism and hence on drug bioavailability may be harnessed for increasing therapeutic effects. Gastrointestinal system is affected by black pepper and piperine in many ways. Both black pepper and piperine have been evidenced to have antidiarrheal property and an effect on intestinal motility and on the ultrastructure of intestinal microvilli improving absorbability of micronutrients. Piperine has been evidenced to protect against oxidative damage by inhibiting or quenching free radicals and lower lipid peroxidation and beneficially influence cellular antioxidant status in different situations of oxidative stress. Piperine also possesses cytoprotective effect by retarding the activation of certain procarcinogens by the drug-metabolizing system. Antimutagenic and antitumor properties of piperine have been evidenced in a few animal and cell line studies. Among other physiological effects piperine exerts, its potential antifertility influence on reproductive system has been clearly established in in vitro and animal systems (Fig. 45.2).

2 Capsaicin of Red Pepper (*Capsicum annuum*)

2.1 Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is the major pungent principle of chili peppers, belonging to the genus *Capsicum*. Dihydrocapsaicin and nordihydrocapsaicin are the other two alkaloids in the order of relative abundance. Chili peppers are extensively used in food as pungent spice, particularly in tropical



Red pepper (chili)

Capsaicin (69%)

Dihydrocapsaicin (22%)

Nordihydrocapsaicin (7%)

Fig. 45.3 Red pepper and capsaicinoids

countries. This alkaloid responsible for the pungency of the spice is also an irritant that produces a burning sensation with which it comes into contact. Capsaicin and several related compounds are called capsaicinoids and are produced as a secondary metabolite by chili peppers, probably as deterrents against certain herbivores and fungi. Pure capsaicin is a hydrophobic, colorless, odorless, crystalline compound. Capsaicin is the main capsaicinoid in chili peppers, followed by dihydrocapsaicin (Fig. 45.3).

Capsaicin is present in large quantities in the placental tissue (which holds the seeds), the internal membranes, and, to a lesser extent, the other fleshy parts of the fruits of Capsicum plants. The seeds themselves do not produce any capsaicin, although the highest concentration of capsaicin can be found in the white pith of the inner wall, where the seeds are attached [95]. Capsaicin is believed to be synthesized in the interlocular septum of chili peppers by addition of a branched-chain fatty acid to vanillylamine; specifically, capsaicin is made from vanillylamine and 8-methyl-6-nonenoyl Coenzyme-A [96].

2.2 Biological Effects of Capsaicin

Many health beneficial physiological effects of red pepper or its bioactive alkaloid – capsaicin – have been reported in recent decades which are summarized below:

2.2.1 Hypocholesterolemic and Hypolipidemic Effects

Red pepper, known for its characteristic pungency and its pungent principle capsaicin have been reviewed for their biological activity [97, 98] was probably the first investigator who studied the metabolic changes caused by feeding red pepper. The beneficial influence of red pepper or its pungent principle capsaicin on lipid metabolism is documented by several investigators. While studying the influence of red pepper and capsaicin on fat absorption in rats on a choline-free high hydrogenated fat (40 %) diet, Sambaiah et al. [99] observed that 5 % red pepper or equivalent levels of capsaicin (15 mg%) included in the diet had a tendency to lower serum and liver cholesterol levels. In another investigation, Srinivasan et al. [100] have reported a reduction in serum total cholesterol levels in rats on a 10 % groundnut oil diet incorporated with 1.5, 3.0, or 15 mg% capsaicin. In yet another study, capsaicin at as low as 0.2 mg% in the diet led to a lowering of serum total cholesterol in both 10 % and 30 % fat-fed rats [101]. An increase in LDL-cholesterol and a reduction in HDL-cholesterol were also observed in the 30 % hydrogenated fat group. In a subchronic toxicity study [102], rats were administered 50 mg/kg/day of capsaicin by gavage or 0.5 g/kg/day of a crude extract of capsicum fruit for 60 days. At 30, 40, 50, and 60 days, plasma total cholesterol levels were significantly reduced along with triglycerides and phospholipids (Table 45.7).

The effect of 14 mg% capsaicin in a diet containing 30 % lard has been studied [103]. The dose of capsaicin fed to rats was reported to be related to that commonly ingested by the Thai people. At the end of the 10-day isocaloric feeding period, serum cholesterol and pre-β-lipoprotein levels were not altered. The influence of capsaicin has also been studied in sucrose-induced hypertriglyceridemia in rats [112]. Capsaicin was fed at 0.15, 1.5, and 15 mg% levels in the diet (the lowest dose is comparable to human intake) for a period of 1 week. Total cholesterol and HDL-cholesterol were either significantly elevated.

The efficacy of capsaicin as a hypocholesterolemic agent has also been investigated in animals fed cholesterol in their diets. Sambaiah and Satyanarayana [104] have reported that the serum cholesterol levels in rats on a 1 % cholesterol + 5 % red pepper diet were lower than those not fed with red pepper. Liver cholesterol was lower in the red pepper- as well as capsaicin (an equivalent level of 15 mg%)-fed groups. Fecal excretion of free cholesterol and of bile acids was enhanced in animals fed the spice and capsaicin. The anti-hypercholesterolemic efficacy of dietary capsaicin has been evidenced in rats fed an atherogenic high-cholesterol diet, and such an influence also resulted in countering of the changes in membrane lipid profile in the erythrocytes [105]. In streptozotocin-induced diabetic situation however, dietary capsaicin did not show any beneficial hypolipidemic property [106].

Intubation of rabbits with 8 mg capsaicin/rabbit (body wt of about 850 g/day for 35 days) did not have any effect with regard to plasma cholesterol, triglyceride, and HDL-cholesterol when they were on a normal diet [108]. In contrast, in rabbits on a 0.5 % cholesterol diet, capsaicin had a beneficial effect in that the plasma cholesterol, triglycerides, and total cholesterol: HDL-cholesterol ratio were significantly lower than in animals fed cholesterol only. Turkeys on a 2–3 mg capsaicin/kg feed for 9 days along with 0.5 % cholesterol had lower total serum cholesterol than the controls [109]. Hypercholesterolemia was produced by feeding a 0.2 % cholesterol-supplemented diet, and capsaicin and dihydrocapsaicin were administered daily via the buccal route at dose of 4 mg per bird for 6 weeks [110]. In

Table 45.7 Hypolipidemic effects of capsaicin and red pepper in animal models

Animal model	Effect demonstrated	References
Rats on 40 % fat	5 % red pepper or 0.015 % capsaicin lowered serum and liver cholesterol	[99]
Rats	1.5, 3, and 15 mg% capsaicin reduced serum cholesterol	[100]
Rats on 10 % or 30 % fat	0.2 % capsaicin effectively lowered serum cholesterol	[101]
Rats	Subchronic levels of capsaicin (50 mg/kg for 60 days) lowered cholesterol and triglycerides	[102]
Rats on 30 % lard	14 mg% capsaicin produced hypocholesterolemic effect	[103]
Rats on 1 % cholesterol	Effective hypocholesterolemic effect; higher excretion of fecal sterols and bile acids	[104]
Rats on 0.5 % cholesterol	15 mg% capsaicin produced anti-hypercholesterolemic effect	[105]
Diabetic rats	15 mg% dietary capsaicin did not reverse hypercholesterolemia and hypertriglyceridemia	[106]
Hypercholesterolemic rats	Dietary capsaicin stimulated activity of hepatic cholesterol-7α-hydroxylase	[107]
Hypercholesterolemic rabbits	Reduced blood cholesterol, triglycerides	[108]
Hypercholesterolemic turkeys	Reduced blood cholesterol; ameliorated aortic atherosclerotic lesions by capsaicin	[109, 110]
Gerbils	75 mg oleoresin/kg decreased blood cholesterol and triglycerides; prevented lipid accumulation in liver and aorta	[111]

animals on a normal diet, total cholesterol, LDL-cholesterol, and HDL-cholesterol concentrations in plasma were increased, whereas VLDL-cholesterol was significantly decreased. Plasma total and LDL-cholesterol were significantly lower in birds on the cholesterol diet administered dihydrocapsaicin. Both the compounds brought about a reduction in VLDL-cholesterol and an increase in HDL-cholesterol in the cholesterol-fed group. Dihydrocapsaicin was more effective than capsaicin. The effect of capsicum oleoresin on dietary hypercholesterolemia was observed in gerbils at a dose of 75 mg/kg body weight/day [111]. The oleoresin reduced serum cholesterol and triglycerides as well as liver cholesterol and triglycerides. Capsaicin oleoresin feeding prevented the accumulation of cholesterol and triglycerides in the liver and aorta. The fecal excretion of cholesterol and triglycerides was significantly increased in oleoresin-fed gerbils.

The possible mechanism of action of capsaicinoids is the net effect of decreased cholesterol absorption and increased excretion of cholesterol and bile acids in the feces which may lead to a decrease in plasma LDL-cholesterol concentration by induced expression of hepatic LDL receptors [110]. These authors also have discussed the differences in response between normal and cholesterol-fed animals to possible hypocholesterolemic compounds. It has been demonstrated that dietary capsaicin stimulates hepatic conversion of cholesterol to bile acids through a stimulation of the activity of cholesterol-7α-hydroxylase, an important pathway for elimination of cholesterol from the body [107]. However, simultaneous stimulation of cholesterol synthesis as well through the activity of HMG-CoA reductase by this spice principle suggests that there may not be any significant contribution of the stimulation of bile acid biosynthesis to the hypocholesterolemic action of this spice principle, and the latter action may solely be due to interference with exogenous cholesterol absorption.

Heat processing of red pepper results in a significant loss of active principle [113, 114]. The hypocholesterolemic potency of raw and pressure-cooked red pepper was evaluated in experimental rats rendered hypercholesterolemic by feeding cholesterol-enriched diet and maintained for 8 weeks on 5 % spice diet [115]. The results suggested that although heat processing of red pepper by pressure cooking resulted in a considerable loss of capsaicin, the hypolipidemic potency of the parent spice was not significantly compromised.

2.2.2 Influence of Capsaicin on Biliary Cholesterol and Bile Acids: Anti-lithogenic Influence

Feeding of 7.5 and 15 mg% capsaicin to rats led to a significant increase in biliary total bile acids [116]. One of the implications of hypocholesterolemic influence is anti-lithogenic potential. Since capsaicin, besides being hypocholesterolemic agent, also enhances bile secretion and influences its composition, its influence on gallstone formation has been examined. Dietary capsaicin (0.015 %) caused a significant reduction in the formation of gallstones in mice and hamsters maintained on a lithogenic diet [117, 118]. Further, capsaicin effected a marked regression of preestablished gallstones in mice [119]. Increased cholesterol saturation index, cholesterol: Phospholipid ratio, and cholesterol: bile acid ratio in the bile caused by lithogenic diet was countered by dietary capsaicin. The anti-lithogenic influence of this spice compound was attributable to the cholesterol-lowering effect of these in blood and liver and their ability to lower cholesterol saturation index by altering the bile composition. When a combination of capsaicin and curcumin were given during experimental induction of cholesterol gallstone (CGS) in mice, there was no additive influence in reducing the incidence of CGS; nevertheless, the combination was more beneficial in reducing the oxidative stress in lithogenic situation [120]. The antilithogenecity of capsaicin has been considered to be due not merely to their ability to lower cholesterol saturation index but also to their influence on biliary proteins [121].

2.2.3 Protective Effect on Erythrocyte Integrity

Hyperlipidemic conditions are believed to affect the fluidity of red blood cells [122]. Hypolipidemic spice compound capsaicin in the diet might offer beneficial protective influence on the integrity of erythrocyte membranes, which are presumably altered in hyperlipidemic situation. In rats rendered hypercholesterolemic by feeding a cholesterol-enriched diet for 8 weeks, erythrocyte membranes were relatively enriched in cholesterol, resulting in elevated cholesterol: phospholipid ratio of their membranes affecting their structural integrity [105]. Inclusion of capsaicin (0.015 %) along with high cholesterol in the diet produced not only the hypolipidemic effect but also countered this altered lipid profile of erythrocyte membranes and thus corrected the increased osmotic fragility of erythrocytes [105]. Dietary capsaicin partially countered the changes in erythrocytes of hypercholesterolemic rats, namely, fatty acid profile of the membranes, phospholipid composition of the membrane bilayer, and reduced Ca^{2+}, Mg^{2+}-ATPase [123]. ESR spectra and fluorescence anisotropy parameters also revealed altered fluidity of erythrocytes in hypercholesterolemic rats which was significantly reversed by dietary capsaicin. In rats rendered hypertriglyceridemic by maintaining them on a high (30 %)-fat diet for 8 weeks, the lipid profile of erythrocyte membranes was not affected, but the erythrocytes displayed a resistance to osmotic lysis [124]. Inclusion of capsaicin (0.015 %) along with high fat in the diet which produced the hypotriglyceridemic effect appeared to beneficially correct this altered osmotic fragility of erythrocytes.

2.2.4 Antioxidant Effects

Lipid peroxidation in human erythrocyte membranes was found to be inhibited by capsaicin [125]. The antioxidant property of capsaicin in terms of inhibiting lipid peroxidation in rat liver [53] and in soybean phosphatidylcholine liposomal biomembrane has been reported [126]. Capsaicin is observed to inhibit copper ion-induced lipid peroxidation of human LDL [55]. The data suggested that capsaicin is an effective antioxidant and offers protection against oxidation of human LDL. Capsaicin inhibited the lipid peroxidation in rat liver mitochondria induced by ADP/Fe^{2+} significantly, more than the well-known antioxidant α-tocopherol [127]. Capsaicin was also found to scavenge 1,1′-diphenyl-2-picrylhydrazyl (DPPH) radicals in membranes. Capsaicin was found to scavenge radicals both at/near the membrane surface and in the interior of the membrane. Vanillin and 8-methyl-6-noneamide were major reaction products of capsaicin with DPPH radicals, thus suggesting that the radical scavenging site of capsaicin is the C7-benzyl carbon. Phenolic compounds of various spices, including capsaicin, modulate 5-lipoxygenase (5-LO) in human PMNL cells, the key enzyme involved in the biosynthesis of leukotrienes [56] (Table 45.8).

Wistar rats administered capsaicin (*i.p.* 3 mg/kg body weight) for three consecutive days showed a reduction of oxidative stress measured as malondialdehyde in the liver, lung, kidney, and muscle [128]. From this study, it is hypothesized that capsaicin can be a potent antioxidant even when consumed for a short period. The influence of capsaicin on the antioxidant status of red blood cells and liver tissue in hyperlipidemic rats is reported [129]. Capsaicin (0.015 %) in the diet which produced the hypotriglyceridemic effect was also effective in reducing the oxidant stress, which was indicated by countering of the depleted antioxidant molecules and antioxidant enzymes in erythrocytes and liver, and decreasing of the elevated lipid peroxide content. The beneficial influence of capsaicin on the antioxidant status of red blood cells and liver in induced hypercholesterolemic rats is also evidenced [130]. The depletion in intracellular thiols and GSH in red blood cells under hypercholesterolemic situation was effectively countered by dietary (0.015 %)

Table 45.8 Antioxidant influence of capsaicin in in vitro and in vivo systems

Animal model	Effect demonstrated	References
Human erythrocyte membranes	Lipid peroxidation was inhibited by capsaicin	[125]
Rat liver microsomes	Ascorbate-Fe^{++}-induced lipid peroxidation was inhibited by capsaicin	[53]
Soybean phospholipid liposomal membrane	Inhibition of oxidation of methyl linoleate micelles by capsaicin	[126]
Rat liver mitochondria	Inhibition of lipid peroxidation induced by ADP/Fe^{2+} and scavenging of DPPH radicals by capsaicin	[127]
Human low-density lipoprotein	Inhibition of Cu^{2+} induced lipid peroxidation by capsaicin	[55]
Human PMNL cells	Inhibition of 5-lipoxygenase	[56]
Rats	Capsaicin administration reduced oxidative stress in the liver, lung, kidney, and muscle	[128]
High-fat-fed rats	Beneficial influence of dietary capsaicin on antioxidant status of red blood cells	[129]
Hypercholesterolemic rats	Beneficial influence of dietary capsaicin on antioxidant status of red blood cells	[130]

capsaicin. Glutathione reductase activity that was lowered in hypercholesterolemic conditions was completely countered by the dietary spice principle. Decreased hepatic total thiols in the hypercholesterolemic situation were partially corrected by dietary capsaicin treatment. Similarly, the lowered activities of hepatic antioxidant enzymes – GSH-reductase, GSH-transferase, catalase, and superoxide dismutase – in hypercholesterolemic rats were effectively countered by the dietary capsaicin.

2.2.5 Anti-inflammatory Property

With increasing interest in alternatives to nonsteroidal anti-inflammatory agents in the management of chronic inflammation, the use of food-based approaches is emerging. Lipid peroxides play a crucial role in arthritis and other inflammatory diseases. Both in vitro and in vivo animal studies have documented the anti-inflammatory potential of capsaicin (of red pepper). Animal studies have revealed that capsaicin also lowers the incidence and severity of paw inflammation and also delays the onset of adjuvant-induced paw edema in rats [131, 132]. This spice principle inhibited the formation of arachidonate metabolites (PgE_2, leukotrienes) and increased the secretion of lysosomal enzymes – elastase, collagenase, and hyaluronidase – by macrophages. It is noteworthy that the levels of 6-keto PgF_{1a} – a vasodilator – increased [133].

Natural anti-inflammatory compound capsaicin appears to operate by inhibiting one or more of the steps linking pro-inflammatory stimuli with COX activation, such as the blocking by capsaicin of NF-kB translocation into the nucleus [134].

It has been shown recently that the natural anti-inflammatory compounds such as capsaicin were as effective as indomethacin (a nonsteroidal anti-inflammatory drug) in inhibiting aberrant crypt foci in the rat.

2.2.6 Chemopreventive Potential

This phytochemical has been found to interact with microsomal xenobiotic-metabolizing enzymes in rodents. Capsaicin has been proposed to inactivate cytochrome P-450 HE1 by irreversibly binding to the active sites of the enzyme [135]. Besides cytochrome P-450 HE1, other isoforms of the P-450 super family are also reported to be inhibited by capsaicin. The inhibition by capsaicin of microsomal monooxygenases involved in carcinogen activation implies its chemopreventive potential.

Studies suggest that capsaicin is able to kill prostate cancer cells by causing them to undergo apoptosis [136]. The studies were performed on tumors formed by human prostate cancer cell cultures grown in mouse models, and showed tumors treated with capsaicin were about one fifth the size of the untreated tumors. There have been several clinical studies conducted in Japan and China that showed capsaicin directly inhibits the growth of leukemic cells [137]. Another study suggests capsaicin is able to trigger apoptosis in human lung cancer cells as well. An epidemiological study has found significantly higher rates for stomach and liver cancer in counties inhabited by groups with high consumption of capsaicin-rich foods [138].

2.2.7 Antidiabetic Potential

Substance P, a neuropeptide released by capsaicin, has been shown to reverse diabetes in mice [139], but the effects to insulin secretion seem to be species dependent. In humans, substance P seems to decrease insulin release and cause fluctuations in blood sugar levels [140]. Capsaicin is also being explored as a possible prophylaxis for type 1 diabetes. Capsaicin was injected subcutaneously in neonatal diabetes-prone NOD mice to permanently remove a prominent subset of pancreatic sensory neurons, which express the transient vanilloid receptor protein (TRPV1). Insulin resistance and β cell stress of prediabetic NOD mice are prevented when TRPV1+ neurons are eliminated. In other words, mice which were genetically predisposed to type 1 diabetes were prevented from developing type 1 diabetes via removal of these neurons, which are thought to attract pathogenic T cells to attacking pancreatic β cells thus causing type 1 diabetes [141].

2.2.8 Thermogenic and Weight-Reducing Influence

According to animal and human studies, the oral intake of capsaicin increases the production of heat by the body for a short time. Dietary red pepper or its pungent principle capsaicin affects satiety and has a promising thermogenic influence that could play an important role in the prevalence and severity of obesity [103], although more data are required to substantiate this benefit. The use of this spice to displace fats and salt in the diet (to make the food palatable) may reduce cardiovascular risk. Although there is no evidence showing that weight loss is directly correlated with ingesting capsaicin, there is a positive correlation between ingesting capsaicin and a decrease in weight regain. Capsaicin is said to cause a shift in substrate oxidation from carbohydrate to fat oxidation [142] which leads to a decrease in appetite as well as a decrease in food intake. Both oral and gastrointestinal exposure to capsaicin increase satiety and reduce energy as well as fat intake [143]. Oral exposure proves to yield stronger reduction suggesting that capsaicin has sensory effects. Short-term studies suggest that capsaicin aids in the decrease of weight regain. However, long-term studies are limited because of the pungency of capsaicin [144]. Another recent study has suggested that the ingestion of capsaicinoids can increase levels of brown adipose tissue through an increase in energy expenditure and oxidation caused by the capsaicin [145]. In yet another recent study, the beneficial effects of dietary tender cluster beans (*Cyamopsis tetragonoloba*) in checking the weight gain and adverse changes in lipid profile in high-fat-fed condition were potentiated by co-administration of capsaicin in rats [146].

2.2.9 Antiulcer Activity

In recent years, infection of the stomach with *Helicobacter pylori* which disrupts the normal inhibitory control for acid secretion resulting in excess acid destroying the mucosal barrier has been understood to be the main cause of gastric ulcers [147]. Excessive acid secretion in the stomach and reduction in gastric mucosal blood flow are considered responsible for ulcer formation. The colonization of *H. pylori* in the stomach is associated with a phospholipase likely to damage the protective layer of stomach. *H. pylori* thrives in the stomach by producing the enzyme urease.

In view of its irritant and likely acid-secreting nature, persons with ulcers were being advised to avoid consumption of red pepper (chili). However, recent studies have revealed that capsaicin of red pepper is not the cause for ulcer formation but a benefactor. Numerous studies suggest that eating hot peppers regularly is protective against stomach cancer [148]. Detailed studies have revealed that capsaicin per se does not stimulate but inhibits acid secretion, stimulates alkali and mucus secretions and gastric mucosal blood flow which help in disposing of acid from the stomach, thus prevention and healing of ulcers [149]. Capsaicin acts by stimulating afferent neurons in the stomach and signals for protection against injury causing agents. An epidemiological study has found three times higher peptic ulcer incidence among Chinese population in Singapore as compared to Malaysians and Indians who are in the habit of consuming more pungent chili in their daily diets [150].

Interestingly, capsaicin has been found to specifically inhibit the growth of *H. pylori*. Capsaicin inhibits also the release of gastrin and stimulates that of somatostatin, the physiological inhibitor of acid secretion. It is also a potent inhibitor of NF-kB whose activation may lead to various pathological conditions and reactive oxygen species. Phosphodiesterase inhibitors are powerful vasodilatory agents and their likely increase of cAMP levels has an antiulcer effect. Capsaicin is a phosphodiesterase inhibitor and may exert its protective effect in this way besides its stimulation of gastric mucosal blood flow. Numerous studies have substantiated the protective role of capsaicin. Seminal to these studies is the discovery about the selective sensitization and desensitization of unmyelinated neurons by capsaicin [151]. Reactive oxygen species are known to be involved in the pathogenesis of gastritis, gastric ulcers, and gastric cancer. Capsaicin has proved to be an antioxidant protecting cellular membranes, cardiac and skeletal muscles, etc., against reactive oxygen species. Capsaicin inhibited lipid peroxidation induced by ethanol in the gastric mucosa [152].

2.2.10 Capsaicin in Pain Relief

Capsaicin has received considerable attention as a pain reliever. In two trials with 70 and 21 patients with osteoarthritis and rheumatoid arthritis, topical application of creams containing 0.025 % or 0.075 % capsaicin was an effective and safe alternative to analgesics employed in systemic medications which are often associated with potential side effects [153, 154]. Capsaicin has also been suggested for the initial management of neuralgia consequent to herpes infection [155].

Capsaicin has been shown to be useful in diabetic neuropathy. In a study involving 219 patients, topical application of 0.075 % capsaicin cream was effective in pain management [156]. Capsaicin is currently used in topical ointments, as well as a high-dose dermal patch (under the trade name *Qutenza*), to relieve the pain of peripheral neuropathy such as post-herpetic neuralgia caused by shingles [157]. It may be used in concentrations of between 0.025 % and 0.075 % as a cream for the temporary relief of minor aches and pains of muscles and joints associated with arthritis, simple backache, strains, and sprains, often in combination with other rubefacients [157]. Capsaicin creams are used to treat psoriasis as an effective way to reduce itching and inflammation [158, 159]. Capsaicin is also the key ingredient in the experimental drug *Adlea*, which is in Phase 2 trials as a long-acting analgesic to treat postsurgical and osteoarthritis pain [157]. Moreover, it reduces pain resulted from rheumatoid arthritis [160] as well as joint or muscle pain from fibromyalgia.

2.2.11 Beneficial Influences on Gastrointestinal System

Beneficial Modulation of Small Intestinal Ultrastructure

The beneficial influence of dietary capsaicin has been examined in experimental rats with respect to (i) the membrane fluidity of intestinal brush-border membranes (BBM), (ii) the activity of intestinal membrane-bound enzymes, and (iii) the ultrastructural alterations in the intestinal epithelium [46]. In this study, Wistar rats were maintained on dietary red pepper (3·0 %) and its bioactive compound capsaicin (0·01 %) for 8 weeks. A membrane fluidity study using an apolar fluorescent probe showed increased BBM fluidity in the spice-fed or capsaicin-fed animals. This was corroborated by decreased cholesterol: phospholipid ratio in the jejunal and ileal regions of the intestine. These dietary spices stimulated the activities of BBM enzymes (glycyl-glycine dipeptidase, leucine amino peptidase, and γ-glutamyl transpeptidase) in the jejunal mucosa, suggesting a modulation in membrane dynamics due to the apolar spice bioactive compound interacting with surrounding lipids and hydrophobic portions in the protein vicinity, which may decrease the tendency of membrane lipids to act as steric constraints to enzyme proteins and thus modify enzyme conformation. Scanning electronic microscopy of the intestinal villi in these spice treatments revealed alterations in the ultrastructure, especially an increase in microvilli length which would mean a beneficial increase in the absorptive surface of the small intestine, providing for an increased bioavailability of micronutrients. Thus, dietary red pepper or capsaicin was evidenced to induce alterations in BBM fluidity and passive permeability property, associated with the induction of an increased microvilli length, resulting in an increased absorptive surface of the small intestine.

Digestive Stimulant Action

The digestive stimulant action of spices is probably exerted through stimulation of the liver to produce and secrete bile rich in bile acids, which play a very important role in fat digestion and absorption. Capsaicin has been examined for its effect on bile secretion in rats, after dietary intake for a period of time or as a one-time oral intake [116]. The hypocholesterolemic spice compound capsaicin stimulated bile acid production by the liver and its secretion into bile. The influence of dietary intake and single-dose administration of capsaicin on the pancreatic digestive enzymes and the terminal digestive enzymes of the small intestinal mucosa has been reported [32, 33]. Dietary intake of capsaicin stimulated pancreatic lipase activity significantly. In contrast to the continued intake, single oral dose consumption of capsaicin failed to exert a stimulatory effect on pancreatic lipase. Pancreatic amylase activity was elevated by dietary capsaicin (72 %) as well as single-dose administration of capsaicin. Capsaicin when incorporated in the diet, stimulated trypsin activity by over 100 %. Chymotrypsin was also significantly higher in animals fed capsaicin. Similar influence of the spice compound on the activity of proteases was not evident when administered as a single oral dose. Capsaicin prominently enhanced the activity of intestinal lipase. The stimulation of this enzyme activity was more than 100 % of the control in spice principle-treated group. Similarly, dietary capsaicin significantly increased the activity of intestinal amylase. Dietary capsaicin moderately stimulated the activities of intestinal disaccharidases.

Based on the evidences from animal studies, the well-recognized digestive stimulant action of red pepper or its active compound capsaicin may be considered to be mediated through two possible modes: (1) stimulation of the liver to secrete more bile enriched in bile acids and (2) stimulation of enzyme activities that participate in digestion, both of pancreatic and intestinal origin. Such stimulation of bile secretion and of the activities of digestive enzymes leads to an accelerated overall digestive process, resulting in a significant reduction in the duration of passage of food through the gastrointestinal tract [35].

Since capsaicin is known to stimulate secretion of bile with higher amount of bile acids which play a major role in digestion and absorption of dietary lipids, capsaicin has been studied to verify if it enables efficient digestion and absorption during high-fat intake [34]. In this context, dietary capsaicin (0.015 %) has been examined for its influence on bile secretion, digestive enzymes of pancreas, and absorption of dietary fat in high-fat (30 %)-fed rats for 8 weeks. Dietary capsaicin enhanced the activity of pancreatic lipase, amylase, trypsin, and chymotrypsin and enhanced dietary fat absorption. It also increased bile secretion with higher bile acid content. Stimulation of lipid mobilization from adipose tissue was suggested by the decrease in perirenal adipose tissue weight by dietary capsaicin. This was also accompanied by prevention of the accumulation of triglyceride in liver and serum in high-fat-fed rats. Activities of key lipogenic enzymes in liver were reduced which was accompanied by an increased activity of hormone-sensitive lipase. Thus, dietary capsaicin enhances fat digestion and absorption in high-fat-fed situation through enhanced secretion of bile salts and a stimulation of the activity pancreatic lipase. At the same time, the energy expenditure is facilitated by this spice compound to prevent the accumulation of absorbed fat.

Enhanced Absorption of Micronutrients

Since dietary pungent spices may alter the ultrastructure and permeability characteristics of intestines, capsaicin has been examined for a possible influence on intestinal absorption of iron, zinc, and calcium by examining their uptake by the intestines from rats pre-fed spice compound for 8 weeks [47]. Everted segments of duodenum, jejunum, and ileum portions of small intestines isolated from these rats were examined for ex vivo uptake of iron, zinc, and calcium from incubations containing digesta of finger millet. Higher in vitro absorption of iron, zinc, and calcium in the intestines was evidenced in capsaicin-fed animals. The positive influence of dietary capsaicin on the mineral uptake by the intestinal segments was highest for calcium. The positive influence of dietary capsaicin was more pronounced on zinc uptake as compared to that of iron. These pungent spices alter permeation characteristics presumably by increasing absorptive surface and thereby enhance intestinal absorption of micronutrients.

Dietary red pepper and capsaicin which alter the ultrastructure and permeability characteristics of intestines are also reported to favorably enhance the intestinal uptake of β-carotene in vitro [48]. In an animal study conducted to evaluate the influence of dietary spice compounds on the absorption of orally administered β-carotene and its conversion to vitamin A, hepatic β-carotene was significantly increased in capsaicin-fed rats suggesting improved absorption of β-carotene [49]. Retinol concentration was not however changed in these animals suggesting that bioconversion of β-carotene to vitamin A was not similarly influenced. Among the two enzymes involved in the bioconversion of β-carotene to vitamin A, activity of intestinal and hepatic β-carotene-15,15′-dioxygenase was lowered in capsaicin treatment, while the activity of intestinal and hepatic retinal reductase was unaffected. Activity of intestinal and hepatic β-carotene-15,15′-dioxygenase was also inhibited by capsaicin in vitro, thus corroborating with in vivo observation.

2.3 Mechanism of Burning and Painful Sensation of Capsaicin

Capsaicin causes a burning sensation when it comes in contact with mucous membranes. The burning and painful sensations associated with capsaicin result from its chemical interaction with sensory neurons. Capsaicin, as a member of the vanilloid family, binds to a vanilloid receptor (TRPV1) [161], an ion channel-type receptor that resides on the membranes of pain and heat sensing neurons [162]. VR1, which can also be stimulated with heat and physical abrasion, permits cations to pass through the cell membrane and into the cell when activated. The resulting depolarization of the neuron stimulates it to signal the brain. By binding to the VR1 receptor, capsaicin produces the same sensation that excessive heat or abrasive damage would cause.

The TRPV1 ion channel has been shown to be a member of the super family of TRP ion channels. There are a number of different TRP ion channels that have been shown to be sensitive to different ranges of temperature. Thus, capsaicin does not actually cause a chemical burn, or indeed any direct tissue damage at all, consequent to exposure to chili peppers. The inflammation resulting from exposure to capsaicin is believed to be the result of the body's reaction to nerve excitement. TRPV1 is a heat-activated calcium channel, which opens between 37 °C and 45 °C. When capsaicin binds to TRPV1, it causes the channel to open at temperature below 37 °C (normal human body temperature), which is why capsaicin is linked to the sensation of heat. Prolonged activation of these neurons by capsaicin depletes presynaptic substance P, one of the body's neurotransmitters for pain and heat. Neurons that do not contain TRPV1 are unaffected. Thus, capsaicin mimics a burning sensation, the nerves being overwhelmed by the influx. Capsaicin will be unable to evoke pain for an extended period of time since with chronic exposure, neurons are depleted of neurotransmitters, leading to reduction in sensation of pain and blockade of neurogenic inflammation. If capsaicin is removed, the neurons recover [163, 164]. The mode of action of capsaicin in inducing bronchoconstriction is thought to involve stimulation of C fiber culminating in the release of neuropeptides [165]. Essentially, the body inflames tissues as if it has undergone a burn or abrasion, and the resulting inflammation can cause tissue damage in cases of extreme exposure.

2.4 Absorption and Metabolism of Capsaicin

Capsaicin fed to rats was rapidly absorbed from the stomach, with 85 % of a 3-mg dose absorbed within 3 h [166]. Doses of 5.12 mg/mouse/week led to maximum plasma concentrations of 51.5 ng/mL and 84.8 ng/mL in male and female mice, respectively [167]. Little absorption of capsaicin also occurs across the skin. When 0.8 g of gel containing 0.075 % of capsaicin was applied to the skin of human volunteers, the average absorbed dose after 8 h of exposure was 22.7 μg/cm^2 [168]. Topical application of pure capsaicin to the skin of mice resulted in peak plasma concentrations occurring 4–12 h later, and capsaicin was detectable in the blood 24 h after dosing [167].

Rats injected intravenously accumulated capsaicin primarily in the brain and spinal cord 3 min after dosing, with lower levels found in the liver and blood, while 10 min after dosing, the greatest concentrations remained in the spinal cord [169]. Subcutaneously injected capsaicin was detected in all tissues of rat 10 min

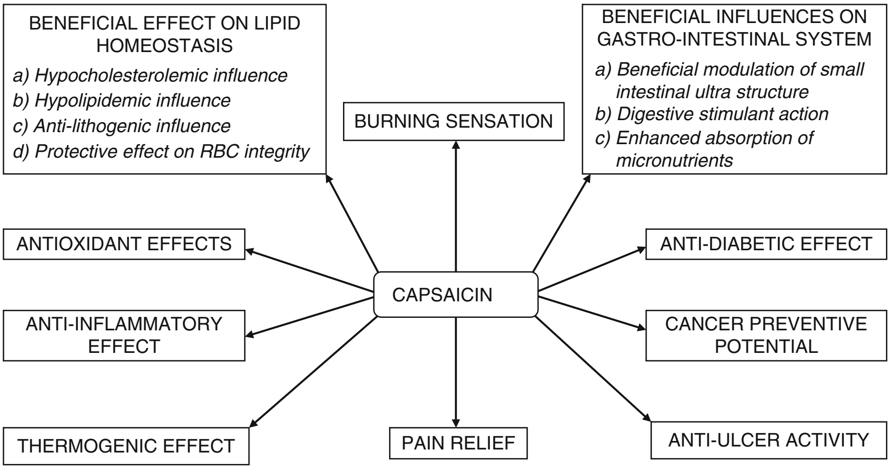

BENEFICIAL EFFECT ON LIPID HOMEOSTASIS
a) Hypocholesterolemic influence
b) Hypolipidemic influence
c) Anti-lithogenic influence
d) Protective effect on RBC integrity
BURNING SENSATION
BENEFICIAL INFLUENCES ON GASTRO-INTESTINAL SYSTEM
a) Beneficial modulation of small intestinal ultra structure
b) Digestive stimulant action
c) Enhanced absorption of micronutrients
ANTIOXIDANT EFFECTS
ANTI-DIABETIC EFFECT
CAPSAICIN
ANTI-INFLAMMATORY EFFECT
CANCER PREVENTIVE POTENTIAL
THERMOGENIC EFFECT
PAIN RELIEF
ANTI-ULCER ACTIVITY

Fig. 45.4 Summary of the diverse physiological effects of capsaicin

following dosing, but residues were undetectable in any tissues 17 h later. Blood concentrations peaked 5 h following dosing, and brain and spinal cord tissue concentrations were somewhat lower. Kidneys contained the greatest concentrations and liver concentrations were low presumably due to metabolic breakdown of the capsaicin [169]. Tissue distribution and elimination of capsaicin has been examined following its oral intake (30 mg capsaicin/kg body weight) in rats [26]. Maximum distribution of 24.4 % of administered capsaicin was seen at 1 h, while no intact capsaicin was detectable after 4 days. Absorption of capsaicin was about 94 % and very rapid.

Metabolism of capsaicin occurs primarily in the liver in the rat [170, 171]. Although the same metabolites were produced, the relative amounts of each metabolite were species dependent. Metabolism of capsaicin by P_{450} enzymes may follow a number of pathways and produce a variety of metabolites, some of which may be associated with increased toxicity [172]. Less than 10 % of an oral dose of capsaicin given to rats was excreted unchanged 48 h after dosing [166].

2.5 Conclusions

The pungent principle of red pepper (hot chili) capsaicin is endowed with several biological activities which are of pharmacological relevance. These include thermogenic influence, effects on gastrointestinal system, cardioprotective influence, anti-lithogenic effect, and anti-inflammatory and pain-relieving effect. The involvement of neuropeptide substance P, serotonin, and somatostatin in the pharmacological actions of capsaicin has been extensively investigated. Tropical application of capsaicin has been proved to alleviate pain in arthritis, postoperative neuralgia, diabetic neuropathy, psoriasis, etc. Contrary to the general belief, capsaicin inhibits acid secretion, stimulates alkali and mucus secretion, and stimulates gastric mucosal blood flow, all of which help in prevention and healing of gastric ulcers. Antioxidant and anti-inflammatory properties of capsaicin are established in a number of in vitro and in vivo studies. Chemopreventive potential of capsaicin has been evidenced in a few animal and cell line studies. The hypocholesterolemic influence of capsaicin has additional implications in the prevention of cholesterol gallstone disease and protection of the structural integrity of erythrocytes under conditions of hypercholesterolemia. Beneficial influences of capsaicin on gastrointestinal system include digestive stimulant action and modulation of small intestinal ultrastructure so as to enhance permeability to micronutrients (Fig. 45.4).

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Antiangiogenic Alkaloids from Plants

46

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Contents

1 Introduction 1442
1.1 Angiogenesis 1442
2 Antiangiogenic Alkaloids from Plants 1445
2.1 Alkaloids Derived from Tyrosine 1446
2.2 Alkaloids Derived from Tryptophan 1451
2.3 Alkaloids Derived from Lysine 1455
2.4 Capsaicin and its Natural Ester Analogues 1456
2.5 Halofuginone 1458
2.6 Pterogynidine 1459
3 Conclusion 1459
References 1460

Abstract

Angiogenesis, the formation of new blood vessels from preexisting capillaries, is an important research field. As the understanding of this process increases, this new knowledge will have a significant impact on several angiogenesis-dependent diseases. A wide variety of plants are rich in alkaloids, and these

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compounds have traditionally been of interest due to their pronounced effects on various physiological activities in animals and humans. Nowadays, it is known that many alkaloids obtained from plants exhibit antiangiogenic activity, and these alkaloids may act through different mechanisms to inhibit angiogenesis. Herein, we will discuss the most important alkaloids obtained from plants, focusing especially in their antiangiogenic activity. Because of the great diversity of plants, certainly, there are many antiangiogenic alkaloids that have yet to be discovered.

Keywords

Angiogenesis • antiangiogenic activity • angiogenesis-dependent diseases • natural products • plants • alkaloids

Abbreviations

Akt	AKT8 virus oncogene cellular homologue
AP-1	Activator protein 1
APL	Promyelocytic leukemia
ATF-2	Activating transcription factor 2
BAEC	Bovine aortic endothelial cells
Bax	B cell lymphoma-associated X
Bcl-2	B cell lymphoma 2 protein
bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
CAM	Chorioallantoic membrane
CD-31	Cluster of differentiation 31
CDK-2	Cyclin-dependent kinase-2
CDK-4	Cyclin-dependent kinase-4
COL1α2	Collagen type 1, alpha 2
COX-2	Cyclooxygenase-2
CREB-1	cAMP response element-binding protein
ECM	Extracellular matrix
ECs	Endothelial cells
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular-signal-regulated kinases 1 and 2
FBS	Fetal bovine serum
FCA	Freund’s complete adjuvant
FGF	Fibroblast growth factor
G6PDH	Glucose-6-phosphate dehydrogenase
GFR-Matrigel	Growth factor reduced-Matrigel
GM-CSF	Granulocyte macrophage colony-stimulating factor

HCC	Hepatocellular carcinoma cells
HIF-1	Hypoxia-inducible factor-1
HIF-1α	Hypoxia-inducible factor-1 – subunit alpha
HIF-1β	Hypoxia-inducible factor-1 – subunit beta
Hsp70	Heat shock p70
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Half maximal inhibitory concentration
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-12	Interleukin-12
iNOS	Inducible oxide nitric synthase
IUPAC	International Union of Pure and Applied Chemistry
JAG2	Jagged-2
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
MMP-2	Matrix metalloproteinase-2
MMP-13	Matrix metalloproteinase 13
MMPs	Matrix metalloproteinases
MVD	Microvessel density
NFκB	Nuclear factor kappa B
NSCLC	Non-small cell lung cancer
p38 MAPK	p38 mitogen-activated protein kinase
p42/p44 MAPK	p42/p44 mitogen-activated protein kinase
$p125^{FAK}$	p125 focal adhesion kinase
PDB ID	Protein data bank identifier
PDGF-$\beta\beta$	Platelet-derived growth factor subunits beta beta
PLCγ1	Protein kinase C-gamma 1
PlGF	Placental growth factor
pRb	Retinoblastoma protein
RT-PCR	Reverse transcription-polymerase chain reaction
TGF-β1	Transforming growth factor beta1
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor-alpha
TrpRS	Tryptophanyl-tRNA synthetase
TRPV-1	Transient receptor potential vanilloid-type 1
TUNEL	Terminal transferase dUTP nick end labeling
VASP	Vasodilator-stimulated phosphoprotein
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A
VEGFR-2	Vascular endothelial growth factor receptor subtype 2

1 Introduction

Angiogenesis research has a solid scientific foundation. It started with the theory of Judah Folkman (1933–2008) that tumor growth is always accompanied by neovascularization. He postulated that in order to survive and grow, tumors require blood vessels, and that by blocking the blood supply, a cancer could be starved into remission [1, 2].

In the past, the blood vessels in the body have long been considered to basically function as a transport compartment of the blood. Nowadays, it is recognized that the vasculature is one of the main organs in the body, extending more than 900 m^2 and playing a major role in supporting the body's integrity in various ways [3].

During the last decade, the field of angiogenesis has been characterized by an enormous increase in research activity [4]. The knowledge of endothelial cells (ECs) physiology and tumor angiogenesis improved, and it provided the necessary background to develop effective antiangiogenic strategies [5].

Nowadays, it is known that many alkaloids obtained from plants exhibit antiangiogenic activity and they can act through different mechanisms to inhibit angiogenesis. In this chapter, we will discuss the most important alkaloids obtained from plants, focusing especially in their antiangiogenic activity.

1.1 Angiogenesis

Angiogenesis, the sprouting of new capillaries from preexisting vessels, is a complex multistep process. It requires extensive interactions between a variety of cells and molecules and is controlled by various peptides and other modulating factors [4, 6].

In order for vascular sprouting to occur, a cascade of events must be concluded. First, extracellular matrix degradation must occur, a process that is facilitated by the activity of matrix metalloproteinases (MMPs). Next, chemoattractants and mitogens are activated in order to assist EC migration and proliferation, respectively. Finally, the tube formation occurs and requires inhibitory signals as well as those that enable formation of junctional complexes and reconstitution of a basement membrane. Formation of the basement membrane, which signals the onset of vessel maturation, involves recruitment by ECs of pericytes that embed within the basement membrane. If this microvessel is to become a larger vessel with a medial layer, then appropriate signals are required for the recruitment of smooth muscle cells [4, 6, 7]. It is known that during angiogenesis, vascular ECs interact with pericytes and such cell–cell contact inhibits EC proliferation. Thus, lack of sufficient number of pericytes around an EC layer may result in an uncontrolled development of microvasculature [8].

Imbalanced expression of pro- and antiangiogenic factors and their receptors on EC may determine the development or regression of new blood vessels. More than 200 pro-angiogenic factors have been identified. Among those, the most important factors include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietins, cytokines, chemokines, and angiogenic enzymes. These factors initiate angiogenesis by modulating the migration and/or proliferation of ECs and the formation of neovasculature [8, 9].

The significance of the angiogenic process increases considerably every year as new findings reveal novel mechanisms through which this process can modulate disease development and recovery [10].

1.1.1 Angiogenesis, Inflammation, and Immune System

It is widely accepted that angiogenesis is the result of a net balance between the activities exerted by positive and negative regulators. This balance is conceptually very similar to that of the pro- and anti-inflammatory mediators that modulate an appropriate and specific inflammatory response [11].

Inflammatory mediators can also, either directly or indirectly, promote angiogenesis. Angiogenesis, in turn, contributes to inflammatory pathology. New blood vessels maintain the chronic inflammatory state by enabling the transport of inflammatory cells to the site of inflammation and supplying nutrients and oxygen to the proliferating inflamed tissue. The increased endothelial surface area also creates a massive capacity for the production of cytokines, adhesion molecules, and other inflammatory stimuli. Although inflammation is essential to protect the body against pathogens, it has adverse effects on surrounding tissue. Some of these effects induce angiogenesis. Inflammation and angiogenesis are thereby linked processes [12].

Therefore, it is well established that the immune system plays an important role in the regulation of angiogenesis. Next to the regulation of angiogenesis by leukocytes, angiogenic processes can influence the cells of the immune system and the development of an immune response as well. Normal EC contributes to the recruitment of immune cells to the site of inflammation by the expression of adhesion molecules. Some authors suggest that several advantages of antiangiogenic therapy for chronic inflammatory diseases can be visualized analogous to those of tumor growth antiangiogenic strategies. First of all, suppression of blood vessel growth leads to a decreased nutrient supply to the metabolically active cells present in inflamed tissue. Second, by preventing blood vessel formation, the entry route of inflammatory cells into the tissue becomes blocked. A third possible advantage of inhibiting EC activation, proliferation, and vascular remodeling in chronically inflamed lesions is the inhibition of the production of EC-derived soluble factors such as MMPs and cytokines [3].

It is also important to mention that monocytes, macrophages, platelets, mast cells, and lymphocytes, cells that are frequently infiltrated in chronic inflammatory sites, directly release or stimulate other cells to produce angiogenic factors [8]. Furthermore, it is well accepted that hypoxia is a potent stimulus for abnormal angiogenesis in these chronic inflammatory sites [13, 14].

1.1.2 Angiogenesis-Dependent Diseases

Angiogenesis occurs in physiologic and pathologic processes. In the physiological condition, the activity of inducers and inhibitors of angiogenesis maintains it in equilibrium. Normal tissue growth, such as in embryonic development, wound healing, and menstrual cycle, is characterized by dependence on new vessel formation for the supply of oxygen and nutrients as well as removal of waste products. However, persistent and upregulated angiogenesis is often found to be a critical causal factor in several diseases, including cancer (both solid and hematologic tumors), cardiovascular diseases (atherosclerosis), chronic inflammation-associated disorders (rheumatoid arthritis, Crohn's disease), diabetes (diabetic retinopathy), psoriasis, endometriosis, and adiposity. These diseases may benefit from therapeutic inhibition of angiogenesis [15–18].

Pathological angiogenesis is closely associated with almost all of the major diseases afflicting human life [19]. Angiogenesis is therefore necessary for tumors and their metastases to grow beyond a microscopic size, and it can give rise to bleeding, vascular leakage, and tissue destruction. These consequences of pathological angiogenesis can be responsible, directly or indirectly, for the symptoms, incapacitation, or death associated with a broad range of "angiogenesis-dependent diseases." In fact, according to Folkman, the concept of angiogenesis-dependent diseases originated in 1972 with the recognition that certain nonneoplastic diseases depend on chronic neovascularization to provide a conduit for the continual delivery of inflammatory cells to the inflammatory site. Examples of such diseases include cancer, autoimmune diseases, age-related macular degeneration, and atherosclerosis. Subsequently, other nonneoplastic disorders were recognized to be in part angiogenesis dependent, for example, infantile hemangiomas, peptic ulcers, ocular neovascularization, and rheumatoid arthritis [20].

1.1.3 The Inhibition of Angiogenesis

The term "antiangiogenesis" was introduced to describe treatments designed to prevent the induction of new blood vessels and perhaps reduce the number of those already present [21]. Angiogenesis inhibition has been proposed as a general strategy to fight cancer and other angiogenesis-dependent diseases [15].

In the treatment of cancer, the inhibition of angiogenesis is a major area of therapeutic development. Whereas conventional chemotherapy, radiotherapy, and immunotherapy are directed against tumor cells, antiangiogenic therapy is aimed at the vasculature of a tumor and will either cause total tumor regression or keep tumors in a state of dormancy [2]. Angiopreventive drugs may have the potential to repress angiogenesis during early steps of carcinogenesis where they might retard the angiogenic switch, preventing unrestrained tumor growth [22].

Moreover, in the treatment of angiogenesis-dependent disease, angiogenesis inhibitors may be useful adjuncts to conventional therapies. When all conventional therapy has failed, an angiogenesis inhibitor may be successfully used as has been demonstrated in the treatment of multiple myeloma by thalidomide [23].

Definitely, angiogenesis can be targeted at several stages. The strategies for therapies are targeting growth factors and their receptors, interrupting the downstream signaling, blocking matrix degrading enzymes, using endogenous inhibitors, or attempting to mimic naturally occurring angiogenesis inhibitors, such as thrombospondin [24, 25].

1.1.4 Evaluating Potential Antiangiogenic Agents Obtained from Plants

Since angiogenesis mainly depends on proper activation, proliferation, adhesion, migration, and maturation of ECs, most approaches to modulate angiogenesis are focused on these endothelial functions during blood vessel formation [3].

Various in vitro and in vivo assays have been utilized to analyze angiogenesis experimentally. In vitro, ECs such as human umbilical vein endothelial cells (HUVECs) cultures are widely used. Migration or invasion of HUVECs could be assayed by transwell, and proliferation could be determined by bromodeoxyuridine (BrdU) incorporation. Differentiation is determined by tube formation assay using HUVECs seeded on Matrigel with or without angiogenic activator. In vivo, the Matrigel plug assay is widely performed in mice subcutaneously inoculated with angiogenic factor treated Matrigel, and the content of hemoglobin is measured in the Matrigel plugs to indirectly quantify functional new blood vessel formation. Taken together, these angiogenesis experimental tools can be useful in studies focused on antiangiogenic activity screening of plants used in traditional folk medicine [10, 26].

Some authors believe that bioactive compounds from natural sources may be used as regulatory agents in the formation of new blood vessels offering an enormous potential for therapeutic intervention of many disorders. An increasing number of bioactive compounds from natural sources and whose chemical structures have already been elucidated are reported as potential inhibitors of angiogenesis [27].

Ng et al. consider that naturally occurring bioactive compounds may be good candidates for the prevention of angiogenic disease, when standard therapy is unsuitable or lacking. They suggest that these products can also complement chemotherapy or radiotherapy, in combination with other anticancer drugs for therapy at different stages, and increase the survival time of the patients [28].

According to Tai-Ping Fan et al., the identification of new drugs from plants has a long and successful history, and certain pro-angiogenic and antiangiogenic plant components have been used in traditional Chinese medicine for thousands of years. Thus, it is rational to explore these medicinal plants as a source of novel angiomodulators [29].

2 Antiangiogenic Alkaloids from Plants

Plant metabolites bearing different structural patterns have showed potent effects on angiogenesis process [30–32]. Several of these substances are alkaloids, and their structures and biological effect on angiogenesis process are detailed below. In this chapter, we have used a broad concept to the alkaloid term, which was based on IUPAC recommendations. Its definition includes any plant metabolite containing basic nitrogen or clearly derived from an amino acid and still retaining its nitrogen atom, whether it demonstrates basicity or not [33]. Herein, the alkaloids have been separated according to their hypothetical amino acid precursor.

2.1 Alkaloids Derived from Tyrosine

2.1.1 Berberine

Berberine (**2**) is a quaternary ammonium salt from the protoberberine class. It is found in several occidental and oriental medicinal plants, including *Hydrastis canadensis* L. (Ranunculaceae) and *Coptis chinensis* Franch var. *chinensis* (Ranunculaceae), also known as Goldenseal and Huanglian, respectively [34].

Berberine has exhibited cytotoxicity against several cancer cell lines from different histological origins, including bone, breast, brain, colon, esophagus, liver, lung, and stomach. This activity was correlated to two main effects: (1) antimitotic, through protein and DNA synthesis inhibitory activities, and (2) proapoptotic, by regulating apoptotic gene expression and decreasing mitochondrial transmembrane potential [35].

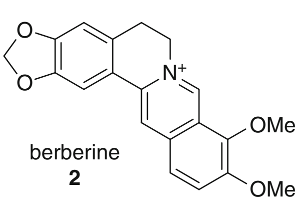
Low micromolar concentrations of **2** reduced the capacity of hepatocellular carcinoma cells (HCC) to stimulate HUVEC proliferation, migration, and endothelial tube formation. In addition, **2** prevented secretion of VEGF from HCC and decreased VEGF mRNA expression. Altogether, these data indicated that **2** showed antiangiogenic properties, which could influence the cross talk between HCC cells and HUVEC [36].

In other experiments, **2** decreased directly the ability of HUVEC migration and capillary tube formation and inhibited gastric adenocarcinoma cancer cell line SC-M1 to stimulate HUVEC migration. The influence of **2** on the cross talk between HUVEC and SC-M1 was studied under hypoxic culture conditions. These experiments indicated that **2** caused a downregulation on the expression of VEGF and hypoxia-inducible factor-1alpha (HIF-1α), two key factors in mediating tumor angiogenesis [37].

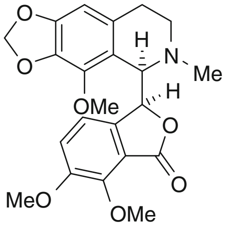
2.1.2 Noscapine

Noscapine or narcotine (**3**) is a phthalideisoquinoline alkaloid from plants of the Papaveraceae family. It is an important alkaloid from the opium poppy (*Papaver somniferum* L.), occurring in variable quantities (level ranges from 2% to 10%) [38]. Unlike opioid morphinane drugs, noscapine lacks hypnoanalgesic, euphoric, and respiratory depressant properties. Its main therapeutic application is as oral antitussive drug, which is indicated to treat nonproductive coughs [39, 40].

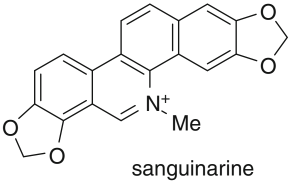
Noscapine displayed HIF-1 inhibitory activity on human glioma cells under different types of hypoxic stress. This activity was determined through variations in the HIF-1α expression. The reductions of the HIF-1α expression were due to accumulation in the nucleus and its degradation through the proteasome pathway [41]. Considering that the expression of HIF-1α protein promotes transcriptional activation of VEGF gene [42, 43], noscapine decreased VEGF production through upregulation of VEGF gene. However, noscapine inhibited the ability of HUVEC to form capillary-like structures in vitro, corroborating its potential as antiangiogenic agent [41].



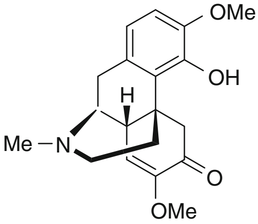
berberine
2



noscapine
3



sanguinarine
4



sinomenine
5

2.1.3 Sanguinarine

Sanguinarine or pseudochelerythrine (**4**) is quaternary ammonium salt belonging to the benzo[*c*]phenanthridine class. It constitutes approximately 50% of redroot latex of *Sanguinaria canadensis* L. (Papaveraceae), an herbaceous medicinal plant popularly known as bloodroot [44]. The main pharmacological application of sanguinarine is in dental formulations, including mouthwashes and toothpastes. It shows antimicrobial and anti-inflammatory properties, reducing gingival inflammation and supragingival plaque formation [45].

Sanguinarine possess antiproliferative activity against several human cancer cell lines, exhibiting differential apoptosis-inducing effect in tumorigenic cells *versus* normal cells [46]. In preclinical experiments, oral treatment with sanguinarine suppressed murine tumor growth in a syngeneic host (B16 melanoma 4A5 cells implanted in C57BL/6 mice) and in a human melanoma tumor xenograft (A37) in athymic mice [47].

Sanguinarine has been recognized as a potent antiangiogenic agent, suppressing VEGF-induced migration, sprouting, and survival in primary cultured ECs, at nanomolar levels. On the other hand, sanguinarine was a relatively mild inhibitor of VEGF-induced DNA synthesis. In vivo assays, using mouse Matrigel plug and chorioallantoic membrane (CAM) of chick embryos, evidenced its potent action on vasculogenesis. Preliminary biochemical experiments revealed that sanguinarine strongly suppressed basal and VEGF-induced Akt (AKT8 virus oncogene cellular homologue) phosphorylation and not interfered on VEGF-induced ERK1/2 (extracellular-signal-regulated kinases 1 and 2) and PLCγ1 (protein kinase C-gamma 1) phosphorylations [48]. More recently, the effects of sanguinarine on Akt phosphorylation inhibition were confirmed by immunoassays [49].

Considering that physiological angiogenesis is a crucial step to ovarian follicle development, under normal conditions, sanguinarine attenuated VEGF production by swine granulosa cells, interfering negatively on female reproductive performance [50].

2.1.4 Sinomenine

Sinomenine or cocculine (**5**) is a morphinane alkaloid identified as antirheumatic chief component from the *Sinomenium acutum* Rehder and Wilson (Menispermaceae). The whole plant has been used to treat joint pain and arthritis for over 2,000 years, and its preparations have been adopted by traditional Chinese medicine [51, 52].

Sinomenine inhibited basic fibroblast growth factor (bFGF)-induced in vitro angiogenesis, acting through four different modes of action on HUVEC: (1) inhibiting of proliferation, (2) arresting their cell cycle in G1 phase, (3) suppressing chemotactic mobility, and (4) disrupting tube formation. Furthermore, in vivo and ex vivo antiangiogenic assays corroborated its in vitro results. It reduced the neovascularization and microvascular outgrowth, which are measured by Matrigel plug and rat aorta ring sprouting experiments, respectively [53].

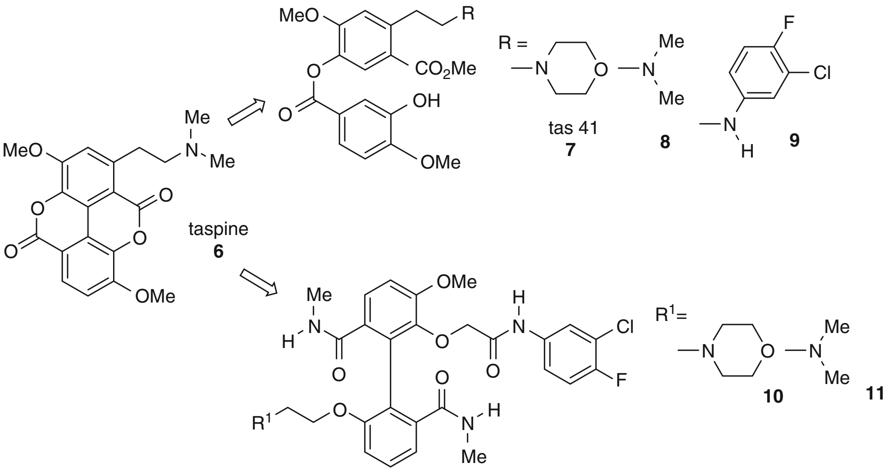
Recent studies revealed that sinomenine showed modulatory effects on gene expression of the IL-1β activated in human synovial sarcoma using Hs701.T cell line. Sinomenine suppressed the expression of genes associated with angiogenesis and vascular remodeling, such as JAG2 (jagged-2), a transmembrane ligand of notch receptor; COL1α2 (collagen, type 1, alpha 2); MMP-13 (matrix metalloproteinase 13); and P1GF (placental growth factor), a member of VEGF family [54].

2.1.5 Taspine and Its Synthetic Analogues

Taspine (**6**) is unusual alkaloid with two lactones, resembling ellagic acid, and a *N*, *N*-dimethylaminoethyl side chain, which hypothetically arisen from tyrosine metabolism [55]. It has been showed several biological activities, such as cicatrizing capacity and acetylcholinesterase inhibition [56, 57].

Taspine has displayed several in vitro antiangiogenic properties on HUVEC, including reduction on its VEGF secretion. It exhibited HUVEC growth inhibition by inducing its apoptosis in a concentration-dependent manner. Its apoptosis-inducing activity was confirmed by transmission electron microscopy, which showed chromatin agglutination, nuclear karyopyknosis, and typical apoptotic bodies. Immunoassays indicated a decreased in the Bcl-2 (B cell lymphoma 2 protein) expression, as well as an increased in the Bax (B cell lymphoma-associated X) expression, anti-apoptotic and pro-apoptotic proteins, respectively [58]. Considering that Bcl-2 overexpression is closely related to the angiogenesis process [59], the dual action of taspine on this target and VEGF could constitute its mechanism of antiangiogenic activity [58]. More recently, taspine showed antiangiogenic and apoptosis-inducing activities against epidermoid carcinoma cells in a manner closely related to HUVEC. Additionally, taspine has increased the expression of caspase-3, cleaved caspase-3, CDK2 (cyclin-dependent kinase-2), and CDK4 (cyclin-dependent kinase-4) [60].

The potential antiangiogenic of taspine encouraged the synthesis of novel analogues (**7**–**9**). Tas 41 (**7**) showed potent antiproliferative activity against human umbilical endothelial (ECV304 cell line) and human epithelial colorectal adenocarcinoma cells (Caco-2 cell lineage), exhibiting IC_{50} values of 2.67 nM and 52.5 nM, respectively. These data indicated that tas 41 was more effective against ECV304 than Caco-2 (approximately 19-fold), suggesting this compound may inhibit vascular endothelial growth factor receptor subtype 2 (VEGFR-2). Docking studies of tas 41 in the active site of VEGFR-2 (PDB ID: 3C7Q), using Sybyl/FlexX, showed its binding mode. It included hydrogen bond interactions to the Asn 923, Arg 1032, and Cys 913 residues [61]. Further studies confirmed the antiangiogenic properties of tas 41, which showed effects closely related to the taspine [62]. More recently, biphenyl taspine analogues (**10** and **11**) showed inhibitory activity against cell lines express abnormally high levels of EGFR (epidermal growth factor receptor). Their predicted physicochemical properties, including distribution and partition coefficients, indicated these compounds were more water-soluble than taspine. Altogether, these studies led to pivotal structural features of ring-opened analogues, including the importance of lactone ring B for antiangiogenic activity. On the other hand, the cleavage of lactone ring A did not interfere to biological activity [63, 64].



2.1.6 Tetrandrine and Its Synthetic Analogues

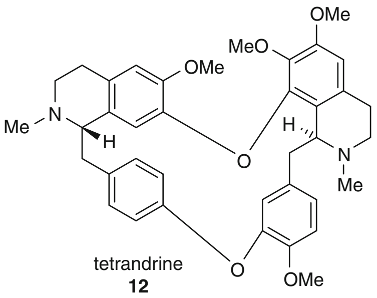
Tetrandrine (**12**) is a *bis*-benzylisoquinoline alkaloid identified as bioactive chief alkaloid from *Stephania tetrandra* S. Moore (Menispermaceae) [65, 66].

It has been traditionally used for the treatment of inflammatory process by several oriental systems of herbal medicine. In the traditional Chinese medicine, this species is among 50 fundamental herbs used, belonging to the Pharmacopoeia of the People's Republic of China [67]. Tetrandrine showed potent antiproliferative activity against a broad panel of human cell lines, including those derived from hepatocellular carcinoma, colorectal, and esophageal cancers [68, 69].

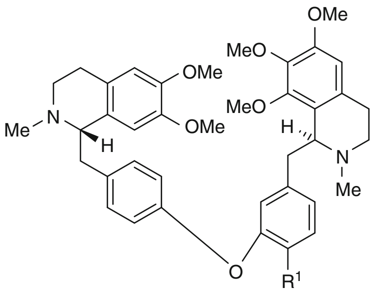
The potential antiangiogenic of tetrandrine was evaluated by several experiments. Tetrandrine was able to decrease the proliferation of HUVEC primary culture and LoVo cells (human colorectal carcinoma). Also, its in vitro activities on HUVEC included migration and tube formation impairment, apoptosis induction, and DNA synthesis suppression. Additionally, in vivo experiments using LoVo cells xenograft in nude mice indicated that tetrandrine significantly reduced the number of hot spots of neo-angiogenesis, which was measured by immunohistochemistry techniques [70].

In other experiments, tetrandrine exhibited inhibitory effect on tube formation of rat vascular ECs, which were stimulated by IL-1α (interleukin-1 alpha) and PDGF-ββ (platelet-derived growth factor subunits beta beta), and FBS (fetal bovine serum). Nanomolar concentrations of tetrandrine were more effective against tube formation stimulated by IL-1α and PDGF-ββ than FBS. In vivo experiments indicated that tetrandrine inhibited air-pouch granuloma angiogenesis, induced by Freund's complete adjuvant (FCA) with croton oil. Similarly to in vitro experiments, tetrandrine exhibited potency similar to the hydrocortisone. These pharmacological results support the traditional use of *S. tetrandra* as anti-inflammatory plant for chronic inflammations and **12** as its responsible biologically active compound [71].

Tetrandrine and its synthetic analogues (**13** and **14**) displayed potent inhibitory effect on FBS-stimulated angiogenesis of cultured choroidal tissues explanted of streptozotocin-diabetic Wistar rats and in vivo air-pouch granuloma angiogenesis in diabetic mice. This study indicated that *bis*[tetrahydroisoquinoline] unit connected by oxy-*bis*[phenylenemethylene] and 2,2-dimethyl groups was important structural feature for antiangiogenic activity. Analogue **13** may be considered as lead compound to treat diabetes-associated choroidopathy and retinopathy [72].



tetrandrine
12



R^1= H **13**
R^1= OMe **14**

2.2 Alkaloids Derived from Tryptophan

2.2.1 Brucine

Brucine (**15**) is a bitter substance closely related to strychnine, which is mainly isolated from the seeds of *Strychnos nux-vomica* L. (Loganiaceae). These compounds are monoterpenoid indole alkaloids and have been classified into type Ib category (or strychnines) [73]. Despite structural similarity between these compounds, brucine was not found be a potent toxic agent [74]. Brucine and strychnine exhibit distinguished historical relevance and have been extensively used as modern research tools, including their application as chiral deriving agents for racemic mixture resolutions [75].

Brucine inhibited tumor angiogenesis process, which was elucidated by in vitro and in vivo assays. It was able to inhibit human breast adenocarcinoma cell proliferation, when administered along VEGF polyclonal antibody, and reduced their pro-angiogenic factors secretion, including VEGF and tumor necrosis factor-alpha (TNF-α). This experiment suggests that brucine binds with VEGF receptors, inhibiting cell proliferation. In vivo experiments, using two Ehrlich tumor models, proved to confirm in vitro results, indicating that brucine downregulated VEGF and TNF-α levels, and increased interleukin-12 (IL-12), another antiangiogenic factor [76].

Brucine showed potent effects on inflammatory angiogenesis process, which was evaluated by in vivo cannulated sponge implant model in Swiss albino mice. It indicated that brucine decreased wet weight granuloma tissue and its hemoglobin content. Also, brucine exhibited inhibitory activity on inflammatory, angiogenic, and fibrogenic cytokines, such as cluster of differentiation 31 (CD-31), transforming growth factor beta 1 (TGF-β1), and VEGF [77].

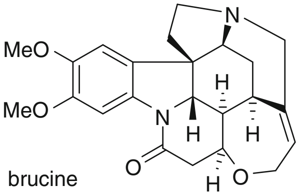
2.2.2 Evodiamine

Evodiamine (**16**) is an indoloquinazoline alkaloid of mixed biosynthetic origin and possesses three nitrogen atoms arising from tryptophan and *N*-methyl-anthranilic acid residues [78]. It is chief alkaloid from fruits of *Evodia rutaecarpa* Bentham (Rutaceae), a plant widely used by traditional Chinese medicine [79].

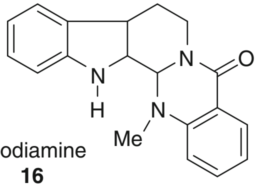
Pharmacologically, evodiamine has been widely studied as innovative anti-obesity agent and was classified as a full TRPV-1 (transient receptor potential vanilloid-type 1) agonist. This receptor is a nonspecific ion channel and is activated by not only chemical compounds containing vanillyl moiety but also heat (≥45 °C) and acid (micromolar range) stimuli. It exhibited binding affinity similar to capsaicin in Chinese hamster ovary cell model. Despite similar in vitro and in vivo anti-obese activities between these compounds, evodiamine has not perceptible taste, including a peppery hot taste [80, 81].

Several evidences demonstrated that evodiamine possesses in vitro and in vivo anticancer properties, such as antiproliferative, anti-metastatic, apoptosis-inducing, and antiangiogenic activities [82]. Evodiamine was able directly to reduce HUVEC capillary tube formation and invasion, using Matrigel assay. In vivo assay, using

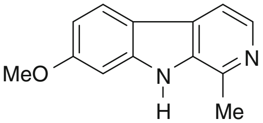
CAM of chick embryo and local administration of evodiamine, confirmed the suppression on tube formation process. Western blot and northern blot experiments, using HUVEC, indicated that evodiamine reduced VEGF and p42/p44 mitogen-activated protein kinases (p42/p44 MAPKs) expression and the mRNA expression of VEGF, respectively. In other experiments, using human lung adenocarcinoma cell lines (CL-1 cells), evodiamine inhibited markedly CL-1-induced tube formation in ECs and their VEGF expression [83].



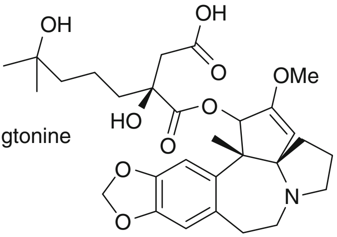
brucine
15



evodiamine
16



harmine
17



homoharringtonine
18

2.2.3 Harmine

Harmine (**17**) belongs to the β-carboline alkaloid class, which occurring in Malpighiaceae family, mainly in South American plants, such as *Banisteriopsis caapi* (Spruce ex Griseb) C. V. Morton [84]. This species is popularly known as "ayahuasca" (Ecuador, Peru), and "caapi" (Brazil) has been used in hallucinogenic preparations. *Banisteriopsis caapi* stems and *Psychotria viridis* Ruiz & Pav. leaves (Rubiaceae) have been widely used in mystic-religious ceremonies. Psychopharmacological and neuropharmacological properties of these infusions and decoctions were related to a complex alkaloid mixture, including harmine [85].

Harmine exhibits potent antineoplastic activity against tumors with high metastatic capacity, including HepA, Lewis lung cancer, and sarcoma 180. It has showed antiproliferative activity against cells of different histological origins, such as epidermoid carcinoma of nasopharynx, osteosarcoma, lung carcinoma, glioblastoma, and breast cancer [86].

Harmine proved to be a potent angiogenic inhibitor, which was extensively evaluated by in vitro and in vivo experiments. In vitro, it was able to reduce different steps of neo-vessel formation by VEGF-induced HUVEC, including proliferation, motility, invasion, and tube formation. Additionally, harmine showed inhibitory effect on transcription factors related to crucial tumor development and angiogenesis process. It reduced the translocation and activation of nuclear factor kappa B (NF-κB) subunits (p65, p50, and c-Rel) and nuclear translocation of important AP-1 (activator protein 1) factors, including c-fos, ATF-2 (activating transcription factor 2), and CREB-1 (cAMP response element-binding protein). Secretion of other factors by B16F-10 melanoma cells, which are involved in angiogenesis, including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and matrix metalloproteinase was also reduced by treatment with harmine. Ex vivo assay, using rat aortic rings and conditioned medium from B16F-10 melanoma cells, demonstrated that harmine inhibited microvessel outgrowth. Experiments using B16F-10 cells intradermally injected on the shaven ventral skin and intraperitoneal administration of harmine (10 mg kg^{-1} body weight) in mice indicated potent reduction of tumor specific neo-vessel formation. The level of serum pro-angiogenic factors of VEGF and nitric oxide, as well as pro-inflammatory cytokines (IL-1β, interleukin-6 (IL-6), TNF-α, and granulocyte macrophage colony-stimulating factor (GM-CSF)), was effectively decreased by harmine treatment. Furthermore, harmine significantly enhanced the levels of interleukin-2 (IL-2) and tissue inhibitor of metalloproteinase (TIMP); both are important antiangiogenic factors [87].

2.2.4 Homoharringtonine

Homoharringtonine (**18**) is an alkaloid of unusual structure and belongs to harringtonine class, which is structurally characterized as cephalotaxine esters. These compounds have been exclusively isolated from *Cephalotaxus* plants, including *C. fortunei* Hook F and *C. harringtonia* K. Koch *var. harringtonia* [88].

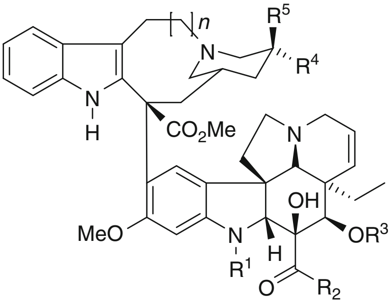
Cephalotaxine and its esters are of particular chemical and medical interest. Among these, **18** and harringtonine are most promising anticancer agents [89]. Homoharringtonine has been submitted to extensive phase I/II clinical studies in patients with different solid tumors, such as malignant melanoma, sarcoma, head and neck carcinoma, breast carcinoma, and colorectal carcinoma. Also, several clinical trials include studies efficacy in patients with acute leukemia, myelodysplastic syndrome (MDS), acute promyelocytic leukemia (APL), and chronic myeloid leukemia [90].

Homoharringtonine exhibited in vitro antiangiogenic effects on endothelial and leukemic cells, ECV304 and K562 cells, respectively. Its effects on ECV304 include proliferation inhibition and apoptosis induction. For leukemic cells, **18** downregulated VEGF mRNA expression and inhibited VEGF protein production, which were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and western blot, respectively [91]. Another in vitro study indicated VEGF antisense oligodeoxynucleotide increased the sensitivity of myeloid leukemia cells to **18** [92]. Experiments using gelatin sponges implanted on top of chick embryo CAM demonstrated that **18** significantly reduced microvessel density (MVD), confirming its in vitro antiangiogenic properties [93]. Studies involving RNA interference and transfected K562 cells suggested that inhibition of H1F-1α expression, at both mRNA and protein after RNAi H1F-1α, decreases VEGF, under hypoxic conditions. Moreover, this HIF-1α inhibition was able to increase K562 cell chemosensitivity to **18**. Altogether, these observations suggested that inhibition of H1F-1α expression is related to antiangiogenic activity through two complementary mechanisms, inhibition of VEGF expression and increase of leukemic cells susceptibility to **18**, an antiangiogenic agent [94].

2.2.5 Vinca Alkaloids and their Semisynthetic Analogues

Vinca alkaloids constitute a group of indole–indoline dimeric compounds from *Catharanthus roseus* (L.) G. Don. (formerly named as *Vinca rosea* L.). It is a pantropical species and belongs to the Apocynaceae family and is popularly known as Madagascar periwinkle [95]. Among these, vinblastine (or vincaleucoblastine, **19**) and vincristine (or leurocristine, **20**) are of clinical oncology interest [96]. Vincristine and vinblastine have been commonly administered in combination with other anticancer drugs in the treatment of hematological malignancies (leukemias and lymphomas) and solid tumors (bladder and breast cancers), respectively [97].

Soon after clinical impact of these phytochemicals in curative and palliative cancer chemotherapies, a number of studies focused on design of their semisynthetic derivatives were developed. These efforts led to important anticancer agents, including vindesine (**21**), vinorelbine (**22**), and vinflunine (**23**). Vinorelbine has been administered for treating non-small cell lung cancer (NSCLC), metastatic breast cancer, and rhabdomyosarcoma [98]. Vindesine is usually used to treat leukemia, lymphoma, melanoma, and breast and lung cancers. Vinflunine is a fluorinated vinblastine derivative and has been submitted to phase III clinical studies in patients with NSCLC and bladder cancer [99].



vinblastine **19** R^1= Me, R^2= OMe, R^3= COMe, R^4= Et, R^5= OH, *n* = 1

vincristine **20** R^1= CHO, R^2= OMe, R^3= COMe, R^4= Et, R^5= OH, *n* = 1

vindesine **21** R^1= Me, R^2= NH_2, R^3= H, R^4= Et, R^5= OH, *n* = 1

vinorelbine **22** R^1= Me, R^2= OMe, R^3= COMe, R^4= Et, R^5= H, *n* = 0

vinflunine **23** R^1= Me, R^2= OMe, R^3= COMe, R^4= CF_2Me, R^5= H, *n* = 0

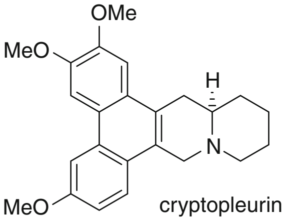
Vinblastine exhibited in vitro antiangiogenic activity. It was able to interfere in HUVEC angiogenic phenotype, at noncytotoxic concentrations, including proliferation, chemotaxis, adhesion, and abnormal vessel morphology. These features were observed when HUVEC was cultured in optimal growth medium alone or supplemented with angiogenic growth factors. Electron microscopy experiments demonstrated morphology alterations caused by **19**. They indicated that the HUVEC elongation was related to thin disturbance of cytoskeleton, in the form of slight depolymerization and addensation, as well as accumulation of microfilaments [100]. In other experiments, **19** inhibited adrenomedullin-induced angiogenesis, using in vitro and in vivo experiments, such as Matrigel tube formation and CAM of chick embryo, respectively [101]. The combination between **19** and rapamycin has exhibited in vitro and in vivo synergistic effects on angiogenesis [102, 103]. Differential proteomic analysis of this combination on ECs indicated crucial variations in protein contents, which were related to angiogenesis process. Among these, downregulation of ATP synthase, annexin A2, heat shock p70 (Hsp70), glucose-6-phosphate dehydrogenase (G6PDH), vasodilator-stimulated phosphoprotein (VASP), proteasome 26S, tryptophanyl-tRNA synthetase (TrpRS), and stathmin/Op18, as well as the up-modulation of carbonyl reductase, RhoGDP-dissociation inhibitor (Rho-GDI), and histone H1.0, was observed [104]. In another in vivo experiment, metronomic vinblastine chemotherapy inhibited VEGF-A-induced-angiogenesis. The continuous vinblastine treatment with an apparently nontoxic dose was able substantially to inhibit vascularized area and microvascular length, which were evaluated by microscopy morphometry and computerized image analysis, respectively [105]. Also, **20** and **23** have been intensively investigated as antiangiogenic agents, including their synergistic effects when combined with other drugs [106–108].

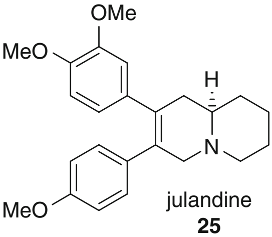
2.3 Alkaloids Derived from Lysine

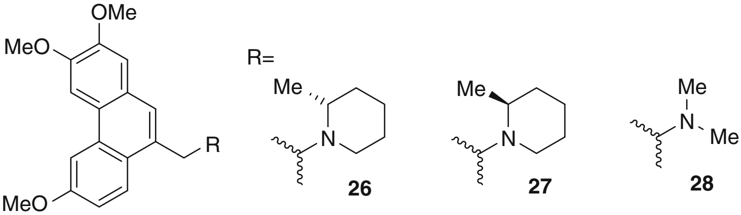
2.3.1 Cryptopleurine, Julandine, and their Synthetic Analogues

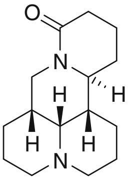
Cryptopleurine (**24**) and julandine (**25**) are phenanthroquinolizidine alkaloids. Together with phenanthroindolizidine alkaloids, they are constituted by over 70 compounds. These alkaloids are commonly isolated from plants of Asclepiadaceae and Moraceae families [109, 110]. They have exhibited similar biological activities, which include antiviral, antifungal, cytotoxic, and vesicant [111].

Cryptopleurine inhibited hypoxic HIF-1 activation in human gastric cancer cells (AGS cells), which were induced by hypoxia using a HIF-mediated reporter gene assay. Furthermore, **24** blocked HIF-1α protein accumulation in AGS cells, but not HIF-β protein, confirming its inhibitory effect on HIF-1 activation under hypoxic conditions [112]. Several C8c–C15 monoseco-analogues of **24** and **25** exhibited antiangiogenic activity, using rat aorta assay. Quinolizidine-ring opened analogues of cryptopleurine **26**–**28** inhibited 100% of blood vessel growth, at 100 μg mL^{-1}, and were more potent than PI-88, a heparin sulfate mimetic used as positive control [113, 114].


cryptopleurine
24


julandine
25




matrine
29

2.3.2 Matrine

Matrine (**29**) is a quinolizidine alkaloid isolated from several species of the genus *Sophora* (Fabaceae), mainly *S. alopecuroides* L. and *S. flavescens* Aiton. The latter has widely been used by traditional Chinese medicine, and its pharmacological properties were correlated to matrine contents [115, 116].

Matrine possesses potent antitumor activities in vitro and in vivo. It exhibits cytotoxic activity on cancer cell lines of different histological origins, including cervical cancer, gastric cancer, glioma, HCC, leukemia, lung cancer, and melanoma. Also, matrine administration reduced the growth of HCC and gastric tumor in mice [117–120].

Matrine showed potent in vivo antiangiogenic effect. Experiments, using breast cancer cell implanted in Balb/c mice, indicated that **29** reduced tumor MVD, which is measured by immunostaining techniques using anti-CD31 antibodies. Corroborating these findings, western blot assays demonstrated that **29** downregulated VEGF and VEGFR-2 expression [121].

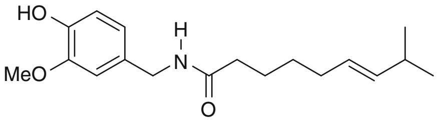
2.4 Capsaicin and its Natural Ester Analogues

Capsaicin (**30**) is an amide belonging to capsaicinoid class, which is formed by condensation between branched-chain fatty acids (C_8–C_{13}) and vanillylamine unit.

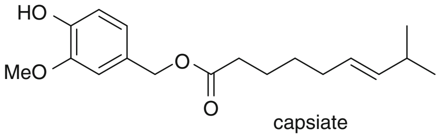
They are isolated from several varieties of *Capsicum annuum* L. and *C. frutescens* L. (Solanaceae), which are commonly named as hot peppers. Their physiological effects, such as lachrymation, coughing, and burning sensation, are directly correlated to capsaicinoid contents [122].

Pharmacologically, **30** has been intensively studied due to high TRPV-1 affinity. After TRPV-1 activation, **30** causes its long-lasting desensitization and loss of pain sensitivity. This unusual analgesia mode became **30** as valuable lead compound for design of innovative painkiller agents. The main therapeutic application of **30** is in cream-topical formulations, used to treat several algias associated with neurological origin, such as osteoarthritis, painful neuropathy linked to the trigeminal nerve, neuralgia caused by herpes infections, and painful diabetic neuropathy [123].

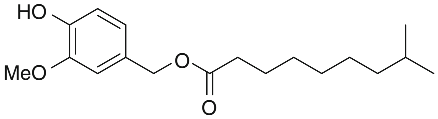
Capsaicin exhibited potent effect on cancer and inflammatory angiogenesis processes. In vitro assays demonstrated that **30** was able to inhibit the proliferation of HUVEC primary culture, which was not related to high TPRV-1 affinity. In other experiments, using HUVEC, capsaicin inhibited their migration and capillary tube formation. Further studies showed that **30** was able to inhibit DNA synthesis in HUVEC, causing G1 phase arrest. Its effect was attributed to downregulation of cyclin D1 expression, leading to inhibition of phosphorylation of retinoblastoma protein (pRb), mediated by CDK-4. Signaling experiments showed that capsaicin inhibited VEGF-induced activation of p38-MAPK (p38 mitogen-activated protein kinase), p125^{FAK} (p125 focal adhesion kinase), and Akt, suggesting its possible antiangiogenic molecular mechanism. Ex vivo assay, using art aortic ring, **30** significantly reduced VEGF-induced microvessel sprouting. Two in vivo experiments confirmed the potential antiangiogenic activity of **30**. It was capable of blocking angiogenesis induced by Matrigel plug containing VEGF, subcutaneously implanted into C57BL/6 mice. In another experiment, using CAM of chick embryo assay, **30** inhibited angiogenesis induced by primate fibrosarcoma (HT1080 cells) [124]. In vivo experiments, using acetic acid-induced kissing gastric ulcer, **30** strongly inhibited the microvessels formation in the granulation tissue at the ulcer margin. Additionally, **30** decreased epidermal growth factor (EGF) levels in salivary gland, serum and gastric mucosa, suggesting its potential antiproliferative activity on several epithelial cells [125].



capsaicin
30



capsiate
31



dihydrocapsiate
32

Capsinoids, which include capsiate (**31**) and dihydrocapsiate (**32**), are esters naturally occurring in red peppers. Although they are structurally similar to capsaicin and its amide-type analogues, capsinoids were not found to be potent pungent compounds. Capsiate and dihydrocapsiate showed in vitro and in vivo antiangiogenic properties closely related to capsaicin performance [124]. Additionally, these compounds blocked VEGF-induced endothelial permeability and loss of endothelial cell–cell junctions facilitated by vascular endothelial cadherin (VE-cadherin). Capsiate was able to suppress not only VEGF-induced activation of Src kinase but also the phosphorylation of its downstream substrates, including p125^{FAK} and VE-cadherin. Molecular modeling studies indicated the binding mode of **31** in Src tyrosine kinase, which interacted to its ATP-binding pocket [126].

2.5 Halofuginone

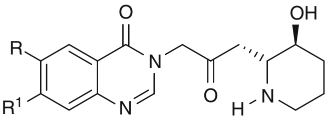
Halofuginone (**33**) is not plant-derived alkaloid. It is halogenated synthetic compound, which shows structural features based on febrifugine (**34**), a *N*3-substituted quinazolin-4-one alkaloid [127].

Febrifugine was first isolated from roots of *Dichroa febrifuga* Lour. (Saxifragaceae), a Chinese medicinal plant traditionally used to treat malaria fevers. This alkaloid showed potent anti-*Plasmodium* activity, which was superior to quinine, and unacceptable side effects, such as hepatotoxicity and severe emesis [128, 129]. Its properties not only precluded its use as drug but also encouraged the synthesis of safer analogues [127].

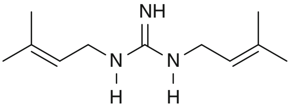
Halofuginone is of particular interest as a drug orphan candidate to treat scleroderma [130, 131]. It has been intensively investigated for control of diseases involving collagen biosynthesis and some cancer types [131, 132]. The main application of its hydrobromide salt is as a curative and prophylactic agent, used to treat protozoan veterinary infections, mainly in commercial poultry and cattle production [131].

Halofuginone was effective in critical steps of in vitro angiogenesis process, which was evaluated on bovine aortic endothelial cells (BAEC). These events include (1) antiproliferative activity, (2) inhibition of MMP-2 gelatinolytic activity, (3) decrease of invasion capacity through Matrigel containing medium conditioned by 3 T3 fibroblasts, (4) inhibition of capillary tube formation, and (5) inhibition of subendothelial extracellular matrix (ECM) deposition. Ex vivo assay, using rat aortic ring, indicated that **33** markedly reduced vascular sprouting. The most relevant antiangiogenic property was evidenced by in vivo experiments, using mouse corneal micropocket experiments. This assay revealed that **33** inhibited bFGF-induced neovascularization, both after intraperitoneally or diet administration [133].

In other studies, **33** exhibited biological activities closely related to key antiangiogenic effects. A potent inhibition on MMP-2 gene transcription activity was demonstrated in human and murine bladder carcinoma cell lines [134]. Highly specific suppression of collagen type α1 gene expression was observed in a broad panel of different cell types, including chicken, mouse, rat, and human origin [135]. Furthermore, **33** showed antiangiogenic properties in several in vivo models [136, 137].



halofuginone **33** R = Cl, R^1= Br
febrifugine **34** R = R^1= H



pterogynidine
35

2.6 Pterogynidine

Pterogynidine (**35**) belongs to guanidine alkaloid class, a quite rare plant-alkaloid category, which is most common in marine organisms. It is formed by guanidine core substituted by two isoprenylated units [138]. This compound was first isolated from *Pterogyne nitens* Tul. (Fabaceae), an ornamental species, which occurs only in South America regions [139].

Pterogynidine has showed cytotoxicity against several human cancer cell lines, including myeloblastic leukemia, glioblastoma, and malignant breast [140]. Its cytotoxic effect has been related to apoptosis-inducing activity, which was evaluated by several in vitro assays, such as Hoechst-propidium iodide, cytometry-based annexin-V, and caspase-Glo 3 and 7 [141].

Pterogynidine showed in vitro antiangiogenic properties, using HUVEC. Among these, **35** was able to decrease proliferation and invasion capacity, which was evaluated by bromodeoxyuridine (BrdU) and double-chamber assay, respectively. The terminal transferase dUTP nick end labeling (TUNEL) experiments indicated that **35** increases HUVEC apoptosis index, corroborating with the effects on cancer cells. In addition, **35** drastically reduced the number of capillary-like structures formation using HUVEC cultured on growth factor reduced-Matrigel (GFR-Matrigel) coated plates. In addition, **35** was shown to act through the inhibition of NFκB [26].

3 Conclusion

Plants are well known to be a rich source of bioactive constituents, and they can provide high potential for discovery of new and effective antiangiogenic agents. Angiogenesis is a process regulated by a balance between pro- and antiangiogenic molecules, and the inhibition of pathological angiogenesis can be important in several diseases. Rapid advances were made in the field of angiogenesis over the past years, and, more importantly, a great attention has been given to angiogenesis as a therapeutic target. The application of this strategy in the treatment of several diseases, including cancer and chronic inflammatory disorders, has now become a reality. At the moment, there are a growing number of alkaloids obtained from plants that may have antiangiogenic properties, suggesting that these naturally occurring compounds may provide significant approaches in treatment or prevention of diseases where excessive angiogenesis is part of the pathology. It can be concluded that antiangiogenic activities can be derived from alkaloids obtained from plants; however, most of the studies using antiangiogenic alkaloids were focused on in vitro studies, and there is a need to carry out in vivo studies to establish their efficacy and evaluate their toxic potentials. Besides that, it is still necessary to continue the search for novel molecules for drug development.

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Prevention of Brain Disorders by Nicotine

47

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Contents

1 Introduction 1471
  1.1 Effect of Nicotine on Structure and Function of the Brain 1473
2 Alzheimer's Disease 1474
  2.1 Effect of Nicotine on Learning and Memory in AD 1475
  2.2 Synaptic Plasticity: The Physiological Correlate of Learning and Memory 1475
  2.3 Molecular Deficits in AD and the Beneficial Effect of Nicotine 1477
  2.4 Possible Mechanisms of Neuroprotective Effect of Nicotine 1478
3 Parkinson's Disease (PD) 1480
4 Attention Deficit/Hyperactivity Disorder (ADHD) 1482
  4.1 Sleep Deprivation 1484
  4.2 Stress 1485
  4.3 Hypothyroidism 1487
  4.4 Aging 1490
  4.5 Schizophrenia 1491
5 Concluding Remarks 1492
References 1493

Abstract

Effective prevention and/or treatment strategies for neurodegenerative and other brain disorders have, so far, not been successful. For example, a number of drugs are available for management of Alzheimer's disease; however, these agents

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produce only transient palliative results that often come with a wide variety of untoward effects. Therefore, there is an urgent need for new therapeutic strategies with better efficacy and fewer side effects. Many plant origin compounds possess properties that can be useful in preventing or retarding neurodegenerative and other neurological diseases. One such compound is nicotine, which has astonishingly varied actions including inhibition of apoptosis and cell cycle progression, stimulation of neurogenesis, restoration of calcium homeostasis, reduction of metal ion overload in the brain, support of mitochondrial respiratory chain, and the possession of substantial anti-inflammatory and antioxidant effects. In this chapter, we summarize some of the published literature on the beneficial effects of nicotine and its potential for the treatment of cognitive deficits associated with selected neuropsychiatric and neurodegenerative disorders.

Keywords

ADHD • aging • Alzheimer’s disease • hypothyroidism • molecular deficit • parkinson’s disease • PTSD • schizophrenia • sleep deprivation • stress • synaptic plasticity

Abbreviations

5-OHDA	5-hydroxydopamine
6-OHDA	6-hydroxydopamine
Ach	Acetylcholine
ACI	Adenylyl cyclase I
AD	Alzheimer’s disease
ADHD	Attention deficit/hyperactivity disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors
APP	Amyloid precursor protein
Aβ	Amyloid-beta
BACE	Beta-site amyloid precursor protein [APP]-cleaving enzyme
BDNF	Brain-derived neurotrophic factor
CA	Cornu ammonis
CaMKIV	Calcium-calmodulin-dependent protein kinase IV
CRE	cAMP response element
CREB	cAMP-responsive element-binding protein C
CSF	Cerebrospinal fluid
DG	Dentate gyrus
DMTS	Delayed matching-to-sample
E-LTP	Early-phase LTP
ERK1/2	Extracellular signal-regulated kinase 1 and 2
fEPSP	Field excitatory postsynaptic potential
FGF-2	Fibroblast growth factor-2
GABA	γ-aminobutyric acid

HFS	High-frequency stimulation
icv	Intracerebroventricular
iNOS	Inducible nitric oxide
L-LTP	Late-phase LTP
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MHFS	Multiple train high-frequency stimulation
MLA	Methyllycaconitine
MWM	Morris water maze
nAChR	Nicotinic acetylcholine receptor
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NMDA	n-methyl-D-aspartate
NS-DA	Nigrostriatal dopaminergic
p-CaMKII	Phosphorylated-Ca^{2+}-calmodulin (Ca^{2+}-CaM)-dependent protein kinase II
PD	Parkinson's disease
PKC	Protein kinase C
PP1	Protein phosphatase 1
PTSD	Post-traumatic stress disorder
RAM	Radial arm maze
RAWM	Radial arm water maze
REM	Rapid eye movement
SUD	Substance use disorders
TM	Transmembrane
TrkB	Tyrosine-related kinase B

1 Introduction

Nicotine, the major alkaloid found in tobacco plant leaves and the prototypical nicotinic acetylcholine receptor (nAChR) agonist, is known to reduce memory impairment associated with chronic stress [1–3], aging [4–8], brain lesions [9], and cognitive disorders, including Alzheimer's disease (AD) [3, 10–14], schizophrenia [14], attention deficit/hyperactivity disorder (ADHD) [15, 16], Parkinson's disease [13, 17], post-traumatic stress disorder (PTSD) [18], sleep deprivation [19, 20], and hypothyroidism [21, 22]. Moreover, nicotine is a potent scavenger of hydroxyl and superoxide free radicals. In fact, it has been reported that nicotine is more of an antioxidant than vitamin C [23].

Craving for nicotine is, perhaps, a form of self-medication and may account for the higher proportion of smokers among people with mental disorders. Individuals with mental disorders have a higher smoking incidence (e.g., up to 60 % of PTSD sufferers; [24]) than the general population (25 %), and about 30 % of the total smokers in the United States suffer from some form of mental illness [25]. For example, up to 90 % of schizophrenic patients [26] and more than 90 % of alcoholics [27] are tobacco users.

The effects of nicotine in the brain basically mimic those of the endogenous neurotransmitter acetylcholine (ACh), which activates nAChR. Acetylcholine itself has a fascinating role as a neuromodulator in memory consolidation in the hippocampal formation [28]. The importance of ACh in cognitive function is indicated by the observation that this neurotransmitter increases significantly in the hippocampus of rats that have learned a memory task [29].

Nicotine produces a variety of effects in the brain by interaction with nAChR. The drug is believed to enhance cognitive function by activating presynaptic nAChR in crucial areas of the brain, facilitating the release of neurotransmitters including those implicated in learning and memory such as ACh, glutamate, dopamine, norepinephrine, γ-aminobutyric acid (GABA), and serotonin [30–34].

Structurally, nAChRs are comprised of homologous or heterologous combinations of five polypeptide subunits. Each subunit is composed of four transmembrane (TM 1–4) domains arranged around a central, water-filled pore. The extracellular N-terminal domain of each of the five subunits makes up the agonist binding site [35]. The pentameric nAChR, formed by various combinations of α and β subunits, is widely distributed in mammalian brain [36, 37]. Eight forms of the α subunit (α2–α10) and three forms of the β subunit (β2–β4) have been identified. The mechanism for the neuroprotective/trophic effects of nicotine is not known but seems to be multifaceted, as the reader will appreciate throughout this chapter.

The cationic selectivity of nAChR arises from the presence of three rings of negative charge, located along the inner pore of the channel. The nAChR belong to a family of ligand-gated ion channels that mediate fast synaptic transmission in the central nervous system (CNS). nAChRs can be classified into three major groups, based on their pharmacological and physiological properties: (a) the heteropentameric nAChRs (subunits α, β, δ, σ, and ε), which exist in endplates of skeletal muscles; (b) the standard neuronal nAChRs formed from α and β combinations; and (c) the homopentameric nAChRs, formed from α_7–α_9 subunits [38]. The hippocampus is highly enriched with a number of nAChR subtypes; however, based on electrophysiological and in situ hybridization studies, the $\alpha_4\beta_2$- and α_7-nAChR subtypes are the most abundantly expressed receptor subtypes in the hippocampus [39, 40]. Receptor subtypes formed with α4β2 subunit combination and those with α7 subunits have been implicated in learning and memory functions [37]. The α4β2 receptor is found in areas that bind nicotine with high affinity (e.g., the cortex, hippocampus, and interpeduncular nucleus [36, 41]). The α7 receptor is distributed in areas with high-affinity binding sites for α-bungarotoxin [42]. Most hippocampal neurons respond to nicotine with a current, characteristic of α7 nAChR [40] that desensitizes rapidly and is blocked by the antagonist methyllycaconitine (MLA) [43]. Outside the central nervous system, in autonomic ganglia, the α3β4 subtype is the most abundant nAChR, and even within the autonomic nervous system, there seems to be differences in the nAChR distribution. For example, the number of neurons containing α3, α4, α5, and α7 subunits in sympathetic ganglia is about three times higher than in parasympathetic ganglia. This may explain the differential effects of certain drugs on neurotransmission in sympathetic and parasympathetic ganglia.

1.1 Effect of Nicotine on Structure and Function of the Brain

Chronic nicotine treatment increases the number of [^{3}H]ACh binding sites in rat brain [44, 45] even though this treatment desensitizes nAChR. This seems paradoxical, but prolonged desensitization may result in an enhanced synthesis rate of nAChR or a reduction in its rate of internalization. However, the seemingly increased number of receptors, indicated by the increase in binding sites, may include a low proportion of functioning receptors [44, 46].

Nicotine has been shown to improve normal memory performance in clinical [16] and animal studies [47, 48]. Furthermore, in vitro electrophysiological studies in brain slices have shown that acute or chronic nicotine treatment facilitates the induction of long-term potentiation (LTP) in area CA1 by lowering its threshold of induction [49–51]. In general, nicotine promotes LTP induction by producing changes in the nAChRs of the hippocampus, which in turn alters local circuit interactions and lowers LTP threshold in the hippocampal area CA1. Later, however, the same group reports that nicotine enhances long-term depression (LTD) as well in area CA1 of hippocampal slices from young rats [52].

The effects of nicotine on memory and LTP are prevented by mecamylamine, a nonselective nAChR antagonist, suggesting that most likely nicotine induces its effects on memory and LTP by acting on nAChRs in the hippocampus [6, 51, 53–55].

Nicotine is known to have neuroprotective and neurotrophic effects as it delays ischemia-induced neuronal death in area CA1 of the hippocampus [56], and it also reverses the effects of proinflammatory cytokines on LTP [57]. Furthermore, nicotine reverses impairment of spatial memory and working memory induced by lesioning of the fimbria [9, 53], hypothyroidism, and chronic stress [2, 58], as well as sleep deprivation [19, 20]. Additionally, clinical and epidemiological studies suggest a neuroprotective/trophic effect of nicotine in AD and Parkinson’s disease [13]. In fact, earlier reports show that the incidence of AD is lower among tobacco smokers and that nicotine treatment improves cognitive function of AD patients [59–61]. Interestingly, chronic nicotine treatment improved working memory performance of young adult rats in the radial arm maze, whereas aged rats treated with the same dose showed no significant improvement [6]. Therefore, depending on the dose, stressor, and individual variation in reaction to the drug, nicotine can reduce or enhance stress-related effects [62].

The role of α_7-nAChRs in memory and LTP is controversial. While blocking α_7-nAChRs by the antagonist methyllycaconitine (MLA) impairs memory and blocks the effect of nicotine on memory in radial arm maze [63], MLA facilitates the induction of LTP in hippocampal slices [49]. The facilitation of LTP by MLA is supported by the finding that chronic nicotine treatment induces desensitization of α_7-nAChRs in GABAergic interneurons [64, 65]. The desensitization of α_7-nAChRs reduces the release of GABA from these interneurons, thus indirectly increasing pyramidal cell excitability [64, 65]. This, in turn, facilitates LTP induction by decreasing its threshold for induction [49, 50]. Thus, changing the dynamics of nAChRs activation and distribution influences the release of neurotransmitters and affects memory and activity-dependent synaptic plasticity. Furthermore, in addition to modulating the activity of neuronal circuits in hippocampal and cortical brain regions, nAChRs appear to be involved equally in neuronal survival. For example, β_2-nAChR knockout old mice exhibit neocortical hypertrophy, hippocampal neuron loss, and astrogliosis and microgliosis, which are correlated with impaired spatial learning [66].

Tobacco smokers indicate that smoking is a way to cope with stress; however, adolescent smokers reported a reduction of stress on smoking cessation [67]. Adolescent exposure to nicotine seems to have gender- and brain region-selective effects that differ substantially from those in adults. Chronic nicotine treatment uniformly produced a longer-lasting upregulation of nAChR in the brain of adolescent rats than in adult rats where receptor upregulation was differential with the highest being in the hippocampus [68].

Nicotine administration has also been reported to improve attention and enhance learning and memory in ADHD as well as schizophrenic patients [14]. These effects are most probably the result of reported enhanced dopamine release [69]. In this report, we reviewed the effect of nicotine on various brain disorders. The number of reports on the neuroprotective action of nicotine is vast; however, due to the restricted space we have, we are only able to cite a limited number of relevant articles.

2 Alzheimer's Disease

Dementia of the Alzheimer's type (AD) is a progressive neurodegenerative disease characterized by a gradual deterioration of memory and other cognitive functions, leading to total incapacity and death within few years after diagnosis [70]. The major pathological hallmarks of AD are marked neuronal loss and the presence of extracellular senile plaques and intracellular neurofibrillary tangles (NFT) in the brain [70].

Dysfunctional cholinergic mechanisms are common in dementia disorders, including AD [71, 72]. The brains of AD patients exhibit marked losses of α_7- and $\alpha_4\beta_2$-nAChRs [73–75] and of presynaptic terminals in neocortical and hippocampal regions that are correlated with progressive cognitive decline [76, 77]. Immunohistochemical, biochemical, and pharmacological data suggest that the high-affinity binding of $A\beta_{1-42}$ to α_7- and $\alpha_4\beta_2$-nAChRs plays a critical role in AD pathogenesis, e.g., formation of AD plaques and degeneration of cholinergic neurons [78, 79]. It is suggested that chronic stimulation of α_7-nAChRs by $A\beta_{1-42}$ facilitates (a) derangement of ERK1/2-MAPK signaling [80], (b) internalization and intracellular accumulation of $A\beta_{1-42}$ [81], (c) modulation of GABAergic signaling [64], and/or (d) excessive glutamate receptor stimulation [82]. Experiments using exogenous Aβ administration, transgenic mice, and gene-targeting mouse models demonstrate correlations between excessive Aβ accumulation, impaired nAChR function [75], and deficits in learning, memory, and LTP [e.g., 3, 83–87].

Numerous epidemiological studies have reported a highly significant negative correlation between cigarette smoking and AD [88; but see 89]. In laboratory and clinical studies, nicotine in cigarette smoke has been shown to improve cognitive function in AD patients [11, 88, 90] and also attenuate Aβ-induced amnesia in animal models [91, 92]. It is well known that chronic exposure to nicotine causes desensitization and upregulation of its receptors [68]. Although the mechanism by which nicotine achieves this is not well understood, several possible mechanisms have been proposed. In the following, we discuss the effects of nicotine on various aspects of AD pathology.

2.1 Effect of Nicotine on Learning and Memory in AD

Nicotine and its metabolites (e.g., cotinine) inhibit β-amyloidosis [93–95]. Several studies have suggested that chronic treatment with nicotine may delay the onset and/or progression of AD by slowing down cognitive decline [93]. Behavioral tests in rodents are consistent with these findings and support the notion that chronic nicotine treatment, if administered prior to Aβ-infusion, inhibits impairment of learning and memory in Aβ-infused animals [3, 86, 87; Fig. 47.1]. Under our experimental conditions, the performance of nicotine-treated animals in the RAWM was not significantly different from that of untreated control animals, which supports previous findings from this lab, indicating that the nicotine dose we used, although completely prevented Aβ and/or stress-induced cognitive decline, had no effect on memory in normal animals [1–3, 96]. This finding is in contrast to that of Levin et al. [14] who reported that the performance of nicotine-treated normal animals was enhanced in the radial arm maze (RAM). However, the improvement of normal spatial memory reported by Levin et al. [14] involved the use of high doses of nicotine (>5 mg/kg/day), whereas the dose used in our studies (2 mg/kg/day) resembles the concentration of nicotine in the blood of chronic smokers [97, 98].

2.2 Synaptic Plasticity: The Physiological Correlate of Learning and Memory

Although LTP has been demonstrated in many parts of the mammalian CNS, hippocampal LTP is the most studied form. Hippocampal LTP requires synaptic glutamate NMDA (n-methyl-ᴅ-aspartate) receptor activation to constitutively activate key protein kinases and/or phosphatases [99]. There are two mechanistically distinct phases of LTP: early-phase LTP (E-LTP; related to short-term memory) and

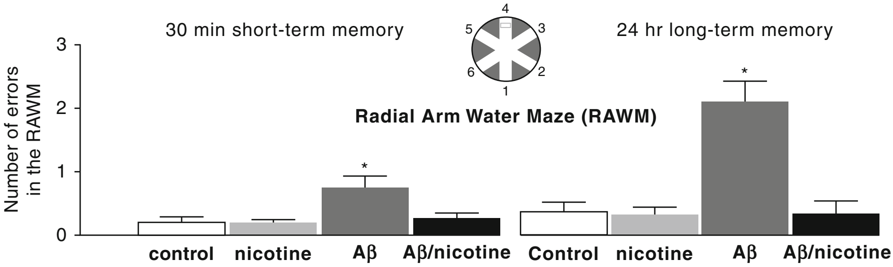

Fig. 47.1 Effect of nicotine on Aβ-induced deficits in spatial short-term memory and long-term memory performance in the RAWM. On days 6–8 of testing, chronic nicotine treatment (1 mg/kg s.c. twice/day for 6 weeks prior to and during Aβ infusion) prevented the Aβ-induced cognitive decline. All values are mean ± S.E.M. (n = 12 rats/group); $*p < 0.05$ compared to all groups

late-phase LTP (L-LTP; related to long-term memory). E-LTP is a transient, protein synthesis-independent phase, which requires constitutive activation of phosphorylated-Ca^{2+}-calmodulin (Ca^{2+}-CaM)-dependent protein kinase II (p-CaMKII) to phosphorylate and enhance the conductivity of postsynaptic glutamate α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPAR) [100–102], which in turn, initiates activation of glutamate NMDA receptors [99].

L-LTP is an enduring, protein synthesis-dependent phase, which requires (a) activation of CaMKIV and mitogen-activated protein kinase (MAPK) to phosphorylate cAMP-responsive element-binding protein (CREB) and (b) cAMP response element (CRE)-mediated transcription of target genes [103]. CRE-mediated gene transcription is absolutely necessary for synapse formation, neuronal survival (brain-derived neurotrophic factor, BDNF), and long-term memory formation [104]. For example, in AD brains, decreased CREB phosphorylation is correlated with decreased neuronal growth and survival, suggesting that Aβ acts through this transcription factor [105].

Altered synaptic plasticity is believed to be an early event that contributes to the cognitive deficits characteristic of AD [76, 106, 107]. Acute and chronic nicotine treatment has been shown to enhance LTP in rat and mouse DG in vivo [108]. The same group reported that co-application of nicotine with 500 nM Aβ prevented Aβ-induced deficits in LTP [108]. In anesthetized rat model of AD, in vivo recording of population spikes consistently showed severely suppressed E-LTP and L-LTP in hippocampal CA1. In contrast, Aβ animals chronically treated with nicotine showed alleviation of LTP impairment [3, 86, 87; Fig. 47.2].

Contrary to the majority of published reports, some labs reported that nicotine neither enhanced nor depressed HFS-induced LTP in normal animals [85, 109]. For example, Itoh et al. [109] found that in slices from Aβ-infused (300 ρmol/day $Aβ_{1-40}$, 11–12 days) rats, 50 μM of nicotine, perfused for 10 min, decreased population spike amplitude in area CA1 of control rats. Similarly, studies by Freir et al. [85] found that coinjection of 3 mg/kg nicotine with 1 or 10 nmol $Aβ_{1-40}$, 1 h prior to HFS, significantly depressed LTP, measured 1 h post-tetanus, more than $Aβ_{1-40}$ alone.

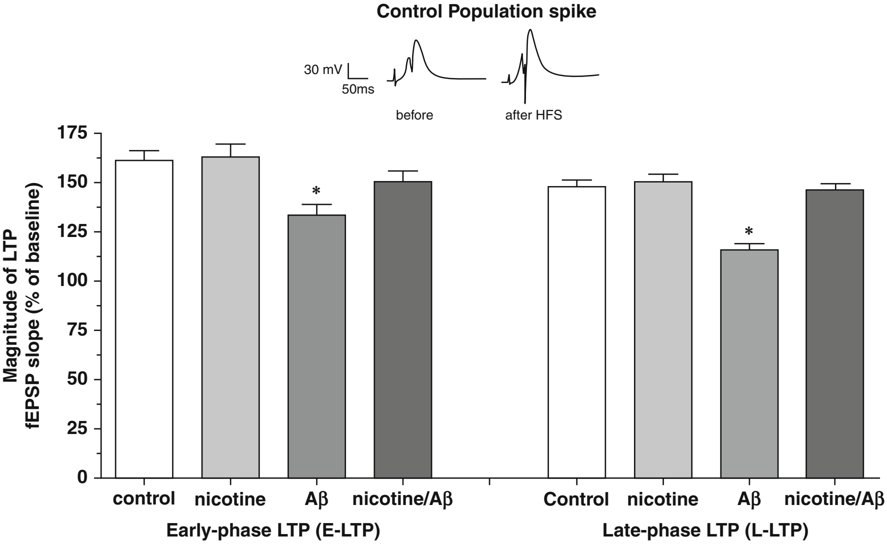

Fig. 47.2 Nicotine prevents Aβ-induced deficits in E-LTP and L-LTP. LTP of the hippocampal area CA1 evoked by repetitive stimulation of the Schaffer collaterals/commissural pathway, measured as increases in the slope of fEPSP. Each bar is mean ± S.E.M. from six anesthetized rats. * indicates significant difference from other groups ($p < 0.05$). Insets are representative pspikes from a normal rat before and after stimulation

2.3 Molecular Deficits in AD and the Beneficial Effect of Nicotine

The molecular processes underlying the expression of LTP in the hippocampus involves a cadre of signaling molecules that are believed to model the consolidation of information storage by this brain region. These signaling molecules play a pivotal role in synaptic plasticity and the processes of learning and memory. Calcineurin and CaMKII play integral roles in converting the initial entry of Ca^{2+} through the NMDA receptor channel, into persistent changes in synaptic activity. Gene-targeting experiments and transgenic animal studies have consistently demonstrated impairment of learning, memory, and hippocampal LTP following altered gene expression of selected protein molecules [110]. In AD, progressive accumulation of Aβ peptides in the brain impairs spatial learning and LTP in area CA1 of the hippocampus. Experiments suggest that Aβ impairs memory and disrupts LTP by perturbing the balance between protein kinase and protein phosphatase activity. Findings from this lab strongly suggest that Aβ interferes with the process of phosphorylation of kinases, downregulates nAChR but increases the levels of the phosphatase calcineurin [3, 86, 87; Table 47.1]. The levels of neurotrophic factors, including BDNF, are initially increased in specific brain regions in response to various types of insults, including AD, ischemia, seizure, traumatic brain injury, and neurotoxins. Consistent with its neuroprotective effects on learning and memory and synaptic plasticity, nicotine shows striking effects in normalizing the levels

Table 47.1 Basal levels of signaling molecules. Summary of the effects of nicotine on the basal levels of signaling molecules in area CA1 of the hippocampus compared to the control. ⇓ significantly decreased; ⇑ significantly increased; ⇔ no change (similar to control)

	Nicotine	Aβ	Nic/Aβ
Aβ	⇔	⇑	⇓
BACE	⇔	⇑	⇔
p-CaMKII	⇔	⇓	⇔
t-CaMKII	⇔	⇔	⇔
Calcineurin	⇔	⇑	⇔
α_7-*nAChR*	⇑	⇓	⇔
α_4-*nAChR*	⇑	⇓	⇔
β_2-*nAChR*	⇑	⇔	⇔
BDNF	⇑	⇑	⇑
p-CREB	⇔	⇓	⇔
t-CREB	⇔	⇔	⇔
CaMKIV	⇔	⇓	⇔

of the signaling molecules in Aβ-infused animals. The drug even reduced the levels of Aβ in these animals [3, 86, 87; Table 47.1].

2.4 Possible Mechanisms of Neuroprotective Effect of Nicotine

It has been suggested that nicotine and cotinine delay or inhibit β-amyloidosis by nonspecifically binding to Aβ and preventing an α-helix to β-sheet conformational conversion [93, 95]. It is postulated that nicotine binds to histidine residues (His6 and His13) on the α-helix [95], or to small, soluble β-sheet aggregates [94] and increases the average separation between Aβ monomers in solution, thus delaying the onset of aggregation [94, 111]. Similarly, studies using nornicotine, a nicotine metabolite, demonstrated that nornicotine-based covalent glycation of lysine-16 on the Aβ peptide occluded the Aβ polymerization domain and thus, delayed formation of the oligomeric β-sheet structure [95]. It is unclear whether nicotine-mediated inhibition of Aβ deposition, aggregation, and/or β-amyloidosis is due to altered processing (toward a non-amyloidogenic Aβ sequence), decreased synthesis, or increased clearance of Aβ peptides. However, a recent study found that chronic nicotine treatment (1 mg/kg/day and 8 mg/kg/day) reduced rat CSF levels of APPγ, which contains the amyloidogenic Aβ fragment, without significantly altering total soluble APP levels [74]. This suggests that nicotine exerts its effects, in part, by altering the processing of APP away from an amyloidogenic route, toward increased production of APP-carboxyl-terminally truncated forms, which do not contain the pathogenic $A\beta_{1-40}$ and/or $A\beta_{1-42}$ fragments.

Furthermore, whereas no single mechanism for nicotine-mediated protection has been determined, studies have shown that nicotine increases the levels of neuronal growth factors [112, 113], decreases the levels of nitric oxide generated in response to neuronal injury [114], and inhibits glutamate-evoked arachidonic acid release from cultured striatal neurons [115]. These and other possible mechanisms are discussed in the following sections:

Overload of metal ions: It has been reported that Aβ interacts with metal ions as indicated by the presence of copper and zinc in the senile plaques of AD brains [116] a finding that implicates these ions in the process of deposition of Aβ and formation of the plaques [117–120]. Nicotine significantly lowered the copper and zinc concentration in senile plaques and in hippocampal area CA1 in the brains of transgenic mice model of AD [121]. The effect of nicotine on metal homeostasis seems to be independent of nAChR activation [121].

Prevention of apoptosis: Shrinking of brain tissue in AD is due to cell death (apoptosis), which may be caused by combination of factors including increased intracellular calcium, oxidative stress and inflammation [122–127]. The caspases are enzymes essential for many cellular functions including growth and development and are crucial in apoptosis for which they have been termed "executioner" proteins. In cultured hippocampal neurons, nicotine effectively inhibited Aβ-induced caspase activity and apoptosis. This protective effect of nicotine is antagonized by mecamylamine indicating the involvement of nAChR.

Mitochondrial dysfunction: Mitochondrial dysfunction in AD is believed to be a major contributing factor that causes Aβ deposition, formation of NFTs and synaptic degeneration [128]. A number of studies have shown impaired mitochondrial dynamics and biogenesis in brains from AD patients, AD mouse models, and APP cell lines [129–131].

It has been suggested that nicotine maintains the intra-mitochondrial redox state and decreases the electron leak at the site of respiratory chain complex I [132, 133]. Therefore, the beneficial effects of nicotine in AD may be related in part, to its supportive actions on mitochondrial function [133].

Nitric oxide (NO) production: Emerging evidence suggests a role for NO in neurodegenerative diseases indicated by the increased expression of the enzymes that catalyze NO synthesis in brain tissue of patients with these disorders [134].

Nicotine treatment decreases the production of NO by downregulating the activity and expression of inducible iNOS in the cortex and hippocampus of APP(V717I) transgenic mice, a model for AD [135]. It is suggested that nicotine, by upregulating and activating α7nAChR, downregulates nitric oxide synthase (iNOS) activity and consequently the production of NO, by preventing activation of NF-κB and c-Myc through inhibition of the activation of MAP kinases (MAPKs). Support for this finding is obtained by the fact that nicotine markedly decreased the expressions of p65 and p50 subunits of NF-κB in the cortex and hippocampus of mice [135]. Incidentally, chronic nicotine treatment of AD rat model prevented Aβ-induced reduction of α7nAChR and impairment of learning and short-term memory [3].

Oxidative stress: Another important aspect of AD, which bespeaks the complexity of this disease, is oxidative stress. Oxidative stress is among the early events in the pathogeny of the disease and has been implicated in the synaptic dysfunction and neuronal loss in AD [136–139]. Moreover, it has been reported that nicotine is a potent antioxidant that can act as a scavenger of hydroxyl and superoxide free radical [23]. Therefore, a possible mechanism for the neuroprotective effect of nicotine in neurodegenerative diseases is its considerable antioxidant effects that may prevent oxidative stress.

Decreased Aβ production: Nicotine treatment effectively lowers the levels of amyloidogenic Aβ in the brains of rodent models of AD [3, 23, 140, 141]. In Aβ-infused rats, chronic nicotine treatment normalized the elevated levels of Aβ and that of BACE, the enzyme responsible for its production [3]. The same report shows that nicotine markedly upregulates the protein levels of α7- and α4β2-nAChR [3], which may suggest the involvement of these receptors in the ability of nicotine to curtail the production of pathogenic Aβ peptides.

In brains of APPs transgenic mice, which show substantial brain Aβ deposits, chronic nicotine treatment produces a significant (80 %) reduction in Aβ 1–42 positive plaques [140]. The same group later reports that the decrease in Aβ peptides is not accompanied by changes in brain α, β, or γ secretase-like activities, NGF (nerve growth factor) or BDNF protein expression [141].

3 Parkinson's Disease (PD)

Parkinson's disease is a debilitating progressive neurodegenerative disorder, which is characterized by a selective loss/damage of dopaminergic neurons that project from the substantia nigra to the caudate nucleus and putamen causing profound dopamine depletion in the striatum [142]. Although the precise cause of this cellular destruction in PD remains unclear yet, several suspected causal mechanisms have been proposed such as; apoptosis, depletion of ATP production by disruption of mitochondrial respiratory chain by endogenous neurotoxins or xenobiotics and production of oxidative stress caused by excessive free radical generation or disruption of antioxidant systems [143–145]. Actually, damage to dopaminergic neurons is proposed to occur through oxidative stress and/or mitochondrial impairment causing activation of an apoptotic, presumably p53-dependent cascade; some neurons experiencing energy failure may not be able to complete apoptosis and end up in necrosis and inflammation [146]. While oxidative stress seems an attractive target, whether it is a causal factor in PD etiology or an outcome of a secondary phenomenon remains to be sorted out. Nicotine has received much attention in the context of this disease primarily due to the fact that smoking, which is considered as a risk factor for most cardiovascular and other diseases, has been identified unexpectedly as a negative risk factor for PD [147]. In fact, since initial epidemiological findings in the early 1960s, several other studies have reported reduced occurrence of PD in smokers [148, 149]. This inverse relationship was revealed from studies that have shown that cigarette smokers are 50 % less likely to develop PD than appropriately matched nonsmoking counterparts [147]. The apparent neuroprotection against PD is reduced with smoking cessation and occurs with different types of tobacco products [148, 149] suggesting that the decline in PD with smoking is a true biological effect. Nicotine, the primary pharmacologically active compound in tobacco, is considered to be responsible for this protective effect. Furthermore, nicotine patches and gum have been reported to relieve some symptoms of PD [13]. Several studies have tested whether nicotine directly improves motor symptoms in PD. Although the etiology of PD is unknown, the symptoms are caused by a loss of dopamine-producing neurons in the pars compacta of the substantia nigra and in the ventral tegmental area of the midbrain. Administration of nicotine to rodents stimulates presynaptic release of dopamine, resulting in activation of dopaminergic receptor postsynaptically [150, 151]. Since nicotine modulates dopamine release from striatum [152], enhanced synaptic dopamine levels could ameliorate motor deficits that arise because of a nigrostriatal dopaminergic deficiency in PD. There is evidence for both in favor and against its role [153–156]. Moreover, works on different PD animal models indicate that nicotine exposure improves dopaminergic markers and function in lesioned striatum, the brain region predominantly affected in PD [144, 157–159]. Additionally, nicotine administration is reported to reduce side effects of L-dopa, the primary treatment for PD [160]. Several mechanisms responsible for the protective effects of smoking on PD have been proposed. The notion of involvement of central nAChR is popular [143, 161], and there is multiple preclinical evidence to support that nicotine is neuroprotective via its action on these receptors [143], but it must be noted that not all of nicotine's effects are receptor mediated [143], and thus neuroprotection via receptors may be partly responsible for the neuroprotective action. Involvement of monoamine oxidase and nitric oxide synthase also has been suggested [143]. The hypothesis that smoking reduces the risk of PD by increasing activity of the ubiquitin–proteasome system, thereby preventing damage associated with aberrant activity of this system, also has received some attention. The idea of nicotine-mediated neuroprotection has led to many hypotheses including that of antiapoptotic effects, regulation of intracellular calcium levels, and release of growth factors like fibroblast growth factor-2 [143, 161]. Whether one, some, or all of these mechanisms act together to afford neuroprotection remains unknown and studies examining the neuroprotective effects of nicotine remain controversial.

Some in vivo studies have reported antioxidant and protective role of nicotine [162, 163], while others have reported a pro-oxidant and deleterious effect [164, 165]. Other studies suggest nicotine might play dual effects: high doses induce neurotoxicity and stimulate oxidative stress, while low concentrations act as an antioxidant and may be neuroprotective. Furthermore, administration of nicotine is reported to reduce, enhance, or can even be ineffective on nigrostriatal degeneration in animal models of PD [162, 166, 167]. Thus, nicotine-independent mechanisms in the protective effects of smoking against PD have not been ruled out [168]. Exactly how nicotine might exert its antioxidant protective effect in PD is unclear. But a consensus is developing regarding its mechanism of action within the framework of oxidative stress. Linert et al. [169] proposed that nicotine may have antioxidant properties due to its Fe^{2+}-binding property and its ability to reduce transferrin-mediated Fe uptake and chelate Fe^{2+} to block the Fenton reaction [170]. Actually, increased levels of Fe accumulate within the substantia nigra of PD brains, which could be a cause of degeneration of nigrostriatal dopaminergic (NS-DA) neurons, a characteristic of PD. It is well known that most NS-DA neurons contain neuromelanin deposits, which bind excessive Fe in PD and increase the vulnerability of NS-DA neurons to degeneration in PD [171]. One mechanism via which nicotine might exert its protective effect on NS-DA neurons involving the above reactions could be its ability to form complexes with Fe(II), perhaps via the pyridine nitrogen although pyrrolidine nitrogen might also be involved. Consistent with this hypothesis, many studies have reported that nicotine inhibits thromboxane synthase enzyme by directly binding to the iron ions of this enzyme [172] and that nicotine reduces transferrin-mediated Fe uptake by reticulocytes and placental cells [173]. Thus, the proposition that nicotine may be protective against PD through complex formation with Fe(II), thus yielding Fenton-inactive Fe(II) and less oxidative stress, seems more convincing. The chemistry involving the reactions of dopamine, 5-hydroxydopamine (5-OHDA), and 6-hydroxydopamine (6-OHDA), with molecular oxygen, with and without the addition of catalytic amounts of iron (Ill) and other metal ions, and the implication of these reactions with respect to the progression of PD have been described. Basically 6-hydroxydopamine (6-OHDA) which is a neurotoxin present in both human brain [174] and urine of PD patients [175] is formed from the oxidation of DA in the presence of free (solvated) Fe(II) ions and H_2O_2 (i.e., Fenton's reagent). In normal brains, most of the Fe is stored as ferritin (Fenton-inactive). However, it has been shown that 6-OHDA is able to free Fe(II) from storage in ferritin [176], thus facilitating the Fenton reaction, which in turn leads to further production of 6-OHDA. Thus, under physiological conditions, 6-OHDA is oxidized by molecular oxygen to give hydrogen peroxide (H_2O_2) and the corresponding *p*-quinone. The *p*-quinone then undergoes a cascade of oxidative reactions, which finally results in the formation of an insoluble polymeric pigment related to neuromelanin [176, 177]. Evidently, the H_2O_2 resulting from the autoxidation of 6-OHDA may generate hydroxyl radicals (-OH) through a metal-catalyzed Fenton-type reaction. Although the precise molecular mechanism of neurotoxicity of 6-OHDA remains uncertain, its neurotoxicity is often related to the oxidative stress caused by the production of (OH) during its autoxidation [178–182]. Furthermore, the stereotaxic injection of 6-OHDA into the medial forebrain bundle or substantia nigra has been long used to produce animal models of PD [183]. Linert and colleagues [169] proposed that in NS-DA neurons, this cycle is continuous, forming strongly oxidizing radicals, which destroy cellular components. However, it has also been reported that 6-OHDA can act directly by inhibiting the mitochondrial respiratory chain at the level of complex I [184, 185]. The antioxidant properties of nicotine may be intracellular through the activation of the nicotinic receptors or extracellular by acting as a radical scavenger in that it binds to iron. The possibility that nicotine might be used to treat some symptoms of certain neurodegenerative diseases underlies the necessity to determine whether nicotine has pro-oxidant, antioxidant, or properties of both.

4 Attention Deficit/Hyperactivity Disorder (ADHD)

Nicotinic systems are vital for cognitive functions, hence, nicotinic system-based therapeutic interventions may be useful for the treatment of a variety of cognitive disorders including attention deficit hyperactivity disorder (ADHD). Although

ADHD is generally regarded as a genetic disorder, environmental and psychosocial factors may aggravate the symptoms but are not considered to be underlying causes of ADHD [186]. The core symptoms are difficulty concentrating, hyperactivity, and impulsivity [187] with prevalence of the disease estimated to be 5–10 % in children and adolescents and 4.4 % in adults. Interestingly, it is reported to be more common in boys than girls [186]. Some observations critical to the present discussion are provided. It has been repeatedly observed that people suffering from ADHD are more likely to become regular smokers [188], begin smoking earlier, become habitual smokers [189], and experience greater quitting difficulty [190] compared to normal subjects. Unexpectedly, it has been noted that cigarette smoking is more common in children [191] and adults with ADHD compared to the general population and/or controls [188]. Moreover, ADHD is not only associated with earlier onset of cigarette smoking [191] but has more severe nicotine dependence [192]. In fact, children with ADHD are at high risk to develop substance use disorders (SUD) in their adolescence and adulthood [193]. Nicotine, consciously or otherwise used as self-medication [194], has been suggested to function as a gateway drug [195, 196]. Actually, prevalence rates for SUD in adolescent and adult ADHD patients range from 30 % to 50 % [197]. This is made even more convincing considering the Minnesota Twin sample study, showing the association between ADHD symptoms in childhood and SUD even when controlled for comorbid disorders [198]. These links however are quite controversial [199] with some researchers suggesting that stimulant treatment induces an increase of SUD [200] while others suggesting a reduced risk of SUD [193]. Moreover, adult ADHD, with a 4.4 % prevalence rate [192], recognizable by inattention, impulsivity, and hyperactivity is associated with poor performance in all spheres of life and also is associated with higher rates of other psychiatric and SUD comorbidity [201]. While adult ADHD is one of the most common mental health disorders of adulthood [202], treatment strategies remain limited [203]. Nicotine has been found to be effective in tackling some of the ADHD symptoms [204], and in fact, several nicotine-like agents are currently being considered as ADHD treatments. It is now generally accepted that nicotine attenuates inattentiveness and improves focus [205, 206]. Recently, in an interesting report, Berlin et al. [207] have highlighted the fact that the core symptoms of ADHD (inattention, hyperactivity, and impulsiveness) are clinically similar to nicotine withdrawal symptoms including lack of focus and restlessness. Some investigators have suggested that perhaps nicotine reduces ADHD-associated deficits in dopaminergic function [208] suggesting a “self-medication” rationale for greater nicotine use among ADHD patients [209]. Little or no mechanistic insights are available to provide a biological basis for such observations.

One interesting concept is the role of oxidative stress in the pathophysiology as well as the association between nicotine and ADHD. Oxidative stress is reported to play an important role in the pathogenesis of many psychiatric disorders. Changes in the oxidant and antioxidant indices have been reported to contribute to autism, developmental disorders, and ADHD [210]. Some investigators have reported a significantly low level of oxidative stress markers in children with ADHD [210, 211], and others have seen low antioxidant levels in children with ADHD [212]. Adult ADHD patients are reported to present with increased levels of oxidative stress and decreased antioxidant activity [213, 214]. More recently, lower level of oxidative stress was detected in ADHD group. The authors of this study suggest that perhaps decreased oxidant level is associated with the hypodopaminergic activity state in ADHD. This is based upon the fact that lower levels of dopamine and noradrenaline have been reported in the cerebrospinal fluid, blood, and urine in ADHD patients [215]. It is believed that the degradation products of these neurotransmitters are important for ADHD etiology. Relevant to this, decreased dopamine secretion in the prefrontal cortex and decreased dopamine turnover and vesicular storage in the substantia nigra, ventral tegmental area, and frontal cortex have been reported in an animal model of ADHD [216]. Additionally, an abnormally active dopamine transporter, which could have increased the reuptake of dopamine and thus depletion of dopamine in the synaptic cleft, has been speculated. Perhaps the antioxidant characteristic of nicotine facilitates the metabolism and excretion of free oxygen radicals and hence exerts a protective effect in ADHD.

4.1 Sleep Deprivation

The National Sleep Foundation reports that 64 % of American adults get less than the 8 h of sleep that experts recommend is required to maintain optimal physical and mental health. One-third of the US population says they get less sleep now than they did 5 years ago. One-half of Americans have experienced insomnia (sleeplessness). Drowsiness due to a lack of a proper night sleep interferes with the daily activities of 37 % of all adults. Due to these pressing demands of modern society, more and more people are tending toward self-medication. While smoking is considered the most important behavioral health risk, its use for coping with anxiety and depression, and increasing focus and alertness is popular. Nicotine, by acting on various neurotransmitter systems, is purported to influence sleep and mood. However, the data regarding a *pro*- or an *antisleep* action of nicotine in both animals and humans is far from being in conformity.

For example, increase in sleep latency, sleep fragmentation and decreased slow-wave sleep with reduced sleep efficiency and increased daytime sleepiness are observed during nicotine consumption. Nicotine-induced rapid eye movement (REM) sleep suppression is also known. Some researchers have not detected any effect of nicotine on sleep [217]. In contrast, when administered intravenously [218], subcutaneously [219], or into the medial pontine reticular formation [220], nicotine is reported to increase REM sleep in cats. Moreover, some human studies employing transdermal nicotine report a decreased total sleep time, sleep efficiency, and REM sleep and an increase in wakefulness [221, 222]. On the other hand, depressive nonsmokers experience a mood improvement under nicotine administration comparable to the effect of antidepressants. Actually, transdermal nicotine patch improves mood in nonsmoking depressed patients. Animal studies using rodent models of depression also have shown that nicotine has antidepressant properties [223–225]. It has been proposed that the improvement in symptoms of depression by nicotine affects sleep architecture and is related to sleep deprivation, especially the deprivation of REM sleep [226]. The inconsistency in findings arguing for a pro- or antisleep role of nicotine is largely a result of different methodologies used, absence of appropriate controls, comorbidity status in human studies, and other criteria.

Clearly, a more unified approach is needed for a better understanding of the role of nicotine in sleep. Simply branding nicotine as a rogue substance and dismissing it on ethical grounds may not be productive. We will have to consider whether it is more important to avoid the severe consequences of debilitating diseases or to fear nicotine dependence. In the absence of other effective therapies for these debilitating diseases, nicotine ought to be taken seriously. Despite its promise, developing nicotine as a treatment is fraught with pitfalls. Regardless of a controversial role of nicotine in this regard, some very interesting data have emerged. For example, during sleep deprivation, the frequency of nicotine consumption is increased among smokers [227]. Nicotine is reported to alleviate impairment of memory associated with psychosocial stress [1, 228], aging [229], hypothyroidism [21, 230], and Alzheimer's disease [12, 231]. In the hippocampus, nicotine facilitates the induction of LTP in the CA1 region of rat hippocampal slices [3, 12] and induces long lasting potentiation in intact mouse dentate gyrus region [232]. In addition, nicotine treatment prevents impairment of hippocampal LTP associated with psychosocial stress [228], hypothyroidism [230], aging [233], as well as other mental diseases. Thus, the reported increase in nicotine consumption among smokers and the initiation of smoking among nonsmokers during sleep deprivation [227, 234] could be a form of self-medication to offset impairment of cognition and synaptic plasticity. Minimal mechanistic insights are currently available regarding the biological underpinnings of these observations. As far as the beneficial effect of nicotine on sleep deprivation is concerned, we suggest a hypothesis that centers on the long debated antioxidant role of nicotine. Recently we and others have reported that SD causes increased oxidative stress in the brain [235], and it also is known that nicotine has antioxidant characteristics. Thus, it is reasonable to suggest that antidepressant-like effects of nicotine perhaps are due to its oxidative stress quenching property.

4.2 Stress

Normal structure and function of the brain are altered by severe and/or long-term stress [reviewed in 236]. The hippocampus is among the brain's regions most vulnerable to stress-related adverse changes [reviewed in 237]. Stress negatively impacts hippocampal neuronal excitability, neurochemistry, and structural and functional plasticity [236, 238]. This has been demonstrated in both animal models [1, 228, 238–240] and in humans [241], where learning and memory are impaired during chronic stress. In addition, memory impairment induced by hypothyroidism [239] and in animal models of Alzheimer's disease [86, 87], is exacerbated during chronic stress conditions. Long-term potentiation (LTP), a cellular correlate for learning and memory in the CA1 area of the hippocampus, is significantly suppressed by chronic stress in adult anesthetized rats [1, 2, 86, 242–246] and freely moving animals [247]. Similar findings have been reported in hippocampal slices from stressed animals [e.g., 248]. At the molecular level, the protein signaling molecules important for memory and LTP including phosphorylated CaMKII [86, 244, 245] and BDNF [245] are reduced during stress.

Nicotine has been shown to attenuate chronic stress-induced impairment of short-term memory [1], and LTP [2, 245], and facilitation of long-term depression (LTD) [96, 249] in area CA1 of the hippocampus in adult anesthetized rats. Thus, the increase in tobacco smoking rate during stress may be a self-medication to counteract the harmful effect of stress on memory [250, 251].

The precise molecular mechanism by which chronic nicotine treatment prevents the effects of stress on memory and synaptic plasticity is not clearly understood. It is known that stress and/or stress hormones downregulate nAChRs [252–254] and that this effect is prevented by treatment with chronic nicotine [254]. This suggests that nicotine is, perhaps, acting through nAChRs to prevent stress-induced impairment of memory. Support for this proposal comes from the finding that the effects of nicotine on memory are prevented by mecamylamine, a nonselective nicotinic receptor antagonist [see 255 for review].

Chronic stress impairs the hippocampus-dependent spatial memory and blocks LTP in area CA1 of the hippocampus possibly by interfering with the function of key signaling molecules involved in memory and LTP including reduction of phosphorylated CaMKII [P-CaMKII], PKCγ, and calmodulin and increase in calcineurin levels [Table 47.2; 244, 245]. These changes are prevented by chronic nicotine treatment during stress [245]. Another important signaling molecule that is markedly impacted by stress is BDNF. The expression of BDNF mRNA [256–258] and protein levels [245, 259] is decreased after stress. Chronic nicotine treatment restores the protein levels of BDNF in CA1 area of the hippocampus of chronically stressed animals [245].

The presence of chronic psychosocial stress in Aβ-infusion rat model of AD induces impairment of short-term and long-term memory that is more severe than either stress or AD alone [86, 260, 261]. This also correlates with more intense suppression of hippocampal CA1 E-LTP and L-LTP by the combination than either condition alone [86, 87, 260]. Chronic nicotine treatment prevents the effects of the combination of these two conditions on memory and LTP [87, 260, 261]. Furthermore, the magnitude of LTD of area CA1 of the hippocampus is higher in the chronically stressed Aβ animals compared to animals with either condition alone [87, 260]. Here again, chronic nicotine administration prevented the effect of the combination of stress and Aβ on LTD and restored the synaptic signal to a magnitude comparable to that of control animals [87, 260].

Table 47.2 Basal levels of signaling molecules. Summary of the effects of nicotine and/or stress on the basal levels of P-CaMKII, CaMKII, BDNF, calmodulin, PKCγ, and calcineurin in CA1 area of the hippocampus. "Decrease/increase"; significantly different from control, "*No change*"; not significantly different from control

Basal levels of signaling molecules in CA1		Stress	Nicotine	Nic/Sts
P-CaMKII (total homogenate)		*Decrease*	*No change*	*No change*
CaMKII (total homogenate)		*Decrease*	*No change*	*No change*
P-CaMKII/CaMKII ratio		*Decrease*	*No change*	*No change*
BDNF (total homogenate)		*Decrease*	*Increase*	*No change*
Calmodulin	Homogenate	*Decrease*	*No change*	*No change*
	membranous	*Decrease*	*No change*	*No change*
	Cytosolic	*No change*	*No change*	*No change*
PKCγ	Homogenate	*Decrease*	*No change*	*No change*
	Membranous	*Decrease*	*No change*	*No change*
	Cytosolic	*No change*	*No change*	*No change*
Calcineurin (total homogenate)		*Increase*	*No change*	*No change*

To elucidate potential mechanisms by which stress exacerbated Aβ-induced impairment of cognitive abilities and synaptic plasticity, the levels of signaling molecules important for these processes have been evaluated. Results show that combination of chronic stress and Aβ reduced the basal levels of P-CaMKII and P-CREB in area CA1 of the hippocampus, more markedly than either condition alone [86, 87, 261]. Chronic nicotine treatment, on the other hand, completely prevents the effect of the combination on P-CaMKII and P-CREB levels [87, 261]. The combination of chronic stress and Aβ also elevates the basal levels of calcineurin in area CA1 of the hippocampus [86, 87, 260]. This elevation is also prevented by chronic nicotine treatment [87, 260]. Combination of chronic stress and Aβ suppresses the expected E-LTP-induced increase in CaMKII phosphorylation and L-LTP-induced increase in CREB phosphorylation and CaMKIV protein expression in area CA1 [86, 87]. These E-LTP- and L-LTP-induced expression of signaling molecules are restored by chronic nicotine treatment [87]. Furthermore, chronic stress has been shown to intensify the increase in protein levels of Aβ and beta-site amyloid precursor protein [APP]-cleaving enzyme [BACE] in area CA1 of Aβ-treated rat. This elevation is normalized by chronic nicotine treatment [262]. Additionally, the levels of the α7, β4, and β2 subunits of nAChRs are significantly decreased in Aβ rats, but these are also normalized in Aβ rats chronically treated with nicotine.

4.3 Hypothyroidism

Hypothyroidism causes a wide range of central nervous system [CNS] dysfunctions, including impairment of cognition [21, 230, 239] and synaptic plasticity in adult onset [21, 22, 229, 230, 243, 246, 263–266] as well as in developmental hypothyroidism [267–270]. In addition, hypothyroidism reduces the basal levels and activity of P-CaMKII and blocks HFS-induced P-CaMKII activation in the CA1 area of the hippocampus, which may, at least partially, explain hypothyroidism-induced impairment of LTP [22, 265]. Hypothyroidism also reduces the basal levels of signaling molecules upstream of CaMKII such as PKCγ, calmodulin, and neurogranin and increases the protein levels and activity of calcineurin in the CA1 area of the hippocampus [22, 265].

Chronic nicotine treatment reverses hypothyroidism-induced impairment of hippocampal learning and short-term memory [21]. It also prevents hypothyroidism-induced impairment of E-LTP and facilitation of LTD in area CA1 [21, 22]. At the molecular level, nicotine normalizes the reduction of basal activity of CaMKII and levels of P-CaMKII in area CA1 [22]. One hour after the induction of E-LTP in CA1, the failure of HFS to increase the levels of P-CaMKII in hypothyroidism is reversed by chronic nicotine pretreatment, suggesting that nicotine by restoring the ability of HFS to activate CaMKII and by normalizing the basal activity and levels of CaMKII reverses hypothyroidism-induced LTP impairment [22]. In addition, chronic nicotine treatment prevents hypothyroidism-induced reduction in protein levels of molecules upstream to CaMKII such as PKCγ, neurogranin, and calmodulin [22].

Calcineurin reduces postsynaptic activity [271], blocks LTP [272], induces and maintains LTD [273], and impairs hippocampus-dependent memory formation in mice [274]. It indirectly activates protein phosphatase 1 (PP1) by inactivating its natural inhibitor "inhibitor 1" [275], thus enabling PP1 to dephosphorylate many molecules including P-CaMKII [271, 276]. During hypothyroidism, calcineurin activity and basal protein levels are increased in CA1 area of the hippocampus [1, 229, 265]. This increase is normalized in nicotine treated hypothyroid rats [22]. It is likely that the increase in the activity and levels of calcineurin in hypothyroid rats may account for the reduced CaMKII activity and consequent impairment of short-term memory and LTP. By normalizing activity and levels of calcineurin in hypothyroid rats, nicotine may have prevented impairment of LTP during hypothyroidism.

Chronic nicotine treatment has been shown to reverse adult-onset hypothyroidism-induced impairment of long-term memory and late-phase long-term potentiation (L-LTP) in area CA1 [21, 263]. In the same area, chronic nicotine treatment of hypothyroid rats prevents the reduction in the basal protein levels of signaling molecules important for long-term memory such as adenylyl cyclase I (ACI), mitogen-activated protein kinases (MAPKp44/42 "ERK1/2"), calcium-calmodulin-dependent protein kinase IV (CaMKIV), and cyclic-AMP response element-binding protein (CREB; phosphorylated (P-) and total) [263]. The expected marked elevation in the levels of P-CREB, P-MAPKp44, P-MAPKp42, and BDNF is not seen in hypothyroid animals after multiple train high-frequency stimulation (MHFS); however, in nicotine-treated hypothyroid, the expected elevation of these molecules is similar to that seen in control animals after MHFS [263]. These findings suggest that prevention of impairment of basal level of CaMKIV, MAPKp44/42, and CREB by nicotine along with the regained ability of MHFS to induce MAPKp44/42 and CREB phosphorylation in nicotine-treated hypothyroid animals may be responsible for the reversal of long-term memory and L-LTP impairment by chronic nicotine treatment during hypothyroidism [263].

Owing to their diversity and multiple combinations, NMDA receptor subunits are differentially regulated in LTP or LTD. Evidence for that comes from pharmacological blockade of certain subunit-containing receptors indicating that selective blockade of the NR2B subunit-containing NMDA receptors abolishes LTD, but not LTP [277, 278]. On the other hand, LTP is impaired, while LTD stays intact when NR2A subunit-containing NMDA receptors are preferentially blocked [277–280]. Recently, it has been shown that NR2B subunit-containing NMDA receptors function by putting an inhibitory restrain on NR2A subunit-containing receptor via activation of calcineurin [280], thus enhancing LTD. Moreover, increased protein levels of NR2B subunit-containing receptor in the membranes of neurons of area CA1 of the hippocampus in hypothyroid rats have been reported [58]. This is accompanied by increased calcineurin activity and facilitated LTD in hypothyroid rats [58]. Therefore, the increase in the levels of NR2B-containing NMDA receptors may be responsible for the enhanced LTD observed in area CA1 of hypothyroid rats. However, in chronic nicotine-treated hypothyroid rats, these increase in the levels of NR2B-containing NMDA receptors, and calcineurin do not take place, which may explain occurrence of normal LTD in these animals [58].

Functional NMDA receptors contain at least one NR1 subunit [281–286]. It has been shown that NR1 transcript [287] and protein [58] levels are reduced in the hippocampus of adult hypothyroid rats. This reduction in the basal protein levels of NR1 in area CA1 is normal in chronic nicotine-treated hypothyroid rats [58]. Chronic nicotine treatment has also been shown to increase NMDA receptor binding sites in area CA1 of the hippocampus [288]. Additionally, nicotine reduces NMDA-induced toxicity in hippocampal cell cultures and slices [289, 290], by increasing calcium buffering capacity in the cells [291]. BDNF, which is increased by nicotine treatment [58], induces NMDA receptor subunit NR1 activation [phosphorylation] in an ERK and PKC-dependent mechanism in rat neuronal tissues and in cell culture [292–296]. It has been suggested that BDNF-induced tyrosine phosphorylation of the AMPA receptor subunit GlutR1 is regulated through NMDA receptor subunits [293]. In addition, BDNF enhances the magnitude of NMDA-mediated synaptic currents [297, 298], an effect that occurs through TrKB receptors [298]. BDNF also restores declining peak amplitude of NMDA-evoked currents in cultured hippocampal pyramidal neurons in a cAMP-dependent manner [299]. Recently, it has been shown that maximal neuroprotection for neuron is achieved by convergence of both NMDA receptor pathway and TrKB pathways to activate MAPKp44/42 [300, 301]. To that end, it has been shown that nicotine, which increases BDNF, normalizes reduction in MAPKp44/42 activation and restores changes in active NMDA receptor subunits induced by hypothyroidism [58]. Thus, nicotine seems to provide maximum neuroprotection for hippocampal neurons, which helps in restoring cognitive impairment during hypothyroidism.

4.4 Aging

Aging is often viewed as a progressive decline in physiological competence with a corresponding inability to adapt to stressful stimuli [302]. Aged animals have increased morbidity and mortality during prolonged exposure to stressful stimuli [302]. The aging process is associated with memory impairment [303, 304], which is alleviated by nicotine treatment [305]. Results from a double-blinded, placebo-controlled, crossover study show that 4-weeks transdermal nicotine patch usage (dose range 3–10 mg/day) in older subjects results in a sustained improvement of age-associated memory impairment measured as improved clinical symptoms and objective scores of computerized tests of attention [8]. In aged monkeys of both sexes, pretreatment with low doses of nicotine improves performance in the delayed matching-to-sample (DMTS) test [306, 307]. Subcutaneous administration of nicotine (0.1 or 0.4 mg/kg) significantly improves accuracy of performance in aged rats on a delayed nonmatching to position paradigm in a T-maze [308]. Additionally, nicotine pretreatment (15 min prior to testing) improves the acquisition and/or memory retention of aged rats in one-way active avoidance pole jumping over 12 days of testing [4, 309]. Moreover, in Lashley III maze training, aged animals are reported to make more alteration errors compared to adult controls, but these errors are prevented by prior nicotine administration in aged animals [4, 309]. In the 17-arm radial maze, both reference and working memories are reported to be impaired in aging. Acute nicotine pre-administration corrects reference, but not working memory performance in aged animals [4, 309]. In agreement, similar experiments in the eight-arm radial maze reveal that chronic nicotine administration (approximately 5 mg/kg per day using osmotic minipump for 4 weeks) does not improve working memory in aged rats. In the Morris water maze (MWM), it has been shown that nicotine administration 3 days prior to the onset of testing and 15 min prior to daily testing improved memory performance in aged rats [5, 310]. However, in the MWM, acute nicotine administration (15 min prior to testing) shows no beneficial effects on reference memory in aged Fischer 344 Rats [311]. Additionally, acute challenge with the nicotinic receptor antagonist, mecamylamine (subcutaneous 0.04–0.16 mg/kg), does not further impair working memory in aged rats [6]. The resistance of aged rats to nicotine-induced working memory improvements and mecamylamine-induced working memory deficits may be the result of the decline in hippocampal cholinergic functions seen in aging [312]. However, using a 5-choice discrimination paradigm, designed to test spatial working memory, Taylor et al. [313] report that chronic nicotine administration (subcutaneous 0.3 or 0.7 mg/kg daily for 5 weeks) improves working memory performance, whereas mecamylamine (subcutaneous 2–8 mg/kg) reverses this impairment in a dose-dependent manner [313]. Another report show that chronic nicotine treatment (0.1 or 0.7 mg/kg daily for 4 weeks in osmotic minipump) improves both working and reference memory in aged rats [314]. The discrepancies in the reported findings concerning the effect nicotine on working memory results may be related to differences in memory testing paradigm, animal strain, route and method for nicotine administration, and doses of both nicotine and mecamylamine.

Interestingly, in aged animals, LTP induction becomes rather difficult. In fact, age-associated decline in LTP induction becomes apparent with minimal stimulation protocol [315, 316], but not with high-frequency tetanic stimulation [315, 317, 318]. Acute nicotine treatment reduces the threshold for LTP induction in hippocampal slices from aged rats. The α7nAChR antagonist methyllycaconitine mimics the effect of nicotine, whereas the non-α7nAChR antagonist dihydro-β-erythroidine blocks nicotine's effect on LTP induction [233]. These results suggest that both nicotine-mediated desensitization of α7nAChRs and activation of non-α 7nAChRs contribute to the effect of nicotine on LTP during aging. The non-α 7nAChR agonist, A85380, facilitates LTP induction in the hippocampus in adult animals; however, it has no effect on LTP in aged animals [233]. This suggests at least one pathway involving non-α7nAChRs is altered during the aging process.

The exact mechanism for the neuroprotective effect of nicotine on aging-induced memory impairment is unknown. Acute nicotine protective effect on memory in aging is reversed by NMDA receptor antagonists or by nicotine antagonist, mecamylamine [310, 319], indicating the involvement of both nAChR and NMDA receptor systems in the effect of nicotine during aging. Additionally, chronic nicotine administration (subcutaneous 0.45 mg/kg twice daily for 4 weeks) prevents aging-induced reduction in the hippocampal protein levels of NR2A and NR2B NMDA receptor subunits [320]. It has also been suggested that nicotine-induced upregulation of fibroblast growth factor-2 (FGF-2) and nerve growth factor (NGF) proteins in several brain areas of rat including the hippocampus [314, 321] may be, at least in part, involved in the neuroprotective effect of nicotine during aging.

4.5 Schizophrenia

The rate of smoking among schizophrenia patients is much higher than the general population [322–324], and smoking withdrawal is associated with worsening of schizophrenia symptoms [325]. Several lines of evidence indicate the possibility of self-medication with nicotine against the disease's symptoms and/or adverse effects of antipsychotic drugs used in schizophrenia [326, 327]. The schizophrenia-induced impairment in visuospatial working memory and attention worsens in schizophrenics with overnight abstinence from smoking [328–330] but is enhanced to pre-quitting levels on smoking reinstatement [328]. Enhancement of visuospatial working memory and attention by smoking reinstatement is blocked by mecamylamine treatment [328], indicating the involvement of nicotine and nAChRs in the effects of smoking on cognitive functions in schizophrenia. However, smoking cessation for 7 days among schizophrenia patients is associated only with slowed motor speed but has no effect on attention, verbal learning and memory, working memory, or executive function [331].

Administration of nicotine nasal spray, although does not affect noncognitive symptoms of schizophrenia, it improves performance on a spatial organization task and working memory and attention, but not verbal memory among smoker schizophrenic patients [332, 333]. Acute nicotine administration also improves smooth pursuit eye movements and visual attention among schizophrenia patients [334] and enhances delayed recognition memory in schizophrenic patients who smoke, but similar performance enhancement is not observed for working memory or for patient who do not smoke [335]. In another study among smoker schizophrenic patients, who are switched from smoking to either nicotine patch or placebo, nicotine improves verbal working memory and selective attention by enhancing activation of and functional connectivity between brain areas that mediate task performance [336]. Similarly, nicotine gum is reported to improve antisaccade tasks performance, which reflects cognitive and neurological functions among schizophrenic patients [337]. Moreover, among nonsmoker schizophrenia patients, nicotine patch application for 4 h results in improved novelty detection and episodic memory [338]. In rat models of cognitive impairment of schizophrenia, nicotine improves working memory span [339]. Therefore, nicotine seems to correct most cognitive aspects among schizophrenia patients.

The α7nAChRs are known to be deficient in brain areas, especially, the hippocampus of patients with schizophrenia [340, 341]. The deficit in α7nAChR is likely to be related to defective sensory gating mechanism often seen among schizophrenics [342, 343]. This defect in the sensory gating mechanism is normalized by cigarette smoking among schizophrenia patients [344]. Acute nicotine can normalize temporal aspects of sensory memory processing in patients with schizophrenia, an effect that may be mediated by activation of α7nAChRs, the function of which is diminished in schizophrenia [345]. Studies in schizophrenia monkey model of memory impairment have shown clear beneficial effects of nAChR agonists [346], especially, the α4β2 and α7nAChRs agonists [347–349].

5 Concluding Remarks

Although nicotine is commonly associated with the stigma of addiction, emerging evidence argues for a potentially "good side" of this often maligned substance. For example, nicotine and nicotine-like compounds have been proposed as treatments for AD, PD, ADHD, and other neuropsychiatric conditions. It has been reported that smokers have a lower risk of developing AD, PD, and ADHD and that schizophrenic patients seemingly relieve the symptoms of their disorder with smoking. These observations however raise serious ethical issues. Obviously pure nicotine is a potentially toxic substance and, as part of tobacco smoke, is a major risk factor for heart and lung disease, but at the same time, it also begs the question of how nicotine can be managed to utilize its therapeutic potential.

The commonly known major psychological effects of nicotine include raising alertness, enhancing attention and information processing while providing a sense of relaxation and calmness of thought. Thus, for someone who is agitated (as in ADHD or schizophrenia), this calming effect might seem therapeutic. For someone who is lethargic and unfocussed (PD or AD), it might serve to increase alertness and focus and sharpness of thought. The fact that the majority of individuals with mental disorders use tobacco products is interesting. Whether this is coincidental or a biologically relevant phenomenon is a debatable issue, and thus causality is assumed but unproven. Relevant to this, nicotine is also known to have antidepressant-like effects in both rats and humans. The reasons behind these observations remain unknown and the scientific relevance unclear, but new research aimed at understanding the biochemical basis for these key observations has provided some persuasive clues into the biological underpinnings of the beneficial effects of nicotine on some of these conditions. Surprisingly, very few studies have examined the effects of nicotine on the CNS with regard to oxidative stress or its role as an antioxidant. This seems even more surprising considering the proposition of using nicotine as a future drug for certain neurodegenerative diseases and some cognitive disorders.

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Aconitum Alkaloids and Biological Activities

48

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Contents

1 Introduction 1504
2 Occurrence and Chemical Classification 1505
3 Pharmacological Activities 1507
3.1 Analgesic and Anesthetic Activities 1508
3.2 Anti-inflammatory Activity 1510
3.3 Arrhythmogenic–Antiarrhythmic Activity 1510
3.4 Anticancer Activity 1511
3.5 Other Biological Activities 1515
4 Mechanism of Action 1515
5 Toxicity 1516
6 Conclusions 1520
References 1520

Abstract

Plants of the *Aconitum* genus have a long-lasting history of use both for their biological and toxicological properties. Most of *Aconitum* plants used in traditional medicine are being extensively explored and their chemical constituents and pharmacological effects are being evaluated.

This chapter summarizes the results of the most recent research works on the pharmacological and toxicological characteristics of alkaloids from various *Aconitum* species and highlights the analgesic, antiarrhythmic, and anticancer effects, attributed to various diterpenoid alkaloids. Diester diterpenoid alkaloids have been characterized as the most toxic compounds because of the presence of acetyl and benzoyl esters in the structures. Their capacity to bind to the sodium channels and activate permanent influx of Na^+ ions through cell membranes was

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described as the principal mechanism underlying toxic, analgesic, and arrhythmogenic effects. Diterpenoid alkaloids responsible for analgesic, antiarrhythmic, and anticancer action are compared on the basis of their therapeutic and toxicological properties.

Keywords

Aconitum • analgesic activity • antiarrhythmic activity • anticancer activity • arrhythmogenic effect • diterpenoid alkaloids

Abbreviations

ED$_{50}$	The calculated estimate of the dose at which the compound is expected to induce 50% of its pharmacological response
i.p.	Intraperitoneal
i.v.	Intravenous
IC$_{50}$	The dose causing 50% inhibition of cell growth as compared to control
LD$_{50}$	The dose causing mortality of 50% of the animals tested
p.o.	per os
s.c.	Subcutaneous

1 Introduction

Plants of *Aconitum* genus, known by various names such as aconite, monkshood, wolf's bane, women's bane, Devil's helmet, or blue rocket, have been used since antique civilizations in Ayurvedic, Chinese, Tibetan, and Greco-Roman medicines for their diverse pharmacological effects [1, 2]. Due to their extreme toxic nature, they have been widely used to eliminate wild animals or enemies and for criminal intents, and have attracted the particular interest of physicians, chemists, and legacy representatives from ancient world to modern times [3, 4]. During Greco-Roman civilization, *Aconitum* species underwent special restrictions to their growth, storage, and use under death penalty [5]. They are still an object of exhaustive investigation in the field of Forensic Sciences [6–8]. Nevertheless, the *Aconitum* species are widely used in Asian traditional medicine; they remain one of the most intriguing genera of plants both for their toxic and curative properties, and the interest to their pharmacological and toxicological characteristics is continuously growing. Their therapeutic potentials originating from traditional medicine are being extensively studied: multiple data on the analgesic and anti-inflammatory activity, the effect on the central nervous system, the arrhythmogenic and antiarrhythmic, antiparasite and anticancer properties of molecules from various *Aconitum* species are continuously reported.

In this chapter, we will overview the pharmacological properties and the therapeutic action and toxicity profile of compounds derived from the different *Aconitum* species in order to determine promising areas for therapeutic applications.

2 Occurrence and Chemical Classification

Plants of the *Aconitum* genus originate from the Northern hemisphere: they grow in the temperate mountainous areas of Europe, Asia, and North American continents. The *Aconitum* genus belongs to the family *Ranunculaceae*; the most known species are *A. napellus, A. lycoctonum* growing in Europe [9, 10], *A. carmichaelii* characteristic for Asian countries [11], *A. ferox* endemic to Himalaya, and *A. columbianum* growing in North America [12]. Presently, the *Aconitum* genus includes over 350 species and varieties [13], and more than 600 molecules isolated from *Aconitum* genus have been characterized [14].

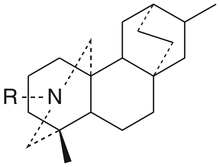
Aconitum species are generally herbaceous perennial plants, with a tall leafy stem (70–130 cm) bearing violet, blue, or yellow (rarely white, rose, or bicolor) zygomorphous flowers in the form of a cylindrical helmet on long, packed racemes. The colors of the flowers and their intensity can vary from pale to deep, depending on the species or intraspecies varieties. The underground part of the plant is represented by tubers, whose size and form depend on the species, or by fibrous roots (*A. lycoctonum*).

The principal group of chemical compounds present in *Aconitum* species consists of diterpenoid alkaloids, detected in all parts of the plant. The maximal accumulation of alkaloids is observed in tubers. Other chemical substances found in plant are flavonol and kaempferol glycosides detected in aerial parts [15–17], polysaccharides, organic acids, etc. [18].

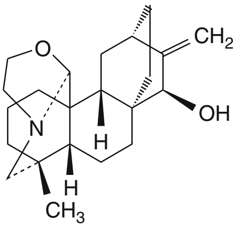
The alkaloids of *Aconitum* genus are divided into three categories: C_{20}, C_{19}, and C_{18} -diterpenoid alkaloids.

C_{20}-diterpenoid alkaloids are classified into four groups according to Wang et al. [19]:

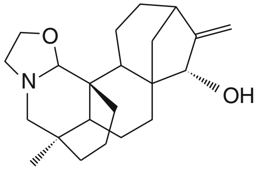
1. The atisane class of alkaloids is based on the pentacyclic atidane **1** skeleton. Atisine **2** is an example of this class.
2. The kauran class of alkaloids consists of four 6-atom rings and one 5-atom ring D. The typical representative of this class is veatchine **3**.
3. The class of rearranged alkaloids, which possess four 6-atom and three 5-atom rings, is considered as rearranged product of hetisine. Delnudine **4** is an example of these type of alkaloids.
4. Bis-diterpenoid alkaloids, which are rare products of dimerization of two diterpene alkaloids.



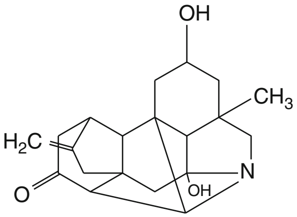
The atidane skeleton **1**



Atisine **2**



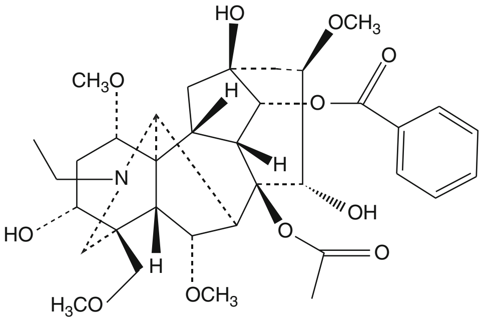
Veatchine **3**


OH
CH3
H2C
N
O
OH

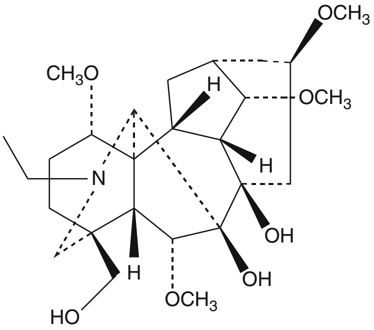
Delnudine **4**

The C_{19} diterpenoid group comprises the most toxic alkaloids and can be classified into four groups on the basis of their structural variations [20]:

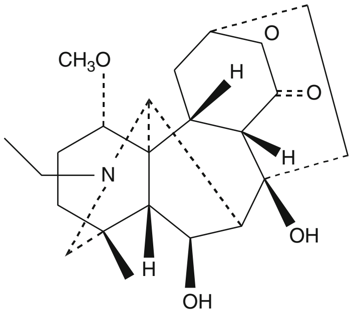
1. The Aconitine type, where the carbon at position 7 of the skeleton does not possess a hydroxyl function: this group is represented by aconitine **5**, condelphine, and delphinine.
2. The Lycoctonine **6** type, where the carbon at position 7 of the diterpene skeleton is hydroxylated.
3. The Heteratisine type, where a δ-lactone moiety is present in the ring C, for example, heteratisine **7**.
4. The Pyroaconitine type, where a double bond is present between the C_{18} and the C_{15} positions, for example, falaconitine **8**.


HO
OCH3
O
CH3O
H
O
H
N
OH
HO
O
O
H
H3CO
OCH3

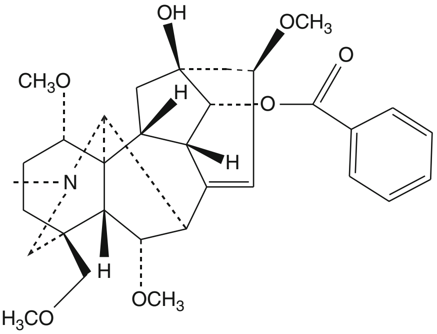
Aconitine **5**


OCH3
CH3O
H
OCH3
N
H
OH
OH
H
OCH3
HO

Lycoctonine **6**

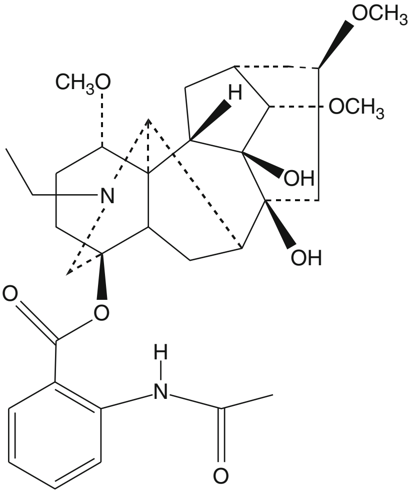

O
CH3O
H
O
N
H
OH
H
OH

Heteratisine **7**


OH
OCH3
O
CH3O
H
O
N
H
H
OCH3
H3CO

Falaconitine **8**

The C_{18}-diterpenoid alkaloid group is represented by lappaconitine **9** or ranaconitine, which are derivatives of C_{19}-alkaloids devoid of the benzoyl moiety at position 14 [21].



Lappaconitine **9**

3 Pharmacological Activities

Preparations from *Aconitum* plants have been used in traditional Chinese medicine as cardiotonics, febrifuges, sedatives, and antirheumatics [22]. They have also been used in traditional medicine of some ex-USSR countries for the treatment of cancer, rheumatism, etc. [18, 23]. *Aconitum* plants were introduced in the medicine of European countries in the nineteenth century. Leaves and roots of *Aconitum* and aconitine powder were included in the British Pharmaceutical Codex (01911) [24], *French Pharmacopoeia* (9th edition) [25], and *USSR Pharmacopoeia* (8th edition) [26] and were applied to relieve neuralgic pain, especially in the face, to allay the pain of sciatica and acute rheumatism. Because of extreme toxicity, aconitine was replaced later by less-toxic anti-inflammatory and analgesic agents in European countries; presently, the use of *Aconitum* plants is limited to the homeopathic medicine [27, 28]. On the contrary, in many Asiatic countries, aconitine-containing plants still remain one of the most popular remedies, and more and more new, naturally occurring or semisynthetic molecules from *Aconitum* species are introduced in medical use. For example, lappaconitine found in several *Aconitum* species, bulleyaconitine isolated from *A. bulleyanum*, and crassicauline A isolated from *A. crassicaule* were introduced in the official Chinese medicine for the treatment of chronic pain, rheumatoid arthritis, and for local anesthesia [29, 30].

3.1 Analgesic and Anesthetic Activities

Various species of *Aconitum* are reported to possess analgesic and anesthetic activities. During the last 30 years, numerous diterpenoid alkaloids originating from plants and used in traditional medicine for the treatment of pain, rheumatisms, and neuralgia have been explored by a number of international groups of researchers for analgesic, anesthetic activities, arrhythmogenic–antiarrhythmic properties, and toxicological characteristics [31–35]. The analgesic activity of substances derived from *Aconitum* is evaluated on the basis of their capacity to relieve the pain produced by chemicals (formalin, acetic acid, and bradykinin), or by acting on thermoreceptors (tail-flick or hot plate test) or on mechanoreceptors in mouse models after s.c., p.o., or i.v. administration of alkaloids. The anesthetic activity of aconitum alkaloids is generally evaluated on mice models by the capacity of alkaloids to block the sensitivity of sciatic nerves [34, 35]. Simultaneously, they are studied for toxicological properties on animals in order to compare their therapeutic versus toxic potentials. The comparative data on analgesic, anesthetic activities and efficacy/toxicity ratios of various aconitum alkaloids obtained from different sources are given in Table 48.1. The therapeutic indices of alkaloids are calculated from the LD_{50} (lethal dose for 50% of animals tested) and ED_{50} (the dose inducing 50% of the pharmacological effect) values, determined by the same route of administration on experimental animals.

Comparing the anti-nociceptive activity of various compounds allows to conclude that there are two groups of *Aconitum* alkaloids exhibiting strong and moderate analgesic effects.

The first group includes aconitine-like diester alkaloids having strong analgesic activity: aconitine, hypaconitine, mesaconitine, 3-acetylaconitine, bulleyaconitine, and yunaconitine. The anti-nociceptive ED_{50} values of these alkaloids are within 0.028–0.097 mg kg^{-1} by i.v. and 0.025–0.14 mg kg^{-1} by s.c. route. The analgesic effect of these substances is about 10–50 times more important than that of morphine in suppressing formalin-induced or tail-flick nociceptive behavior [32]. The opioid receptor antagonist levallorphan does not interfere with the pharmacological effect of mesaconitine. The same results are obtained in experiments attempted to antagonize the effect of 3-acetylaconitine by naloxone. Moreover, in contrast to morphine, daily administration of 3-acetylaconitine in mice neither induced tolerance, nor physical dependence after a sudden withdrawal. These results prompted to suppose, that the anti-nociceptive activity of aconitine-like alkaloids is not mediated by stimulation of opioid receptors [33].

The second group includes less-toxic alkaloids with moderate analgesic effect. One of them, lappaconitine, is a well-studied compound already used in practical medicine, with analgesic ED_{50} of 2.7 mg kg^{-1} (i.v.). It is generally assumed that lappaconitine and its deacetylated analogue have lower toxicity than aconitine and, therefore, can be safely used as analgesic or anesthetic agents. However, the comparison of their analgesic and toxic properties has shown that even lappaconitine is about 40 times less toxic; its LD_{50}/ED_{50} ratio does not differ from that of aconitine, which characterizes it as an unfavorable drug candidate.

Table 48.1 The analgesic and anesthetic activities of *Aconitum* alkaloids

	Anti-nociceptive effect ED_{50} (mg kg^{-1})	LD_{50}/ED_{50} (analgesic effect) ratio	Local anesthetic effect ED_{50} (mg kg^{-1})	LD_{50}/ED_{50} (anesthetic effect) ratio	References
Aconitine	i.v. 0.028	3.57	0.007	43.63 (38.03)	[31, 32, 34]
3-Acetylaconitine	i.v. 0.097	0.87	0.003	510 (310)	[32, 34]
Mesaconitine	i.v. 0.025	2.724			[31]
Yunaconitine	s.c. 0.04	9.25			[34]
Bulleyaconitine	s.c. 0.05	18.8	0.003	303.23	[34]
Beiwutine	s.c. 0.06	6.48	0.05	7.78	[34]
Nagarine	s.c. 0.14	8.7	0.01	122	[34]
8-*O*-cynnamoyl-neoline	s.c. 0.86	13.8			[36]
Lappaconitine	i.v. 2.7 s.c. 3.50	2.51	0.04	292	[32, 34]
N-deacetyl-lappaconitine	s.c. 2.3	15.83	0.076	479.21	[34]
Ranaconitine	s.c. 4.2	2.14	0.1	90.1	[34]
N-deacetyl-ranaconitine	s.c. 8.6	3.2	0.1	274.5	[34]
Heteratisine	i.v. 100	1.47	–		[31]
6-Benzoyl-heteratisine	i.v. 2.0	1.58	–		[31]
Songorine	i.v. 46.4	2.284	–		[31]
Napelline	i.v. 68.1	>2.158	–		[31]
1-Benzoyl-napelline	i.v. 21.5	2.158			[31]

All data in table were obtained in experiments with mice.
The routes of administration of substances were i.v.-intravenous and s.c.-subcutaneous.
The same route of administration was used for calculation of LD_{50}/EC_{50} (analgesic effect) values.

After evaluation of the analgesic versus toxic potential of some *Aconitum* alkaloids, Gutser et al. [32] have arranged the therapeutic indices (LD_{50}/ED_{30}) of molecules in the following order: aconitine (6.0)>hypaconitine (2.4)> 3-acetylaconitine (2.0) and lappaconitine (2.0). They reported that, because of their high toxicity, these alkaloids cannot be considered as effective anti-nociceptive agents. Friese et al. [31] and Ameri [33] have reached the same conclusions, emphasizing that the therapeutic indices of *Aconitum* alkaloids remain quite low, which characterizes them as unfavorable to become effective analgesic agents.

More recent studies on new *Aconitum* alkaloids allowed to identify a range of molecules possessing analgesic activity on different pain models [34–36]. Among them, four alkaloids, bulleyaconitine, *N*-deacetyllappaconitine, yunaconitine, and

8-*O*-cinnamoylneoline, may be selected as favorable candidates having better therapeutic indices of anti-nociceptive effect with LD_{50}/ED_{50} ratios of 18.8, 15.83, 9.25, and 13.8 respectively.

Evaluation of anesthetic versus toxic properties of diester diterpenoid alkaloids revealed that they possess good anesthetic potential and high therapeutic indices, with LD_{50}/ED_{50} ratios of 510 for 3-acetylaconitine, 479 for *N*-deacetyllappaconitine, 303 for bulleyaconitine, 292 for lappaconitine, and 274 for *N*-deacetylranaconitine.

The less-toxic groups of C_{19}- and C_{20}-diterpenoid alkaloids have been reported to possess no analgesic activity [31, 33].

3.2 Anti-inflammatory Activity

The anti-inflammatory activity of diterpenoid alkaloids has been determined experimentally on various inflammation models: acetic acid–induced edema, carrageenan-induced hind-paw edema, etc. Both mesaconitine and 3-acetylaconitine have been shown to inhibit the increased vascular permeability induced by acetic acid in the mouse peritoneal cavity and by histamine in rat intradermal sites. They were both reported to exert anti-inflammatory action in sham-operated mice as well as adrenalectomized mice, confirming that the anti-inflammatory effect was not mediated via stimulation of the hypophysis-adrenal system. Mesaconitine did not interfere with prostaglandin biosynthesis in either guinea-pig lung homogenate, or rat peritoneal-activated macrophages, thus excluding the hypothesis about similarity of their mechanism of action with that of nonsteroidal anti-inflammatory agents. It prevents hind-paw edema induced by injection of carrageenin, or by subplantar injection of histamine, serotonine, or prostaglandine, indicating that it is effective at an early, exudative stage of inflammation [33]. However, diterpenoid alkaloids are less effective than nonsteroidal anti-inflammatory drugs. For example, aconitine is 400–500 times less active than phenylbutazone, and the efficacy of 3-acetylaconitine is reported to be 50–100-fold less than that of indomethacin and 1,000–2,000-fold less than that of salicylic acid [37].

3.3 Arrhythmogenic–Antiarrhythmic Activity

The most toxic alkaloids of aconite, such as aconitine, mesaconitine, hypaconitine, etc., are known to possess arrhythmogenic effect. Aconitine itself has long been used to induce arrhythmia in experimental models [38, 39]. Aconitine and its structural analogues induce ventricular ectopics, ventricular tachycardia, torsades de pointes, and ventricular fibrillation in a dose-dependent manner. Aconitine has a positive inotropic effect on the heart. It has hypotensive and bradycardic actions [40]. Another group of diterpenoid alkaloids possessing C_{18} and C_{19} skeleton, such as lappaconitine, *N*-deacetyllappaconitine, heteratisine, and 6-benzoylheteratisine, are

Table 48.2 Antiarrhythmic activity of alkaloids from plants of *Aconitum* genus

	ED_{50} (mg kg^{-1})	LD_{50}/ED_{50}
Lappaconitine	i.v. 0.05	118
N-deacetyllappaconitine	i.v. 0.05	146
Ranaconitine	i.v. 0.05	124
6-Benzoylheteratisine	i.v. 0.035	142.9

known to exert antiarrhythmic action on the heart muscle. Lappaconitine is a well-studied compound in China and ex-USSR countries, introduced into medical use as an antiarrhythmic agent [41–43].

The data on the antiarrhythmic effect of some alkaloids isolated from various *Aconitum* species are presented in Table 48.2 [41].

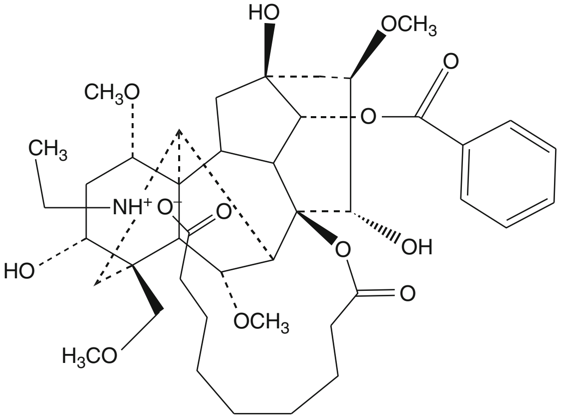
Experimental studies of the effect of lappaconitine and *N*-deacetyllappaconitine on guinea-pig-isolated atria have shown that these substances exert an antiarrhythmic effect on ouabain- and aconitine-induced arrhythmia in a dose-dependent manner. These substances decrease the contractile force of atria, increase excitation threshold, cause bradycardia at low doses (0.2 and 0.06 μM respectively), and can provoke asystolia of right atria at higher doses (4.5 and 10 μM respectively) [44]. 6-benzoylheteratisine, the main alkaloid of *A. tanguticum*, incubated with guinea-pig-isolated atria, exerts a negative inotropic effect on left atria and reduces the contractile force to about 50% at 0.6 μM, elevates the excitation threshold and causes bradycardia at the dose of 1 μM. It reduces significantly the maximum rate of action potential rise as well as the action potential amplitude of guinea-pig papillary muscle. Preincubation of atria with 6-benzoylheteratisine suppresses arrhythmia induced by aconitine, ouabain, and veratridine at a dose 0.06 μM [45]. It can be concluded that 6-benzoylheteratisine is a naturally occurring class-I antiarrhythmic substance. On the basis of the information that *A. tanguticum* has been used in Chinese and Tibetan folk medicine as a poison antidote, it can be inferred that the antidote effect might be at least due to antiarrhythmic property of 6-benzoylheteratisine, which antagonizes with the arrhythmogenic action of aconitine, ouabain, and veratridine.

3.4 Anticancer Activity

In spite of various and detailed studies of *Aconitum* alkaloids, very little information is available on their antitumor activity. De Ines et al. [46] have screened 43 C_{19}-diterpenoid alkaloids on CT26 (murine colon adenocarcinoma), SW480 (human colon adenocarcinoma), HeLa (human cervical adenocarcinoma), SK-MEL-25 and SK-MEL-28 (human melanomas), and on the non-tumor cell line CHO (Chinese hamster ovary cells). Several compounds (neoline, 8-*O*-methylcolumbianine, 18-*O*-demethylpubescenine, lycoctonine, acetylajadine, etc.) exhibit a selective cytotoxic effect to cancer versus non-cancer cells. Dasyukevich and Solyanik [47] have studied the anticancer activity of an *Aconitum* extract on in vivo experimental models using mice with ascites and solid form of Erlich's carcinoma. The product tested has no effect on the ascites form of tumor, but has shown substantial growth inhibition effect on the solid form of Erlich's carcinoma with 77.3% decrease of the tumor size compared to control. Hazawa et al. [48–50] reported that semisynthetic derivatives of C_{20}-norditerpenoid alkaloids, 11-veratroylpseudokobustine, 11-(*m*-trifluoromethylbenzoyl)kobustine, 11-(*m*-trifluoromethyl-benzoyl) pseudokobustine, and 11,15-dianisoylpseudokobustine, exhibited cytotoxic activity against A172, A549, HeLa, and Raji cell lines. Certain *Aconitum* alkaloids have been screened for anticancer activity at the Developmental Therapeutic Program of National Cancer Institute, NIH, USA.

Ajaconine, aconine hydrochloride, dehydroheteratisine, and heteratisine have been tested on the P388 leukemia model and isoatisine on the L1210 leukemia model in mice. Atisine has been studied on a yeast anticancer drug screen. Aconitine, jaconine, 8-*O*-ethylaconine, 8-*O*-ethyl-14-benzoylaconine, and delphinine have been screened on the panel of 60 human tumor cell lines. Among these, only two compounds exhibited cytotoxic activity: 8-*O*-ethyl-14-benzoylaconine was active against 786–0 (human renal cancer) with IC_{50} of 7.9 μM and delphinine was moderately cytotoxic on SK-OV-3 (ovarian cancer) with IC_{50} of 39.8 μM [51–53].

Aconitum karakolicum is widely used in traditional medicine against cancer in Kyrgyzstan. In order to explore its anticancer activity, we undertook a project aimed to isolate alkaloids from this plant and tested them on human tumor cell lines. Extraction and purification of alkaloids guided by in vitro cytotoxicity assay has resulted in the isolation of the compound 8-*O*-Azeloyl-14-benzoylaconine **10**. It is active against three human tumor cell lines with IC_{50} of 16.8 μM on HCT15 (colon cancer), 19.4 μM on A549 (lung cancer), and 10.3 μM on MCF7 (breast cancer). The comparison of IC_{50} values of this molecule with that of some clinically used anticancer drugs has shown that it is more active than melphalan (Table 48.3) [54].



8-*O*-Azeloyl-14-benzoylaconine **10**

Further, the naturally occurring 8-*O*-azeloyl-14-benzoylaconine was used as a basis for the design and hemisynthesis of new structural analogues [55]. A series of mono- and bifunctional acyl compounds, centered around the 8-*O*-azeloyl-14-benzoylaconine scaffold and differing by the length of the acyl chain, were synthesized and evaluated against three human tumor cell lines: A-549 (lung cancer), MCF-7 (breast cancer), and HCT-15 (colon cancer). Among bis-[*O*-(14-benzoylaconine-8-yl)]esters, only three molecules linked by azelaic, suberic, and pimelic acids presented a noticeable in vitro cytotoxic activity (IC_{50} ranging from 4 to 28 μM) (Table 48.4). The length of the linker chain in the structure seems to be important; a compound having a suberic acid moiety between two benzoylaconine skeletons was the most active, while shortening or lengthening of the diacid chain decreased the activity of compound.

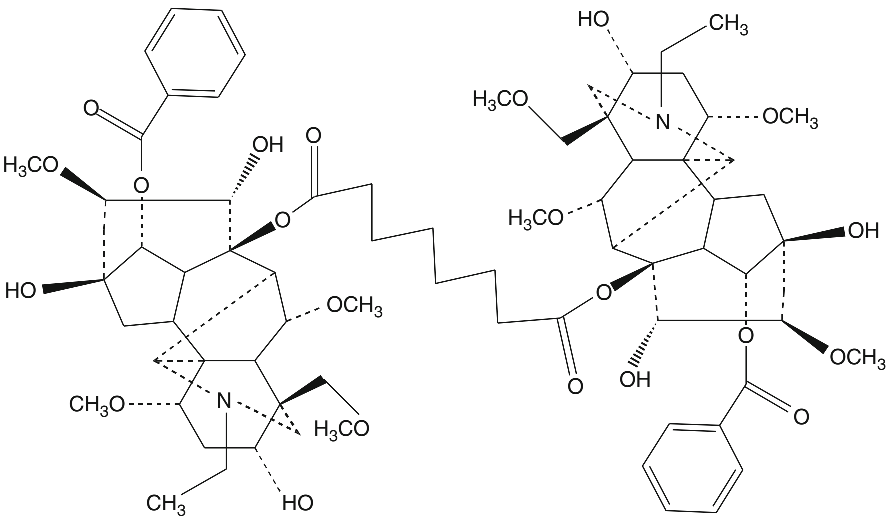
The most active compound, bis-[*O*-(14-benzoylaconine-8-yl)-suberate **11**, was selected for further in vivo studies with breast cancer (MCF-7) and colon cancer (HCT-15) cells. It was evaluated for anticancer activity in immunodeficient mice xenografted subcutaneously with human tumor cells, at the dose of 10 mg kg^{-1} injected i.v.

Table 48.3 Comparison of the growth inhibitory activities of 8-*O*-azeloyl-14-benzoylaconine with those of standard anticancer drugs

	Tumor cell lines, IC_{50} (μM)		
Cytotoxic agents	HCT15	A549	MCF7
8-*O*-azeloyl-14-benzoylaconine	16.8	19.4	10.3
5-Fluorouracil	2.11	5.69	1.75
Cisplatin	3.08	7.21	3.01
Etoposide	3.61	17.76	5.73
Melphalan	28.7	39.4	11.1

Table 48.4 In vitro cytotoxicity of bis[*O*-(14-benzoylaconine-8-yl)]esters with different diacyl chain length (2≥n≥7) against human tumor cell lines

		IC_{50} (μM)		
Compound	*n*	A549	MCF7	HCT15
bis-[*O*-(14-benzoylaconine-8-yl)]-succinate	2	>60	>60	>60
bis-[*O*-(14-benzoylaconine-8-yl)]-glutarate	3	>60	>60	>60
bis-[*O*-(14-benzoylaconine-8-yl)]-adipate	4	>60	>60	>60
bis-[*O*-(14-benzoylaconine-8-yl)]-pimelate	5	9.50±3.21	7.56±0.84	4.64±1.53
bis-[*O*-(14-benzoylaconine-8-yl)]-suberate	6	7.53±3.08	6.90±1.62	4.01±0.51
bis-[*O*-(14-benzoylaconine-8-yl)]-azelate	7	19.5	16.9	28



bis[-O-(14-benzoyl-8-yl)]-suberate **11**

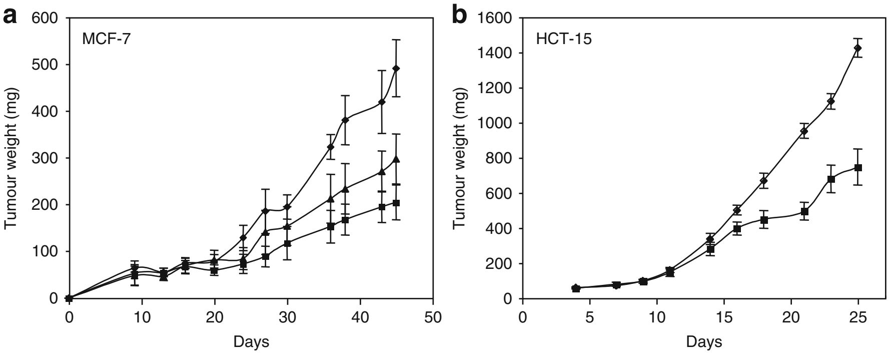


Fig. 48.1 In vivo antitumor activity of bis[*O*-(14-benzoylaconine-8-yl)]suberate on MCF-7 (**a**) and HCT-15 (**b**) tumor models grown in immunodeficient NOG mice. Two schedules were used for MCF-7 tumors

Antitumor activity was clearly detected at the dose of 10 mg/kg, a dose largely below the maximum tolerated dose (15 mg/kg). For MCF-7 cells, administration of five doses every 4 days, as well as weekly administration of four doses, resulted in T/C percent values of 36% ($p = 0.001$) and 56% ($p = 0.02$) on day 45, respectively (Fig. 48.1a). For HCT-15 cells, administration of five doses every 3 days resulted in 49% tumor regression on the 25th day (Fig. 48.1b, $p = 0.00001$).

3.5 Other Biological Activities

Lappaconitine has been shown to possess antiepileptic activity on rat hippocampal slices [56].

Gonzalez et al. have reported that three molecules of the C_{20}-diterpenoid alkaloid group: 15,22-*O*-diacetyl-19-oxo-dihydroatisine, azitine, and isoazitine exerted antiparasitic activity on promastigotes and intracellular amastigotes of *Leishmania infantum* [57, 58].

4 Mechanism of Action

The mechanism of action of aconitine-like alkaloids has been studied by many researchers [31, 33, 44] on synaptosomes of rat cerebral cortex and on guinea-pig-isolated atria. It was established that aconitine and its structural analogues have strong affinity to the voltage-gated Na^+ channels at epitope 2. Their binding to the open sodium channel provokes activation of this channel and permanent influx of Na^+ ions through the cell membrane, which finally results in prolonged depolarization of neurons and inhibition of neuronal conductivity. Consequently, the intracellular concentration of Ca^{2+} increases, resulting in transient enhancement of the contractile force of isolated atria. This mechanism underlying analgesic, local anesthetic, and arrhythmogenic properties is also responsible for the extreme toxicity of diester alkaloids. The arrhythmogenic action of aconitine is in part due to its anticholinergic effect, mediated by vagus nerve. Through binding to the axonal Na^+ channel site, aconitine inhibits also neuromuscular transmission by decreasing the release of acetylcholine [40].

The quantitative structure-activity relationship analysis has established a strong correlation between the toxicity and analgesic activity ($r = 0.96$), toxicity and local anesthetic activity ($r = 0.71$) of aconitine and its analogues [35]. These results are in line with the conclusions made by various authors [31–33]. It is obvious that the local anesthetic activity of these alkaloids is conditioned by a direct action on the receptors in the administration site. However, the mode of their anti-nociceptive action and the details of signal-ascending and/or signal-descending mechanisms participating in the pain-inhibition process are being explored. Ameri [33] hypothesizes that aconitine-like alkaloids are centrally acting analgesic agents. It was established that they do not interact with opioid receptors, which allows to suspect that they use a pain-suppressing pathway different from that of narcotic opioid analgesics [58]. Various experimentations on pain models using α- and β-adrenoreceptor agonists and antagonists together with aconitine-like alkaloids helped to determine that the anti-nociceptive effect of *Aconitum* alkaloids is potentiated by β-adrenoreceptor agonists, and on the contrary, inhibited by antagonists. Moreover, the mesaconitine-induced anti-nociception is potentiated by 3′,5′-cyclic monophosphate and phosphodiesterase inhibitors [59]. It was reported that the analgesic effect of mesaconitine is blocked by selective α_2-adrenoreceptor antagonists, as well as by 5-hydroxytryptamine receptor antagonists [60]. Taken together, these results suggest that the nociceptive effect of mesaconitine is exerted via stimulation of β-adrenoreceptors. It activates also descending inhibitory signals of α-adrenoreceptors and serotonergic neurons in nociception transmission pathways from the brain stem to the spinal cord [33, 61].

In contrast to aconitine, the less-toxic diterpenoid alkaloids with antiarrhythmic action, such as lappaconitine, its N-deacetylated analogue, and 6-benzoylheteratisine, selectively block the tetrodotoxin-sensitive Na^+ channels, decrease the intracellular concentration of Ca^{2+}, increase the activation threshold of Na^+ channels, resulting in bradycardia and significant negative inotropic action on the heart right atrium [44, 45]. The capacity of 6-benzoylheteratisine to prevent arrhythmia induced by aconitine prompted to conclude that these compounds compete for the same receptors, the former being characterized as a blocker, and the latter as a stimulator of Na^+ channels. The mode of anti-nociceptive and, probably, antiepileptic action of lappaconitine is due to its inhibitory action on inward tetrodotoxin-sensitive sodium currents, which is quantitatively and qualitatively different from the effect of aconitine [33, 56].

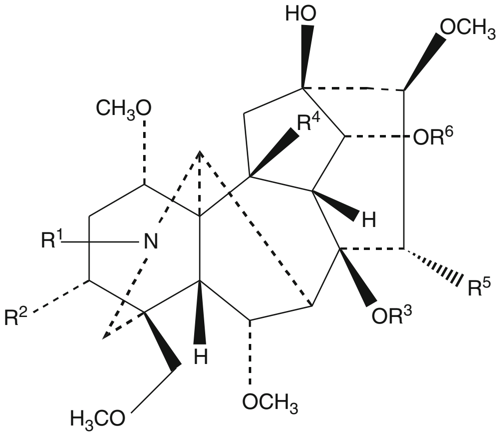
5 Toxicity

The onset and intensity of clinical symptoms of intoxication with *Aconitum* alkaloids usually depend on the quantity ingested. The first sign of poisoning may appear within 10 min after administration as paresthesia, numbness in the area of contact with poison (perioral, skin, then face, and limbs), followed by weakness, nausea and vomiting, dizziness, palpitation, hypotension, arrhythmia, systemic paralysis, shock, and coma. The death can occur within 2–6 h, or immediately, if a high dose of alkaloid is introduced. The main causes of death are ventricular arrhythmias, and paralysis of cardiovascular and respiratory systems [40].

Treatment of intoxication is supportive. No specific antidote is presently known. M-cholinoblockers (atropine) are indicated to treat bradycardia. Amiodarone and flecainide can be recommended for the treatment of aconitine-induced arrhythmia. Ventricular arrhythmias provoked by aconitine are often refractory to direct current cardioversion and antiarrhythmic drugs. In refractory cases of ventricular arrhythmias and cardiogenic shock, the cardiopulmonary bypass is recommended.

Even if all the plants of the *Aconitum* genus are considered to be poisonous, the toxicity of different species varies depending on the qualitative and quantitative content of alkaloids, plant origin, times of harvest, etc. There are multiple investigations that have been made by Japanese and German researchers to determine the toxicity of various alkaloids found in *Aconitum* genus. The toxicological characteristics of some alkaloids and related products given in Table 48.5 have shown that C_{19}-diterpenoid alkaloids can be divided into three groups depending on their toxicity.

The first group consists of the most toxic alkaloids such as aconitine, mesaconitine, hypaconitine, 3-acetylaconitine, jesaconitine, yunaconitine, beiwutine, bulleyaconitine, etc. Their molecular structures are relatively similar (**12**, Table 48.6). The common feature of all these alkaloids is the presence of two ester groups on position C-8 formed by acetyl moiety and on position C-14 formed by benzoyl or anisoyl moiety.



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Numerous studies on the toxicological properties of these alkaloids have shown that the presence of these two ester groups is indicative of their toxicity; de-esterification drastically reduces the toxicity of all compounds, the LD_{50} of monoesterified benzoylaconine being about 200-fold less than that of aconitine. The same tendency is observed for benzoylmesaconine and benzoylhypaconine (210- and 49-fold less toxic than mesaconitine and hypaconitine, respectively). Elimination of both acetyl and benzoyl groups from the molecule of aconitine by alkaline hydrolysis results in the formation of the least toxic product aconine, with LD_{50} of 120 mg kg^{-1} (1,000-fold less than that of aconitine).

Indeed, *Aconitum* roots used in traditional medicine of many Asian countries should undergo a special processing in order to decrease their toxicity. Even if the methods of processing vary from one country to another (e.g., soaking in saline solution, heating, or autoclaving [62]), they are all intended to hydrolyze highly toxic alkaloids, transforming them into nontoxic products. These so-called processed tubers lose their toxicity by 65–90% in comparison with raw tubers, and are designed for internal administration [41]. Recent investigations of Chinese and Korean researchers have shown [63, 64] that processing of tubers gives not only the products of hydrolysis, but also by-products of trans-esterification. These reactions might give the lipo-alkaloids formed by replacement of the acetyl moiety on the C-8 position of aconitine, mesaconitine, hypaconitine, neoline,

Table 48.5 The toxicity of *Aconitum* alkaloids

	Highest dose without mortality (mg kg^{-1})	LD$_{50}$(mg kg^{-1})	References
Aconitine	i.v. 0.0681	i.v. 0.1–0.13	[31, 65]
		i.p. 0.38	
		s.c. 0.27	
		p.o. 1.8	
3-Acetylaconitine		i.v. 0.138	[32]
		s.c. 0.87	
Mesaconitine	i.v. 0.464	i.v. 0.068; 0.1	[31, 65]
		i.p. 0.213	
		s.c. 0.204	
		p.o. 1.9	
Hypaconitine		i.v. 0.47; 0.215–0.316	[31, 32, 65]
		i.p. 1.1	
		s.c. 1.19	
		p.o. 5.8	
Jesaconitine		i.p. 0.2–0.25	[65]
		p.o. 1–2	
Yunaconitine		s.c. 0.37	[34]
Bulleyaconitine		s.c. 0.92	[34]
Beiwutine		s.c. 0.39	[34]
Nagarine		s.c. 1.22	[34]
Lappaconitine		i.v. 4.6–6.8;	[32–34]
		i.v. 5.9–11.5	
		s.c. 11.7	
N-deacetyllappaconitine		i.v. 7.3	[19, 34]
		s.c. 36.42	
Ranaconitine		i.v. 6.2	[19, 34]
		s.c. 9.01	
N-deacetyl ranaconitine		s.c. 27.5	[34]
Benzoylaconine		i.v. 23;	[65]
		i.p. 70	
Benzoylmesaconine		i.v. 21	[65]
Benzoylhypaconine		i.v. 23	[65]
Aconine		i.v. 120	[65]
Lipoaconitine		s.c. 100–200	[62]
Neoline		i.v. 215–261	[66]
Lycoctonine		i.v. 100–147	[32]
Heteratisine	i.v. 600	i.v. 147; 180	[31, 33]
6-Benzoylheteratisine	i.v. 2.15	i.v. 2.15–3.16; 5.0	[19, 31, 33]
		30	
Songorine	i.v. 68.1	i.v. 106	[31]

(*continued*)

Table 48.5 (continued)

	Highest dose without mortality (mg kg^{-1})	LD$_{50}$(mg kg^{-1})	References
Napelline	i.v. 147	i.v. >147; 87.5	[31, 33]
Karakoline		i.v. 46.4–68.1	[32]

All data in table were obtained in experiments with mice
The routes of administration were *i.v.* intravenous, *s.c.* subcutaneous, *i.p.* intraperitoneal, and *p.o.* per os

Table 48.6 The structural differences of aconitine-like diester diterpenoid alkaloids

	R^1	R^2	R^3	R^4	R^5	R^6
Aconitine	–C_2H_5	–OH	–OAc	–H	–OH	–OBz
3-Acetyl-aconitine	–C_2H_5	–OAc	–OAc	–H	–OH	–OBz
Hypaconitine	–C_2H_5	–H	–OAc	–H	–OH	–OBz
Mesaconitine	–CH_3	–OH	–OAc	–H	–OH	–OBz
Yunaconitine	–C_2H_5	–OH	–OAc	–H	–H	–OAs
Bulleyaconitine	–C_2H_5	–H	–OAc	–H	–H	–As
Beiwutine	–CH_3	–OH	–OAc	–OH	–OH	–OBz
Nagarine	–C_2H_5	–OH	–OAc	–OH	–OH	–OBz

Ac acetyl, *Bz* benzoyl, *As* anisoyl

or bikhaconine, by palmitoyl, linoleoyl, stearoyl, or oleoyl moieties. Examples of such lipo-alkaloids are 8-linoleyl-14-*O*-benzoylmesaconine, 8-nonadecenoyl-14-*O*-benzoylhypaconine, 8-palmitoyl-14-*O*-benzoylaconine, 8-stearoyl-14-*O*-anisoylbikhaconine, etc. Mixtures of lipo-aconitines are reported to be less toxic than aconitine, hypo- or mesaconitine [62].

The second group of diterpenoid alkaloids comprises moderately toxic C_{19}-alkaloids having a benzoyl moiety on the C-6, C-4, or C-14 positions, and without an acetyl moiety on the C-8 position. The representatives of alkaloids with a C-6 or a C-4 benzoyl moiety are 6-benzoylheteratisine, lappaconitine, and ranaconitine. Even if the LD$_{50}$ values of lappaconitine and 6-benzoylheteratisine found in the literature are not identical [32, 33, 35], these compounds remain about 20–50 times less toxic than diester alkaloids. However, they are more toxic than the de-esterified derivatives of aconitine-like alkaloids having a benzoyl group at C-14, such as benzoylaconine, benzoylmesaconine, and benzoylhypaconine, which indicates the importance of the position of the benzoyl moiety for toxicity.

The third group of C_{19}-diterpenoid alkaloids comprises lycoctonine, neoline, and heteratisine. They are devoid of any ester group on C-8 or C-14 positions; consequently, they have been reported to be among the least toxic of all *Aconitum* alkaloids.

The LD$_{50}$s of songorine, napelline, and karakoline presented in Table 48.5 allow to conclude that C_{20}-diterpenoid alkaloids are among the least toxic alkaloids.

6 Conclusions

The interest to the pharmacological activities of alkaloids extracted from the *Aconitum* genus is continuously growing. Most of *Aconitum* plants used in traditional medicine are being extensively explored and their therapeutic versus toxic potentials are being evaluated. Numerous investigations on the chemical constituents of raw and processed tubers of *Aconitum* plants allowed to explain the nature of the "Processed *Aconitum* preparations" used in traditional medicine: high-temperature treatment of *Aconitum* roots destroys highly toxic diester alkaloids to less-toxic derivatives by hydrolyzing or transforming them to lipo-alkaloids. The structure-toxicity relationship studies clarified the role of acetyl and benzoyl esters in the structure of diterpenoid alkaloids.

The pharmacological activities of *Aconitum* alkaloids principally have been evaluated in three axes: analgesic, antiarrhythmic, and anticancer effects. Aconitine-like alkaloids possess strong analgesic activities. They were determined as centrally acting anti-nociceptive agents, more potent than narcotic analgesics. However, there is a strong correlation between analgesic, arrhythmogenic activity and toxicity of diester diterpenoid alkaloids, which limits their use in medicine. More recent studies allow to find a number of alkaloids having wider therapeutic interval, for example, yunaconitine, *N*-deacetyllappaconitine, bulleyaconitine with LD_{50}/ED_{50} ratios of about 10–18. Among diterpenoid alkaloids evaluated for antiarrhythmic activity, three compounds, lappaconitine, *N*-deacetyllappaconitine, and 6-benzoylheteratisine, were characterized as the best antiarrhythmic agents. It was supposed that their antiarrhythmic action is due to the block of Na^+ channels, which is antagonistic in relation to the effect of aconitine-like alkaloids.

Interesting results were obtained on the cytotoxic activity of semisynthetic derivatives of C_{20}-diterpenoid alkaloids: 11-(*m*-trifluoromethylbenzoyl)kobustine, 11-(*m*-trifluoromethylbenzoyl)pseudokobustine, and 11,15-dianisoylpseudokobustine. The anticancer activity of synthetic bis [*O*-(14-benzoylaconine-8-yl)-suberate has been demonstrated on xenografted tumor models in mice with two human tumor cell lines: MCF7 (breast cancer) and HCT15 (colon cancer). The diverse biological activities of alkaloids from various *Aconitum* species are very encouraging and warrant further investigations.

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Alkaloids and Inhibitory Effects Against Enzymes Linked to Neurodegenerative Diseases (Physostigmine, Galanthamine, Huperzine, etc.)

49

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Contents

1 Introduction 1526
2 Physostigmine 1527
3 Galanthamine 1529
4 Huperzine A 1530
5 Examples of Other Promising Alkaloids with Anticholinesterase Effect 1532
5.1 Lycorine 1532
5.2 Dehydroevodiamine 1532
5.3 Visoltricin 1533
5.4 Narciprimine 1533
5.5 Berberine 1534
6 Conclusion 1535
References 1535

Abstract

Alkaloids are one of the largest natural products with low molecular weight found in plants, fungus, animals, and microorganisms. Many reports have confirmed that alkaloids have a desired therapeutic potential against human diseases. Acetylcholinesterase (AChE, EC 3.1.1.7) is an enzyme that has ability to inhibit the hydrolysis of the neurotransmitter called acetylcholine and elevate its level in the synaptic cleft, associated with several neurological disorders such

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as mostly Alzheimer's disease (AD) and myasthenia gravis. AChE inhibition in addition to butyrylcholinesterase (BChE) has been the most widely accepted treatment strategy against AD, which is characterized by shortage of acetylcholine in the brain. The current AChE inhibitors used in clinical application (rivastigmine, donepezil, and galanthamine) are classified under alkaloids, and consequently, alkaloids have become a popular target in discovery of novel cholinesterase inhibitors. In this chapter, current cholinesterase-inhibiting alkaloids of natural origin (physostigmine, galanthamine, and huperzine A) will be mentioned in addition to some promising ones with marked cholinesterase inhibition.

Keywords
Acetylcholinesterase • alkaloid • Alzheimer's disease • cholinesterase • plant

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
BCh	Butyrylcholine
BChE	Butyrylcholinesterase
FDA	Food and Drug Administration
IC	Inhibitory concentration
PD	Parkinson's disease

1 Introduction

Neurodegeneration is a term describing progressive loss in structure or malfunctioning of nerve cells called neurons. Neurodegenerative diseases have quite complex pathogenesis and therefore, the full pathology has not been elucidated for most of them up to date. The prevalence of neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) is on the increase as they activate neuronal cell death through endogenous suicide pathways [1]; however, effective treatments are still lacking. Although AD was discovered more than a century ago by the German physician Alois Alzheimer, the precise physiologic changes that trigger the development of AD largely remain unknown [2]. At the moment, approximately 33.9 million people worldwide are suffering from AD and, unfortunately, prevalence is expected to triple over the next 40 years [3]. PD, the second most common neurodegenerative disorder especially beyond the age of 60, was firstly described in 1817 by James Parkinson, a British physician. In the United States, PD has been estimated to affect at least 500.000 people, and is expected to double in the next 20 years [4].

Thus far, two hypotheses have been proposed for AD pathogenesis: "cholinergic hypothesis" and "amyloid hypothesis" [5]. Cholinergic hypothesis describes the biochemical and histopathological changes of neurotransmitter acetylcholine (ACh), whose shortage has been found to occur in the brains of patients with AD [6, 7]. Cholinesterase inhibitors provide a great contribution to elevation of ACh levels by inhibiting the enzymes implicated in its degradation, namely, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [8]. BChE (EC 3.1.1.8), also known as pseudocholinesterase, nonspecific cholinesterase, or simply cholinesterase, preferentially acts on butyrylcholine (BCh), in addition to its ability to hydrolyze ACh [9]. However, AChE is distinguished from BChE by its greater specificity toward ACh. Needless to say, the selectivity of cholinesterase inhibitors for AChE *versus* BChE may have an impact on both therapeutic and adverse effects of these compounds [10]. Consequently, inhibition of AChE, the key enzyme which catalyzes hydrolization of ACh into acetic acid and choline, is critical and AChE inhibitors have become the most prescribed drug class for the treatment of AD [11]. PD is often associated with dementia with Lewy bodies and, therefore, use of AChE inhibitors is also suggested for PD treatment [12].

An extensive research has been going on finding new cholinesterase inhibitors, whose efficacy has been confirmed in delaying the deterioration of symptoms of AD, a valuable treatment target considering the progressive nature of the disease. On the other hand, alkaloids that are nitrogen-containing natural compounds have been an attractive target for anticholinesterase drug research due to their structural similarity to all of the clinically approved anticholinesterase drugs (physostigmine, tacrine, rivastigmine, donepezil, and galanthamine) which also contain nitrogen in their structures. In consistent with this fact, the PubMed search using “alkaloids with anticholinesterase activity” as the keyword yields 2,562 results which underlines the popularity and efficacy of alkaloids in cholinesterase inhibition [13].

In this chapter, the main three alkaloids with cholinesterase inhibitory activity which have become approved drugs (physostigmine, galanthamine, and huperzine A) will be highlighted and some examples of promising alkaloids (lycorine, dehydroevodiamine, visoltricin, narciprimine, and berberine) with marked cholinesterase inhibition that may have potential in both therapy and drug development will be given.

2 Physostigmine

Physostigmine (also known as eserine) (Fig. 49.1) is a parasympathomimetic alkaloid isolated in 1864 by Jobst and Hesse from the calabar bean *Physostigma venenosum* Balf. (Fabaceae) growing in tropical forests of Africa [14]. The first therapeutic use of this drug was in 1877 in the treatment of glaucoma, which is still one of its clinical uses. Besides, it was the first discovered AChE inhibitor, which led to start practice of AChE inhibitors in clinical conditions in 1980s. Physostigmine [1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethyl-*N*-methylcarbamate,(3a*S*,8a*R*)-pyrrolo(2,3-b)indol-5-ol] is a pyrroloindole type of alkaloid having a carbamate moiety which has been considered to be responsible for cholinesterase inhibition [15]. Due to its reversible inhibitory effect toward AChE, physostigmine has been used to treat myasthenia gravis, glaucoma, AD, atropine-induced coma along with delayed gastric

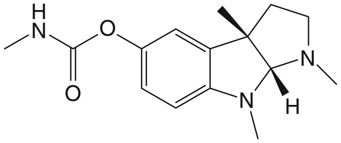

Fig. 49.1 Physostigmine

emptying [16]. In the late 1800s, physostigmine was utilized against atropine intoxication and glaucoma while its total synthesis was achieved in 1935 by the chemists Percy Lavon Julian and Josef Pikl [11]. The compound having lipophilic features has ability to pass blood-brain barrier that is probably due to its tertiary structure.

However, short half-life (30 min) of physostigmine and unsolicited side effects has put a limit to its widespread use as anticholinesterase agent. A number of clinical studies investigated efficacy of physostigmine in dementia patients by oral and intravenous applications, which was due to its cholinesterase-inhibiting properties. Development of an oral form with extended-release formula of the compound afforded sustainable blood levels [17]. Relevantly, a multicenter, double-blind, and large study showed efficacy and safety of extended-release formula of physostigmine salicylate after following 6 weeks use at the doses of 18, 24, and 30 mg *per* day [18]. In a later multicenter, double-blind, and placebo-controlled clinical trial, extended-release formula of physostigmine salicylate was evaluated in 850 subjects diagnosed with mild to moderate AD with dose-enrichment phase [19]. In the end of total 16 weeks, physostigmine salicylate was modestly effective in the AD patients at the daily doses of 12 and 15 mg according to body index. On the other hand, 204 patients with AD diagnosis were given the patches containing 30 mg and 60 mg of physostigmine with a placebo patch in a double-blind, randomized, multicenter study performed in Germany [20]. The evidence after a treatment period of 24 weeks showed that the efficacy of physostigmine was not superior to that of placebo and physostigmine did not cause any significant alteration in plasma cholinesterase activity. Therefore, it was concluded that the physostigmine patch application in doses of 30 mg and 60 mg actually did not lead to sufficient level of physostigmine concentrations in plasma that were to compensate for cholinergic deficiencies in affected brain areas and produce clinical benefits.

Despite its efficacy as a cholinesterase inhibitor, physostigmine has a narrow therapeutic index, short duration of action, and side effects such as nausea, dizziness, vomiting, headache, diarrhea, etc., and hence, it is not in clinical application any more for the treatment of neurological disorders [21]. Nevertheless, novel derivatives and analogs of physostigmine have been synthesized by many researchers and the structure of this compound has been a starting molecule for design of new anticholinesterase drug candidates. One of the earliest derivatives of physostigmine was neostigmine [3-(dimethylcarbamoyloxy)-*N*,*N*,*N*-trimethylbenzenaminium], which was used in the 1930's to treat gastrointestinal or urinary bladder atony and afterward approved for treatment of myasthenia gravis and glaucoma [11, 22, 23]. Later on, some other carbamate analogs of physostigmine have been synthesized including phenserine, tolserine, cymserine, and phenethylcymserine, which possess longer durations of action concerning cholinesterase inhibitory effect compared to their parent molecule; physostigmine as well as the other derivatives, neostigmine and pyridostigmine [24]. Eptastigmine (heptyl physostigmine; heptylcarbamic acid [(3aS,8aα)-1,2,3,3a,8,8a-hexahydro-1,3aα,8-trimethylpyrrolo [2,3-b]indol-5-yl] ester), a carbamate derivative of physostigmine in which the carbamoylmethyl group in position 5 of the side chain has been substituted with a carbamoylheptyl group [25]. The evidence underlined that eptastigmine is also a long-lasting reversible inhibitor of both AChE and BChE. Phenserine and tolserine are among the third generation cholinesterase inhibitors derived from physostigmine, entering in clinical trials.

Briefly, physostigmine representing the first generation of cholinesterase inhibitors has been a good model molecule for designing new derivatives with stronger cholinesterase inhibitory action with specific advantages over the earlier ones.

3 Galanthamine

Galanthamine [(4a*S*,6*R*,8a*S*)-5,6,9,10,11,12-hexahydro-3-methoxy-11-methyl-4a*H*-[1]-benzofuro[3a,3,2-ef][2]benzazepin-6-ol] (Fig. 49.2) is an alkaloid firstly isolated from *Galanthus woronovii Losinsk* (Amaryllidaceae) (snowdrop) and it is also known to be present in *Leucojum aestivum* L. (summer snowflake) and *Narcissus pseudonarcissus L.* from the same plant family, found widespread in the eastern parts of Europe mainly including Bulgaria and Turkey [5, 26]. Presence of galanthamine was also shown in *Lycoris radiata (L'Hér.) Herb.* and *Ungernia victoris Vved.* growing naturally in Asian countries [27]. The first work on discovery of AChE-inhibiting properties of galanthamine was published in 1951 by the Russian scientists Mashkovsky and Kruglikova-Lvova, who used an *ex vivo* system and smooth muscles (isolated rabbit small intestine and guinea pig uterus) [28]. Galanthamine is a central acting reversible AChE inhibitor, which has a good ability to penetrate into the blood-brain barrier and acts by dual mechanisms that can inhibit AChE and allosterically modulates human α3β4, α4β2, and α6β4 nicotinic ACh receptors [29, 30]. (-)-Galanthamine was developed as a drug for AD treatment and has been clinically used in Europe and the USA approved by FDA. Total synthesis of galanthamine that can yield in kilogram scale was achieved [31] and has been marketed as its hydrobromide salt (Razadyne®, Reminyl®) for the treatment of AD. Although nausea and vomiting are the common side effects of galanthamine, it is possible to eliminate these effects by increasing the galanthamine dose gradually and besides, no liver toxicity has been reported for galanthamine up to date [32, 33].

Galanthamine was reported to exert 50-fold selectivity against AChE than BChE by in vitro and in vivo experiments [28] and shown to inhibit AChE activity in human brain 10-fold less potent than that in human erythrocytes [34]. The pharmacokinetic data obtained from human subjects revealed that application of galanthamine hydrobromide at doses of 5 and 10 mg caused first-order pharmacokinetics, complete oral bioavailability, and a mean terminal half-life of 5.68 h [35].

The comparatively weakest one of the three clinically available cholinesterase inhibitors (rivastigmine, donepezil, and galanthamine) is galanthamine with IC_{50}

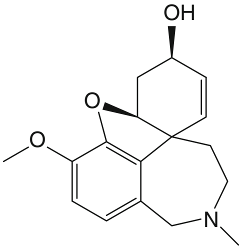

Fig. 49.2 Galanthamine

value of ~ 2.8–3.2 μM for the frontal cortex and the hippocampus [36]. However, the galanthamine dose should be escalated to 16 and 24 mg/day at 4-week intervals to achieve maximal tolerability [37]. Clinically, cognitive improvements are observed following 8-week treatment with galanthamine, which characteristically continues for 3–6 months [38]. Galanthamine was also shown to provide neuroprotection through antioxidant mechanisms, which was assessed by an in vitro model of H_2O_2-induced oxidative stress [39]. It reduced the release of reactive oxygen species (up to 50 %) and prevented loss in mitochondrial activity.

Many researchers have put effort in synthesizing novel analogs of galanthamine in order to obtain higher efficacy on cholinesterase inhibition. Those studies also furnished some key points for structure and activity relationship for galanthamine. For instance; new bis-interacting ligands of galanthamine were synthesized and length of the alkyl linkers was stated to play an important role to promote inhibitory potentials of these derivatives [40]. In this series, the highest inhibition was observed with the twelve methylene (-CH_2-) groups-containing derivatives. Moreover, *N*-alkylammonium derivatives exerted stronger inhibition than *N*-phthalimido derivatives. The results indicated that iminium moiety and *N*-alkylation of the nitrogen atom in galanthamine molecule enhanced inhibitory effect of these compounds against AChE. In several similar studies [41, 42], it was concluded that the alkylene moieties attached to galanthamine improved AChE inhibitory action due to lipophilicity of the alkyl and alkylene groups. Conversely, the anticholinesterase activities of new glucosyl and nitroxy derivatives prepared from galanthamine were shown to be lower as compared to the parent molecule (100 %), which was found below 70 % of inhibition [43].

From the evidence obtained with galanthamine, it can be pointed out to the fact that unique combination of AChE inhibition and nicotinic ACh receptor modulation for galanthamine provides an extra advantage and more significant benefit for this drug as compared to other cholinesterase inhibitors.

4 Huperzine A

(−)-Huperzine A [(5*R*, 9*R*, 11*E*)-5-amino-11-ethylidine-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta-[*b*]-piridin-2 (1H)-one] Fig. 49.3 is an alkaloid isolated

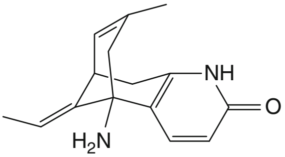

Fig. 49.3 (−)-Huperzine A

from *Lycopodium serratum* Thunb. (*syn. Huperzia serrata* (Thunb. ex Murray) Trevis.) (Lycopodiaceae/Huperziaceae), a Chinese plant used in traditional medicine for memory enhancement [11]. It was discovered in 1986 by the Chinese scientists at Shanghai Institute of Materia Medica [44]. It is an alkaloid with sesquiterpene skeleton that exhibits a potent and reversible inhibition toward AChE and has been shown to be effective against AD in preclinical and clinical studies [45, 46]. Huperzine A has been demonstrated to display a powerful, highly specific, and reversible inhibition against AChE [47, 48]. A tablet formulation with huperzine A, called as shuangyiping in China, was licensed in 1996 by the Chinese Health Authority for the treatment of AD [49]. Huperzine A was reported to improve memory deficits significantly in elderly people with benign senescent forgetfulness and in patients with AD or vascular dementia in phase IV clinical trials in China along with the findings of minimal peripheral cholinergic side effects and no unexpected toxicity [50–52]. Phase I studies in the United States indicated efficacy of increasing doses up to 400 μg twice daily in healthy older individuals [53]. In a phase II study under randomized, double-blind, placebo-controlled, dose-escalation conditions to investigate safety, tolerability, and efficacy of huperzine A in mild to moderate AD patients, the compound was generally well tolerated at doses of up to 400 μg for 24 weeks [54]. The results pointed out to the fact that huperzine A at dose of 200 μg is ineffective in the treatment of AD, whereas at a higher dose (400 μg) may improve cognition to some extent.

Huperzine A has a good penetration into the blood-brain barrier, higher oral bioavailability, prolonged half-life, and longer duration of AChE inhibitory action as compared to other cholinesterase inhibitors [55]. Total synthesis of huperzine A was achieved [56] and, using this compound as the parent molecule, a good number of analogs have been synthesized and compared with that of huperzine A. Among several novel fluorinated derivatives of huperzine A containing one, three, and six fluorine atoms in the molecule, one-fluorine containing analog; (±)-12-fluorohuperzine A was revealed to be the most active with IC_{50} value of 0.2 mM, whereas synthetic (+)-huperzine A and natural (−)-huperzine A had the IC_{50} values of 10 and 0.05 mM in the same experiment [57]. Among the new derivatives (±)-7-ethylhuperzine A as well as its two regioisomeric derivatives, (±)-7-ethyl derivative exhibited the highest inhibition although its effect was approximately 12-fold less than (−)-huperzine A [58]. In this regard, it definitely needs to be mentioned that ZT-1, a Schiff base derivative from natural huperzine A. ZT-1, *N*-[2-hydroxy-3-methoxy-5-chlorobenzilidene] huperzine A, is a pro-drug and rapidly transformed into the active huperzine A [59]. Because of its optimal profile and potency, ZT-1 was selected out of approximately 100 derivatives of huperzine

A identified at Shanghai Institute of Materia Medica, Chinese Academy of Sciences. It is currently being developed as a new drug candidate for AD treatment by Debiopharm S.A. (Switzerland) and also in China. On the other hand, the development of novel huperzine A-tacrine hybrids (huprins) led to discovery of a new class of very potent and selective AChE inhibitors [60–62].

On the basis of the reports on huperzine A, it seems to be most promising drug molecule with cholinesterase action as the last generation of this class.

5 Examples of Other Promising Alkaloids with Anticholinesterase Effect

5.1 Lycorine

Alkaloids are a large chemical class of bioactive compounds from natural sources. Various alkaloids including galanthamine, isolated especially from several members of Amaryllidaceae family, have been identified with remarkable cholinesterase inhibitory features [63–68]. Lycorine (1,2,4,5,12b,12c-Hexahydro-7*H*-[1, 3]dioxolo [4,5-j]pyrrolo[3,2,1-*de*]phenanthridine-1,2-diol) (Fig. 49.4), an isoquinoline alkaloid, isolated from the bulbs of *Crinum jagus* (J. Thomps.) Dandy (swamp lily) and *C. glaucum* A. Chev. (Amaryllidaceae) that are used in traditional medicine in southern Nigeria for memory loss and other mental symptoms, was reported to be a notable inhibitor of AChE with IC_{50} 450 μM and two free hydroxy groups in this structure was suggested to be associated with AChE inhibitory activity [69]. McNulty et al. synthesized a number of analogs of lycorine and stated that the effect of lipophilic substitution at C2 and polar H bond acceptor groups at C1 is additive in that the most potent analogs have both of these functionalities, which may help to design novel derivatives from the lycorine series [70].

5.2 Dehydroevodiamine

Dehydroevodiamine (21-methyl-3,13,21-triazapentacyclo[11.8.0.$0^{2,10}$.$0^{4,9}$.$0^{15,20}$] henicosa-2(10),4,6,8,15,17,19-heptaen-14-one) (Fig. 49.5) is a quinazolinocarboline alkaloid isolated from the fruits of *Evodia rutaecarpa* Bentham (Rutaceae), a well-known plant that has been used for a long time in Chinese traditional medical practice. It was identified as the active principal of the extract from *E. rutaecarpa* that was shown to have strong anti-AChE activity [71]. Dehydroevodiamine displayed a potent inhibition towards AChE in a concentration-dependent and noncompetitive manner with IC_{50} value of 37.8 μM. Anti-amnesic effect of dehydroevodiamine was also demonstrated by the same research group in scopolamine-induced amnesia model of the rat using the passive avoidance and eight-arm radial maze tests [72]. An in vivo pharmacokinetic study revealed that dehydroevodiamine is transported from the systemic circulation to the brain *via* the blood-brain barrier by linear kinetics [73]. Efficacy of this compound has been partially attributed to its AChE inhibitory

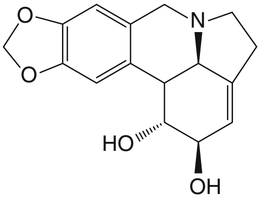

Fig. 49.4 Lycorine

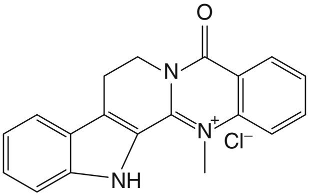

Fig. 49.5 Dehydroevodiamine

effect, but also to long-lasting facilitation of synaptic transmission due to activation of both muscarinic and NMDA receptors [74].

In a study that investigated whether the indole moiety was essential for AChE inhibitory activity of dehydroevodiamine [75], a series of compounds were synthesized, in which the indole was replaced by a benzene moiety. In some compounds of this series, the amidine structure was found to be responsible for moderate to strong AChE-inhibiting properties.

5.3 Visoltricin

Visoltricin (2-propenoic acid, 3-[1-methyl-4-(3-methyl-2-buten-1-yl)-1 H-imidazol-5-yl]-methyl ester) (Fig. 49.6) is an alkaloid isolated from the microfungus *Fusarium tricinctum* [76]. It was reported to have potent AChE-inhibiting properties with IC_{50} values of 2.6×10^{-4} M and 4.0×10^{-4} M in human serum and erythrocyte AChE, respectively [77]. Kinetic studies indicated that visoltricin is a reversible inhibitor of AChE with a mixed-type inhibition in concentration-dependent manner, but independent of time.

5.4 Narciprimine

Narciprimine (Fig. 49.7) is the known phenanthridone alkaloid isolated from *Cyrtanthus contractus* N.E. Br. (fire lily) (Amaryllidaceae). In in vitro experiments [66], it exhibited strong AChE inhibition with IC_{50} value of 78.9 μM. In that study, presence of narciprimine was shown for the first time in the genus

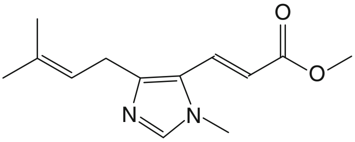

Fig. 49.6 Visoltricin

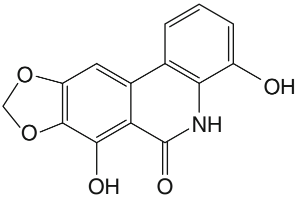

Fig. 49.7 Narciprimine

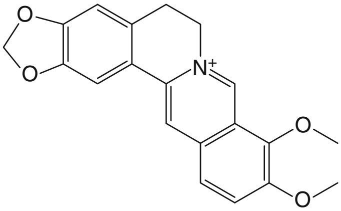

Fig. 49.8 Berberine

of *Cyrtanthus*, in addition to its known sources such as *Zephyranthes*, *Narcissus*, and *Lycoris*, the genera endemic to the Americas, Europe, and Asia, respectively.

5.5 Berberine

Berberine (Benzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium, 5,6-dihydro-9,10-dimethoxy-chloride) (Fig. 49.8) is from the protoberberine group of isoquinoline alkaloids isolated from many plant sources such as some *Berberis* sp. (Berberidaceae) such as *Berberis vulgaris* L. (barberry) and *B. aristata* D.C. (tree turmeric) [78, 79], *Mahonia aquifolium* (Pursh) Nutt. (oregon grape) (Berberidaceae) [80], *Hydrastis canadensis* L. (goldenseal) (Ranunculaceae) [81], *Phellodendron amurense* Rupr. (Amur Cork Tree) (Rutaceae) [82], *Coptis chinensis* Franch. (Chinese goldthread, Huang-Lian, Huang-Lien) (Ranunculaceae) [83], *Tinospora cordifolia* (Thunb.) Miers (Menispermaceae) [84], as well as to a smaller extent in *Argemone mexicana* L. (prickly Poppy) (Papaveraceae) [85] and *Eschscholzia californica*

Cham. (californian poppy) (Papaveraceae) [86]. One of the earliest records on AChE inhibitory activity of berberine obtained from *Macleaya microcarpa* (Maxim.) Fedde (plume poppy) (Papaveraceae) growing in Ukraine was reported by Kuznetsova et al. [87]. Berberine isolated from the aerial parts of *Corydalis speciosa* Maxim. ex Regel of Korean origin (Papaveraceae) was shown to inhibit AChE strongly at IC_{50} value of 3.3 μM in concentration-dependent manner [88]. The tuber methanol extract of *Corydalis yanhusuo* W.T. Wang of Chinese origin also afforded berberine as the active principle of this plant with a high anti-AChE effect ($IC_{50} = 1.01$ μM) [89]. In a recent study, a new derivative of berberine identified as (-)-2,9-dihydroxyl-3,11-dimethoxy-1,10-dinitrotetrahydroprotoberberine displayed a potent inhibitory activity against AChE with IC_{50} value below 10 μM [90].

In a series of 9-N-substituted berberine derivatives synthesized, (o-methyl-phenethyl)amino derivative linked at the 9-position of berberine was demonstrated to possess a very high AChE-inhibiting feature ($IC_{50} = 0.027$ μM) [91]. Jiang et al. also reported that, among the benzenediol-berberine hybrids synthesized, berberine-pyrocatechol hybrid ($IC_{50} = 0.123$ μM) exerted a much higher inhibition against AChE than the parent molecule berberine ($IC_{50} = 0.374$ μM) [92].

6 Conclusion

Great number of alkaloids has been discovered from natural sources, especially the plant kingdom. The literature survey clearly points out to the fact that a good number of alkaloids possess remarkable anticholinesterase effect and some of them made it to clinical application as the approved drugs. The research going on finding new cholinesterase inhibitors has given to an assumption that many more alkaloids are waiting to be discovered for this purpose. Therefore, alkaloids need a special emphasis in the design and discovery of cholinesterase inhibitors.

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Part V

Phenolics: General Biology and Biotechnology

Phenolic Compounds: Introduction

50

Vincenzo Lattanzio

Contents

1 Introduction 1544
2 The Major Classes of Phenolics in Plants 1548
3 Physiological and Ecological Role of Plant Phenolics 1565
4 Carbon Fluxes from the Primary to the Secondary Metabolic Pathways 1570
5 Conclusions 1573
References 1573

Abstract

"Plant phenolics" and "polyphenols" are secondary natural metabolites arising biogenetically from either the shikimate/phenylpropanoid pathway, which directly provides phenylpropanoids, or the "polyketide" acetate/malonate pathway, which can produce simple phenols, or both, thus producing monomeric and polymeric phenols and polyphenols, which fulfill a very broad range of physiological roles in plants. Higher plants synthesize several thousand known different phenolic compounds. The ability to synthesize phenolic compounds has been selected throughout the course of evolution in different plant lineages, thus permitting plants to cope with the constantly changing environmental challenges over evolutionary time.

Plant phenolics are considered to have a key role as defense compounds when environmental stresses, such as high light, low temperatures, pathogen infection, herbivores, and nutrient deficiency, can lead to an increased production of free radicals and other oxidative species in plants. Both biotic and abiotic stresses stimulate carbon fluxes from the primary to the secondary metabolic pathways,

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thus inducing a shift of the available resources in favor of the synthesis of secondary products. An interesting link between primary and secondary metabolism couples the accumulation of the stress metabolite proline with the energy transfer toward phenylpropanoid biosynthesis via the oxidative pentose phosphate pathway. The alternating oxidation of NADPH by proline synthesis and reduction of $NADP^+$ by the two oxidative steps of the oxidative pentose phosphate pathway lead to the simultaneous accumulation of phenolic compounds.

Keywords

Secondary metabolism • plant phenolics • phenolic classes • ecological and physiological role • trade-off

1 Introduction

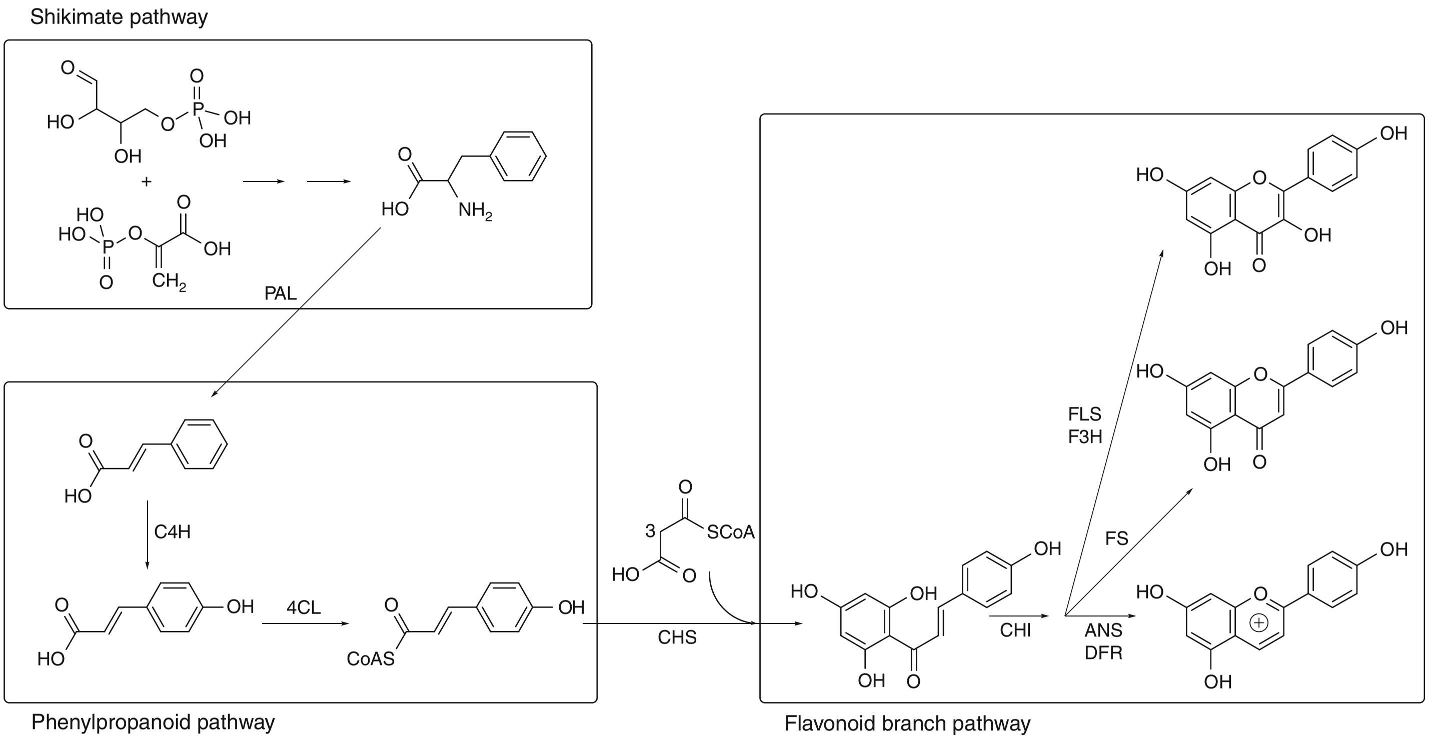
There are approximately 300,000 documented species of higher plants on the planet, which synthesize an enormous number of chemicals of diverse structure and class (more than 200,000 isolated and identified individual chemical entities). These compounds can be further divided into primary and secondary metabolites. The primary metabolites include metabolites such as sugars, fatty acids, amino, and nucleic acids, as well as chemicals considered ubiquitous to all plants for growth and development [1, 2]. Secondary metabolites are structurally and chemically much more diverse than the primary metabolites and refer to compounds present in specialized cells that are not directly essential for basic photosynthetic or respiratory metabolism but are thought to be required for plants' survival in the environment. Plants have metabolic pathways leading to tens of thousands of secondary products capable of effectively responding to stress situations imposed by biotic and abiotic factors. These pathways, often recruited from essential primary metabolism pathways upon initial gene duplication leading to duplicated genes showing new functions and optimized and diversified roles in new pathways, are an integral part of the developmental program of plants. Accumulation of secondary metabolites often marks the onset of developmental stages. A strict spatial and temporal control of gene expression ensures the correct accumulation pattern of various secondary products. The required transport of metabolic intermediates constitutes an additional level of regulation. Ontogeny and circadian clock-controlled gene expression are also important features of plant secondary metabolism, as are master regulatory transcription factors [3–8]. Secondary metabolites apparently act as defense (against herbivores, microbes, viruses, or competing plants) and signal compounds (to attract pollinating or seed dispersing animals), as well as protecting the plant from ultraviolet radiation and oxidants. The pattern of secondary metabolites in a given plant is complex; it changes in a tissue- and organ-specific way; differences can be seen between different developmental stages (e.g., organs important for survival and reproduction have the highest and most potent secondary metabolites), between individuals, and between populations. These secondary metabolites are classified into several groups according to their biosynthetic routes and structural features [9–14].

Phenolic compounds are the most widely distributed secondary metabolites, ubiquitously present in the plant kingdom, even if the type of compound present varies according to the phylum under consideration. Phenolics are uncommon in bacteria, fungi, and algae. Bryophytes are regular producers of polyphenols including flavonoids, but it is in the vascular plants that the full range of polyphenols is found [15, 16]. It is estimated that about 2% of all carbon photosynthesized by plants is converted into flavonoids or closely related compounds [17]. Higher plants synthesize several thousand known different phenolic compounds, and the number of those fully characterized is continually increasing. Leaves of vascular plants contain esters; amides and glycosides of hydroxycinnamic acids; glycosylated flavonoids, especially flavonols; and proanthocyanidins and their relatives. Lignin, suberin, and pollen sporopollenin are examples of phenolic-containing polymers. Some soluble phenolics are widely distributed, for example, chlorogenic acid, but the distribution of many other structures is restricted to specific genera or families, making them convenient biomarkers for taxonomic studies.

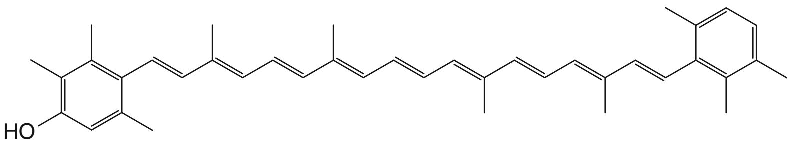
The ability to synthesize phenolic compounds has been selected throughout the course of evolution in different plant lineages when such compounds addressed specific needs, thus permitting plants to cope with the constantly changing environmental challenges over evolutionary time [18–21]. For example, the successful adaptation to land of some higher members of the Charophyceae – which are regarded as prototypes of amphibious plants that presumably preceded true land plants when they emerged from an aquatic environment onto the land – was achieved largely by massive formation of "phenolic UV light screens" [9, 15, 22, 23]. The phenylpropanoid pathway leading to lignins involves a common set of biochemical reactions in vascular plants already present 400 million of years ago with the emergence of erect vascular land plants. From an evolutionary point of view, these metabolic backbones have been progressively enriched to provide specific adaptations to different plant families and the remarkable biochemical diversity we can observe [21]. Accounting for about 40% of organic carbon circulating in the biosphere, these phenolic compounds are biosynthetically formed by way of either the shikimic acid pathway or the malonate/acetate pathway, also known as the polyketide pathway, and related biochemical pathways. Furthermore, it is their reassimilation back to carbon dioxide during biodegradation (mineralization) that presents the rate-limiting step in recycling biological carbon [24, 25].

Plant phenolics are, in themselves, a fascinating group of substances and have attracted some of the most distinguished of the Nobel-Prize-winning organic chemists, including Emil Fischer, who studied chemical substances used in tanning; Richard M. Willstätter, who in 1913 proposed the first chemical hypothesis concerning blue flower color development; Robert Robinson (copigmentation theory); Richard L.M. Synge (interested in interactions of tannins with proteins); and Alexander R. Todd who worked from 1931 to 1934 on anthocyanins and other coloring matters together with Sir Robert Robinson [26–29]. The continuing fascination of polyphenols for chemists involved with plant constituents is shown by the fact that a "plant phenolics group" was established in France (Narbonne 1970: the first meeting; Avignon-Montfavet 1971: the first International Conference on Polyphenols). This "Groupe Polyphénols" was constituted in an international society in Narbonne (France) in 1972 with the aim of promoting research on plant polyphenols, while providing members worldwide with a unique forum to exchange information on all aspects of these fascinating natural products, from their most basic and fundamental biophysicochemical properties to their most diverse applications in food and agricultural, pharmaceutical, and cosmetic sciences and technologies (http://www.groupepolyphenols.com/).

Broadly, as far as the definition of plant phenolics is concerned, the term 'phenol' is a chemical term that defines a phenyl ring bearing one or more hydroxyl substituents. The term 'polyphenol' could thus be used to define natural products featuring at least two phenyl rings bearing one or more hydroxyl substituents, including their functional derivatives (e.g., esters and glycosides), but in the context of plant phenolics such a definition is not satisfactory, since it would include compounds such as the gossypol, the phenolic carotenoid 3-hydroxyisorenieratene (I) or the phenolic female sex hormone oestrone (II), which are principally terpenoid in origin [30]. Thus, as a general rule recently proposed by Quideau et al. [31], the terms 'plant phenolics' should be strictly used to refer to secondary natural metabolites arising biogenetically from either the shikimate/phenylpropanoid pathway, which directly provides phenylpropanoids (Scheme 50.1), or the 'polyketide' acetate/malonate pathway, and which fulfill a very broad range of physiological roles in plants [30, 31]. In fact, although the bulk of these compounds play cell wall structural roles, plant tissues synthesize a vast array of nonstructural constituents that have various roles in plant growth and survival. Thus, the expression "plant phenolics" embraces a highly diverse group, the chemically known members of which can be counted in several thousand with a large range of identified structures: monomeric, dimeric, and polymeric phenolics have been identified. Several classes of phenolics have been categorized on the basis of their basic skeleton: C_6 (simple phenol, benzoquinones), C_6—C_1 (phenolic acid), C_6—C_2 (acetophenone, phenylacetic acid), C_6—C_3 (hydroxycinnamic acid, coumarin, phenylpropanes, chromones), C_6—C_4 (naphthoquinones), C_6—C_1—C_6 (xanthones), C_6—C_2—C_6 (stilbenes, anthraquinones), C_6—C_3—C_6 (flavonoids, isoflavonoids, neoflavonoids), $(C_6$—C_3—$C_6)_{2,3}$ (bi-, triflavonoids), $(C_6$—$C_3)_2$ (lignans, neolignans), $(C_6$—$C_3)_n$ (lignins), $(C_6)_n$ (catechol melanins), and $(C_6$—C_3—$C_6)_n$ (condensed tannins). Low-molecular-weight phenolics occur universally in higher plants; some of them are common in a variety of plant species and others are species specific. Higher-molecular-weight proanthocyanidins (also called condensed tannins) are the most abundant polyphenols in woody plants but are usually absent in herbaceous plants. Hydrolyzable tannins have a more restricted occurrence than proanthocyanidins, being found in only 15 of the 40 orders of dicotyledons [16, 32–40].


Shikimate pathway
PAL
Phenylpropanoid pathway
C4H
4CL
CoAS
3
SCoA
CHS
Flavonoid branch pathway
CHI
ANS
DFR
FS
FLS
F3H

Scheme 50.1 Schematic of the major branch pathways of (poly)phenol biosynthesis. *PAL* phenylalanine ammonia-lyase, *C4H* cinnamate-4-hydroxylase, *4CL* 4-coumaroyl:CoA-ligase, *CHS* chalcone synthase, *CHI* chalcone isomerase, *ANS* anthocyanidin synthase, *DFR* dihydroflavonol reductase, *FS* flavone synthase, *FLS* flavonol synthase, *F3H* flavanone 3-hydroxylase



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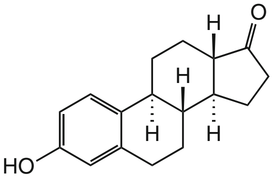
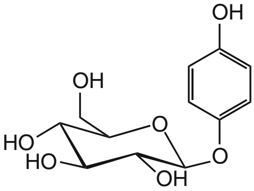


II

2 The Major Classes of Phenolics in Plants

Examples of simple phenols (C_6) include catechol and phloroglucinol. Although most of the more complex plant polyphenols contain these two simple phenols as a parts of their structures, catechol and phloroglucinol are uncommon in plant tissues. Catechol has been found in leaves of *Gaultheria* species, while phloroglucinol has been found as glucoside in the peel of various *Citrus* fruits. Arbutin (**III**) is found in leaves of various *Vaccinium* spp., such as blueberry, cranberry, cowberry, and pear trees (*Pyrus communis* L., *Rosaceae*) [41–43].

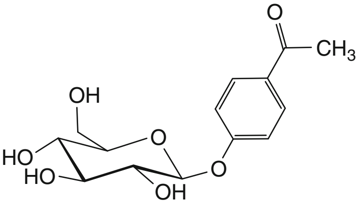


III

Phenolic acids (C_6—C_1) are commonly represented by gallic, *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids. Phenolic acids are usually present in the bound soluble form conjugated with sugars or organic acids and are typically components of complex structures such as lignins and hydrolyzable tannins. Gallic acid is the base unit of gallotannins, whereas gallic acid and hexahydroxydiphenoyl moieties are both subunits of the ellagitannins, which are classified as hydrolyzable tannins. Free and bounded phenolic acids are found in cereals. Hydroxybenzoic acid glycosides are also characteristic of some herbs and spices. Aldehydes related to C_6—C_1 acids are also found in plants. Salicylaldehyde, *p*-hydroxybenzaldehyde (*Sorghum* spp.), *p*-anisaldehyde (*Vanilla*, *Mimosa*), and *p*-protocatechualdehyde

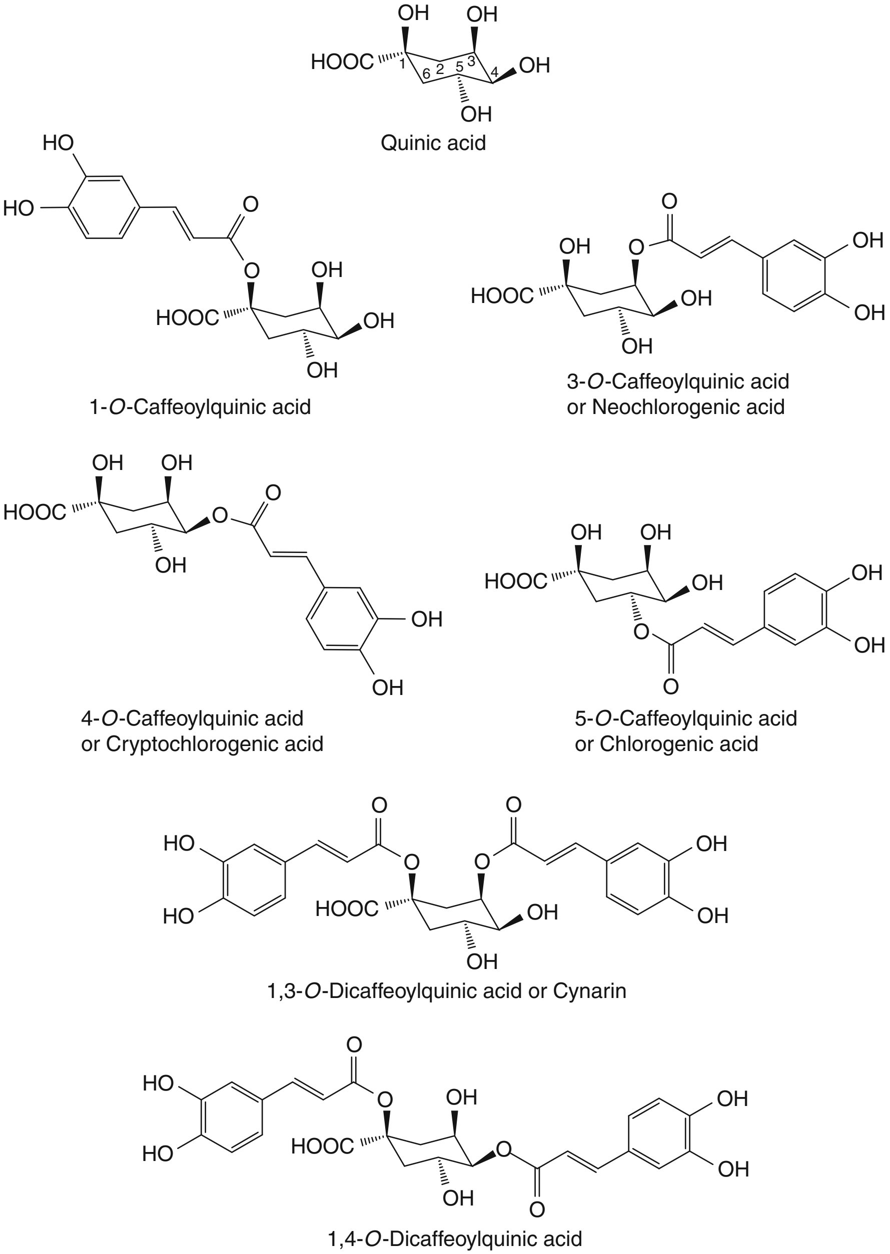
(*Cichorium intybus*) are also quite common in plants. Vanillin, the odor principle occurring in *Vanilla* pods, is certainly of very widespread origin [42, 44]. Ellagic acid has been reported to be present in berries, particularly raspberries (*Rubus idaeus*), strawberries, and blackberries. However, free ellagic acid is normally present in low levels in berries that more commonly contain ellagitannins [38, 45].

Among the less common C_6—C_2 compounds, phenolic ketones have occasionally been found as plant constituents. Picein (**IV**) the main component of all investigated spruce needles (*Picea abies* (L.) Karst.), also occurs in *Larix decidua* Mill., *Populus balsamifera*, and *Salix* spp. *p*-Hydroxyphenylacetic acid occurs free and as a glucoside in bamboo shoots. Xanthoxylin, a phloroacetophenone derivative, has been found in *Xanthoxylum* spp. [42, 46–48].



IV

A ubiquitous phenolic unit in plants is one with an aromatic ring attached to a C_3 aliphatic side chain. Such phenylpropanoids occur universally in plants and are also the precursors of many other classes of plant phenolics. For example, by *o*-hydroxylation and subsequent cyclization, *p*-coumaric acid can give rise to hydroxycoumarins. On reduction, it yields *p*-coumaryl alcohol, which is one of the monomeric building rocks of the lignin. β-Oxidation of *p*-coumaric acid yields *p*-hydroxybenzoic acids. Dimerization of *p*-coumaryl alcohol can give rise to a lignin. Finally, the *p*-coumaric moiety is an important unit in the structure of flavonoids, stilbenoids, and xanthones. The most widely distributed hydroxycinnamates (C_6—C_3) are *p*-coumaric, caffeic, ferulic, and sinapic acids, which usually occur in various conjugated forms and are seldom found in the free state except as artifact due to chemical or enzymic hydrolysis during tissue extraction. In aqueous solvents, they isomerizes from the more stable *E* (*trans*) form to the *Z* (*cis*) form, especially under the action of UV light. Hydroxycinnamates frequently accumulate as their respective tartrate esters, coutaric, caftaric, and fertaric acids. Quinic acid conjugates (Fig. 50.1) of caffeic acid, namely, 3-, 4-, and 5-*O*-caffeoylquinic acid, are commonly found in fruits and vegetables. Green coffee beans (*Coffea arabica*) are one of the richest dietary sources of caffeoylquinic acids. Chlorogenic acid (5-*O*-caffeoylquinic acid) (**V**) is the dominant caffeoylquinic derivative accounting for 50% of the total [49, 50]. Coumarins, which are also C_6—C_3 derivatives, are benzo-α-pyrones (lactones) formally derived from *o*-hydroxycinnamic acids by cyclization and ring closure between the *o*-hydroxy and carboxyl groups. This group of phenolic compounds can be found free in nature or in combined form with sugars as heterosides and glycosides in many dicotyledonous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Quinic acid

1-*O*-Caffeoylquinic acid

3-*O*-Caffeoylquinic acid
or Neochlorogenic acid

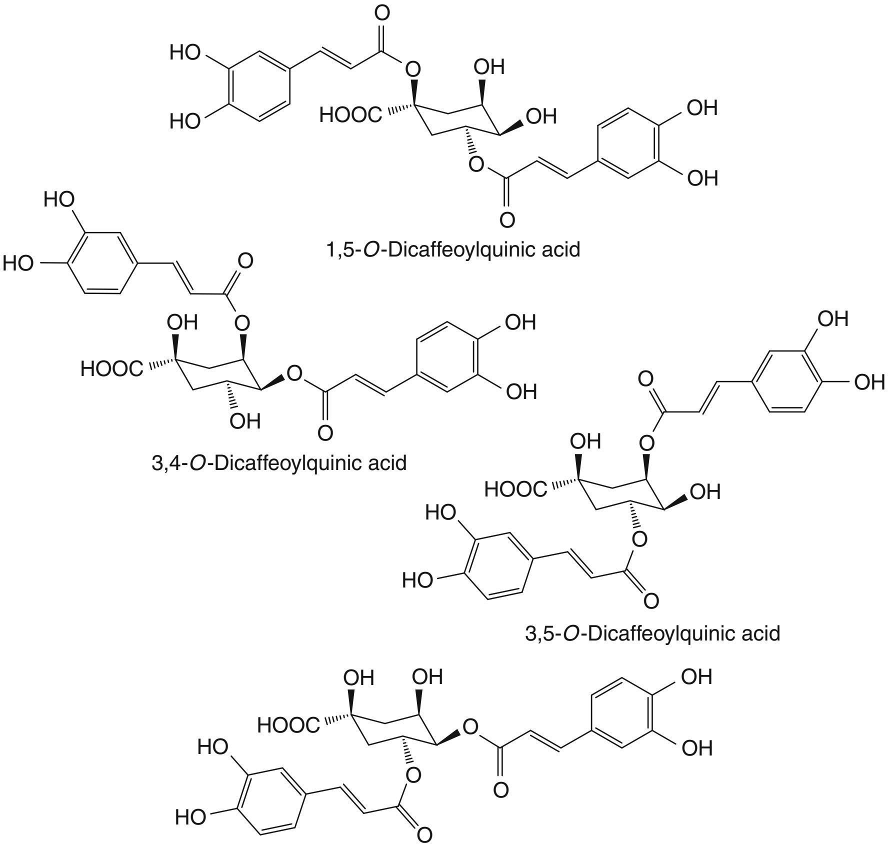
4-*O*-Caffeoylquinic acid
or Cryptochlorogenic acid

5-*O*-Caffeoylquinic acid
or Chlorogenic acid

1,3-*O*-Dicaffeoylquinic acid or Cynarin

1,4-*O*-Dicaffeoylquinic acid

Fig. 50.1 (continued)



1,5-*O*-Dicaffeoylquinic acid

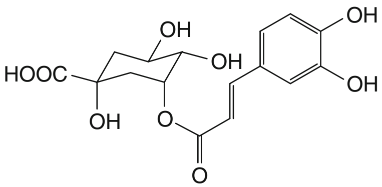
3,4-*O*-Dicaffeoylquinic acid

3,5-*O*-Dicaffeoylquinic acid

4,5-*O*-Dicaffeoylquinic acid

Fig. 50.1 Mono- and di-caffeoylquinic acids nomenclature according to IUPAC rules 1976 [149]

families, including the *Apiaceae*, *Asteraceae*, *Fabaceae*, *Moraceae*, *Rosaceae*, *Rubiaceae*, and *Solanaceae*. Although mainly synthesized in the leaves, coumarins occur in high levels in fruits, roots, and stems. Coumarins can be roughly categorized as (i) simple hydroxycoumarins, the most common ones being umbelliferone, esculentin, and scopoletin (**VI**), which occur naturally as their β-O-glucosides; (ii) furanocoumarins (bergaptene, (**VII**)) that consist of a five-membered furan ring attached to the coumarin nucleus; and (iii) finally the pyranocoumarins (calanolide B, (**VIII**)) that are analogous to furanocoumarins but contain a six-membered pyran ring [51, 52]. Chromones are isomeric with coumarins but differ in the biosynthesis and position of the keto group. Their biosynthesis seems to proceed *via* the polyketide pathway by the condensation of five acetate units and subsequent *O*- and *C*-methylation. Most naturally occurring chromones contain a methyl group or an alkyl group at C-2 and hydroxyl or alkoxyl groups at C-5 and C-7. Only few simple chromones are known to occur naturally. Eugenin (**IX**) is an example of a simple 2-methyl-chromone present in *Eugenia aromatica* [49].



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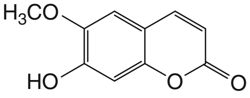
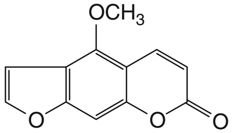
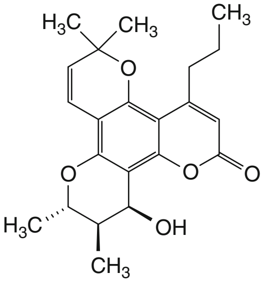


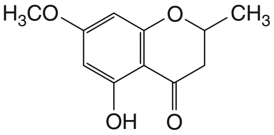
VI



VII

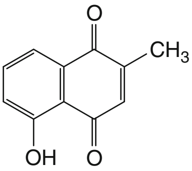


VIII

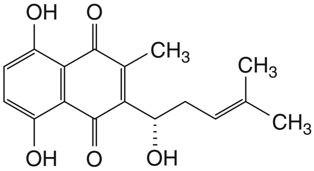


IX

Naphthoquinones (C_6—C_4) represent a class of quinone pigments widespread in nature. The most important higher plant families containing naphthoquinones are *Avicenniaceae*, *Bignoniaceae*, *Boraginaceae*, *Droseraceae*, *Ebenaceae*, *Juglandaceae*, *Nepenthaceae*, and *Plumbaginaceae*. They are biosynthesized *via* a variety of pathways including polyketide pathway (plumbagin, **X**) shikimate/ succinyl-CoA combined pathway [lawsone (2-hydroxy-1,4-naphthoquinone)], and shikimate/mevalonate pathway (alkannin, **XI**). Although intensely colored naphthoquinones are not often apparent as plant-coloring matters. They are often present in heartwood or bark, where their presence is masked. When present in living tissues, leaves, and roots, they are usually in colorless form, and color is produced only after extracts have been treated with acid to bring about hydrolysis of sugar linkages and oxidation of quinol to quinone as observed for plumbagin, an orange pigment identified in *Plumbago capensis* [16, 53–55].



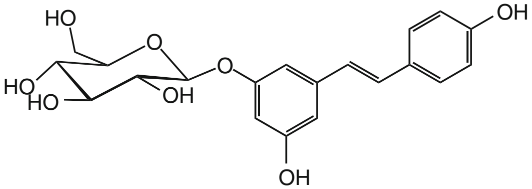
X



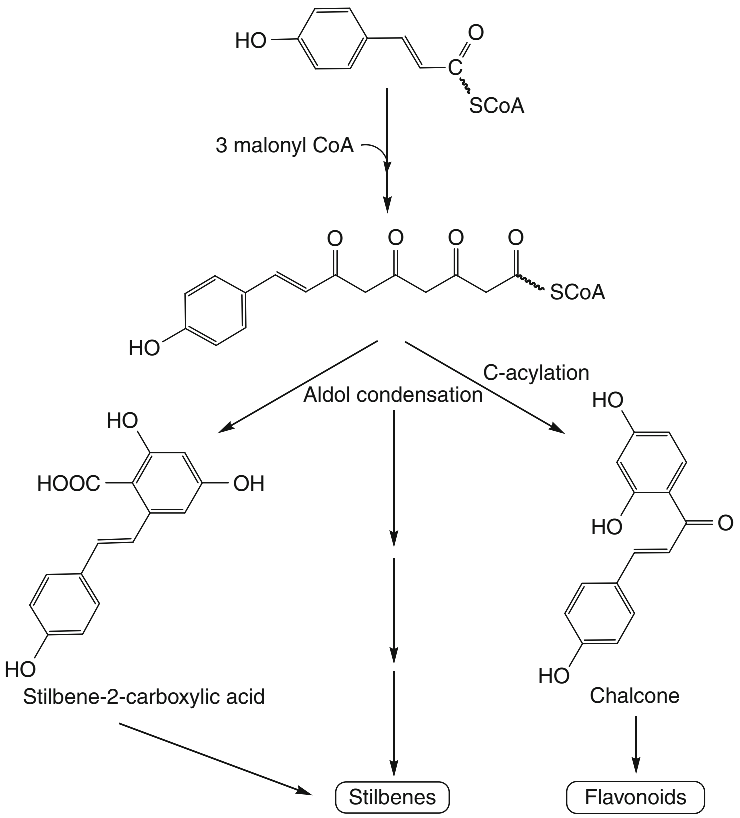
XI

Xanthones (C_6—C_1—C_6) are a class of plant phenolics occurring in a few higher plant families (Gentianaceae, Guttiferae, Loganiaceae, Podostemaceae, and Polygalaceae); therefore, they have a high taxonomic value in such families. The majority of natural xanthones have been found in just two families of higher plants, Guttiferae and Gentianaceae. Xanthones may be classified into five major groups: simple oxygenated xanthones (this group can further be subdivides into six groups according to the degree of oxygenation), xanthone glycosides, prenylated and related xanthones, xanthonolignoids, and miscellaneous. As far as the biosynthetic pathways to xanthones is concerned, ring A and attached carbonyl group are provided by the shikimic acid pathway, whereas ring B arises via the acetate-malonate polyketide route. Mangiferin is unique among the natural xanthones in having a much wider natural occurrence than that of any of the others. This 2-*C*-glucoside of 1,3,6,7-tetrahydroxyxanthone was first found in the leaves of *Mangifera indica*. It has since been found in many ferns and angiosperms such as *Asplenium* spp., *Hypericum* spp., *Cratoxylum pruniflorum*, *Senecio* spp., and *Dahlia* spp. [56–59].

The members of the stilbene family have the C_6—C_2—C_6 structure and are widely distributed in the plant kingdom, although some structures are characteristic of particular plant families. They are found in liverworts, in some ferns, in gymnosperms, and in many dicotyledonous angiosperms, ranging from the unsubstituted *trans*-stilbene from *Alnus* and *Petiveria* to the hexasubstituted combretastatin A-1 from *Combretum caffrum* [60]. There are also increasing numbers of reports of prenylated stilbenes, stilbene glycosides, and polymeric stilbenes. Stilbenes are biosynthesized via phenylpropanoid-polymalonate pathway. The first part of the pathway is common to both stilbenoids and flavonoids, the two biosynthetic routes diverging at the point of cyclization of a styryl-3,5,7-triketoheptanoic acid (Scheme 50.2). A C-acylation produces a chalcone, and subsequent modifications lead to the flavonoids, while an aldol condensation of the same intermediate polyketide produces a stilbene-2-carboxylic acid that is an unstable intermediate in pathways to a range of stilbenoids [61]. The main physiological roles of stilbenes include those of phytoalexins and growth regulators. Preexisting stilbenes may help to protect plant tissues from the attack by fungi, insects, and other organisms. In addition, synthesis of a number of antifungal stilbenes can be induced by infections by appropriate organisms, or by a number of abiotic stimuli such as UV light. These stilbene phytoalexins include resveratrol and its derivatives (**XII**) in *Trifolium*, *Arachis*, and members of Vitaceae [54].



XII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Aldol condensation
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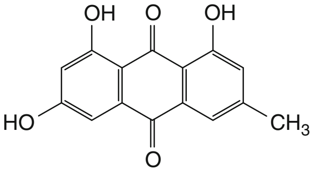
Stilbene-2-carboxylic acid
HO
HO
O
HO
Chalcone
Stilbenes
Flavonoids

Scheme 50.2 Schematic of stilbene biosynthesis

The tricyclic C_6—C_2—C_6 anthraquinones represent the largest group of natural quinones, and a wealth of structures with varying numbers of hydroxyl groups and other substituents have been identified. More than half of the natural anthraquinones are found in lower fungi, particularly in *Penicillium* and *Aspergillus* species, and in lichens. Over 200 structures have been found in flowering plants, especially in the families of Leguminosae, Liliaceae, Polygonaceae, Rhamnaceae, Rubiaceae, and Scrophulariaceae. Anthraquinones have been encountered in all parts and organs of plants. Thus, they have been isolated from leaves and stems, pods, seed coats, and embryos of *Cassia* plants and from leaves and roots of *Digitalis* spp. Anthraquinones may either be formed via the acetate-malonate pathway in Polygonaceae and Rhamnaceae or *O*-succinylbenzoic acid in the Bignoniaceae and Verbenaceae. In plants, anthraquinones are mostly present as glycosides but the free aglycones are widely distributed as well. Among the most common naturally occurring anthraquinone aglycones in higher plants are emodin (**XIII**), rhein, chrysophanol, aloe-emodin, and physcion. The anthraquinone emodin (1,3,8-trihydroxy-6-methylanthraquinone), mainly reported in three plant families, Fabaceae

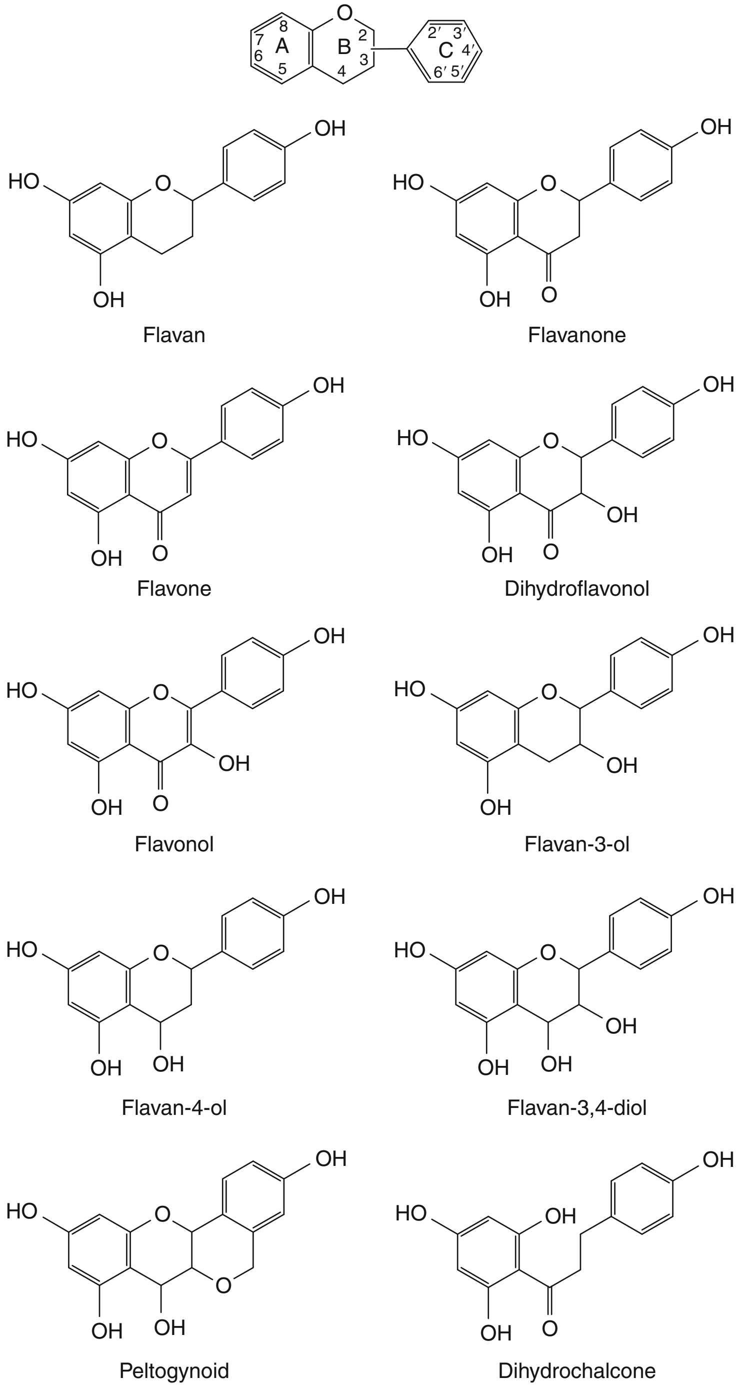
(*Cassia* spp.), Polygonaceae (*Rheum*, *Rumex*, and *Polygonum* spp.), and Rhamnaceae (*Rhamnus* and *Ventilago* spp.), is present in both vegetative organs (stem, foliage) as well as in reproductive organs (flower, fruit, seeds, pods) [16, 62, 63].



XIII

Flavonoids and their conjugates form a very large group of natural products; over 8,000 different flavonoids have been identified. They are found in many plant tissues, where they are present inside the cells or on the surfaces of different plant organs. The chemical structures of this class of compounds are based on a C_6—C_3—C_6 skeleton. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes: the flavonoids (2-phenylbenzopyrans), the isoflavonoids (3-benzopyrans), and the neoflavonoids (4-benzopyrans). In addition, these chemical structures may differ in the saturation of the heteroatomic ring C and in the overall hydroxylation patterns. All these groups usually share a common chalcone precursor and, therefore, are biogenetically and structurally related. The flavonoids may be modified by hydroxylation, methoxylation, or *O*-glycosylation of hydroxyl groups as well as *C*-glycosylation directly to carbon atom of the flavonoid skeleton. In addition, alkyl groups (often prenyls) may be covalently attached to the flavonoid moieties, and sometimes additional rings are condensed to the basic skeleton of the flavonoid core. Various subgroups of flavonoids are classified according to both the substitution pattern and the degree of oxidation and saturation present in the heterocyclic C-ring. The flavonoids *sensu stricto* may be divided into 13 subgroups (Fig. 50.2), while isoflavonoids are subdivided into 11 subgroups (Fig. 50.3). The neoflavonoids, structurally and biogenetically closely related to the flavonoids and the isoflavonoids, comprise the 4-arylcoumarins (4-aryl-2*H*-1-benzopyran-2-ones), 3,4-dihydro-4-arylcoumarins, and neoflavenes [35, 39, 64–69].

Together with the proanthocyanidins, the bi- and triflavonoids constitute the two major classes of complex C_6—C_3—C_6 plant phenolics. These compounds arise from the oxidative coupling of various flavonoid structures and thus predominantly possess a carbonyl group at C-4 or its equivalent in every constituent unit. Anyhow, it should be emphasized that the terms bi- and triflavonoids are used loosely and that there is no commonly accepted trivial nomenclature for these classes of flavonoids. A multitude of compounds that do not arise via the phenol oxidative coupling of monomeric flavonoid structures possessing C-4 carbonyl functional groups are also classified as bi- and triflavonoids. For example, the bichalcone (**XIV**) isolated from *Dorstenia zenkeri* is a biflavonoid generated via an intramolecular Diels-Alder process [70].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O

Flavan

Flavanone

Flavone

Dihydroflavonol

Flavonol

Flavan-3-ol

Flavan-4-ol

Flavan-3,4-diol

Peltogynoid

Dihydrochalcone

Fig. 50.2 (continued)

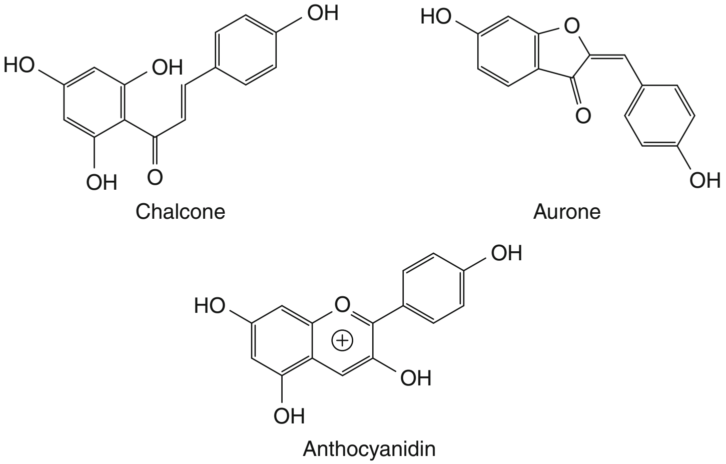
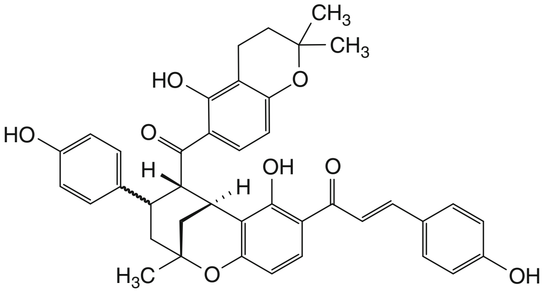
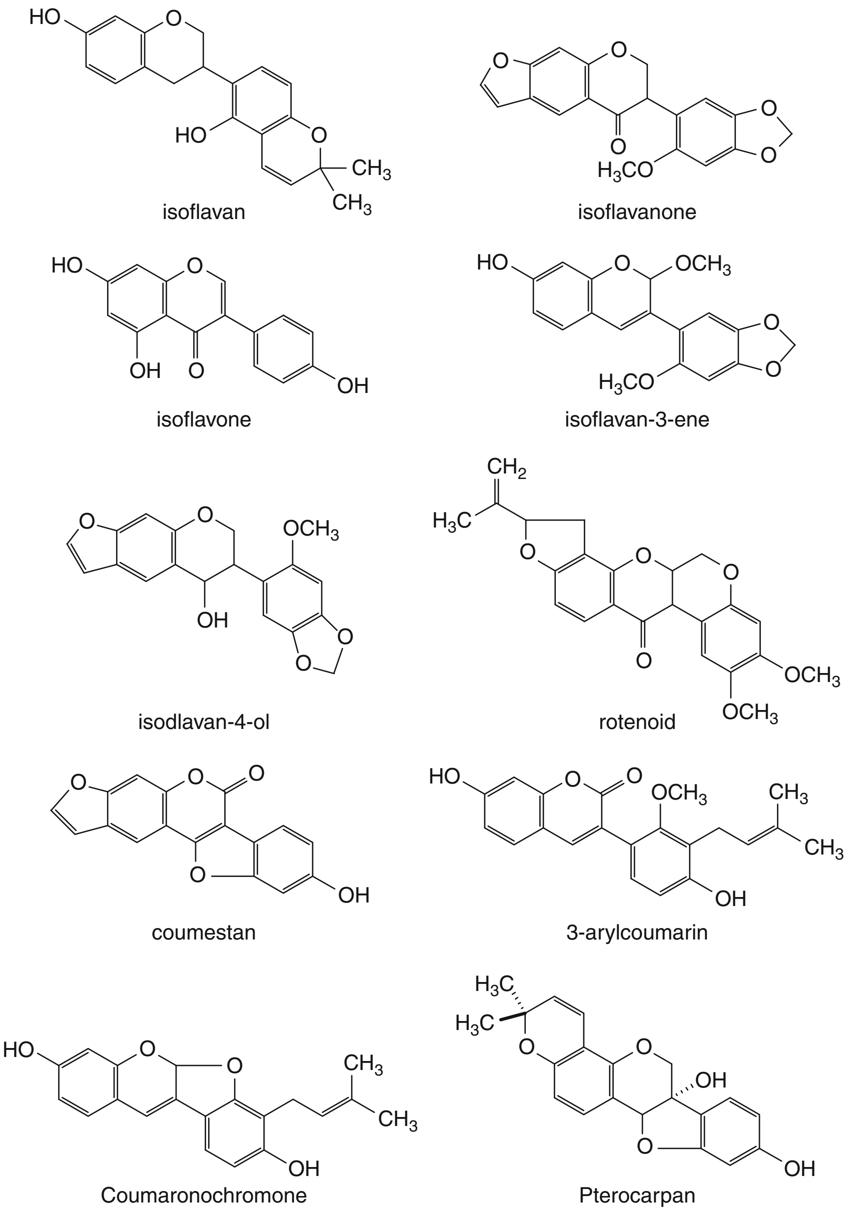


Fig. 50.2 Flavonoid subgroups



XIV

Lignans and neolignans (C_6—C_3)$_2$ are a large and varied group of plant phenolics produced by the oxidative dimerization of two phenylpropanoid units, which occur in a wide range of plant species. For the purpose of numbering the various parent structures, the C_6—C_3 unit is numbered 1–9 where the α position is the 9, β is the 8, and γ is the 7. When the two C_6—C_3 units are linked by a β,β′-bond (or 8,8′-bond), the parent structure lignane is used as the basis for naming the lignan. If the two C_6—C_3 units are linked by a bond other than a β,β′-bond, the parent structure, neolignane, is used as the basis for naming the neolignan (Fig. 50.4) [71]. (+)-Pinoresinol (**XV**), for example, is a lignan derived by a tail-to-tail linkage in the β-position of two coniferyl alcohol residues, which has been extracted from various plant sources such as *Saussurea medusa*, *Pinus*, and *Picea*. Related dimers, called neolignans, can be formed by other condensations between two C_6—C_3 units, for example, joining head-to-tail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H3CO
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isoflavan-3-ene
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OCH3
OH
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isodlavan-4-ol
CH2
H3C
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OCH3
OCH3
rotenoid
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OH
coumestan
HO
O
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OCH3
CH3
CH3
OH
3-arylcoumarin
HO
O
O
CH3
CH3
OH
Coumaronochromone
H3C
H3C
O
O
OH
O
OH
Pterocarpan

Fig. 50.3 Isoflavonoid subgroups

instead of tail-to-tail. One example of dimer formed by other condensations between two C_6—C_3 units, for example, joining head-to-tail instead of tail-to-tail, is eusiderin (**XVI**). This and related structures occur in heartwoods of Magnoliaceae, Piperaceae, and Lauraceae [72, 73].

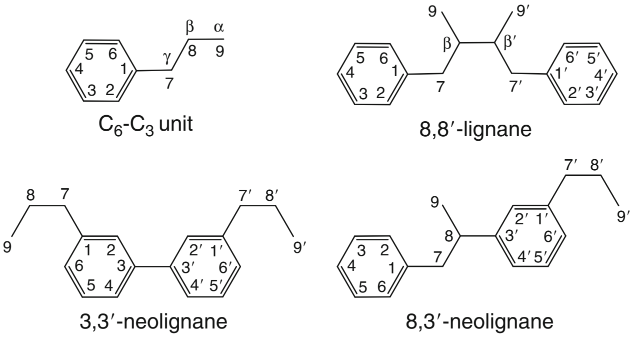
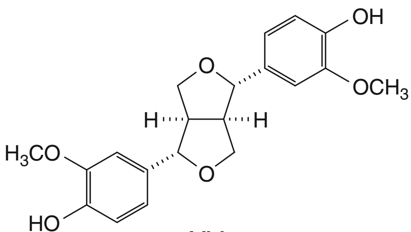
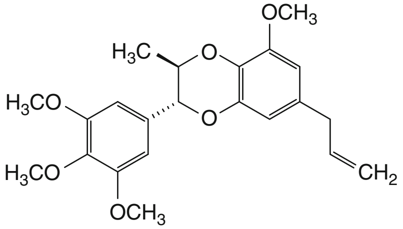


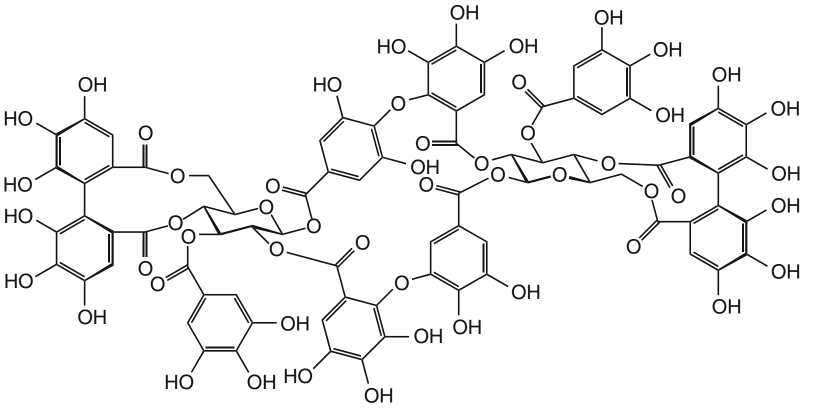
Fig. 50.4 Parent structures of lignane and neolignanes


XV

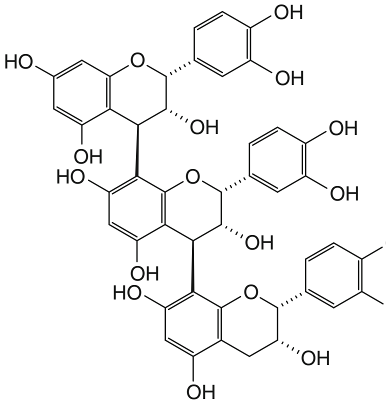

XVI

The plant *tannins* are a unique group of phenolic compounds of relatively high molecular weight which have the ability to complex strongly with carbohydrates and proteins. The name “tannin” is derived from the French “tanin” (tanning substance) and is used for a range of natural polyphenols. Probably the most acceptable definition of vegetable tannins is still that of Bate-Smith and Swain, formulated in 1962. They adopted the earlier ideas of White and classified these higher plant metabolites as “water-soluble phenolic compounds having molecular weights between 500 and 3,000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins.” In higher plants, tannins consist of two major groups of metabolites: the hydrolyzable tannins and condensed tannins. More recently, a third class of tannin, the phlorotannins, has been isolated in several genera of algae. Hydrolyzable tannins are split by acids, bases, and in some cases by hydrolytic enzymes (tannase) into sugars (usually D-glucose) or related polyols and a phenolic acid. In the case of gallotannins, this is gallic acid, and the vegetable tannins are simply polygalloyl esters. Ellagitannins (**XVII**) can be defined in a narrow sense as hexahydroxydiphenoyl esters of carbohydrates or cyclitols. When the hexahydroxydiphenoyl group is cleaved from the molecule, the parent acid rapidly lactonizes to yield the insoluble dilactone ellagic acid. The oligomeric and polymeric proanthocyanidins (*syn*. condensed tannins) (**XVIII**) constitute one of the most ubiquitous groups of all plant phenolics.

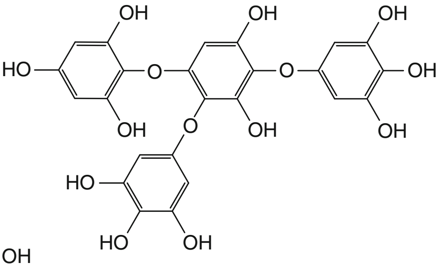
The proanthocyanidins usually originate by coupling at C-4 (C-ring) of an electrophilic flavanyl unit, presumably generated from a flavan-3,4-diol or a flavan-4-ol, most commonly to C-8 or C-6 (A-ring) of a nucleophilic flavanyl unit, for example, a flavan-3-ol. This classification of the two major groups of tannins suggested by Freudenberg, based on structural considerations, also represents a subdivision based on biogenetic origins: proanthocyanidins are products of the flavonoid pathway of biosynthesis (polyketide and cinnamate) and gallo- and ellagitannins are both derived from the shikimate pathway. Phlorotannins consist of phloroglucinol units linked to each other in various ways and are of wide occurrence among marine organisms, especially brown and red algae. Based on the means of linkage, phlorotannins can be classified into four subclasses, namely, phlorotannins (i) with an ether linkage (fuhalols and phlorethols), (ii) with a phenyl linkage (fucols), (iii) with an ether and a phenyl linkage (fucophlorethols), and (iv) with a dibenzodioxin linkage (eckols and carmalols). 3-Phloroeckol (**XIX**), isolated from *Eisenia arborea*, is an example of phlorotannins with a dibenzodioxin linkage [37, 74–81].



XVII



XVIII



XIX

Lignin (Latin: *lignum* = wood) [$(C_6—C_3)_n$], the essential structural polymer of wood and second only to cellulose as the most abundant organic substance in plants, is found as an integral cell wall constituent of all vascular plants including herbaceous species. Gymnosperm lignins are primarily derived from coniferyl alcohol and, to a lesser extent, *p*-coumaryl alcohol, whereas angiosperms contain coniferyl and sinapyl alcohols in roughly equal proportions. As a biomacromolecule, lignin is unusual in having a complex network-type structure that at a first glance appears chaotic. In fact, it is optically inactive. "Normal" biomolecules, such as cellulose and proteins, generally are synthesized in the active site of an enzyme (or enzyme complex) in nucleotide triphosphate-driven condensations, and the polymers formed have very defined structures. It is unlikely that lignin is synthesized in this way. First, lignin polymerization from monolignols is not a condensation. Second, many different types of bonds connect the monolignol residues in a somewhat random pattern. Third, lignin is racemic, which does not accord with enzyme-controlled biosynthesis.

As far as in vivo formation of lignins is concerned, investigators originally proposed that monolignols were transported into the cell walls and that the only subsequent enzymatic requirement for biopolymer formation was the one-electron oxidation of the monolignols to give the corresponding free radical intermediates. The free radical intermediates formed by oxidation were initially believed to couple together in a manner requiring no further enzymatic control or input. These nonenzymatic free radical coupling reactions were thought to generate dimeric lignan structures that underwent further reoxidation and coupling to yield the lignin biopolymer. Freudenberg [82] demonstrated in a classic work that a polymer with the same types of chemical bonds as those in lignin could be obtained through the oxidation of coniferyl alcohol to resonance-stabilized radicals by a peroxidase. He suggested that the coupling of two unpaired electrons formed the covalent bonds. The polymerization could continue if a phenolic group on the lignin polymer became oxidized to a radical, either by a peroxidase or by a peroxidase-generated monolignol radical, and the phenolic radical on the polymer underwent coupling to a second monolignol radical. According to this model, nature's second most abundant substance is the only natural product for which its formation is not under enzymatic control. However, although it is rarely recognized, natural and synthetic lignins differ in terms of bonding frequency, bonding type, and macromolecular size. For example, for lignins in vivo, the 8–*O*–4′ interunit linkage predominates (more than 50%), with the 8–5′ substructure found in much lower amounts (about 9–12%). In contrast, in synthetic *in vitro* preparations, the 8–*O*–4′ substructure is present to only a very small extent, and the 8–5′ and 8–8′ linkages predominate [83–85]. Currently, there are two models for coupling radicals to produce a functional lignin molecule. One, the random coupling model, which emerged during the early studies on the structure of lignin, centers on the hypothesis that since lignins are not optically active, the optical centers must be generated randomly, that is, under simple chemical control, and then lignin formation proceeds through coupling of individual monolignols to the growing lignin polymer in a near-random fashion. In this view, the amount and type of individual phenolics available at the lignification site and normal chemical coupling properties regulate lignin formation (Fig. 50.5) [86, 87]. The second model, the dirigent protein model proposed by Lewis group, is more recent and suggests that a protein putatively harboring an array of dirigent (monolignol radical binding) sites assembles primary lignin chains. Such chains are then hypothesized to act as templates for replication of the chain. The rationale for this new model is the belief that nature would not leave the formation of such an important molecule as lignin "to chance." It is argued that the only way to explain the high proportion of 8—*O*—4 linkages in lignin would be through regulation by specific dirigent proteins [88, 89].

Suberin is an example of hydroxycinnamic acid-derived polymer that comprises both a poly(aliphatic) and a poly(aromatic) lignin-like domain. Suberized tissues are formed as multilamellar domains consisting of alternating polyaliphatic and polyaromatic layers. The aliphatic domain (aliphatic suberin) consists of a glycerol-based fatty acid–derived polyester that, on transesterification, releases small amounts of p-hydroxycinnamic acid (mainly ferulic) together with aliphatic monomers and glycerol. The major aliphatic constituents are esterified ω-hydroxy fatty acids and 1,ω-dicarboxylic acids. Structural knowledge of the aromatic part is not well established. Although it has been reported that esterified hydroxycinnamic acids are the major constitutive components, a lignin-like polymer of oxidatively cross-linked phenolics has also been postulated for the polyaromatic domain. Suberin is an abundant, complex, intractable plant cell wall polymeric network that forms both protective and wound-healing layers. Suberin is found in specialized plant cell walls (e.g., in dermal tissues of most underground plant organs, the periderm of aerial tissues that undergo secondary thickening, and the Casparian band of the endodermis), where it is laid down between the primary wall and plasmalemma. It is essential for water retention by plants and functions in the overall control of water movement through the apoplastic stream, as well as in providing a physical barrier to opportunistic pathogens. From an evolutionary perspective, suberization was of utmost importance in plant adaptation to living on land and may even have preceded lignifications [90–93].

Finally, melanins are pigments of high molecular weight formed by the oxidative polymerization of phenolic compounds and usually are dark brown or black. They are widely distributed in the living world. In general, they are conjugated polymers of ortho-dihydroxyphenols. The individual residues of polymeric melanin likewise contain two *ortho* oxygens. Melanins are among the most stable, insoluble, and resistant biochemical materials, and they enhance the survival and competitive abilities of organism in certain environments. Melanins constitute a mechanism of defense and resistance to stress such as UV radiations, free radicals, gamma rays, dehydration, and extreme temperatures and contribute to the fungal cell wall resistance against hydrolytic enzymes in avoiding cellular lysis. It has also been shown to chelate metal ions, to function as a physiological redox buffer, to provide structural rigidity to cell walls, and to help to store water and ions. The more general

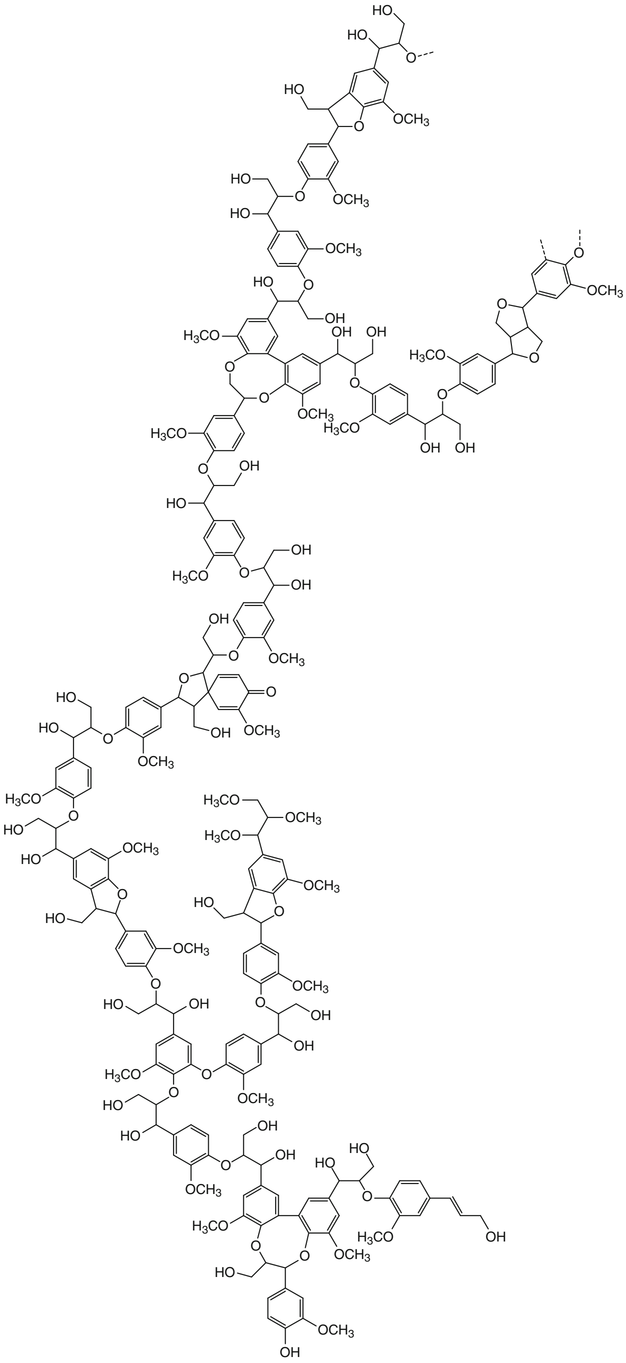
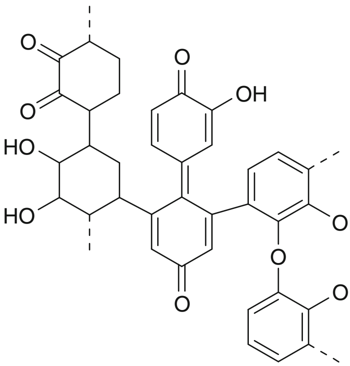


Fig. 50.5 Spruce lignin model (Redrawn from [87])

classification of such compounds, including all their types in pro- and eukaryota, contains three main types of such polymers:

1. Eumelanins (black or brown) that are produced in the course of oxidation of tyrosine (and/or phenylalanine) to 3,4-dihydroxyphenylalanine (DOPA) and dopaquinone, which further undergoes cyclization to 5,6-dihydroxyindole or 5,6-dihydroxyindole- 2-carboxylic acid
2. Pheomelanins (yellow, red, or brown) that are initially synthesized just like eumelanins, but DOPA undergoes cysteinylation, directly or by the mediation of glutathione, then polymerizes
3. Allomelanins (black), the most heterogeneous group of polymers, which emerge through oxidation/polymerization of dihydroxynaphthalene or tetrahydroxynaphthalene, homogentisic acid, γ-glutaminyl-4-hydroxybenzene, 4-hydroxyphenylacetic acid, as well as of catechols

The eumelanins and pheomelanins are found mainly in animal species, whereas the allomelanins are found in plants. For example, they constitute an important part of the black protective coating of many ripe seeds, such as seeds of sunflower and watermelon, and fungi, where they are found primarily in the gills and spores, as in mushrooms or in the black hyphae of molds. The characteristic feature of allomelanins is that they appear to be polymers of simple phenols, such as catechol, and their quinones and are considered as catechol melanins [$(C_6)_n$]. The available evidence indicates that, in the presence of the enzyme polyphenol oxidase (PPOs, EC 1.14.18.1 and EC 1.10.3.1), extensive condensation may occur between catechol units in various stages of oxidation to give polymeric structures in which the monomeric units are joined by C—C and C—O linkages, for example, partial structures such as (**XX**). Broadly, since the polymerization does not follow a precise pattern, as does the synthesis of most other biopolymers, a given sample of melanin contains molecules with various structures; therefore any diagramed structure represents an oversimplification. However, catechol also appears to be the main degradation product of plant allomelanins. The allomelanin of the fungi *Aspergillus niger*, *Ustilago maydis*, and *Daldinia* appears to contain perylene (**XXI**) units derived from 1,8-dihydroxynaphthalene (**XXII**) [94–99].



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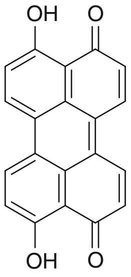
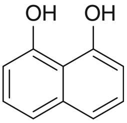


XXI



XXII

Unless they are completely esterified, etherified, or glycosylated, plant phenolics are normally soluble in polar organic solvents. Most phenolic glycosides are water-soluble, but the corresponding aglycones are usually less so. With a few exceptions, water solubility increases with the number of hydroxyl groups present. Some phenolics are solubilized by sodium hydroxide and sodium carbonate, but in alkaline media their oxidation is enhanced, and therefore treatment with alkaline solvents should either be performed under N_2 or, preferably, avoided. Phenolics with only a few hydroxyl groups are soluble in ether, chloroform, ethyl acetate, methanol, and ethanol. Methanol, ethanol, water, and alcohol–water mixtures are most commonly used for dissolving phenolic compounds for analytical purposes. All phenolic compounds exhibit intense absorption in the UV region of the spectrum and those that are colored absorb strongly in the visible region as well. Each class of phenolic compounds has distinctive absorption characteristics. For example, phenols and phenolic acids show spectral maxima in the range 250–290 nm, cinnamic acid derivatives have principal maxima in the range 290–330 nm, flavones and flavonols exhibit absorption bands of approximately the same intensity at about 250 and 350 nm, chalcones and aurones have an absorption peak of high intensity above 350 nm and a much less intense band at 250 nm, and anthocyanins and betacyanins show rather similar absorption in the visible region (475–560 nm and 535–545 nm, respectively) and a subsidiary peak at about 270–275 nm [14, 100–102].

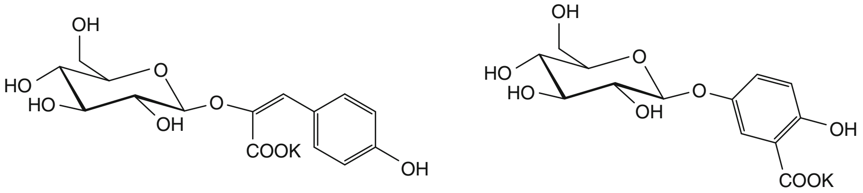
3 Physiological and Ecological Role of Plant Phenolics

The highly ordered interactions between plants and their biotic and abiotic environments have been a major driving force behind the emergence of specific natural products. In this connection, the accumulation of phenolics in plant tissues is considered a common adaptive response of plants to adverse environmental conditions, therefore increasing evolutionary fitness. Plant phenolics are considered to have a key role as defense compounds when environmental stresses, such as high light, low temperatures, pathogen infection, herbivores, and nutrient deficiency, can lead to increased production of free radicals and other oxidative species in plants. A growing body of evidence suggests that plants respond to these biotic and abiotic stress factors by increasing their capacity to scavenge reactive oxygen species. The induction of secondary metabolism gene expression by biotic and abiotic stress is often mediated by integrating signaling molecules such as salicylic acid, jasmonic acid, and their derivatives [8, 103, 104].

Nonfreezing low temperature stress on phenolics metabolism has been considered in several papers. These studies have shown that the phenolic metabolism is enhanced under chill stress and that the behavior of the same metabolism is further dependent on the storage temperature. There is a low critical temperature below which an increase of phenolic metabolism is stimulated, and this temperature is related to the threshold temperature at which chilling injury is also induced. It has been also observed that the low temperature effect involves a cold-induced stimulation of the phenylalanine ammonia-lyase activity (PAL, EC 4.3.1.5) as well as other enzymes important in the phenolic biosynthetic pathway. Cold treatments caused an increase in levels of hydroxycinnamic acid derivatives in artichoke heads (*Cynara cardunculus L.* subsp. *scolymus (L.) Hayek*) as well as of phloretin and quercetin glycosides in Golden Delicious apples (*Malus domestica* Borkh). These responses in phenolic metabolism to low temperature (increase in the activity of the enzymes, as well as in the level of phenolic compounds) could combine with the temperature-dependent phase changes in the cellular membrane, to affect the shelf life of stored fruit and vegetables by providing an adequate substrate to the browning reactions [105–107].

Plant growth depends on the supply of recycled nutrients; external nutrient inputs generally contribute only with a minor proportion to the total requirement. Nutrient mineralization by soil microorganisms is generally viewed as the rate-limiting step in the nutrient cycle, and the factors involved in the control of this process include climate, substrate (litter) quality, and decomposer organisms. Polyphenols, which enter the soil mainly as leachates from above- and belowground parts of plants and/or within above- and belowground plant litter, have been recognized as regulators of soil processes, where it has been suggested that they could control the pool and the form of nutrients available for plants and/or microbes. For example, phenolic compounds can directly affect the composition and activity of decomposer communities, thus influencing the rates of decomposition and nutrient cycling. Different types of soluble polyphenols, such as ferulic acid, gallic acid, or flavonoids, have been found to either stimulate or inhibit spore germination and hyphal growth of saprotrophic fungi. Plant mycorrhizal infection, nutrient uptake, and plant growth can be impaired by specific phenolics released by competitors in a process referred to as allelopathy [10]. Furthermore, plants depend on the ability of roots to communicate with microbes. The converse is also true; many bacteria and fungi are dependent on associations with plants that are often regulated by root exudates. For example, isoflavonoids and flavonoids present in the root exudates of a variety of leguminous plants activate the rhizobium genes responsible for the nodulation process and might be responsible for vesicular–arbuscular mycorrhiza colonization. Nodule formation is initiated by the host plant roots exuding phenolic flavonoid compounds into the rhizosphere. The exudate partly determines the specificity of the symbiotic relationship as each rhizobia species responds to specific flavonoids. The flavonoid perception attracts the bacteria to the root and activates the rhizobia *nod* (nodulation) gene expression, leading to the production and secretion of strain-specific lipo-chito-oligosaccharides, also known as nod factors (NF). NFs have an oligosaccharide backbone of *N*-acetyl-D-glucosamine units with a fatty acyl group attached to the nonreducing sugar. A major determinant of host–symbiont specificity is attributed to the different NF substituents attached to the oligosaccharide backbone. The presence of compatible rhizobia species and their corresponding NF is generally sufficient to trigger nodule development [108–111].

In general, plants are rooted and are unable to demonstrate mobility. However, a variety of plants are able to move in certain ways. Some plants are known to open their leaves in the daytime and "sleep" at night with their leaves folded. This circadian rhythmic leaf movement known as nyctinasty is widely observed in



Potassium lespedezate
(Leaf-opening compound)

Potassium 5-*O*-β-D-glucopyranosyl gentisate
(Leaf-closing compound)

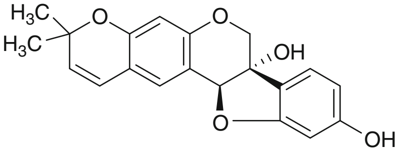
Fig. 50.6 Leaf-movement factors from nyctinastic plants

leguminous plants. Nyctinastic leaf movement is induced by the swelling and shrinking of motor cells in the pulvinus, an organ located in the joint of the leaf. A flux of potassium ions across the plasma membranes of the motor cells is followed by a massive water flux, which results in the swelling and shrinking of these cells. At the heart of such a mechanism is the regulation of the opening and closing of the potassium channels involved in the nyctinastic leaf movement, a process which is under metabolic control. Many attempts have been made to isolate the endogenous bioactive substances that control nyctinasty. It has been found that nyctinastic plants have a pair of endogenous bioactive substances that control the nyctinastic leaf movement. One of these is a leaf-opening factor that “awakens” the plant leaves, and the other is a leaf-closing factor that reverses this process so that the plant leaves “sleep.” Figure 50.6 shows a pair of bioactive substances showing a phenolic structure. Five sets of leaf-closing and -opening factors in five different nyctinastic plants have been identified. All the leaf-opening factors have a common structural feature, the *p*-coumaroyl moiety, and this result suggests that this structural feature would be deeply involved in the common mechanism for leaf-opening [112–114].

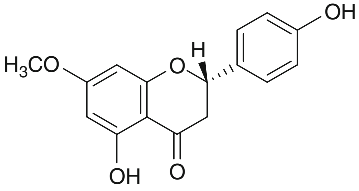
Anthocyanins represent a class of flavonoids providing the red and blue/purple colors familiar in many flowers and fruits. These compounds are synthesized as visual cues, to attract pollinators and other animals for seed dispersal, as well as molecular cues protecting plants from various stress conditions, and are stored in the acidic vacuole of specialized cells. Anthocyanins are also the pigments responsible for spectacular displays of variable red to reddish-orange color in the leaves of deciduous trees. Leaf color change is not simply a side effect of leaf senescence, and, in the past decade, several hypotheses have emerged to explain the evolution of autumn colors. Autumn colors are due mainly to carotenoids (yellow-orange) and anthocyanins (red-purple). Although carotenoids are present all year round in the leaves, they are masked in mature leaves by the green of chlorophyll; in autumn, they become visible because of the breakdown of green chlorophyll molecules into colorless metabolites, but there is no evidence for a de novo synthesis. Anthocyanins, by contrast, are newly generated in autumn, shortly before the leaf fall. Thus, red is produced actively in autumn and is not simply the side effect of leaf senescence. Why leaves that are about to be shed turn red? The adaptive value of the autumn colors of leaves is still a matter of controversy. Red may protect the leaf from the damaging effects of light at low temperatures (photoinhibition and photooxidation), allowing a more efficient resorption of nutrients, especially nitrogen (“photoprotection theory”). Alternatively, red might be a warning signal of the status of the tree (indicating high levels of defenses or low nutritional capacity) to animals, particularly feeding insects like aphids (“coevolution theory”). During winter, the combination of cold, dry, and bright sunlight conditions can result in excess energy capture relative to processing, photoinhibition of photosynthesis, formation of ROS, and greater photooxidative damage. Red pigments are thought to alleviate these stress factors by intercepting green sunlight (light attenuation) and/or neutralizing ROS directly as antioxidants. On the other hand, recent studies on autumn and juvenile leaves suggest that red pigments reduce the leaf damage either by making leaves less palatable or less visible to animals lacking a red visual receptor (camouflage) or by signaling a low leaf quality. According to the coevolution theory, red is a signal of the status of the tree to insects that migrate to (or move among) the trees in autumn. Migrating insects avoid red leaves and colonize preferentially green leaves. Trees with red leaves have better chemical defenses or a worst nutritional capacity that induces a lower fitness in the insects. In this scenario, therefore, color and preference coevolve in an arms race: autumn colors are an adaptation of the trees to reduce their parasite load, and insect preference for green is an adaptation to find the most suitable host trees [115–120].

Plants encounter numerous pests and pathogens in the natural environment. An appropriate response to the attack by such organisms can lead to tolerance or resistance mechanisms that enable the plant to survive. Resistance mechanisms refer to traits that inhibit or limit attack, while tolerance strategies do not limit attack but reduce or offset consequences on the plant fitness by adjusting its physiology to buffer the effects of herbivory or diseases. In this connection, plants produce a broad range of phenolic metabolites that serve a dual function of both repelling and attracting different organisms in the plant’s surroundings. The role of plant phenolics in chemoecology, especially on the feeding behavior of herbivores, has been recognized since 1959 when Fraenkel [121] described phenolic compounds as “trigger” substances, which induce or prevent the uptake of nutrients by animal herbivores. They act as protective agents, inhibitors, natural animal toxicants, and pesticides against invading organisms, that is, herbivores, nematodes, phytophagous insects, and fungal and bacterial pathogens [54, 122, 123]. Preformed antibiotic compounds that occur constitutively in healthy plants are likely to represent inbuilt chemical barriers to herbivorous and fungal enemies and may protect plants against attacks by a wide range of potential pests and pathogens. In contrast, induced defense compounds are synthesized in response to biotic stresses as a part of the plant defense response. These induced defense mechanisms are expressed at the site of attack (hypersensitive response) as well as at a distance (signaled by salicylates, jasmonates) from the site of primary infection and protect the plant from the spread of infection and future attacks. Induced resistance is regulated by a network of interconnecting signal transduction pathways in which phenolic acids are key signaling molecules [124, 125].

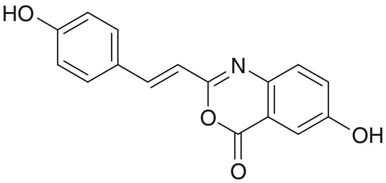
In an environment rich in potentially harmful microbes, plant survival depends on an efficient microbe perception and fast defense responses. Plant immunity relies on the ability of each cell to recognize pathogens. A first level of microbe recognition is performed by membrane proteins termed pattern recognition receptors (PRRs), which perceive the molecular signatures characteristic of a whole class of microbes, termed pathogen-associated molecular patterns (PAMPs). Phenolics are synthesized when plant PRRs recognize potential pathogens by conserved PAMPs, leading to a PAMP-triggered immunity [126–132]. The plant's recognition of pathogens induces its endogenous multicomponent defense system. The multicomponent defense response induced after the pathogen attack requires a substantial commitment of the cellular resources, including extensive genetic reprogramming, because the induced expression of a large number of defense-related genes is essential for plants to counter the pathogen attack. Many defense-related genes encode enzymes that catalyze defense metabolites, known as phytoalexins, for example, antimicrobial, low-molecular-weight secondary metabolites that are both synthesized by and accumulated in plant cells as a result of the interaction between the metabolic systems of the host and a fungal parasite. The structures of the phytoalexins are often unique at the family level: the majority of phytoalexins produced by the members of the family Leguminosae are isoflavonoids, and the commonest isoflavonoid subclass is the pterocarpans such as medicarpin and glyceollin II (**XXIII**), whereas phytoalexins from Vitaceae seem to constitute a rather restricted group of molecules belonging to the stilbene family, the skeleton of which is based on the *trans*-resveratrol structure (3,5,4′-trihydroxystilbene). Dicotyledonous species represent the majority of plants from which such compounds have been identified. Some monocotyledonous species producing phenolic phytoalexins are rice (*Oryza sativa*) (sakuranetin, **XXIV**), oat (*Avena sativa*) (avenalumin I, **XXV**), and sorghum (*Sorghum bicolor*) (3-deoxyanthocyanidins). Sorghum phytoalexins have been identified as 3-deoxyanthocyanidins: luteolinidin, 5-methoxyluteolinidin, apigeninidin, caffeic acid ester of arabinosyl 5-*O*-apigeninidin (**XXVI**), and 7-methoxyapigeninidin [36, 54].



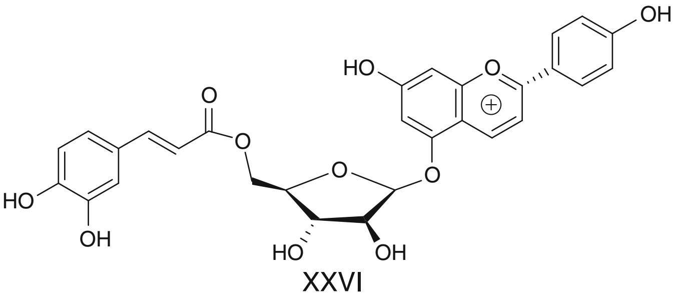
XXIII



XXIV



XXV



XXVI

The ecological relationship between plants and insects is a complex one with physical as well as chemical interactions. This relationship is also affected by plant factors, insect factors, and by some insect–plant factors, including hypersensitive reaction and plant resistance to insect-borne diseases. Plant constituents that make unpalatable a host are secondary metabolites in sufficient concentration to exert an undesirable physiological effect. It is now generally accepted that plant phenolics play a role in protecting plants from insects. For example, some cotton flavonoids are feeding stimulants for the boll weevil, *Anthonomus grandis* [133], or oviposition stimulants of a *Citrus*-feeding swallowtail butterfly, *Papilio xuthus* L. [134] or, finally, antibiotics effective against phytophagous insects [135]. Tannins, also, may affect the growth of insects in three main ways: they have an astringent taste which affects palatability and decreases feed consumption; they combine with proteins to form complexes of reduced digestibility; and they act as enzyme inactivators. Recent work by Raymond Barbehenn and coworkers about tannin oxidation in insects suggests that tannin activity cannot be explained quite this simply, as tannin oxidation should also be taken into account as a defense mechanism for plants [136–138].

4 Carbon Fluxes from the Primary to the Secondary Metabolic Pathways

Chemical defenses represent a main trait of the plant innate immune system. Besides regulating the relationship between plants and their ecosystems, plant phenolics are involved both in resistance against pests and pathogens and in tolerance toward abiotic stresses. Both biotic and abiotic stresses stimulate carbon fluxes from the primary to the secondary metabolic pathways, thus inducing a shift of the available resources in favor of the synthesis of secondary products, for example, reallocation of host resources. Therefore, defensive chemicals are considered to be costly for plants, reducing the fitness of the host in the absence of disease, because resistance genes might impose metabolic costs on plants (e.g., lower growth rates than their sensitive counterparts) [18]. When resistant genotypes have relatively low fitness in the absence of pests, it is often assumed that their poor performance must be explained by the energetic drain involved in building and maintaining a chemical or structural defense, metal hyperaccumulation, and temporal or spatial escape: all these mechanisms include allocation costs (the costs due to diversion of limited energy and resources away from primary metabolism), storage costs, ecological (i.e., environmental) costs (negative effects of resistance on one of the myriad of interactions between a plant and its environment that affect a plant's fitness under natural growing condition), etc. One way for a plant to reduce these costs is to synthesize defense compounds only after there has been some degree of initial damage by a pathogen or insect: this strategy is inherently risky because the initial attack may be too rapid or too severe for an effective defense response. Therefore, plants that are likely to suffer frequent and/or serious damage may be better off investing mainly in constitutive defenses, whereas plants that are attacked rarely may rely predominantly on induced defenses [139–142].

The negative correlation between concentrations of secondary metabolites and plant growth rate is assumed to indicate a trade-off between plant growth and the production of defensive compounds. Plants, in fact, have limited resources to support their physiological processes; hence, all requirements cannot be met simultaneously and more carbon is diverted from growth toward secondary metabolism when plant growth is restricted by any physiological and/or ecological constraint.

An interesting link between primary and secondary metabolism has been recently proposed by Lattanzio et al. [143] which couples the accumulation of the stress metabolite proline with the energy transfer toward phenylpropanoid biosynthesis via the oxidative pentose phosphate pathway [144] (Fig. 50.7). In many plants, free proline accumulates in response to the imposition of a wide range of biotic and abiotic stresses, such as water deprivation, salinization, high/low temperature stress, heavy metal toxicity, pathogen infection, nutrient deficiency, atmospheric pollution, and UV irradiation. In this connection, it has been also suggested that the value of stress-induced proline accumulation may be mediated largely *via* the effects of its synthesis and degradation on cellular metabolism [145–148]. Proline synthesis is accompanied by the oxidation of NADPH. An increased $NADP^+$/NADPH ratio is likely to enhance the activity of the oxidative pentose phosphate pathway providing precursors for the phenolic biosynthesis via the shikimic acid pathway. The alternating oxidation of NADPH by proline synthesis and reduction of $NADP^+$ by the two oxidative steps of the oxidative pentose phosphate pathway serve to link both pathways and thereby facilitate the continuation of high rates of proline synthesis during stress and lead to a simultaneous accumulation of phenolic compounds.

Furthermore, it has been also shown that an exogenous application of proline to the nutrient medium of *in vitro* grown oregano plants elicited the accumulation of rosmarinic acid and other phenolic compounds in that plant. This suggests that mitochondrial proline oxidation could drive the oxidative pentose phosphate pathway by recycling glutamic acid into the cytosol to generate a proline redox cycle.

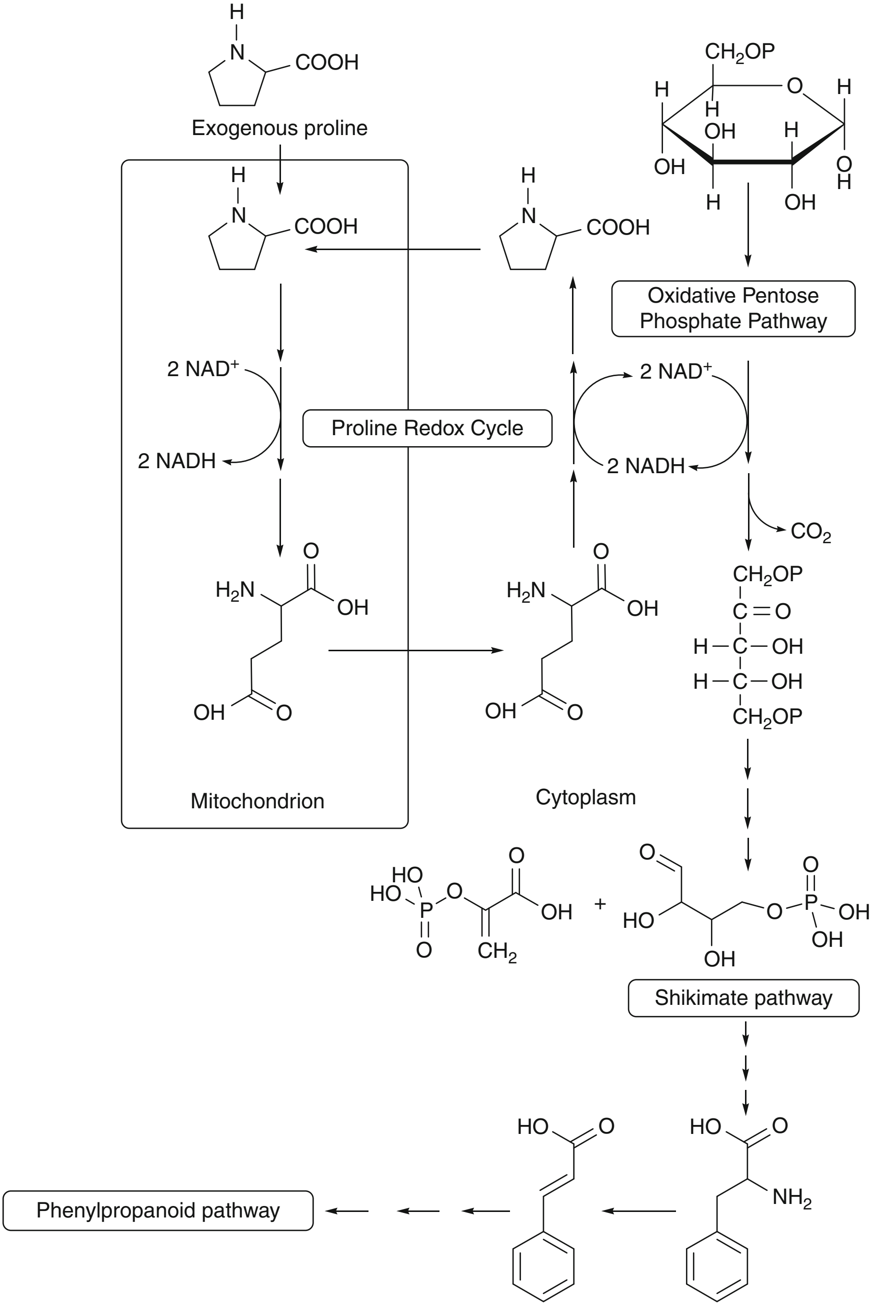

Exogenous proline
COOH
Oxidative Pentose Phosphate Pathway
2 NAD+
2 NADH
Proline Redox Cycle
CO2
CH2OP
Mitochondrion
Cytoplasm
Shikimate pathway
Phenylpropanoid pathway
NH2

Fig. 50.7 Relationship among proline redox cycle, oxidative steps of cytosolic pentose phosphate pathway, and phenylpropanoid pathway

5 Conclusions

(Poly)phenols are plant secondary metabolites that constitute one of the most common and widespread groups of substances in plants. They constitute a large reservoir of natural chemical diversity that encompasses an enormous range of compounds and enzymes and a wide spectrum of mechanisms of gene regulation and of transport of metabolites and enzymes. Levels of phenolic compounds in plants are both environmentally induced as well as genetically controlled. Despite a basic knowledge of the main biosynthetic pathways, some open questions, such as the biosynthesis, the intracellular transport, and functions of plant phenolics, need additional study. Until now, it appears that the phenolic patterns of higher plants have been shaped by a dialog between plants and their environment for the benefits of plants and their better adaptation to external conditions. Plants adapt themselves their phenolic patterns to a changing environment through the emergence of new genes brought about by gene duplication and mutation and subsequent recruitment for adaptation to specific functions. Plants also have the ability to synthesize specific chemical compounds which can act as toxins and deterrents to pathogens/herbivores and other competitors and are also able to attract needed symbionts for procreative purposes. Primary metabolism is an important source of precursors for the synthesis of secondary phenolic metabolites. Central metabolism requires high levels of limited plant resources, and during intense growth, the synthesis of phenolic metabolites may be substrate- and/or energy-limited. On the other hand, either abiotic or biotic stresses divert substantial amounts of substrates from primary metabolism into secondary defensive product formation, and this could lead to constraints on growth. Adjustments in resource allocation are the major mechanism by which plants respond to environmental constraints. The allocation pattern of a plant defines its ecological roles and is therefore an important factor in understanding plant distribution and adaptation. On the other hand, as far as the development of a new strategy to enable the production of useful secondary metabolites on a commercial scale is concerned, any progress made in the basic understanding of metabolic pathways and regulatory mechanisms may be addressed to exploit the plant cell and tissue culture potentials to produce food additives, such as antioxidant phenolics.

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51 Metabolic Engineering for Functional Foods: Tomato Fruits and Stilbenes

Giovanna Giovinazzo, Ilaria Ingrosso, Marco Taurino, and Angelo Santino

Contents

1 Introduction 1582
1.1 Overview on Genetics, Biochemistry, and Molecular Biology of Stilbene Synthase 1582
1.2 Stilbenes Functions in Plants and in Humans 1583
2 Resveratrol Synthesis in Transgenic Tomato Fruits 1585
2.1 Biochemical Analysis of Flavonoids in Fruit 1587
3 Antioxidant Parameters 1588
3.1 Effect of Resveratrol Synthesis on the Level of ASA and GSH in Green and Red Tomato Fruits 1588
3.2 Antioxidant Capability 1589
4 Anti-inflammatory Activity of Resveratrol-Producing Tomato 1590
5 Effects of Stilbene Synthase Expression on Reproductive Development of Tomato 1591
6 Concluding Remarks 1593
References 1594

Abstract

The metabolic engineering of plants offers the opportunity to change the content of specific phytonutrients in plant – derived foods.

The plant polyphenol *trans*-resveratrol (3, 5, 4′-trihydroxystilbene), mainly found in grape, peanut, and other few plants, displays a wide range of biological effects. Numerous in vitro studies have described various biological effects of resveratrol. In order to provide more information regarding absorption, metabolism, and bioavailability of resveratrol, different research approaches

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have been performed. In recent years, the induction of resveratrol synthesis in plants which normally do not accumulate such polyphenol has been successfully achieved by molecular engineering.

In this context, the ectopic production of resveratrol could have positive effects on the enhancement of the nutritional value of several widely consumed fruits and vegetables. This chapter focuses on the latest findings regarding on resveratrol bioproduction in tomato (*Solanum lycopersicum*) fruits.

Keywords
Biological activity • Functional foods • Metabolic engineering • *Solanum lycopersicum* • Stilbenes

Abbreviations

ASA	Ascorbate
CHS	Chalcone synthase
DHA	Dehydroascorbate
GSH	Glutathione
GSSG	Glutathione disulfide
ROS	Reactive oxygen species
STS	Stilbene synthase

1 Introduction

Because of resveratrol capacity to confer disease resistance in grapevine and given its clinically useful, most interest has centered, in recent years, on STS gene transfer from grapevine to the genome of numerous plants, with the objective of increasing their tolerance to pathogenic microorganisms and/or improving the of food products through the expression of resveratrol in plants incapable of synthesizing this compound. Studies have been published describing STS-encoding genes isolated from grapevine or other plant species and their use for plant genetic transformation. This chapter explores recent research on the gene transfer of grape STS-encoding genes to tomato plants.

1.1 Overview on Genetics, Biochemistry, and Molecular Biology of Stilbene Synthase

Bioactive ingredients of functional foods help to overcome nutritional deficiencies, and the demand of healthy food products will certainly increase in the future. Plant secondary metabolites are nutritional components of our daily diet.

Stilbenes are small naturally occurring phenolic compounds found in a wide range of plant – derived food – among which berries are important sources [1, 2]. Resveratrol is biosynthetically correlated to stilbenes, but its biosynthesis is restricted to only a few plant species commonly used for human consumption [3–9].

The most abundant levels of naturally occurring resveratrol are found in the roots of Japanese *Polygonum cuspidatum* which has been used in traditional Asian herb medicine, for hundreds of years, in the treatment of inflammation [5]. Grape (*Vitis vinifera* L.) is probably the most important source of resveratrol, since the compound is also found in wine. The economic importance of grapevine has encouraged many researchers to study the physiological and molecular basis of berry physiology, particularly those processes affecting wine quality [10]. In this species, a large array of STS genes has been identified thus suggesting the importance of stilbene metabolism [11].

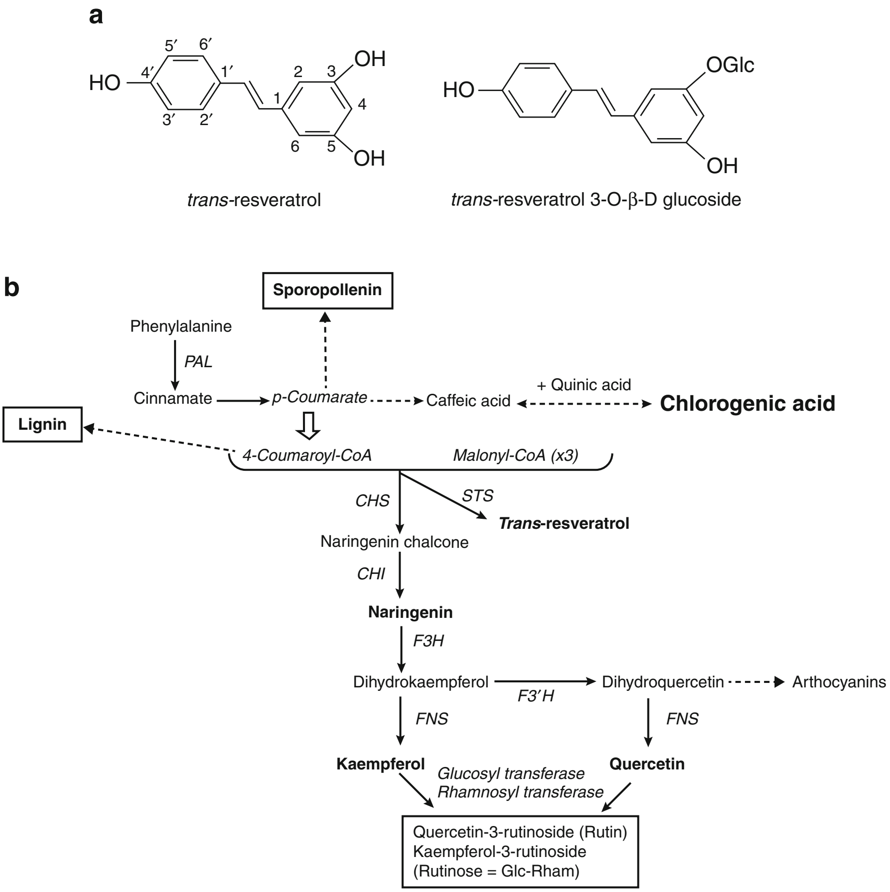
Resveratrol and its 3-*O*-β-D-glucoside derivative share a chemical structure similar to that of other polyphenol-type stilbenes (Fig. 51.1a) and are controlled by the key enzyme, stilbene synthase (STS), which belongs to a multigene family of the type III group of the polyketide synthase superfamily [3, 4]. STS catalyzes the condensation of three molecules of coumaroyl-CoA to form resveratrol. Synthesis of resveratrol takes place in a single enzymatic step with CoA-esters of cinnamic acid derivatives and 3 malonyl-CoA units as starting blocks (Fig. 51.1b). STS and chalcone synthase (CHS) that are key enzymes of the flavonoid pathway share a high degree of homology and competing for the same substrates.

1.2 Stilbenes Functions in Plants and in Humans

In nature, the most abundant form of resveratrol would appear to be the 5,3,4′-dihydroxystilbene-3-*O*-*β*-D-glucopyranoside [12]. Resveratrol exists in two stereoisoforms with *cis*- or *trans*-configuration, the latter being the most widely studied, although *cis*-resveratrol may also possess health-promoting properties. The number as well as the position of moieties play an important role in the biological activity of the compound [13].

Synthesis of resveratrol in plants tissues is either constitutive or inducible being strongly enhanced by fungal attacks, UV irradiation, and other environmental stress conditions [3–5].

Resveratrol possesses numerous important bioactivities including anti-inflammatory, antioxidant, anti-aggregatory functions, and modulation of lipoprotein metabolism [14]. It has also been shown to possess chemopreventive properties against certain forms of cancer and cardiovascular disorders [15]. Subsequent work has shown that resveratrol extends the life spans of lower eukaryotes [16–18]. In mice, long-term administration of resveratrol-induced gene expression patterns that resembled those induced by calorie restriction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Caffeic acid
+ Quinic acid
Chlorogenic acid
Lignin
4-Coumaroyl-CoA
Malonyl-CoA (x3)
CHS
STS
Trans-resveratrol
Naringenin chalcone
CHI
Naringenin
F3H
Dihydrokaempferol
F3′H
Dihydroquercetin
Arthocyanins
FNS
FNS
Kaempferol
Glucosyl transferase
Rhamnosyl transferase
Quercetin
Quercetin-3-rutinoside (Rutin)
Kaempferol-3-rutinoside
(Rutinose = Glc-Rham)

Fig. 51.1 Chemical structures of stilbenes (**a**); flavonoids biosynthetic pathway (**b**). The expression of a stilbene synthase gene in transgenic plants competes with substrates used in the first step of the complex route of flavonoids

and delayed aging-related deterioration [19]. Resveratrol also decreased insulin resistance in type 2 diabetic patients [20], suggesting that the pathway targeted by resveratrol might be important for developing therapies for type 2 diabetes.

An important mediator of the metabolic effects of resveratrol [21, 22] is the peroxisome proliferator-activated receptor g coactivator, PGC-1α [23]. Consistent with the known ability of Sirt1 to deacetylate and activate PGC-1α [24, 25], resveratrol increased Sirt1 and PGC-1α activity in mice fed a high-fat diet [21, 22].

AMPK, an emerging key regulator of whole-body metabolism, has been shown to activate Sirt1 and PGC-1α [26–28]. Recently it was shown that AMPK-deficient mice are resistant to the metabolic effects of resveratrol, providing evidence that AMPK is a key mediator of the metabolic benefits produced by resveratrol [27]. Resveratrol directly inhibits cAMP-specific phosphodiesterases and the cAMP effector protein Epac1, which leads to the activation of AMPK and Sirt1 [27].

2 Resveratrol Synthesis in Transgenic Tomato Fruits

Plant metabolic engineering has provided a means to improve polyphenol composition and levels. For example, tomato plants with enhanced flavonols have been recently developed through the overexpression of a transcription factor able to activate flavonol biosynthesis [30–32].

STS structural genes have been transferred to a number of crops, either to improve the resistance to stresses or the nutritional value of the plant (for a review see ref. [33] and Table 51.1).

Few articles reported on the production of stilbenes in transgenic tomato, suggesting that it is indeed possible to introduce new branches of the flavonoid pathway, at least at its first step, by introducing foreign structural genes [34–36]. In order to further explore the possibilities of flavonoid engineering in tomato fruits, some authors [35] have targeted this pathway toward classes of flavonoids, which are normally not present in tomato. Using structural genes from several plant sources and combinations thereof, they were able to produce transgenic tomatoes accumulating stilbenes, deoxychalcones, or flavones. These fruits displayed altered antioxidant profiles and an up to threefold increase in total antioxidant activity of the fruit peel (Table 51.1).

Transgenic tomato plants (*Solanum Lycopersicum*, cv money maker), expressing stilbene synthase gene under constitutive 35S, and mature fruit-specific promoter (*TomLoxB*) were obtained by genetic transformation through *Agrobacterium* infection of cotyledons [34]. The phenotype of all transformed lines was similar to that of the wild-type plants, showing a regular development, flowering, and fruit maturation. However, high resveratrol-producing 35SS tomato fruits were seedless, whereas low resveratrol LoxS fruit showed a normal seed set, comparable to wild type (Fig. 51.2) [38]. Protein analyses revealed that the highest accumulation of stilbene synthase was at green stage in constitutively transformed 35SS tomato line [34] and at the red mature stage in the LoxS line [37]. Resveratrol accumulation showed some quantitative differences since the levels recorded in the LoxS were about 20 times lower than those found in 35SS fruits (Fig. 51.3a and b, respectively).

Qualitative and quantitative comparisons between the different transgenic plants synthesizing resveratrol and related stilbenes are difficult, as different analytical methods are used to assay these compounds. These analyses have shown various stilbene levels and spatiotemporal distributions, leading to a considerable variability in terms of relative amounts of different forms.

In particular, the glycosylation of polyphenolic compounds occurs frequently in plant to protect the cell from their potential toxic effects and to prevent their

Table 51.1 Stilbene synthase gene expression in transgenic plants for quality improvement

Plant species	Gene	μg/g	Biological activity	References
Tomato (*Solanum lycopersicum* L.)	*StSy*	53 (in fruit tissues)	Increased antioxidant capability	[34]
	STS	30 (in fruit tissues)	Food quality improvement	[35]
	StSy	50–120 (in fruit tissues)	Modulation of other polyphenols	[36]
	StSy	10–120 (in fruit tissues)	Increased antioxidant and anti-inflammatory capabilities and male sterility	[56]
		50–180 (in flower tissues)		[55]
Rapeseed (*Brassica napus* L.)	*Vst1*	361–616	Food quality improvement	[57]
Strawberry (Fragaria axananassa)	*NS-Vitis3*	–	–	[52]
Hop (*Humulus lupulus* L.)	*Vst1*	490–560	Modulation of other polyphenols	[58]
Orobanche (*Rehmannia glutinosa* Libosch)	*AhRS3*	22–116 Up to 650 (under stress)	Antioxidant capability	[59]

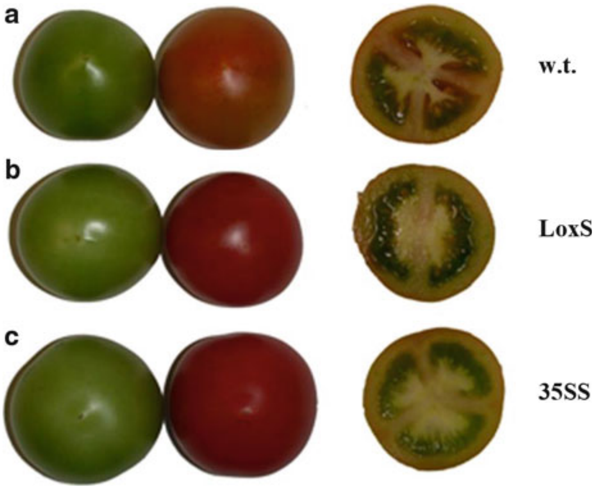


Fig. 51.2 Tomato fruits of wild-type (**a**) LoxS (**b**) and 35SS transformed line (**c**). The 35SS fruits were seedless and in some cases showed the presence of few aborted seeds

oxidation and enzymatic degradation [13]. In the case of resveratrol, the free compound is first synthesized, before being glycosylated by endogenous glycosyl-transferases. Free resveratrol and its glycosylated forms have been both detected in transgenic plants [13]. Stilbenes content also depends strongly upon plant species, probably on account of different endogenous pools of enzymes or

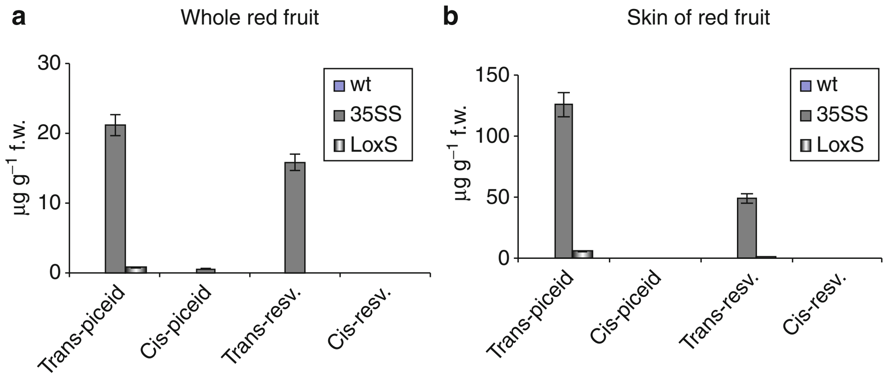


Fig. 51.3 Stilbenes accumulation in 35SS and LoxS whole mature red fruits (**a**) and skin (**b**). The amount of *trans*-resveratrol, *trans*-piceid, and their correspondent *cis*-forms were simultaneously detected on the basis of their retention time and UV spectra of the additional peaks observed after UV irradiation at λ: 366 nm of *trans*-resveratrol and *trans*-piceid, respectively

precursors, as well as differences in secondary metabolic pathways. In resveratrol synthesizing tomato lines, the free to glycosylated resveratrol ratio was related to the fruit ripening stage [34]. Furthermore, these related compounds accumulate differentially in different fruit tissues at the mature stage [36].

The comparison of different resveratrol levels in LoxS and 35SS fruits allowed the authors to study the effect of the differential depletion of substrates and, in the meantime, to clarify the effect of a novel metabolite as resveratrol on related and unrelated pathways committed to secondary metabolism.

2.1 Biochemical Analysis of Flavonoids in Fruit

The main flavonoids which had accumulated in tomato fruits were naringenin and rutin (quercetin-3-rutinoside), predominantly detected in the peel [43, 44].

Both these compounds, together with kaempferol-rutinoside (Kae rut), were quantified in the peel extracts from both the resveratrol synthesizing tomato lines (35S and LoxS lines; Fig. 51.4).

In order to clarify whether the novel biosynthetic pathway introduced in transgenic tomato caused competition for the utilization of the common substrates, levels of main flavonoids in tomato fruit were analyzed. The overall levels of these flavonoids were not impaired in either the transgenic lines. On the contrary, the 35SS line showed an increased level of Kae rut, whereas the LoxS line showed an increased naringenin content. The levels of the other flavonoids did not show any significant differences in comparison with the control tomato line. As far as the other soluble phenolics are concerned, HPLC analysis indicated that significant differences in coumaric and cinnamic acids content, which decreased in 35SS tomato fruit in all the ripening stages [34, 37].

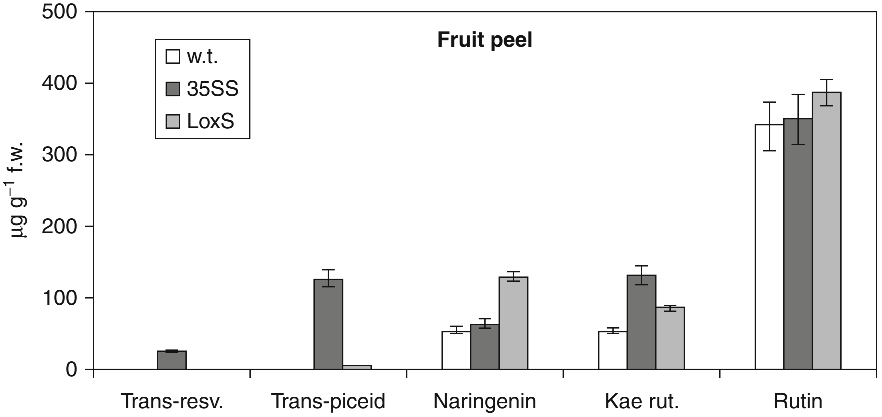


Fig. 51.4 Comparison of flavonoid levels in wild type and transgenic skin of fruit at the *red* stage of ripening. Naringenin levels (λ:.290 nm) in the wild type and transgenic skin (of almost three) red fruits. Outline of flavonols biosynthesis and profiles of accumulation of rutin and kaempferol-rutinoside (λ:.370 nm) in skin of transgenic tomatoes compared with those of wild type

3 Antioxidant Parameters

Fruit ripening has described as an oxidative phenomenon which requires a turnover of ROS, such as H_2O_2 and superoxide anion. A balance between the production of ROS and their removal by antioxidant systems has been proposed. It is likely therefore that the antioxidant system will play a crucial role in the ripening process.

3.1 Effect of Resveratrol Synthesis on the Level of ASA and GSH in Green and Red Tomato Fruits

The production of resveratrol in transgenic plants increased the levels of ASA and GSH in edible tissues thus improving the nutritional values of tomato fruit (Fig. 51.5a).

Given the importance of ROS in the ripening process, the authors have examined the changes in some oxidative parameters and in the antioxidant system during transgenic tomato fruit ripening. It is worth noting that the highest ASA and GSH contents were detected in fruits at green and red stage of ripening concomitantly to the highest level of resveratrol. In ripening tomato fruits, a transient increase in ROS production occurs during the intermediate phases of maturation. The relevance of antioxidant enzymes and metabolites during ripening has been widely investigated [45]. The acquisition, in tomato fruits, of a new biosynthetic pathway leading to the synthesis of resveratrol, which, as other phenolic compounds, has antioxidant properties, induces an increase in the ASA and GSH pools in the transformed fruits [34, 37]. In the red phase, the transformed plants (both 35S and LoxS) had ascorbate content higher than wild type. Therefore the ex novo synthesis

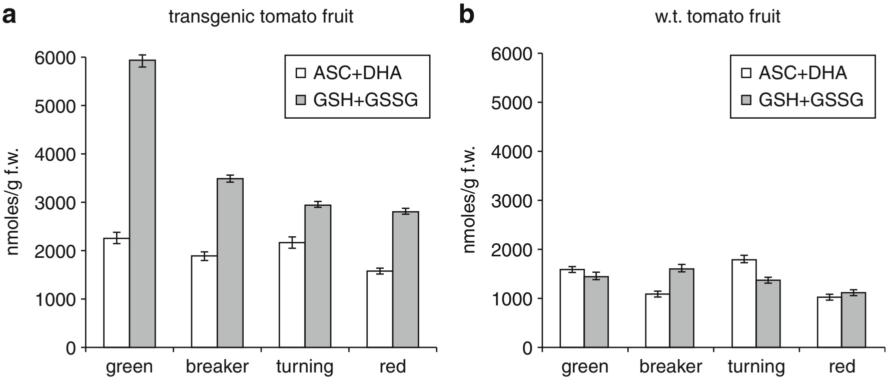


Fig. 51.5 Behavior of the ascorbate and glutathione redox pairs during tomato fruit maturation in transgenic 35SS (**a**) and wild type (**b**). Ascorbate pool (ascorbate plus dehydroascorbate) and glutathione pool (glutathione plus glutathione disulfide) were analyzed in the four different stages of fruit ripening

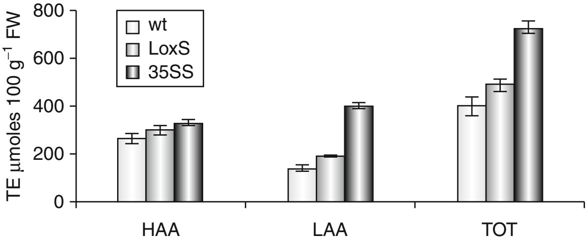
of resveratrol affected the biosynthesis/storage of other antioxidants although these molecules do not have any biosynthetic correlation. Interestingly, only the 35SS fruits, in which stilbene synthase was constitutively expressed, showed increased ascorbate level and enzyme activities altered in the green phase (data not shown). This was in agreement with the fact that stilbene synthase was under the control of *spatio*temporal-specific promoter (LoxS), which allowed gene expression preferentially in the late phase of fruit maturation [36, 37].

In transformed fruits, the increase in GSH levels was much higher than that observed in ASA levels at all the ripening stages analyzed, and the GSH redox state was at least twice that of the control [34, (Fig. 51.5b)]. The increase of glutathione biosynthesis could be a consequence of the presence, in the transformed cells, of the foreign molecules. Indeed, besides a role in ROS scavenging, GSH is also the co-substrate of glutathione transferase and is involved in phenol and xenobiotic conjugation and in their segregation into the vacuole [46, 47]. On the other hand, plant transformation with the STS gene does not seem to affect the level of membrane-associated molecules mainly present in the plastids, since their levels do not significantly differ in either transformed or non-transformed fruits, at any stage of ripening [34]. Since ASA and GSH are soluble antioxidants widely distributed in all cellular compartments, the different effect of resveratrol on these two types of metabolites could be the effect of their different cellular localizations.

3.2 Antioxidant Capability

In transgenic tomato, resveratrol synthesis was able to increase the overall antioxidant properties of the fruit, as well as the ascorbate/glutathione content with

Fig. 51.6 Antioxidant power of mature fruit of wild type, LoxS and 35SS tomato fruits. Antioxidant capability of hydrosoluble (HAA) and liposoluble (LAA) fraction of whole mature fruit



a consequent two to threefold increase in antioxidant activity of fruits and a correlation was found between resveratrol concentrations and antioxidant capacities in the ripening stages accumulating high resveratrol levels [34].

The higher antioxidant capability of resveratrol synthesizing fruits, compared to wild type, has also been confirmed by the analysis of total antioxidant activity (Fig. 51.6). Resveratrol contributes to such an increase since both hydrosoluble and liposoluble fractions have higher antioxidant activities in the transformed in comparison with wild-type fruits, whereas ASC and GSH only affect the hydrosoluble fractions. Interestingly, the increase in the antioxidant activity of the fruits affects lipid peroxidation, the value of which is significantly lower in transformed fruits in respect to that in wild types [34].

4 Anti-inflammatory Activity of Resveratrol-Producing Tomato

Several studies have reported that resveratrol has cardioprotective effects because of its ability to increase plasmatic antioxidant capacity, inhibit platelet aggregation and coagulation, reduce low-density lipoprotein oxidation, and suppress the pro-inflammatory response [48]. The anti-tumorigenic, anti-inflammatory, and cardioprotective effects of resveratrol seem to be related, at least in part, to its ability to suppress prostaglandin production through its interference with the expression and activity of COX-2, the rate-limiting enzyme in prostaglandin biosynthesis. In particular, macrophages are prominent producers of prostaglandin during inflammatory processes in response to signals that trigger macrophage activation, such as bacterial lipopolysaccharide or phorbol ester [49].

Altered COX-2 levels and consequent abnormally high prostaglandin secretion are thought to be involved in diverse pathological processes, and COX-2-specific inhibitors represent important challenges for cancer treatment, as well as chronic inflammatory diseases such as atherosclerosis [50]. The effects of resveratrol-enriched tomato extracts on COX-2 expression induced by phorbol ester in monocyte–macrophage U937 cells indicated that resveratrol reduces the level of the inducible, but not constitutive, COX isoform, thus confirming and expanding the anti-inflammatory activity of resveratrol (Fig. 51.7), as previously suggested [51–53]. Both 35SS and LoxS fruits, which contained different levels of resveratrol, showed higher antioxidant and anti-inflammatory properties than wild-type fruits [38].

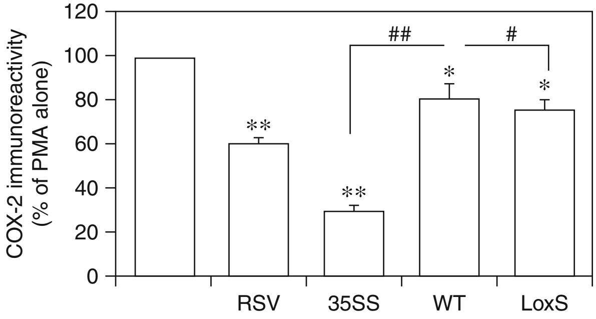


Fig. 51.7 Resveratrol synthesizing tomato fruits inhibited PMA-stimulated COX-2 expression in monocytoid cells. U937 cells were pre-treated with vehicle (ethanol), resveratrol synthesizing tomato fruits (35SS, LoxS) or wild-type fruit, and then stimulated with PMA. Cells extracts, at equal amount of proteins, were immunoblotted with monoclonal antibodies against COX-1 or COX-2. These results are obtained from three independent experiments with similar results. * $p < 0.05$ and ** $p < 0.01$, compared with PMA treated cells; # $p < 0.05$ and ## $p < 0.01$ compared with wild-type fruit treated cells

Remarkably, the extracts of transgenic resveratrol containing tomato fruits displayed an anti-inflammatory effect greater than that of chemically synthesized resveratrol. In conclusion, these results indicate that the presence of a new biosynthetic route responsible for resveratrol biosynthesis improves the health-giving biological activities of tomatoes.

5 Effects of Stilbene Synthase Expression on Reproductive Development of Tomato

The novel STS activity and the significant changes in the levels and/or redox state of soluble primary antioxidants do not have any apparently adverse effect on the normal vegetative growth and development of the transgenic tomato lines. As far as reproductive development is concerned, 35S transformed plants produced seedless fruits.

The development of tomato fruit in the absence of pollination and/or fertilization (parthenocarpy) was achieved using different approaches [54]. STS-induced male sterility has been demonstrated to be a successful approach to genetically engineer male sterility in a model species (tobacco). The resveratrol synthesis in transgenic tomato plants may provide a new method to obtain parthenocarpic fruits. Indeed, pollen development in 35S STS flowers was hampered, resulting in a strongly reduced seed set and all strong yielded parthenocarpic fruits (Fig. 51.2), prompting the authors to hypothesize that specific polyphenols might be efficiently down-regulated by STS gene expression in tomato flowers.

Flavonoids belonging to the class of flavonols have especially been shown to have strong stimulatory effects on pollen development, germination, pollen tube growth, and seed set [55]. When wild-type pollen was applied on transgenic

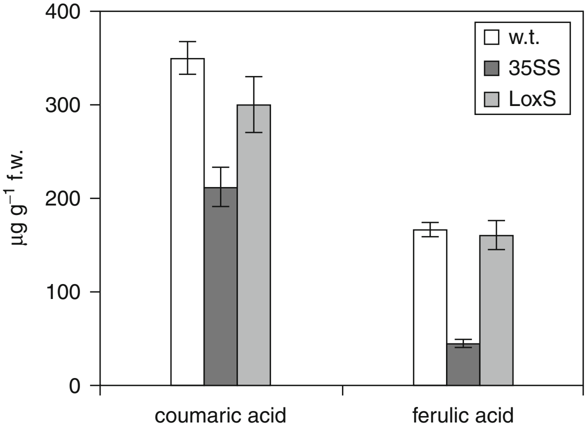


Fig. 51.8 Comparison of coumaric acid and ferulic acid accumulation in wild-type 35SS and LoxS tomato transgenic open flowers

35S STS stigmas, pollen tube growth and seed set were fully rescued thus indicating that fertilization was abolished due to the lack of metabolites in the male reproductive organ [56].

Parthenocarpic tomatoes with suppressed chalcone synthase obtained by using RNA interference [55] showed abolished production of flavonoids. Several studies [55, 57] have clearly demonstrated the importance of flavonols for normal pollen tube development and male fertility. Flavonols have been detected in the pollen of all plant species tested so far and clearly play an important role in pollen tube development. In tomato plants overexpressing STS, the analysis of flavonols showed that pollen ablation was independent of the production of detectable amounts of flavonols in flowers [38].

The authors have introduced an alternative hypothesis for STS -induced male sterility, which, if correct, would significantly contribute to the conclusions drawn by other authors [58]. When expressed in tobacco anthers, STS depletes the substrate for CHS which are key molecules in several other pathways, including those linked to sporopollenin or lignin biosynthesis. Therefore, these pathways may be affected as a result of the decreased availability of precursors. Pollen grains are surrounded by a complex cell wall comprising three layers with an outer exine, a multilayered structure primarily made of sporopollenin; an inner intine, made primarily of cellulose; and a lipid- and protein-rich pollen coat. Several studies have indicated that sporopollenin is a complex polymer composed of fatty acids and phenolic compounds [59]. The analyses of coumaric and ferulic acids, precursors of sporopollenin and lignin, levels suggested impairment in their synthesis in STS sterile male tomato flower tissues (Fig. 51.8). Therefore conclude that when constitutively expressed in tomato, STS competes with CHS for common substrates, in particular in flower tissues where leaded to reduced synthesis of naringenin. Similarly, these compounds are key molecules for other important pathways such as the synthesis of exine layer constituents and lignin.

6 Concluding Remarks

Metabolic engineering is generally defined as the redirection of one or more enzymatic reactions to improve the production of existing compounds, produce new compounds, or mediate the degradation of undesirable compounds. It involves the redirection of cellular activities by modifying the enzymes, endocellular localization, and regulatory functions within cell. Even more sophisticated metabolomic tools and analysis systems will offer the possibility to study both the primary and secondary metabolic pathways in an integrated fashion. Some interesting and important developments may be expected from plant transformation with STS gene.

Therefore, further progress in better understanding of the metabolic pathways and our ability to manipulate gene expression, in genetically modified plants, can be envisaged. Success of this approach depends upon the possibility to change the host metabolism and will depend primarily on a far more sophisticated knowledge of plant metabolism, especially the nuances of interconnected cellular networks.

Identifying rate-limiting steps in the synthesis of specific metabolites could provide targets for genetically engineering biochemical pathways to produce increased amounts of compounds as well as new compounds. Together with traditional plant breeding, genetic engineering provides great opportunities to develop plants with the desired levels and/or composition of specific polyphenols. New insights into stilbene synthase expression restricted to flower tissues may provide a novel hybrid seed system for the development of a nutritionally fortified tomato. Moreover, the consequences of the unintended spread of genetic material through pollen and the risk of cross-pollination with non-transformed plants would be avoided using genetically modified sterile male plants.

Metabolite profiling is an essential tool to analyze the effects of intervention on flavonoid composition itself but also on other related or unrelated metabolic pathways. The availability of phenolic precursors toward specific pathways of the phenylpropanoid network is also controlled by biochemical, genetic, environmental, and developmental parameters [60]. The beneficial effect of *trans*-resveratrol on human health has directed research toward the production of this metabolite in staple plants [61]. The presence of a novel biosynthetic route responsible for resveratrol biosynthesis improved the biological activities of transformed tomatoes [62]. Comparison of the effects of diets containing tomatoes enriched in different polyphenol classes upon onset and progression of diseases in cell assays or animal disease models will allow a quantitative assessment of the efficacy of stilbenes within a common food matrix. The nutritional context could influence the effects of polyphenols, by affecting their bioavailability, or be the result of various dietary phytonutrients acting synergistically, once absorbed [63, 64].

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Cocoa Cultivation, Directed Breeding and Polyphenolics 52

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Contents

1 Introduction 1600
2 Cocoa: Cultivation and Genetic Diversity 1600
2.1 Cultivation Aspects 1601
3 Directed Breeding Approaches 1602
4 Cocoa Bioactive Molecules with Special Focus on Polyphenolics 1603
4.1 Fresh Cocoa Seeds 1604
4.2 Biotechnology, Postharvesting Processes: Fermentation and Drying 1608
5 Medicinal Importance: Health Support by Phenolics 1610
5.1 Bioavailability and Metabolism 1611
6 Conclusion 1611
References 1611

Abstract

Seeds of Theobroma cacao are worldwide in use for production of cocoa butter and confectionary products. The production of raw cocoa from fresh seeds is based on a complex fermentation process, which leads to the aroma precursors. This process enhances the amount of peptides and free amino acids in the seeds, but it also reduces the amount of phenolic compounds, especially the proanthocyanidins. These antioxidative compounds are mostly composed of catechin and epicatechin monomers and oligomers up to decamers. The fermentation has to take into account that both factors, production of aroma precursors as well as

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maintenance of health-supporting phenolic factors, are guaranteed. The worldwide rising consumption of high-quality cocoa leads to strong international efforts to develop elite clones of trees with high field performance in resilience, quality, and yield.

Keywords

Antioxidative compounds • breeding aims • fermentation • health claims • phenolics • selection for quality markers

1 Introduction

Theobroma cacao is the unique plant source for production of chocolate. It is a traditional plant product which is in use since more than 3,000 years. Its use is accompanied by myths, and also its scientific name is a reference to the valuable and important seeds, “food of the gods.” In modern science, it is proven that the seeds are delivering health-supporting antioxidative compounds in very high concentrations. Besides these valuable compounds, the cocoa seeds deliver about 50 % of dry weight of fat, cocoa butter, which is in use in cosmetics, confectionary, and pharmacy. Furthermore, cocoa trees are ideal components of small-scale agriculture and are of high importance for about 50 million families that depend economically on this plant for their sustainable lifestyle on the global tropical belt.

Cocoa development organizations are underway to select new, modern cocoa genotypes with good disease resistance, high tolerance to ecological factors like drought and climatic changes, and special flavors and other quality factors. This chapter will present these aspects in detail.

2 Cocoa: Cultivation and Genetic Diversity

Theobroma cacao is a tree native to the eastern Andean slopes in the tropical range of the Northern Amazon basin and is now found on the whole tropical belt [1]. The first wave of selection by man dates back for more than 2,000 years [1, 2], when migrating Indians introduced cocoa trees from the primary center of origin to Central America and Mexico. Their selections formed the basis for the cacao populations in the Circum-Caribbean region and in Central America which are considered to be “the region of secondary distribution of cacao” [1]. Plant material deriving from these populations was used for cultivation and breeding later on by the Mayas in Central America [3]. No exchange with the cacao populations (the gene pool) in the primary center of origin took place. Therefore, the cacao populations in the Circum-Caribbean region and in Central America developed considerable morphological and physiological differences compared to the entire South American cacao populations. These morphological and physiological differences led Cuatrecasas [4] to the formulation of two subspecies, *T. cacao* ssp. *cacao* or “Criollo” (Central America) and *T. cacao* ssp. *sphaerocarpum* or “Forastero”

(South America), which developed independently, separated by the Panama Isthmus [3, 5]. According to the distribution in the Amazonian basin, "Forastero" is further grouped into Upper and Lower Amazon "Forastero." Nowadays, "Criollo" is considered to be a traditional cultivar which has developed in pre-Columbian times due to breeding by the Indians [2]. Besides "Criollo," further traditional cultivars are known, the "Nacional" cacao of Ecuador, a result of intensive selection and translocation from the primary center of origin to the Ecuadorian highlands; the "Trinitario," a group of "Criollo" x "Forastero" hybrids [6]; as well as "Amelonado," a Lower Amazon "Forastero" often planted in West Africa. These cultivars are younger than "Criollo," with "Nacional" being ca 600 years old, "Trinitario" ca 450 years old, and "Amelonado" ca 300 years in use [7, 8].

Under international trading aspects the "Criollo," the "Nacional," and the "Trinitario" are the sources of fine cocoa characterized by special aroma notes (e.g., fruity, floral) [9]. They are grouped as fine or flavor cocoa, whereas Upper and Lower Amazon "Forastero," having, after standard fermentation, a general, but not a specific, chocolate aroma, is classified as bulk cocoa. The latter produces about 95 % of the annual world cacao harvest. Fine or flavor cocoas are generally lower in yield per area than "Forastero" is. Moreover, they are lower in resistance against fungal pathogens and have less growth vigor. Yet, fine or flavor cocoa contains specific aroma components and delivers the material for special high-priced chocolate products.

There are intensive studies on factors that are responsible for fine cocoa aroma development. The main results so far collected revealed that several genetic components are involved [10]. However, fine aroma seems to be achieved only when also an adapted postharvest treatment (e.g., fermentation) of the cocoa seeds is applied [5, 9]. Other research approaches are underway [11], which are aimed at the identification of the key substances for fine aroma. They reveal that some of them may be located in the fruit pulp.

With the molecular methods for differentiation of the genetic constitution of cocoa genotypes, the old nomenclature is under revision, and the most rigorous new approach separates the species T. *cacao* into 10 genetic clusters [12]. This classification together with the recently published genome sequences of a "Forastero" [13] and a "Criollo" [14] genotype allows formulating a completely new era of directed breeding strategies and selections, regarding for instance yield, resistance, and fine aroma. Reevaluation of the cacao germplasm is starting with precise and concise information and thus on an enlarged broad basis of knowledge [5].

2.1 Cultivation Aspects

Cacao is a perennial tree crop revealing special features like a juvenile nonproductive status and a productive mature status. It originates from the understory of the ombrophilic humid tropical rain forest and is naturally adapted to high relative humidity and to low light intensity. Light saturation is given at a light intensity of 400 μM photons m^{-2} s^{-1} [15] and under irrigated conditions up to 600 μM photons m^{-2} s^{-1} [16]. These aspects of high tolerance to environmental conditions led to alternative environmental growth concepts and many plantations are run as full-sun plantation systems with high water input and high fertilizer input with trees pruned in such a way to form a sun crown over a productive shade crown.

Zuidema et al. [17] presented a physiological production model for cacao in which the yield-determining parameters were identified as fruit morphology, photosynthesis and maintenance respiration, the influence of shade was estimated to reduce yield when 60 % or more shade were applied.

3 Directed Breeding Approaches

The development of new characters by breeding is oriented to various fields, especially in high productivity, high resistance against the most important diseases, and on high quality. High productivity comprises a series of factors, starting with optimal photosynthetic parameters, good water use efficiency, drought resistance, tolerance to windbreaks, tree architecture, and suitability for pruning.

Breeding for yield is a complex task, because the final measure is the dry seed, the raw cacao after complex postharvest treatment. Before harvesting ripe pods, there are many steps which depend on the interaction of the plant with environmental factors. This field is still under intensive evaluation, especially under management aspects for the decision if a cacao plantation shall be run under shade trees or in direct sun. Directly combined with these decisions is the shorter lifetime of plantations in direct sun, the high investment costs for high fertilizer input, and the potential need for irrigation [5, 17].

Factors like number of fruits per plant and number of seeds per fruit and per plant, and weight of fresh seeds and dry seeds per tree have been used for evaluation, but there are doubts in their suitability, as cultivars can differ in seed moisture [1]. A comprehensive discussion of the evaluation parameters and their complexity is given by Dias and Resende [7].

Resistance breeding is a wide field, as the diseases which reduce the cocoa production vary with the cultivation area. On global scale, the epidemiologically important diseases are witches broom caused by the basidiomycete *Moniliophthora perniciosa* [18] and frosty pod disease caused by *Moniliophthora roreri* in South and Central America [19]; in West Africa it is dominated by *Phytophthora species* (pod rot) [20] and swollen shoot virus [21]; in Asia it is vascular streak disease (VSD), caused by *Oncobasidium theobromae* [22]. In addition, some pests are severely reducing the production, especially the pod borer in Southeast Asia [23]. Some additional diseases are of local importance like cacao wilt (*Ceratocystis fimbriata*), Verticillium wilt (*Verticillium dahliae*), and pink disease (*Corticium salmonicolor*), though restricted in distribution, these diseases may be of considerable economic importance [24].

The breeding approaches now are directed to combine various resistance factors against pests and diseases in one plant (e.g., Dias [7]). Using the molecular indicators, it is hoped to develop genotypes with accumulated resistance patterns against more than only one disease. This concept of pyramidizing resistance genes is based on intercrossing of resistant genotypes, followed by recurrent selection cycles, but new concepts look forward to use biotechnological approaches and in vitro gen transfer. The basic data for somatic embryogenesis in cacao are under worldwide development [25, 26].

Concerning phenolics as general factors of plant resistance to diseases, especially in control of *Phytophthora*, the amount and composition of preformed phenolic compounds is under study [27]. The ripe cocoa seeds contain high amount of polyphenols and a very active polyphenol oxidase. The feeding deterring action of these substances is well described [28]. In addition, the anthocyanins and anthocyanidins are antifeeding compounds due to their astringency. Pure alkaloids, especially theobromine and coffein, reveal a drastic bitter taste and prevent the seeds from being consumed by herbivores. Cinnamic acid amides and high concentrations of monomers and oligomers of procyanidins act also as repellents and biochemical control agents [29].

The phenolic compounds are protective compounds with respect to herbivores and diseases; they are also highly important as quality factors for consumers due to their health support actions in the vascular systems of chocolate consumers based on the antioxidant properties of these compounds. In directed breeding, these compounds are considered to be very important quality factors. In fact reveals cocoa with 12–18 % polyphenols per whole seed dry weight [30], the highest amount of phenolic substances reported for plant tissue. Also the kinetics of accumulation, the location in the storage tissue, and the free enzymes for oxidative degradation must be regarded as regulatory factors in breeding strategies.

Cocoa butter or Theobroma oil is an edible vegetable fat obtained by hydraulic pressing of whole cocoa seeds or broken nibs [31]. This fat is widely used in cosmetics, pharmaceutical products, and in chocolate products. The melting point is near to 37 °C. The fat market is still demanding for this fat, and the fat content, which may exceed 50 % of dry bean weight, is an important breeding aim [32].

4 Cocoa Bioactive Molecules with Special Focus on Polyphenolics

Under the aspects of their biological function, fresh cocoa seeds are highly effective physiological storage tissues. They contain more than 50 % of fat, the cocoa butter, about 10 % protein, and 8 % starch per dry weight [33], but they also are characterized by a high amount of polyphenols, methylxanthines, minerals, organic acids, and small amounts of amines, terpenoids, and other organic compounds. Many of these compounds are known to act as bioactive ingredients with an expressed impact on human metabolism.

The compounds found in fermented and dried raw cacao, the basis for chocolate production, differ considerably from the compounds present in fresh-harvested cocoa seeds. The transition of fresh seeds to raw cocoa takes place in the fermentation. In order to illustrate the biochemical changes which take place in the course of seed transformation to raw cocoa, the fresh seed conditions will be compared to the fermented and dried raw cocoa sample.

4.1 Fresh Cocoa Seeds

About 12–18 % of defatted dry matter of cocoa seeds is phenolic compounds [30]. These compounds are concentrated in polyphenol cells of the cotyledon storage tissue. About 10–20 % of the storage tissues are special idioblasts which contain the polyphenolics [33–35]. The idioblasts are filled by a vacuole which contains about 14 % of purine alkaloids and over 66 % of phenolic compounds [36].

Figueira et al. [37, 38, 39] assume that the composition of the phenolic compounds of the fresh seeds varies with the genotype and has an influence on the cocoa flavor. In general, there is no significant difference in total phenols in the major groups of cacao, but there are seed-to-seed variations in the amounts of phenolics [40].

The early studies of phenolic compounds have been carried out in 1965 [41] and in 1978 [42]. They reported on phloroglucin, protocatechuic acid, 4-hydroxybenzoic acid, syringic acid, vanillic acid, phenylacetic acid, 4-hydroxyphenylacetic acid, and 2-hydroxyphenylacetic acid.

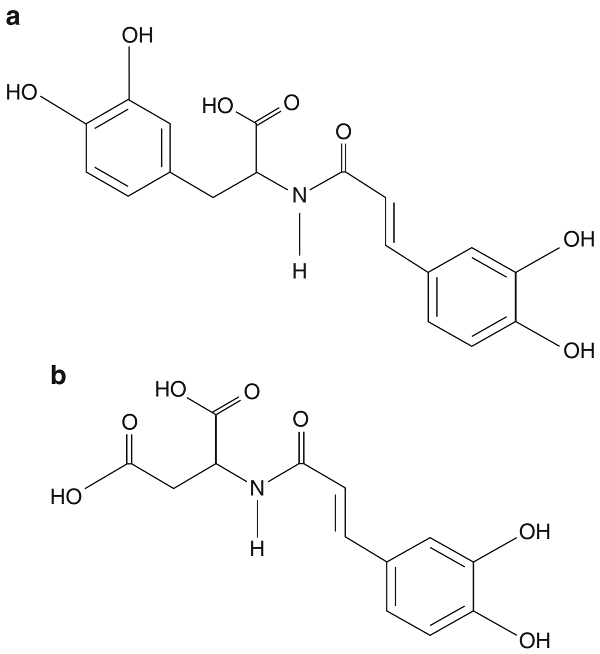
The free hydroxyl cinnamic acids in raw cocoa seeds were p-coumaric acid [41, 42], caffeic acid [43, 44], ferulic acid [41], chlorogenic acid, and neochlorogenic acid [43, 45] in raw cocoa extracts. The existence of chlorogenic acid was also stated by Wollgast [46]. Aesculetin was found 1965 [41].

Hydroxycinnamic acid amides (Fig. 52.1), proanthocyanidins, and flavonol-glycosides are the largest groups of phenolic compounds in cocoa seeds.

Clovamid (N-*trans*-caffeoyl-L-DOPA) and Dideoxyclovamid (N-*trans*-p-cumaryol-L-tyrosin) were described in 1998 [47]. Monodeoxyclovamid (N-*trans*-caffeoyl-L-tyrosine) was found in cell cultures, fresh seeds, and callus cultures of *T. cacao* [26]. N-*trans*-coumaroyl-DOPA was identified by Wollgast [46].

In roasted cocoa powders, 13 Cinnamic acid amides [48–50] have been quantified, which are also found in fresh seeds, but unfortunately there are no quantitative data for these amides in unprocessed fresh cacao seeds:
Caffeoyl-L-DOPA (= clovamid) (58.6 mg kg^{-1} in processed raw cocoa)
Caffeoyl-L-tyrosin (19.07 mg kg $^{-1}$)
p-coumaroyl-L-DOPA (9.04 mg kg^{-1})
p-coumaroyl-L-tyrosin (=Dideoxyclovamid (55.34 mg kg^{-1}))
p-coumaroyl-L-tyrosin (1.1 mg kg^{-1})
(+)-N-(E) caffeoyl-L-aspartate (428.4 mg kg^{-1})
(+)-N-(E)-coumaroyl-L-aspartate (120.4 mg kg^{-1})
(+)-N-(E)-feruloyl-L-aspartate (18.8 mg kg^{-1})
(+)-N-(E)-cinnamoyl-L-aspartate (2.26 mg kg^{-1})
(−)-N-(E)-caffeoyl-L-glutamate (5.55 mg kg^{-1})
(−)-N-(E)-coumaroyl-L-glutamate (3.28 mg kg^{-1})
(+)-N-(E)-caffeoyl-L-tryptophan (0.45 mg kg^{-1})
(+)-N-(E)-coumaroyl-L-tryptophan (0.39 mg kg^{-1})

Fig. 52.1 Cinnamic acid amides: (**a**) Caffeoyl-L-DOPA = clovamid, (**b**) Caffeoyl-L-aspartate



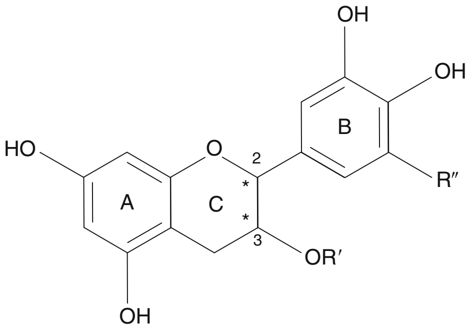
Caffeic acid aspartate with about 430 mg kg^{-1} and coumaric acid aspartate (120 mg kg^{-1}) are of quantitative importance in raw cocoa.

The biological importance of these substances is not really elucidated. They are discussed to reveal antimicrobial or antiviral activity [51], and some of them are astringent and slightly bitter [50]. They may act as feed repellents, but they act also as antioxidants.

The procyanidins are a mixture of oligomers and polymers of the monomers (+)-catechin and (−)-epicatechin (Figs. 52.2 and 52.3). Most of them are linked by position 4 and 8. With normal-phase HPLC, polycyanidins up to decamers can be separated [52, 53], as cited in Gu et al. [54]. Molecules consisting of two to six flavan-3-ol units are generally water soluble; longer chains are either soluble (about 6–12 units) in methanol or are really insoluble.

Kim and Keeny [55] detected between 43,270 and 21,890-mg kg^{-1} epicatechin in eight different cacao genotypes; Tomas-Barberán et al. [56] quantified 25,650-mg kg^{-1} fat-free dry matter; Gotti et al. [57] reported on 15,300–5,700 mg kg^{-1}. It is still unclear whether environmental or genotypic factors dominate the variation. The diastereoisomer (+) catechin is lower in mature cocoa seeds: 646 mg kg^{-1} [56], 450–140 mg kg^{-1} [57].

The occurrence of (+) gallocatechin and (−) epigallo catechin is not really proved for cocoa seed tissue [58].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2
*
A
C
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3
R″
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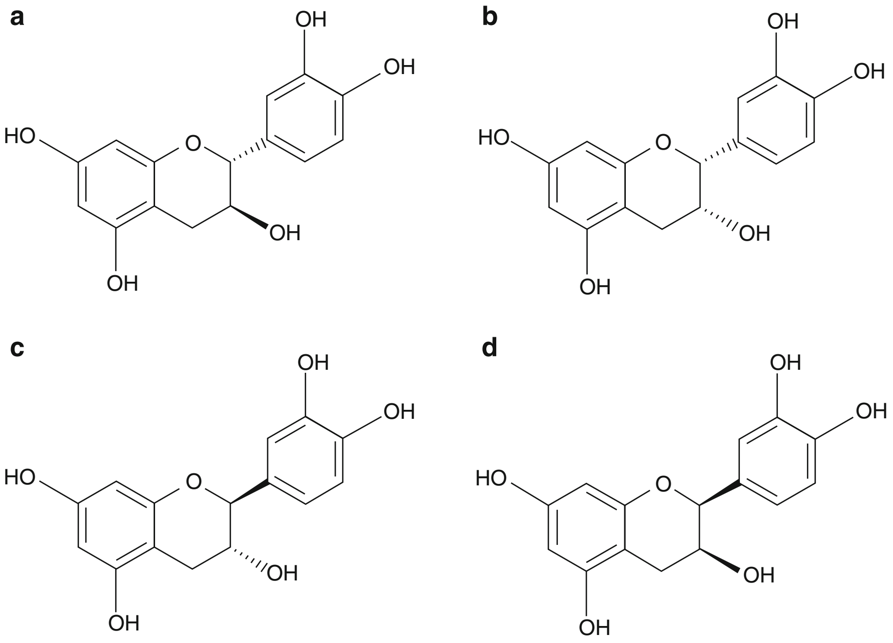
Fig. 52.2 General formula of Flavan-3-ol


a
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b
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c
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OH
OH

Fig. 52.3 Stereoisomers of Flavan-3-ol monomers (**a**) (+) catechin, (**b**) (−) epicatechin, (**c**) (−) catechin, (**d**) (+) epicatechin

Proanthocyanidins or condensed tannins [59] are oligomers of flavan-3-ol units. Many dimers are formed (Fig. 52.4).
Procyanidin B-1: Epicatechin [4β → 8] catechin
Procyanidin B-2: Epicatechin [4β → 8] epicatechin
Procyanidin B-3: Catechin [4β → 8] catechin
Procyanidin B-4: Catechin [4β → 8] epicatechin.

Many steps of proanthocyanidin biosynthesis have recently been reviewed [59]. The genes encoding the key enzymes of biosynthesis of flavonols, the transport

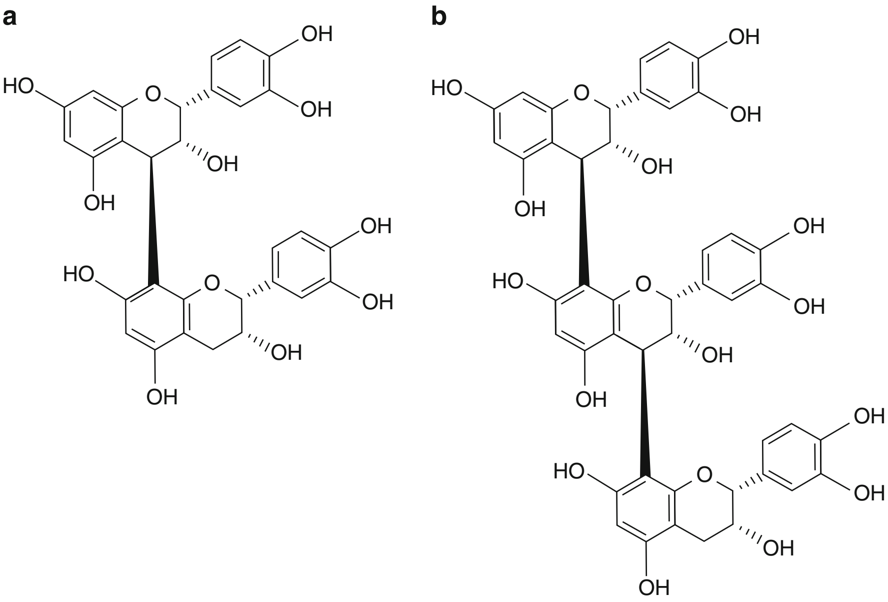

Fig. 52.4 Proanthocyanidins. (**a**) Dimer Procyanidin B-2 Epicatechin [4ß-8] epicatechin. (**b**) Procyanidin *n* = 3 (condensed tannin) trimer

factors, and the factors for transcriptional regulations are presented [60], but some steps remain still unclear [59].

Raw cocoa seeds contain approximately 6.9–18 % proanthocyanidins of defatted dry weight [61, 62]. Porter et al. [63] described (2R,3S)-(+)-catechin, (2R, 3R)-(−)-epicatechin, and the dimers epicatechin-(4β → 8)-catechin (procyanidin B1, see above) procyanidin B, epicatechin-(4β → 6)-epicatechin, and epicatechin-(4β → 8)-epicatechin (procyanidin C1) as the dominating compounds in cacao seeds [63]. Two glycosides 3-0-β-D galactopyranosyl-ent-epicatechin-(2α → 7, 4 α → 8) epicatechin and 3-0-L-arabinopyranosyl ent-epicatechin-(2α → 7, 4α → 8)-epicatechin were found in addition to epicatechin-(2β → 5, 4β → 6)-epicatechin [63, 64].

Griffiths [65] reported about anthocyanidins and anthocyanidin glycosides, which give rise to violet to light red coloring of cocoa seeds, depending on the genetic clusters they belong to. Anthocyanins occur as 4 % [36, 66] or 4.5 % [67] of the polyphenols of cocoa seeds. The highest amounts are cyanidin-3-β-D galactoside and cyanidin-3-α-L-arabinoside with lower amounts of a rutinoside and a pentoside [68]. An exception for extraordinarily high amounts of anthocyanins is the genotypes of the Cameroon group of German cacao, a regional “Trinitario” hybrid population [69]. The seeds of this traditional group contain very high activity of polyphenol oxidases compared to other cocoa seeds and very high amounts of anthocyanidin glycosides, three to five times more than reported for other cocoa seeds [70]. This group of cacao seeds delivers especially red-colored raw cocoa powder.

Quercetin, a flavonol, and corresponding quercetin-3-0-glucoside, quercetin-3-0-galactoside, quercetin-3-0-arabinoside, quercetin-3-0-glucuronide, and free quercetin are found in amounts of some milligrams to more than 300 mg kg^{-1} (e.g., [56]).

Using high sensitive detection LC/ESI/MS, the flavones apigenin, vitexin, isovitexin and luteolin as well as luteolin-7-0-glucoside, isoorientin, and orientin have been detected [71]. They also found flavanone naringenin and its 7-0-glucoside as minor components. Stark et al. [50] also reported on naringenin-7-0-glucoside, luteolin-7-0-glucoside, apigenin-8-c-glucoside, and apigenin-6-C-glucoside. Isovitexin, vitexin, apigenin-7-0-glucoside, luteolin-7-0-glucoside, and chrysoeriol-7-0 glucoside had also been found in cocoa tree leaf extracts [44].

Due to the well-described positive physiological action of phenolic cocoa ingredients on human health, intensive studies on the impact of processing of ripe, fresh cocoa seeds to the fermented and dried raw cocoa as a traded good are underway.

The biological function of the phenolic compounds in the seeds seems to be a repellent function of astringent-tasting flavan-3-ols, of proanthocyanidins, and of the bitter-tasting cinnamic acid amides against herbivores. In addition to the tanning properties of the proanthocyanidins and the rapid enzymatic oxidation of *o*-dihydroxyphenolic compounds by a very high plant polyphenoloxidase [72], these compounds act as repellent, but they also reduce the nutritive value of cocoa seeds by reactions of *o*-diquinones (as oxidation products) with proteins of the seeds and by the inactivation of salivary proteins and proteolytic enzymes. Mono- and oligomeric Flavan-3-ols are described to be bitter (monomeric fraction) and astringent (oligomeric fraction) [50, 73]. Cocoa seeds are consumed by monkeys, rodents, and parrots. These distributors of seeds consume the sweet–sour pulp surrounding the seeds and spit out the bitter seeds.

The antimicrobial property of tannins is assumed to be an important control factor against fungal attack to the germinating seeds [74].

4.2 Biotechnology, Postharvesting Processes: Fermentation and Drying

Cocoa pods are hard-shelled berries which contain 30–50 seeds in five adjacent rows. The seeds consist of two large storage cotyledons and a small embryo axis, surrounded by a flexible, elastic multilayered testa [75]. On the outer testa layer, slimy elongated cells of the endocarp form the pulp. This pulp has to be degraded during postharvest processing (e.g., fermentation) in order to produce the raw cocoa, which can be stored and shipped. Moreover, the characteristic chocolate flavor is only generated during roasting of well-fermented cocoa seeds, whereas no chocolate flavor is formed from roasting of unfermented seeds [76, 77]. Thus, the essential precursors of chocolate aroma are developed in the course of fermentation. Flavor development also is influenced by the genetic constitution of seed and pulp.

During fermentation, the carbohydrates and organic acids of the pulp are degraded to ethanol and further transformed to acetic acid and to lactic acid [78, 79].

The acidification of the seed tissue together with a significant rise in temperature caused by the exergonic process of acetic acid formation leads to the cocoa-specific aroma precursor formation. There are various factors influencing the biochemical outcome of the fermentation process and thus the quality of the raw cocoa. Important factors are, for instance, fruit ripeness, overall amount of seeds used, degree of acetic and lactic acid formation, and rise as well as homogeneity in temperature and oxygen.

The components produced during fermentation are distributed all over the tissue of the storage cotyledons. During drying after end of fermentation, these substances are mixed, and by migration with the water leaving the seeds, they undergo changes which result in various ways, especially in browning of the cocoa seeds. Most of these browning processes are based on oxidation of phenolic compounds and follow reactions of the oxidation products.

4.2.1 Standard Fermentation, Drying, Storage

The fermentation process is characterized by a sequence of biotechnological steps and is carried out in different traditional ways. Standard fermentation time for “Forastero” cacao is about 6–7 days. The time for fine and flavor cacao is 3–5 days [80]. Widely used fermentation methods are heap and box fermentation:

Freshly harvested cocoa pods are opened mechanically to extract the seeds with the pulp. This fresh material is heaped and covered with banana leaves, or transferred into wooden boxes. Because of its high content in free sugars and organic acids, the pulp is an ideal substrate for yeasts. Their high metabolic activity and the insufficient penetration of air into the fermentation mass result in anaerobic conditions consequently leading to ethanol formation. The secretion of pectinases by the yeast causes pulp liquidation and drainage. Subsequently, air enters the fermentation mass, and ethanol is oxidized to acetic acid by acetic acid bacteria. Temperature rises to more than 50 °C, and the combined action of tissue acidification and high temperature kills the embryo (see 79 for details). The entry of acetic acid into the seeds occurs after seed swelling and opening of the micropyle [75]. In the same time also, a transport of matter out of the seeds takes place. In this phase, the first losses of phenolics, purine alkaloids, and further organic compounds occur. Induced by tissue acidification, the preformed proteases in the protein storage vacuoles [81] are activated and deliver peptides and amino acids, which serve as important aroma precursors during later roasting [81–83]. As long as the pH of the seed tissue is around pH 4–5, the reaction of the phenolic compounds is very low, but with the onset of buffer action caused by peptides and free amino acids, the pH rises and first enzymatic oxidations start. The seeds begin to turn from violet to brown. This process explains the losses of procyanidins [84]. After the fermentation, the seeds are spread to thin layers on wood or concrete for drying. The drying phase includes loss of water and water-soluble compounds from the seeds. It enhances the enzymatic oxidation, especially by cell wall immobilized, stable phenolases and leads to further losses of proanthocyanidins (e.g., [85]). The physical cut test [86] is a good indicator for the fermentation and drying-related oxidation of phenolic compounds. After processing the seeds are cut in half, and the color of the seed tissue is evaluated. Slaty seeds are non-fermented, violet seeds are acidified, and red- to violet-colored anthocyanins and glycosides are distributed in the tissue. Most of these substances are tightly bound to the tissue and cannot be extracted anymore by aqueous or organic solutes. In this stage most of the astringent components are not anymore disturbing aroma formation. Other components like cinnamic acid amides may still interfere as quality-reducing factors. The drying process of cocoa seeds will be stopped at about 7 % water content [86, 87]. Aroma-relevant components also are produced in the course of drying like pyrazines, and methylpyrazines from the reactions of amino acids and reducing sugars [88].

The reaction of phenolic compounds during fermentation and drying differs between "Criollo" genetic cluster and other genotype groups [61]. The reduction of the phenolic monomer (−)-epicatechin during fermentation and drying is over 92 % of the content of unprocessed seed. The decrease of (+)-catechin is even more expressed, but in contrast, the diminution of caffeic acid aspartate is far lower. The "Criollo" seeds and the "Forastero" seeds differ in this aspect [61]. This may be due to the fact that the fermentation time reported for "Criollo" is shorter (3–4 days) than for "Trinitario" or "Forastero."

For chocolate production, the raw cocoa is stored, shipped, and processed. The processing steps are roasting and liquor production. The heat treatment induces Maillard reactions, caramelization of sugars, protein degradation, and formation of volatile aroma components [85, 89]. An often applied step to cocoa is the "dutching," the alkali treatment of cocoa powder in order to modify the color, and other physiochemical properties. The pH values of cocoa powders are adjusted from ph 5.3–5.8 in natural powders to higher than 7.6 in heavily dutched materials. The total flavanol contents are reduced from more than 34 to 3.9 g kg^{-1}. In the same way, the antioxidative properties of the powders are diminished [90].

5 Medicinal Importance: Health Support by Phenolics

Cocoa products are rich in phenolics, especially in flavanoids. These substances provide a strong protection against cardiovascular diseases; they are effective antioxidative compounds and scavengers of free radicals. The flavanols reveal beneficial effects on low-density lipoproteins oxidation [91] platelet aggregation [92], endothelial function [93], and blood pressure [94]. Consumption of flavanol-rich diet also reverses dysfunction in diabetes [95].

The mechanisms which are causing the biological effects of flavanols include the modulation of cell signaling pathways [95] and the regulation of nitric oxide (NO) homeostasis, which in turn influences the flow-mediated dilation in capillaries. The antioxidative potential of the flavanols leads to scavenging free radicals and inhibits lipid peroxidation [96].

5.1 Bioavailability and Metabolism

The health benefits of flavanol-rich foods have widely been described. The dominating flavanol in cocoa product is epicatechin and its oligomers from dimer to decamers [54]. The in vitro action of these compounds on the health-supporting reaction like alteration of cellular redox environment, modulation of cellular signaling pathways, and radical scavenging has been analyzed in many experiments, but for the in vivo action, the substances have to be taken up in the blood serum. After administration of flavanol-containing diets to humans within 2 h, specific flavanol metabolites are found. Various *O*-methylated, *O*-glucuronidated, and *O*-sulfated flavanol derivatives occur in the blood serum [97]. These flavanol derivatives form a bioactive circulating flavanol pool, and it remains to be elucidated which of the metabolites contributes to which activity in vivo. The stability of epicatechin and catechin and the related dimeric procyanidins under gastric condition allows to calculate the bioavailability and the uptake kinetics of cacao active ingredients [98].

The uptake of oligomeric proanthocyanidins from a complex diet is influenced by various interacting components and is still under study.

6 Conclusion

The international research on *Theobroma cacao* L. starts into a highly effective selection phase. The elucidation of the complete cocoa genome allows new and rational strategies for directed breeding and gives also rise to develop regional gene pool collections for international plant exchange and for the development of new agricultural qualities of new genotypes. In addition, the analysis of aroma precursors, of fine flavor components, and of health-supporting ingredients supports the use of cacao in many new fields of application. New food qualities, based on antioxidative properties, will be developed for dietary use. The step into a new molecular era of cocoa breeding and cultivation is on its way, and opens a wide activity in quality management for a perennial tropical crop.

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Genetics of Flavonoids

53

Ramesh Prasad Pandey and Jae Kyung Sohng

Contents

1 Introduction 1619
2 Biosynthesis of Flavonoids in Plants 1622
3 Production of Flavonoids from Microbial System 1623
3.1 Production of Flavonoids from Yeast 1628
3.2 Production of Flavonoids from *E. coli* 1631
3.3 Production of Flavonoids from *Streptomyces* and Fungi 1637
3.4 Production of Flavonoids from Transgenic Plants and Plant Tissue Cultures 1639
4 Conclusion 1640
References 1641

Abstract

Flavonoids are structurally diverse plant pigments that comprise the largest group of natural products and are ubiquitously present in the plant kingdom. They exhibit a broad range of functions in plants. Interestingly, these polyphenolic compounds have also shown beneficial health effects in humans by inhibiting or delaying the onset of diseases. Though the biosynthetic pathway of flavonoids in plants is well known, many studies have explored the significance of the flavonoids. In the last 10 years, flavonoids have been produced in

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Saccharomyces cerevisiae, *Escherichia coli*, and *Streptomyces* species by heterologously expressing partial or entire plant flavonoids biosynthetic gene clusters and rewiring the native host metabolic networks. Currently, a few natural and novel flavonoids have been produced in significant amounts in microbial systems. However, the development of a microbial factory for the efficient biosynthesis of diverse flavonoids remains incomplete. The integrated use of modern metabolic engineering, proteomics, and synthetic biology tools could aid the design of robust microbial cells for the production and biotransformation of flavonoids in the near future. In this chapter, we will discuss recent progress in the production and biotransformation of flavonoids from engineered microbial cells, transgenic plants, and whole cells of plants and *Streptomyces*.

Keywords

Biosynthetic pathway • flavonoids • metabolic engineering • microbial factory • natural products • novel flavonoids • production and biotransformation of flavonoids • proteomics • synthetic biology

Abbreviations

4CL	4-coumaryl-CoA-ligase
ACC	Acetyl-CoA carboxylase
ACS	Acetyl-CoA synthase
ANS	Anthocyanidin synthase
C4H	Cinnamic-4-hydroxylases
cDNAs	Complementary deoxyribonucleotides
CHI	Chalcone isomerases
CHR	Chalcone reductase
CHS	Chalcone synthase
CPR	Cytochrome P450 reductase
DFR	Dihydroflavonol reductase
F3′5′H	Favonoid 3′ 5′hydroxylase
F3H	Flavanone 3-hydroxylase
F3′H	Flavonoid 3′ hydroxylase
FHT	Flavanone 3*β*-hydroxylase
FLS	Flavonol synthase
FS I and II	Flavone synthase I and II
IFS	Isoflavone synthase
LCR	Leucoanthocyanidin reductase
LDOX	Leucoanthocyanidin dioxygenase
PAL	Phenylalanine ammonia lyase
STS	Stilbene synthase

TAL	Tyrosine ammonia lyase
TCA	Tricarboxylic acid
UGT	UDP-glucose: flavonoid 3-*O*-glucosyltransferase

1 Introduction

Flavonoids are the largest group of naturally occurring polyphenolic compounds with low molecular weight. These compounds are ubiquitous, found in almost all plants in the kingdom. Flavonoids are also known as plant pigments or copigments that are responsible for various colors and combination of colors exhibited by different parts of the plants such as fruits, vegetables, grains, bark, roots, stems, and flowers, as well as in tea, wine, etc. Structurally, these plant secondary metabolites bear C6-C3-C6 skeletons derived from a phenylpropanoid (C6-C3) and three molecules of C3 elongation units of malonyl-CoA. Flavonoids are characterized by two aromatic cycles (A and B rings) linked with a heterocycle (C-ring) (**2, 3, 4**). This C15 phenylpropanoid core is extensively modified by rearrangement, methylation, methoxylation, alkylation, oxidation, glycosylation, and hydroxylation [1, 2] resulting over 9,000 structural variants [3–6].

Based on the position of the aromatic group attached to the chromano (benzopyrano) moiety(**1**), flavonoids are classified into three classes [7, 8].

1. Flavonoids (2-phenylbenzopyrans) (**2**)
2. Isoflavonoids (3-phenylbenzopyrans) (**3**)
3. Neoflavonoids (4-phenylbenzopyrans) (**4**)

All these groups share a chalcone precursor.

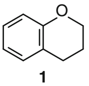
The degree of oxidation and saturation in the heterocyclic C-ring further divides the flavonoids into different subgroups. The subgroups and selected examples are presented in Table 53.1 with the general structures of each group [2, 3, 7] (**5–10**).

The second class of flavonoids, isoflavonoids, possesses a 3-phenylchroman (**3**) skeleton that is biogenetically derived by 1, 2-aryl migration in a 2-phenylchroman

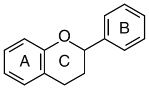
Table 53.1 Examples of flavonoid subgroups and related compounds

Flavonoid subgroups	Selected examples
Flavan	Tupichinol, kazinol Q, acutifolin A
Flavan-3-ol	Elephantorrhizol, guibourtinidol, epirobinetinidol
Flavanone	Liquiritigenin, (2S)-naringenin, (2S)-eriodictyol, (2S)-pinocembrin
Dihydroflavonol	Diosalol, plumbaginol, floranol
Flavone	Chrysin, baicalein, luteolin, velutin
Flavonol	Kaempferol, fisetin, morin, rhamnetin, isorhamnetin, myrecetin

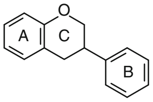
precursor. These compounds are also divided into different subgroups (**11–20**), and the isoflavones are one of the largest subgroups because of the varieties of modifications. These compounds are widely found in leguminous plants. However, some rare nonleguminous plants also biosynthesize isoflavonoids.



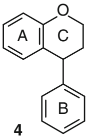
1
Chromano/
Benzopyrano moiety



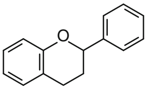
2
2-Phenylbenzopyrans/
Flavonoids



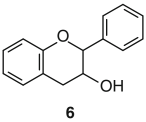
3
3-Phenylbenzopyrans/
Isoflavonoids



4
4-Phenylbenzopyrans/
Neoflavonoids



5
Flavan



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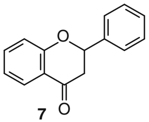
Flavan-3-ol



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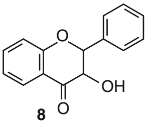
Flavanone



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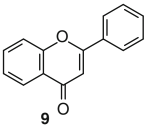
Dihydroflavonol



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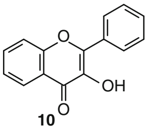
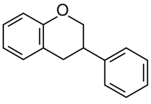
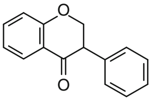
Flavone



10
Flavonol



11
Isoflavan



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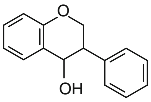
Isoflavone



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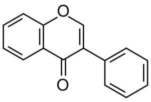
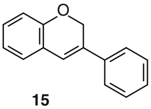
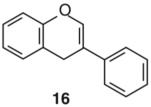
Isoflavonol



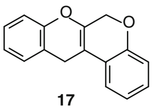
14
Isoflavone



15
3-Arylcoumarin



16
Isoflav-3-ene



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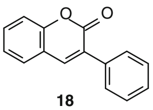
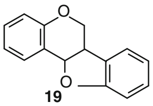
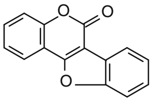
Isoflav-2-ene



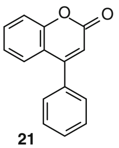
18
Rotenoid



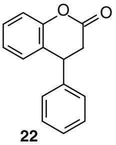
19
Pterocarpan



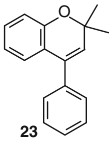
20
Coumestan



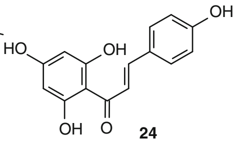
21
4-Arylcomarin



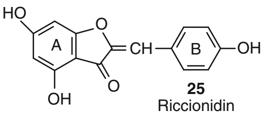
22
3,4-Dihydro-4-arylcoumarin



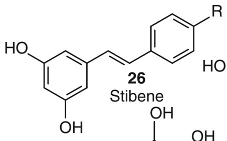
23
Neoflavene



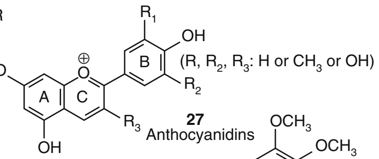
24
Chalcone


HO
O
A
CH
B
OH
O
OH

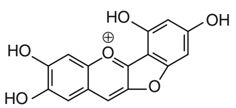
25
Riccionidin



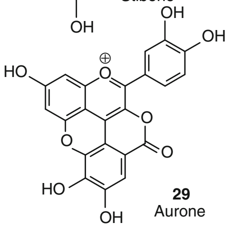
26
Stibene


R1
OH
⊕
B
(R, R2, R3: H or CH3 or OH)
R2
HO
O
A
C
R3
OH

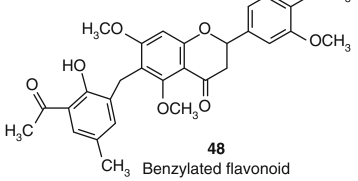
27
Anthocyanidins


HO
OH
⊕
HO
O
HO
O

28
Rosacyanin


OH
OH
⊕
HO
O
O
O
O
HO
OH

29
Aurone


OCH3
OCH3
H3CO
O
OCH3
HO
O
OCH3O
H3C
CH3

48
Benzylated flavonoid

The third class of flavonoids, neoflavonoids, is closely structurally related to flavonoids and isoflavonoids. They have a 4-phenylchroman (**4**) skeleton. 4-Arylcoumarin (**21**), 3,4-dihydro-4-arylcoumarin (**22**), and neoflavene (**23**) fall into this group.

Chalcones and aurones also contain a flavonoid backbone frame (C_6-C_3-C_6) and were considered as minor flavonoids. Importantly, chalcones are the immediate precursors of all other classes of flavonoids. 2′-OH-chalcone, 2′-OH-dihydrochalcone, 2′-OH-*retro*-chalcone, isoliquiritigenin, hamilcone, and tepanone belong to the chalcones (**24**); aureusidin, bracteatin, broussoaurone A, and auronols are aurones (**25**). Resveratrol and related compounds belong to the stilbene (**26**) family, a nonflavonoid group with a (C_6-C_2-C_6) structure. A number compounds belonging to these groups is significantly increasing day by day. Therefore, the biosynthesis and production of minor flavonoids and stilbenes are also mentioned briefly in this chapter along with flavonoids. Anthocyanidins (**27**), a separate group of flavonoids, are polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts. Complex types of anthocyanidins, like riccionidin (**28**) and rosacyanin (**29**), are also found in plants [8]. The variety of colors in the different parts of plants is mainly due to anthocyanidins. The number of hydroxyl groups, the nature and number of sugar moieties attached to the aglycone molecule, and the position of the attachment render this group among the largest water-soluble flavonoids. Pelargonidin, cyanidin, delphinidin, peonidin, malvidin, and petunidin are the major subgroups of anthocyanidins. The glycosylated forms of anthocyanidins are called anthocyanins [4, 7–9].

Different conjugated forms of flavonoids are also found in plants. However, the complex flavonoids are not ubiquitously present in all plant species. Biflavonoids (anacarduflavanone, licoagrone, cordigone, leucaediflavone, delicaflavone, licoagrodin, and bicaryone A, B, C, and D are unusual biflavonoids), triflavonoids (aulacomniumtriluteolin), tetraflavonoids (lophiroflavan A, taiwanhomoflavone C), pentaflavonoids (ochnachalcone), and hexaflavonoids (azobechalcone) [7] have been reported from different plant extracts.

Flavonoids play different roles in plant physiology and plant interactions with the environment. They provide flower coloring to attract pollinators and seed dispersers, influence the transport of the plant hormones, offer UV protection, and balance the levels of reactive oxygen species. Moreover, flavonoids also have key roles in signaling between plants and microbes and in male fertility of some species. Flavonoids play crucial roles in legumes-bacterium symbiosis and root-nodule organogenesis. In addition to these properties, flavonoids have antioxidant, antiproliferative, antitumor, anti-inflammatory, antiallergic, antiviral, and anticarcinogenic properties that have been proven by in vitro and in vivo studies. Flavonoid consumption through plant-derived foods and from medicinal plants has beneficial health effects in humans. Flavonoids prevent neurodegenerative diseases associated with oxidative stress such as Alzheimer's and Parkinson's diseases [9–11] by modifying protein kinase-mediated signal transmission, thereby inducing antioxidant and anti-inflammatory genes expression [12] and immune-modulating properties without the potential toxic and adverse side effects [13, 14]. Since both the flavonoid aglycones and the glycol-conjugates can pass the blood–brain barrier, several flavones can bind to the benzodiazepine site on the γ-amino butyric acid-A (GABA-A)-receptor in central nervous system resulting in sedation, anxiolytic, or anticonvulsive effects [15, 16]. Recently, many flavonoids were found to act on biological targets involved in type 2 diabetes mellitus, such as α-glycosidase, glucose cotransporter, or aldose reductase. Therefore, flavonoids are promising lead compounds in type 2 diabetes mellitus [17] drug design and development.

Although a diet rich in polyphenols seems to offer hope in delaying the onset of different disorders, it is still too early to define their exact clinical benefits for treating diseases [18]. However, flavonoids are incorporated as dietary components by different food industries. In this chapter, we will discuss in detail the recent developments in flavonoid biosynthesis and modifications in different prokaryotic and eukaryotic systems for flavonoid production using genetic engineering approaches.

2 Biosynthesis of Flavonoids in Plants

Since Mendel's classical genetic analysis of flower color inheritance patterns in the 1850s and the experimental studies using radiolabeled precursors at the end of 1950s, recently invented sophisticated chemical analyses, enzymology, and gene technology have led to a vast number of studies and detailed information about the genetics of flavonoid biosynthesis in several plant species. The first experimental model plants in this system, maize, snapdragon (*Antirrhinum majus*), and petunia, were established and work on these species led to the isolation of many flavonoid structural and regulatory genes [19]. The development of *Arabidopsis* later as a model plant helped to explore the regulation and subcellular organization of the flavonoid pathway [20]. The flavonoid pathway is well characterized in *Arabidopsis* and is probably the best characterized of all the secondary metabolic pathways. Mutants of *Arabidopsis* lacking genes encoding each enzymatic step or within regulatory genes encoding transcription factors that affect flavonoid biosynthesis are currently available.

Recently, much effort has been directed at elucidating the flavonoid biosynthetic pathway from a molecular genetics point of view by identifying and analyzing genes or cDNAs for flavonoid biosynthetic enzymes and regulatory factors. Moreover, analysis of enzyme structures and functions involved in flavonoid biosynthesis, generation of transgenic plants, development of protein engineering tools, and heterologous production of flavonoids in microbial systems has allowed tremendous progress in the characterization of the flavonoid biosynthesis pathway. This pathway produces a large number of secondary metabolites, such as lignins, stilbenes, phenolic acids, and other polyphenols [7, 8].

Chalcones are the major intermediates of flavonoid biosynthetic pathways; they are produced by the condensation of three molecules of malonyl-CoA and a single molecule of 4-coumaryl-CoA. The major precursor malonyl-CoA is derived from citrate, an intermediate product of the TCA cycle. Acetyl-CoA is produced in mitochondria, plastids, peroxisomes, and cytosol via various routes. The cytosolic acetyl-CoA, produced by the multiple subunit enzyme ATP-citrate lyase, is used by acetyl-CoA carboxylase (ACC) to form malonyl-CoA for flavonoid biosynthesis. Another precursor, 4-coumaryl-CoA, is available via the polypropanoid pathway, in which phenylalanine generated via the shikimate and aerogenate pathway is converted to 4-coumaryl-CoA in three sequential enzyme-catalyzed reactions. In a few species, caffeoyl-CoA and feruloyl-CoA may also be used as substrates for chalcone formation. Phenylalanine ammonia lyase (PAL) catalyzes the conversion of amino acid L-phenylalanine to *trans*-cinnamate by the *trans*-elimination of ammonia and the pro-*3S* proton; *trans*-cinnamate is hydroxylated by cinnamic-4-hydroxylases (C4H) and is finally activated by the 4-coumarate/cinnamate coenzyme, 4-coumaryl-CoA-ligase (4CL), for condensation with malonyl-CoA. The condensation of 4-coumaroyl-CoA and malonyl-CoA is carried out by chalcone synthase (CHS) to form either tetrahydroxychalcone or trihydroxychalcone. The formation of trihydroxychalcone requires one extra enzymatic step catalyzed by chalcone reductase (CHR). Chalcones are converted to the (*2S*)-flavanone naringenin by chalcone isomerases (CHI) in a ring-closing step that forms the heterocyclic C-ring. From these central intermediates, the pathway diverges into several side branches for the synthesis of a variety of other classes of flavonoid molecules that are produced through the combined actions of functionalizing enzymes that hydroxylate, reduce, alkylate, oxidize, and glycosylate the phenylpropanoid core structure [20–23] as shown in Fig. 53.1. Unlike otherflavonoids, stilbenes are produced directly by stilbene synthase (STS) enzyme using 4-coumaryl-CoA and malonyl-CoA without chalcone intermediates.

Flavanone 3-hydroxylase (F3H) catalyzes the stereospecific 3-hydroxylation of (*2S*)-flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, dihydroflavonol reductase (DFR) catalyzes the stereospecific conversion of dihydroflavonols to the respective flavan-3,4-diols (leucoanthocyanins) through NADPH-dependent reduction at the 4-carbonyl. The leucoanthocyanins are further converted to anthocyanidins by leucoanthocyanidin dioxygenase (LDOX) or anthocyanidin synthase (ANS). Flavan-3,4-diols are converted to flavan-3-ols by leucoanthocyanidin reductase (LCR) and finally form proanthocyanidins or condensed tannins in different steps. DFR also catalyzes the conversion of flavanones to flavan-4-ols, which form phlobaphenes in different sequential steps. Moreover, dihydroflavonols are converted to flavonols and their glycosides by the corresponding flavonol synthase (FLS) and glycosyltransferases (GTs). The intermediate flavanones are further diverted to another class of flavonoid, flavones, by flavone synthase I and II (FS I and II) enzymes and to isoflavones by isoflavone synthase (IFS) enzyme. These flavones and isoflavones are further modified by various hydroxylases, methyltransferases, reductases, and glycosyltransferases to form diverse flavonoids and isoflavonoids.

The flavonoid end products are transported to various subcellular or extracellular locations, while those flavonoids involved in pigmentation are generally transported into the vacuole.

3 Production of Flavonoids from Microbial System

Since ancient times, knowingly or unknowingly, humans have used microorganisms to supply products like beverages, fermented foods, or breads. During World

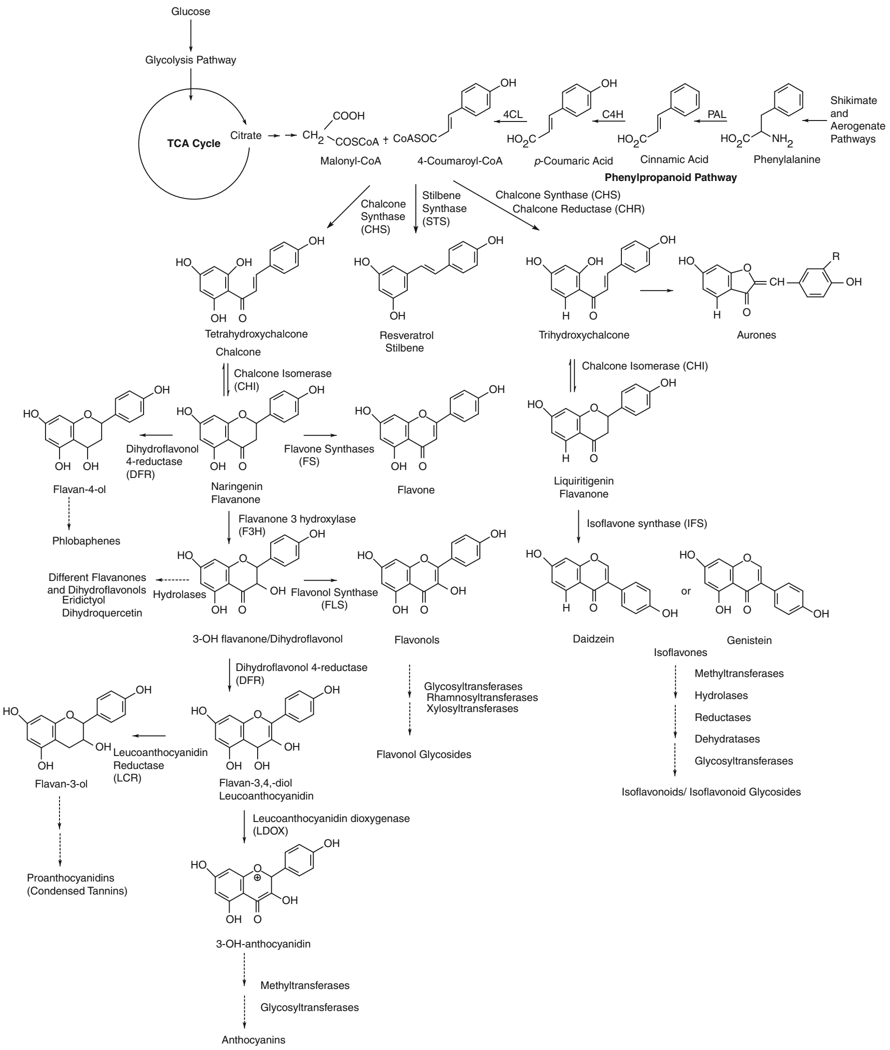

Glucose
Glycolysis Pathway
TCA Cycle
Citrate
COOH
CH2 COSCoA + CoASOC
OH
4CL
C4H
PAL
HO2C
NH2
Shikimate and Aerogenate Pathways
Malonyl-CoA
4-Coumaroyl-CoA
p-Coumaric Acid
Cinnamic Acid
Phenylalanine
Phenylpropanoid Pathway
Chalcone Synthase (CHS)
Stilbene Synthase (STS)
Chalcone Synthase (CHS)
Chalcone Reductase (CHR)
HO
OH
O
H
R
CH
Tetrahydroxychalcone
Chalcone
Resveratrol
Stilbene
Trihydroxychalcone
Aurones
Chalcone Isomerase (CHI)
Chalcone Isomerase (CHI)
Dihydroflavonol 4-reductase (DFR)
Flavone Synthases (FS)
Flavan-4-ol
Naringenin
Flavanone
Flavone
Liquiritigenin
Flavanone
Phlobaphenes
Flavanone 3 hydroxylase (F3H)
Isoflavone synthase (IFS)
Different Flavanones and Dihydroflavonols
Eridictyol
Dihydroquercetin
Hydrolases
Flavonol Synthase (FLS)
or
3-OH flavanone/Dihydroflavonol
Flavonols
Daidzein
Genistein
Isoflavones
Dihydroflavonol 4-reductase (DFR)
Glycosyltransferases
Rhamnosyltransferases
Xylosyltransferases
Methyltransferases
Hydrolases
Reductases
Dehydratases
Glycosyltransferases
Leucoanthocyanidin Reductase (LCR)
Flavan-3-ol
Flavan-3,4,-diol
Leucoanthocyanidin
Flavonol Glycosides
Isoflavonoids/ Isoflavonoid Glycosides
Leucoanthocyanidin dioxygenase (LDOX)
Proanthocyanidins (Condensed Tannins)
⊕
3-OH-anthocyanidin
Methyltransferases
Glycosyltransferases
Anthocyanins

Fig. 53.1 Biosynthetic pathway network of different types of flavonoids and related compounds in plants

War I and II, the second phase of traditional microbial biotechnology began with the development of acetone, butanol, and glycerol fermentations, followed by processes yielding citric acid, vitamins, and antibiotics. Industrial microbiology later merged with different interrelated areas of biology, chemistry, and engineering to lead to the development of more than 40 biopharmaceutical products during the early 1970s. Today, microbiology is a major participant in global industry, especially in the pharmaceutical, food, and chemical industries [24], to produce value-added compounds with numerous applications.

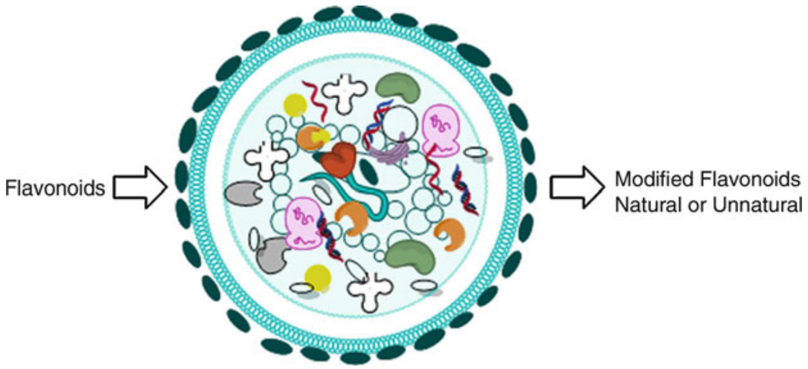


Fig. 53.2 Whole cell biotransformation of flavonoids by wild-type cells of *Streptomyces*, fungi, and plant cells

In general, three different strategies are applied for the production of natural products by microbial systems.

1. Use of wild-type whole cells for biotransformation
2. Use of purified natural or engineered enzymes
3. Use of genetically engineered microbial cells as a microbial cell factory for combinatorial biosynthesis

The first approach is normally applied with *Streptomyces*, fungal mycelia, and plant cells. All these cells contain a wide range of natural product-modifying native enzymes within their cytoplasm. Such enzymes could be constitutively expressed inside the cell. The supplementation of purified natural products, such as flavonoids, in these growing cells at a specific incubation temperature could lead to spontaneous modification of the compound by the cells' own available cofactor(s) or donor molecule(s) (Fig. 53.2). Since this strategy of modification of natural compounds is the simplest, the cheapest, and the easiest, the approach could be scaled up to industrial level upon optimization of the biotransformation conditions for high-level production of target compounds. Importantly, the production of desired products should be significantly high for industrialization. But, the demerit of this method lies with the production of a wide range of unexpected by-products.

The second approach uses purified enzymes and in vitro reactions for target modification of compounds (Fig. 53.3). Such practice is routinely done in the laboratory, although rarely at the industrial level, for the functional characterization of biosynthetic and modifying enzymes from different sources. Moreover, such study could help to determine the exact reaction mechanisms of enzymes, the interaction between different cofactors, ligands, and other molecules with enzymes, and finally the structure of the target modifying proteins. Since this method of natural product modification requires expensive cofactors and donors, efficient enzyme activity, and optimized reaction conditions, application at the industrial

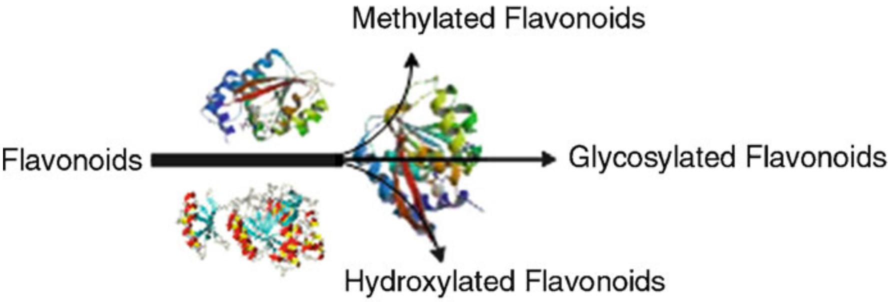


Fig. 53.3 In vitro conversion of flavonoids by recombinant proteins

level is comparatively costly and difficult. However, by this technique, the regiospecifically modified product can be obtained with few or even without by-products.

The last approach is relatively applicable for the biotransformation of flavonoids and other natural products. In this method, the microbial cell factory is constructed by using metabolic engineering, protein engineering, and synthetic biology tools (Fig. 53.4). The genetically well-known microbial cell is modified according to a particular purpose with high cell growth rate and efficient bioconversion of target compounds without a metabolic burden in the cell. The model microbial cells, like *E. coli*, *Streptomyces*, or *Saccharomyces cerevisiae*, are modified by introducing a partial or entire biosynthetic pathway gene cluster for the target compound. The genes of the pathway might be entirely of heterologous origin or partially native to the host. In certain cases, the host can be modified by deleting or blocking some essential genes for enhanced carbon flux through the pathway of interest. The heterologous genes will be functionally expressed and, finally, the biosynthetic pathway and growth conditions can be optimized for high titer product formation or biotransformation. In rare cases, by-products might be produced in very low amount in this method. This strategy of biosynthesis of natural products could be applicable for high-level production of different compounds in industry because of the low cost and the use of a simple fermentation technique.

The development of metabolic engineering, protein engineering, and synthetic biology approaches has revolutionized the use of efficient microbial systems for biosynthesis of value-added products, leaving synthetic chemistry methodologies far behind. Environmentally friendly routes of production, formation of lesser quantities of by-products due to high enzyme specificity, cost efficiency, improved yield and productivity of the target compounds, and easy development of novel derivatives are advantages of microbial biosynthesis over chemical synthesis of different natural products [25]. Obtaining compounds from their native host requires extensive optimization of the growth conditions and metabolic pathways within the native host, which is very difficult because of the adverse growth conditions and lack of ability to grow in laboratories. Therefore, well-characterized microbial cells like

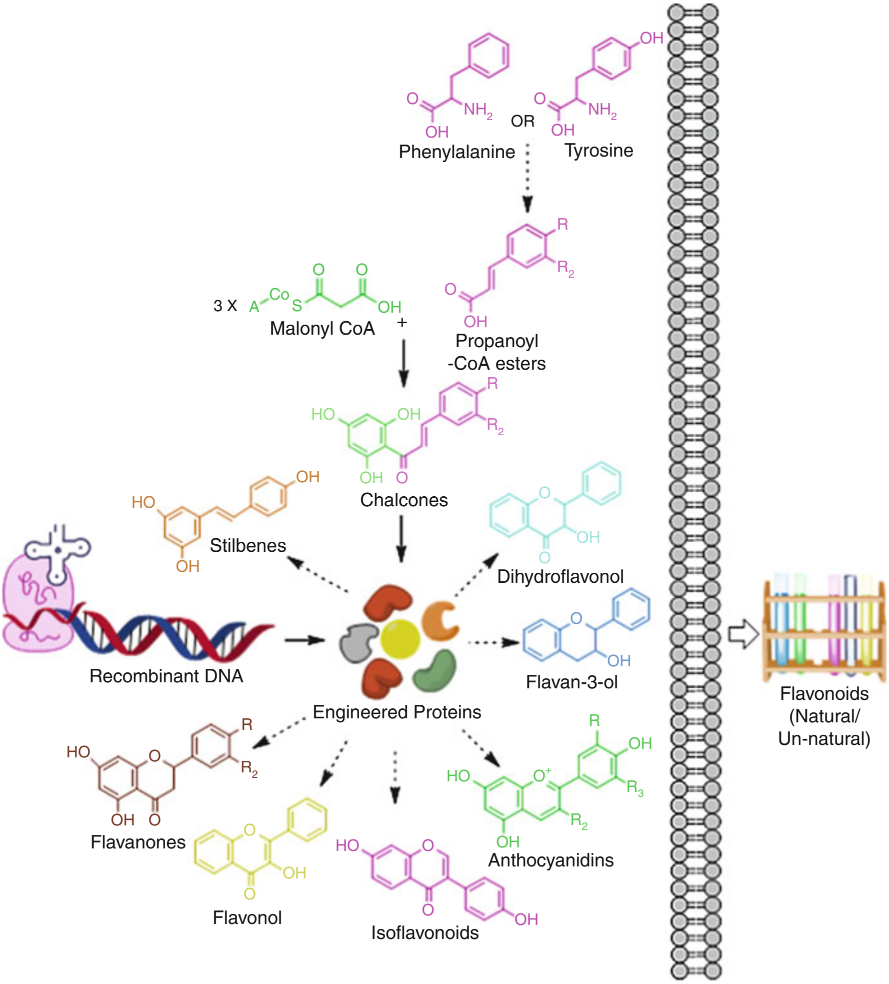

Phenylalanine
OR
Tyrosine
NH_2
OH
O
Propanoyl
-CoA esters
R
R_2
3 X A Co S
Malonyl CoA
+
Chalcones
HO
Stilbenes
Dihydroflavonol
Recombinant DNA
Engineered Proteins
Flavan-3-ol
Flavonoids
(Natural/
Un-natural)
Flavanones
R_3
O^+
Anthocyanidins
Flavonol
Isoflavonoids

Fig. 53.4 Engineered microbial cells for biosynthesis of different types of flavonoids

Escherichia coli, *Saccharomyces cerevisiae*, *Streptomyces* species, and *Bacillus subtilis* as universal platform organisms are highly desired due to their well-known physiology and genetics, high growth rates, and availability of abundant genetic tools [25]. Recently, numbers of natural products, their derivatives, and even unnatural compounds have been produced by using such established microbial hosts. Alkaloids, terpenoids, flavonoids, polyketides, and nonribosomal peptides, lantibiotics, antibiotics, organic acids, amino acids, and vitamins are some examples of microbially produced compounds from combinatorial biosynthetic approaches. In this chapter, we will discuss the recent developments in the production of flavonoids from different microbial systems and different approaches in detail.

3.1 Production of Flavonoids from Yeast

Like *E. coli* and other model microbial systems, the yeast *Saccharomyces cerevisiae* was developed as a model microbial factory for the production of different valuable products. Not only flavonoids, but also other terpenoid groups of compounds like artemisinic acid, a precursor of antimalarial drug artemisinin [26], alkaloids like magnoflorine and (*S*)-scoulerine, and taxol, were produced by coculturing a recombinant system of *E. coli* and *S. cerevisiae* [27]; berberine and morphine were produced from *S. cerevisiae* [28]. Metabolites from yeast cells, as well as whole yeast cells, have been used for animal feeding and in the baking industry for a long time. However, rapid developments in the biological sciences in the late 1980s and 1990s led scientists to use microbial hosts for high-level production and generation of new derivatives of pharmaceutical compounds. Recently, by using *E. coli*, Ajikumar and his group [29] produced more than 1 g l^{-1} of taxadiene, a precursor of taxol, by a metabolic engineering approach. Though the production is significantly higher, the drug supply is still insufficient to meet the demand of the world market. Previously, different approaches were applied in *E. coli* and *S. cerevisiae* for the production of taxadiene; unfortunately, titers have been limited to less than 10 mg l^{-1}. Taxol and its derivatives are the most potent and commercially successful anticancer drugs. However, the production of taxol still depends on plant sources.

The rate of development of antibiotic resistance is reaching a crisis point around the globe. Thus, there is an urgent need for replacement with new types of anti-infective or antimicrobial agents. However, developing new adjuncts to lower or reduce the resistance against a particular drug or to inhibit cellular components essential for cell viability is also an effective strategy. Recently, plant-derived natural compounds with antibacterial properties are attracting attention in the development of new drugs. Traditionally, flavonoid-rich medicinal plants are used for treatment and prevention of various infectious diseases, like sores, wound infections, acne, respiratory infections, and gastrointestinal diseases [30]. In vivo and in vitro studies have proven the broad spectrum of biological activities of flavonoids. Therefore, the health-protecting effects of flavonoids have stimulated significant research toward the elucidation of their biosynthetic networks, as well as the development of production platforms using well-characterized hosts, such as *E. coli* and *S. cerevisiae*.

Expression of plant genes involved in flavonoid biosynthesis is not easy in *E. coli*, but the ability of yeast *S. cerevisiae* to express such genes makes it an attractive production platform. However, the functional expression of many plant genes has recently been reported for the heterologous production of value-added products from *E. coli*.

First, Ro and Douglas constructed the initial steps of the plant phenylpropanoid pathway by connecting two enzymes, PAL and C4H, with a cytochrome P450 reductase (CPR) from a plant source in *S. cerevisiae* [31]. This heterologous transfer of plant phenylpropanoid pathway genes into yeast became the platform for the biosynthesis of various flavonoids and related compounds in microbial systems. Within a decade from the beginning of biosynthesis of flavonoids in microbes, a wide variety of derivatives of this group had been produced from yeast *S. cerevisiae* (Fig. 53.5). Monohydroxylated naringenin and unhydroxylated pinocembrin were produced by introducing phenylpropanoid pathway genes of different plant origins, that is, PAL from *Rhodosporidium toruloides*, 4CL from *Arabidopsis thaliana*, and CHS from *Hypericum androsaemum*, into *Saccharomyces cerevisiae*. The yeast produced approximately 7 mg l^{-1} of naringenin (**30**) and 0.8 mg l^{-1} of pinocembrin (**31**) without expression of C4H. This shows that PAL enzyme has tyrosine ammonia lyase (TAL) activity. Several by-products, such as 2′,4′, 6′-trihydroxydihydrochalcone and phloretin, were also identified in the culture broth in trace amounts [32].

In a similar study, different phenylpropanoid acids, such as cinnamic acid (**32**), *p*-coumaric acid (**33**), caffeic acid (**34**), and ferulic acid (**35**), were fed to recombinant yeast containing four different initial flavanone biosynthetic plant genes, C4H, 4CL, CHS, and CHI, at different time intervals to produce flavanones. In cinnamic acid-supplemented culture, 16.3 mg l^{-1} of pinocembrin (**31**) and 0.2 mg l^{-1} of naringenin (**30**) were produced. Naringenin (28.3 mg l^{-1}) and (2*S*)-eriodictyol (6.5 mg l^{-1}) were detected in *p*-coumaric acid- and caffeic acid-supplemented cultures, respectively. No flavanones were produced with ferulic acid precursor substrate [33]. The production of pinocembrin and naringenin in *S. cerevisiae* was 22- and 62-fold higher compared to that of respective flavanones in *E. coli* [34].

5-hydroxyflavanone and 5-deoxyflavanone were also produced from recombinant strains of *E.coli* and *S. cerevisiae* by a similar approach [35]. The same yeast strain producing flavanones was reconstructed by expression of FSI and II, resulting in the production of the flavones, chrysin (**36**), apigenin (**37**), and luteolin (**38**), as major products, along with the intermediate flavanones, pinocembrin, naringenin, and eriodictyol (**39**). Overexpression of yeast CPR1 and the use of acetate as the sole carbon source, rather than glucose or raffinose, led to overall increases in flavone-specific production in the FSII-expressing recombinant strain [36].

Stilbene, a defense compound, is only produced by specific plants, such as *Vitis vinifera*, during fungal infection, wounding, or UV irradiation. Resveratrol (**40**), a stilbene, was produced by using 4CL and STS genes from different plant sources in *S. cerevisiae* with maximum titer of 6 mg l^{-1} from polypropanoid acids as precursors [37, 38]. A transitional fusion protein, joining 4CL and STS, enhanced the production of resveratrol by 15-fold compared to that in the cotransformed yeast cells. This experiment emphasizes the importance of the physical localization of 4CL and STS for facilitated production of resveratrol. Resveratol was also successfully produced in human HEK293 cells by introducing the entire resveratrol pathway [39].

Isoflavonoid production was also achieved in *S. cerevisiae* by using different types of isoflavonoid biosynthetic genes from plants [40]. The supplementation of the intermediate product naringenin (**29**) in growing culture of recombinant *S. cerevisiae* with IFS and CPR genes produced 20.8 mg l^{-1} of genistein (**41**) [41], which was around threefold higher than the production from the *S. cerevisiae*

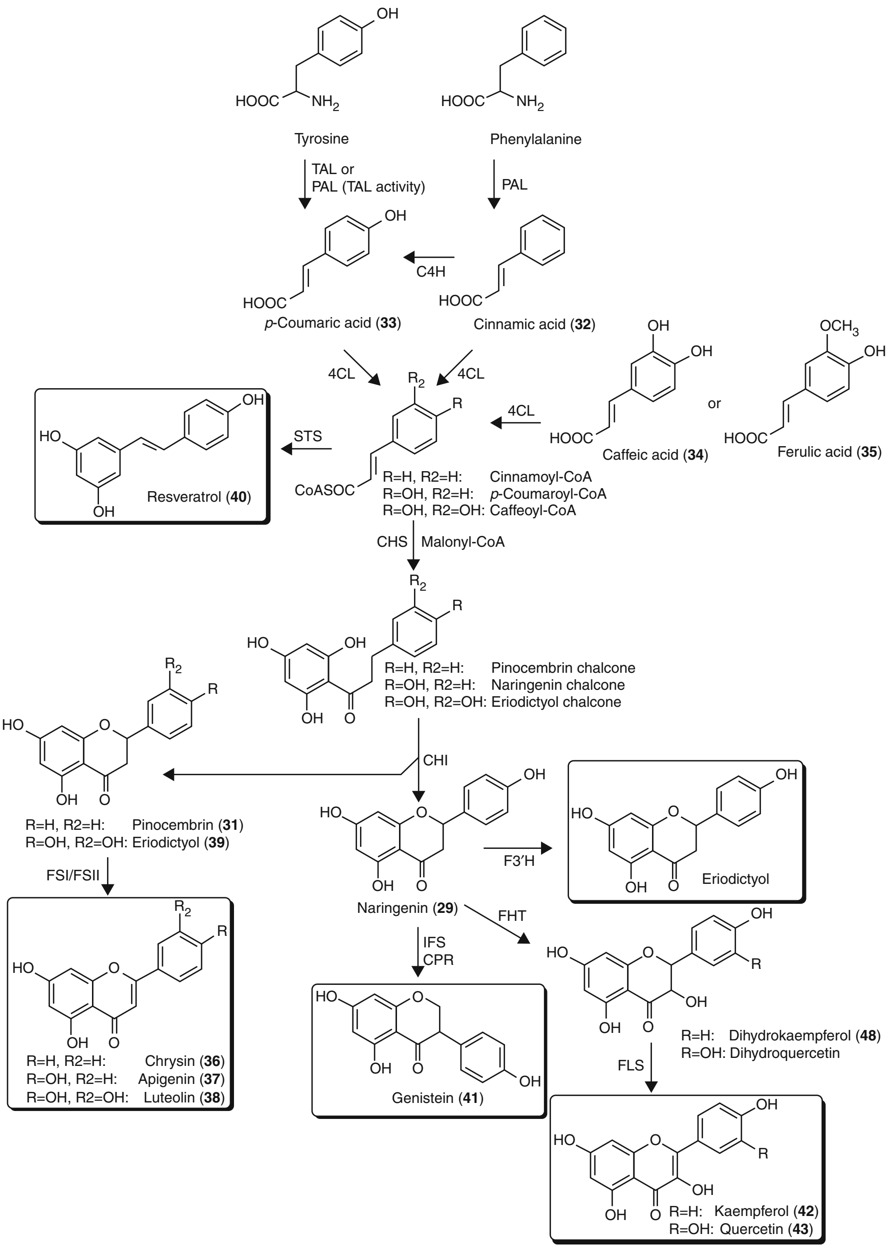

Tyrosine
Phenylalanine
TAL or
PAL (TAL activity)
PAL
C4H
p-Coumaric acid (**33**)
Cinnamic acid (**32**)
4CL
4CL
4CL
Caffeic acid (**34**)
or
Ferulic acid (**35**)
STS
Resveratrol (**40**)
R=H, R2=H: Cinnamoyl-CoA
R=OH, R2=H: *p*-Coumaroyl-CoA
R=OH, R2=OH: Caffeoyl-CoA
CHS
Malonyl-CoA
R=H, R2=H: Pinocembrin chalcone
R=OH, R2=H: Naringenin chalcone
R=OH, R2=OH: Eriodictyol chalcone
CHI
R=H, R2=H: Pinocembrin (**31**)
R=OH, R2=OH: Eriodictyol (**39**)
FSI/FSII
R=H, R2=H: Chrysin (**36**)
R=OH, R2=H: Apigenin (**37**)
R=OH, R2=OH: Luteolin (**38**)
Naringenin (**29**)
F3′H
Eriodictyol
IFS
CPR
Genistein (**41**)
FHT
R=H: Dihydrokaempferol (**48**)
R=OH: Dihydroquercetin
FLS
R=H: Kaempferol (**42**)
R=OH: Quercetin (**43**)

Fig. 53.5 Biosynthetic pathways engineered in *S. cerevisiae* for the production of varieties of flavonoids, isoflavonoids, and stilbenes

YPH499 strain GEN23 carrying PAL, C4H, CPR, 4CL, CHS, CHI, and IFS genes from different plants [42]. Insignificant production of genistein was reported from coculture of *E. coli* and *S. cerevisiae* recombinants with tyrosine precursor [43]. *S. cerevisiae* YPH499 strain RESV11 (PAL, C4H, CPR, 4CL, and STS) produced resveratrol (**40**), strain NAR12 (PAL, C4H, CPR, 4CL, CHS and CHI) produced naringenin (**29**), strain KAE34 (PAL, C4H, CPR, 4CL, CHS, CHI, F3H, and FLS) produced kaempferol (**42**), and strain QUE44 (PAL, C4H, CPR, 4CL, CHS, CHI, F3H, F3′H, and FLS) produced quercetin (**43**) [42].

The whole cell biotransformation assay with recombinant yeast carrying a single gene revealed a significant bioconversion rate. For example, yeast carrying flavonoid 3′ hydroxylase (F3′H) from Gerbera hybrid produced 200 mg l^{-1} of eriodictyol from naringenin [44]. However, the conversion of aglycone to glycoside is dose dependent, increasing as the initial substrate concentrations increase up to a certain limit. Variation of the glucose concentration in the medium during bioconversion reaction controls the selectivity to produce the desired glycoside. For example, the major product will be either naringenin-7-*O*-glucoside or naringenin-4′-*O*-glucoside depending upon the glucose concentration in the medium when naringenin is fed to yeast carrying flavonoid glucosyltransferase from *Dianthus caryophyllus*. Orotic acid in the medium enhanced the yield and shifted the selectivity toward naringenin-7-*O*-glucoside in the same experiment [45, 46].

3.2 Production of Flavonoids from *E. coli*

The functional expression of plant cytochrome P450 monooxygenases, such as flavonoid 3-hydroxylase, flavonoid 3, 5-hydroxylase, and other genes of flavonoid biosynthetic pathways from plant origin, is difficult in prokaryotic cells [47]. This ultimately results in low titer production of the desired compound. However, the application of recently developed molecular biology, metabolic and protein engineering, and synthetic biology tools significantly increased the production of different natural products in *E. coli*. The high-level production of such value-added products from simple hosts like *E. coli* using renewable substrates has garnered the attention of the entire world. Therefore, the techniques of constructing efficient microbial factories for the production of natural compounds could be applied to generate novel compounds with unique structures and functions by introducing highly catalytic enzymes. Recent data analysis shows that a number of modified natural compounds have been produced from *E. coli*. In this section, the recent approaches used for flavonoid production from *E. coli* will be discussed in detail.

Almost a decade ago, the heterologous expression of anthocyanin biosynthetic genes with individual promoters and ribosome binding sites (RBS) [34] from different plant sources, i.e., *flavanone 3β-hydroxylase*(FHT) from *Malus domestica*, DFR from *Anthurium andraeanum*, ANS from *M. domestica*, and UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UGT) from *Petunia hybrid*, was achieved successfully in *E. coli* (Fig. 53.6). Such recombinant *E. coli* can

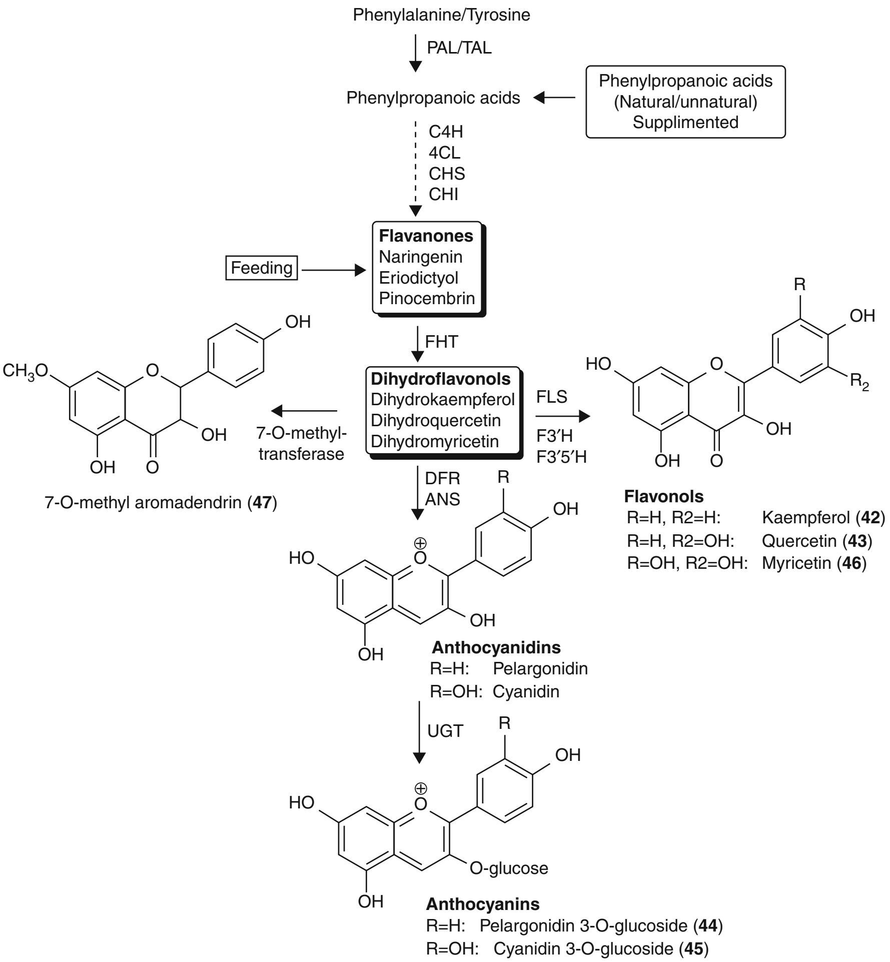

Phenylalanine/Tyrosine
PAL/TAL
Phenylpropanoic acids
Phenylpropanoic acids (Natural/unnatural) Supplimented
C4H
4CL
CHS
CHI
Feeding
Flavanones
Naringenin
Eriodictyol
Pinocembrin
FHT
Dihydroflavonols
Dihydrokaempferol
Dihydroquercetin
Dihydromyricetin
7-O-methyl-transferase
FLS
F3′H
F3′5′H
DFR
ANS
UGT
CH3O
OH
O
7-O-methyl aromadendrin (47)
HO
R
R2
Flavonols
R=H, R2=H: Kaempferol (42)
R=H, R2=OH: Quercetin (43)
R=OH, R2=OH: Myricetin (46)
Anthocyanidins
R=H: Pelargonidin
R=OH: Cyanidin
O-glucose
Anthocyanins
R=H: Pelargonidin 3-O-glucoside (44)
R=OH: Cyanidin 3-O-glucoside (45)

Fig. 53.6 Biosynthetic networks of flavonoids engineered in *E. coli*

convert the colorless flavanones, naringenin (**29**) or eriodictyol (**39**) to the corresponding colorful glycosylated anthocyanins, pelargonidin 3-*O*-glucoside (**44**) or cyanidin 3-*O*-glucoside (**45**) [34, 48]. Similarly, a strain expressing plant 4CL, CHS, CHI, ANS, and 3-*O*-glycosyltransferase produced flavanones up to 700 mg l^{-1} and anthocyanins up to 113 mg l^{-1} [1].

The coexpression of flavonol biosynthetic structural genes 4CL, CHS, CHI, flavanone 3β-hydroxylase (FHT), and FLS, along with plant P450 flavonoid 3′,5′hydroxylase (F3′5′H) as a fusion protein with a P450 reductase, produced three different flavonols: kaempferol (**42**), quercetin (**43**), and myricetin (**46**) [49]. Later, chimeric cytochrome P450 enzymes were developed and used for the bioconversion of naringenin to genistein (**41**), and liquiritigenin to daidzein. The expression of the chimera in bacteria catalyzed fivefold higher production of isoflavones than in yeast expressing wild-type enzymes. The artificial enzyme design could potentially be used to increase the availability of natural pharmaceuticals and for the synthesis of other plant chemicals by industrial microbes [50]. Currently, mutant enzymes are generated to enhance the bioconversion as well as for broad modifications of the compounds by the same enzyme. For example, engineered 7-*O*-methyltransferase from *Populus deltoids* converted quercetin to rhamnetin with an increased conversion rate [51].

Different approaches have been applied to produce a number of natural and unnatural flavonoids in *E. coli*. For example, 4CL-1, ACC, CHS, and CHI genes from plants and bacterial sources were used to develop recombinant *E. coli* as an enzyme bag that converted exogenous fed natural carboxylic acids (cinnamic acid, *p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid, caffeic acid) and unnatural carboxylic acids (fluorocinnamic acids, furyl, and thienyl and naphthylacrylic acids) to the corresponding natural and unnatural flavanones (Fig. 53.7). When FSI was introduced into the same *E. coli*, 13 flavones were synthesized from the corresponding flavanones, including four unnatural ones. Further introduction of F3H and FLS to the flavanone-producing *E. coli* generated eight different types of flavonols with two unnatural ones. The yields of the natural flavanones were roughly 70–90 mg l^{-1} and those of the unnatural flavanones reached 50–100 mg l^{-1}, whereas the production of natural and unnatural flavones was 30–50 mg/l. Only trace amounts of flavonols were detected. Reconstruction of the same *E. coli* cell with 4CL, ACC, and STS resulted in the production of 15 stilbenes, including five unnatural, upon supplementation with different carboxylic acids. The overall production of unnatural stilbenes was 55–130 mg l^{-1}, while that of stilbenes derived from natural carboxylic acids was 130–170 mg l^{-1}. Overall, 87 polyketides, including 36 unnatural flavonoids and stilbenes, were produced by exogenously feeding different carboxylic acids to this recombinant *E. coli*. This system could be promising for construction of a larger library if it employed other modification enzymes [52].

In many cases, the biosynthetic efficacy is greatly limited by the precursor and cofactor availability in the host. The initial precursors of the phenylpropanoid and flavonoid biosynthetic pathways, L-tyrosine, L-phenylalanine, and malonyl-CoA, are derived from native metabolism in both *E. coli* and *S. cerevisiae*. When the flavonoid biosynthetic pathway is heterologously introduced and expressed in such hosts, the precursor molecules become limited within the cell [53]. This results in relatively low production of the target compound and cell growth rate because of the metabolic burden within the cell [33], which remains a bottleneck for large-scale applications and low-cost production of different flavonoids. Therefore, introduction of alternative carbon assimilation pathways, such as the malonate utilization pathway, and inhibition or deletion of competitive reactions, including fatty acid synthesis and UDP-glucose consumption pathways, could improve the availability of malonyl-CoA and UDP-glucose. The overall modification carried out in yeast, *E. coli* or *Streptomyces* for enhanced carbon flux through malonyl-CoA is presented in Fig. 53.8.

This limitation was partially addressed by rational modification of the acetate-acetyl-CoA–malonyl-CoA metabolic node in order to improve malonyl-CoA

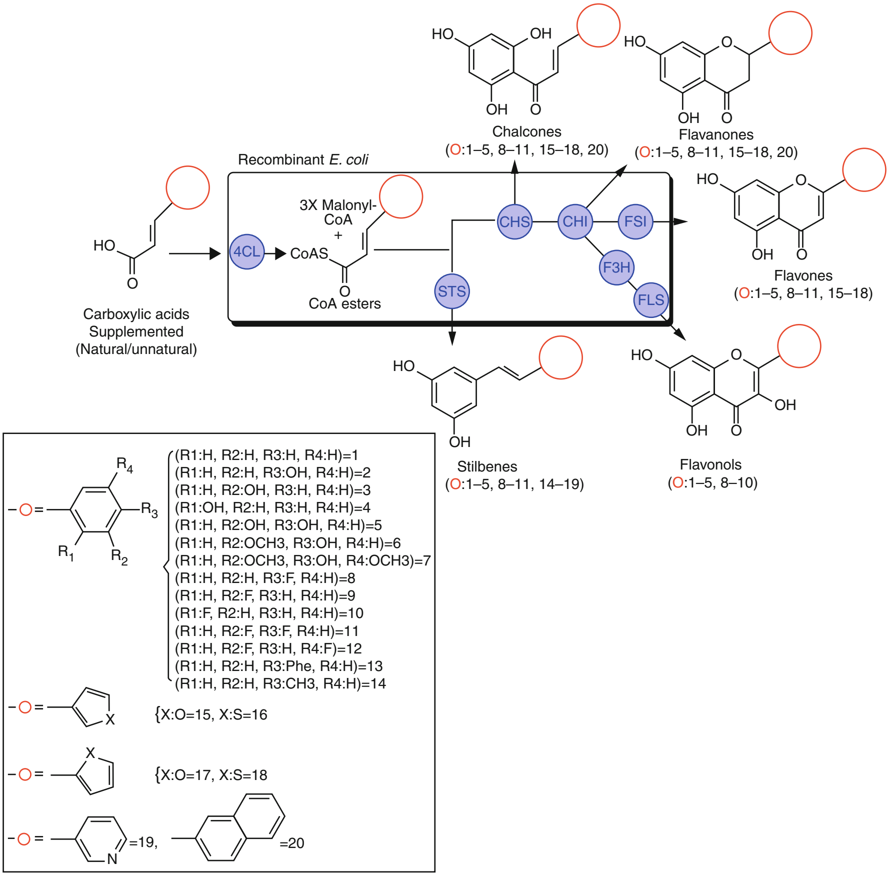

HO
OH
OH
O
Chalcones
(O:1–5, 8–11, 15–18, 20)
HO
O
OH
O
Flavanones
(O:1–5, 8–11, 15–18, 20)
Recombinant E. coli
3X Malonyl-
CoA
+
HO
O
Carboxylic acids
Supplemented
(Natural/unnatural)
4CL
CoAS
O
CoA esters
CHS
CHI
FSI
F3H
FLS
STS
HO
O
OH
O
Flavones
(O:1–5, 8–11, 15–18)
HO
OH
Stilbenes
(O:1–5, 8–11, 14–19)
HO
O
OH
OH
O
Flavonols
(O:1–5, 8–10)
R4
R3
R1
R2
(R1:H, R2:H, R3:H, R4:H)=1
(R1:H, R2:H, R3:OH, R4:H)=2
(R1:H, R2:OH, R3:H, R4:H)=3
(R1:OH, R2:H, R3:H, R4:H)=4
(R1:H, R2:OH, R3:OH, R4:H)=5
(R1:H, R2:OCH3, R3:OH, R4:H)=6
(R1:H, R2:OCH3, R3:OH, R4:OCH3)=7
(R1:H, R2:H, R3:F, R4:H)=8
(R1:H, R2:F, R3:H, R4:H)=9
(R1:F, R2:H, R3:H, R4:H)=10
(R1:H, R2:F, R3:F, R4:H)=11
(R1:H, R2:F, R3:H, R4:F)=12
(R1:H, R2:H, R3:Phe, R4:H)=13
(R1:H, R2:H, R3:CH3, R4:H)=14
X
{X:O=15, X:S=16
X
{X:O=17, X:S=18
N
=19,
=20

Fig. 53.7 Biosynthesis of natural and unnatural flavonoids and stilbenes in *E. coli*. Supplementation of unnatural synthesized carboxylic acids and natural carboxylic acids in *E. coli* recombinants produced unnatural and natural respective chalcones, flavanones, flavones, flavonols, and stilbenes. The *red circle* shown in the figure is one of the structure/groups shown in the *inset*

precursor availability in flavanone-producer recombinant *E. coli* strains by increasing carbon flux toward malonyl-CoA, which was achieved after overexpression of multisubunit complex of acetyl-CoA carboxylase (ACC)-biotin ligase (BirA) and enzymes of acetate assimilation pathways. Auxiliary expression of ACC with a chimeric biotin ligase (BirA) consisting of the N-terminus from *E. coli* and C-terminus from *Photorhabdus luminescens* increased the production of pinocembrin, naringenin, and eriodictyol up to 1,379 %, 183 %, and 373 % compared to production in strains expressing only the flavonoid pathway; these corresponded to 429, 119, and 52 mg l^{-1}, respectively [54]. Previously, Miyahisa and coworkers [53] reported that the expression of the two-subunit ACC from the gram-positive bacterium *C. glutamicum* resulted in pinocembrin and naringenin synthesis of approximately 1 and 0.7 mg l^{-1}, respectively, from a flavanone-producing *E. coli*.

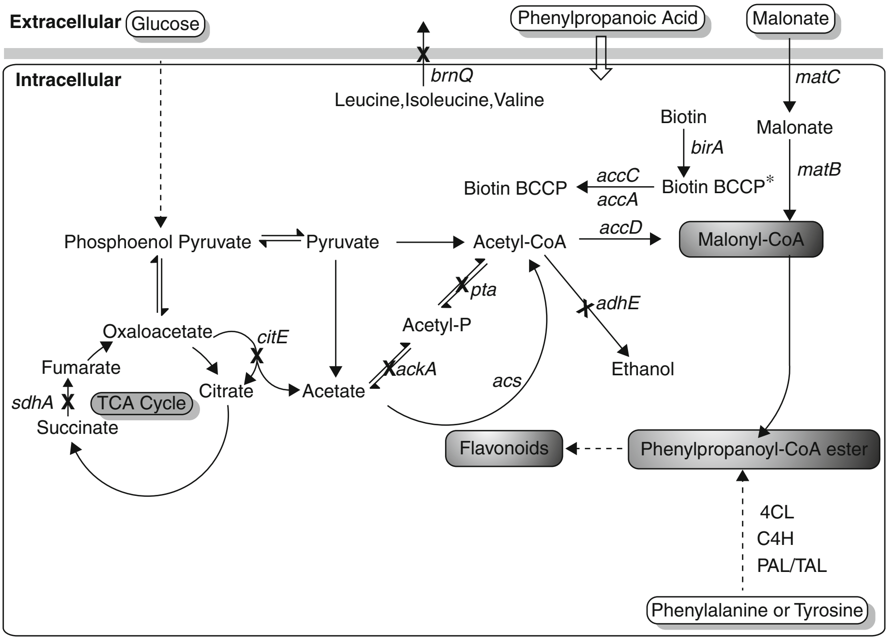

Extracellular
Glucose
Phenylpropanoic Acid
Malonate
Intracellular
brnQ
Leucine,Isoleucine,Valine
matC
Biotin
Malonate
birA
matB
Biotin BCCP
accC
accA
Biotin BCCP*
Phosphoenol Pyruvate
Pyruvate
Acetyl-CoA
accD
Malonyl-CoA
pta
adhE
Acetyl-P
Oxaloacetate
citE
Fumarate
ackA
acs
Ethanol
sdhA
TCA Cycle
Citrate
Acetate
Succinate
Flavonoids
Phenylpropanoyl-CoA ester
4CL
C4H
PAL/TAL
Phenylalanine or Tyrosine

Fig. 53.8 Diagrammatic representation of engineering of microbial cells for increased carbon flux through malonyl-CoA for enhanced supplementation of flavonoid biosynthetic intermediates. *Dashed arrow* represents multiple steps, *crossed arrow* represents deletion of gene (e.g., *sdhA*, *cite*, *ackA*, *pta*, *adhEbrnQ*), and other genes are overexpressed (e.g., *matB*, *matC*, *acs*, *acc*, *birA*). Extracellular components are supplied to the cell culture growth medium (e.g., Glucose, malonate, phenylpropanoic acids). A detailed explanation of the cell modification for carbon flux through malonyl-CoA and the effect in flavonoid production is given in the text

In another similar study, Cipher of Evolutionary Design (CiED) model was used to generate improved *E. coli* genotypes that more effectively channeled carbon flux toward malonyl-CoA and other cofactors. The deletion of *sdhA*, *adhE*, *brnQ*, and *citE* genes and overexpression of the enzymes acetyl-CoA synthase (ACS), ACC, biotin ligase (BPL), and pantothenate kinase (PNK) along with plant-derived flavanone biosynthetic genes increased the naringenin titer to 270 mg l^{-1} and to 150 mg l^{-1} of eriodictyol in the engineered construct [55].

Recently, naringenin-producing *E. coli* [54] was reconstructed with F3H, a FeII-dependent dioxygenase enzyme from *Arabidopsis thaliana*, and 7-*O*-methyltransferase from *Streptomyces avermitilis* to produce 7-*O*-methyl aromadendrin (**47**) Fig. 53.6. The maximum titer of 7-*O*-methyl aromadendrin (2.7 mg l^{-1}) was achieved by increasing the availability of malonyl-CoA precursor in the pathway by overexpressing ACC α and β subunits (*nfa9890* and *nfa9940*), biotin ligase (*nfa9950*), and acetyl-CoA synthetase (*nfa3550*) genes from *Nocardia farcinica* upon supplementation with *p*-coumaric acid. But when naringenin was supplemented in the growing recombinant culture producing 7-*O*-methyl aromadendrin, the product was enhanced by 12-fold. Dihydrokaempferol (**48**) and sakuranetin were also detected as intermediate products from the same strain [56]. A similar strategy of higher carbon flux through malonyl-CoA has been applied to overcome the shortage of native precursors and cofactors of flavonoids in the cell without retarding cell growth. The deletion of competing pathway enzymes (ackA, pta, adhE) using acetyl-CoA for the production of acetate and ethanol, and overexpression of acetate assimilation enzyme genes (*acs*, *acc*, and *fabD*) led to the creation of an *E. coli* strain with 15-fold higher cellular malonyl-CoA level [57]. Such recombinants would be useful for the high-level production of flavonoids and other natural products that use malonyl-CoA as a precursor or intermediate.

A different approach has been used to produce glycosylated flavonoids from *E. coli* by enhancing the pool of different activated sugars. For example, UDP-glucose dehydrogenase (CalS8) and UDP-glucuronic acid decarboxylase (CalS9) from *Micromonospora echinospora* spp. *Calichensis* were expressed together with an integrated copy of *E. coli* K12 UDP-glucose pyrophosphorylase (GalU) in the background strain *E. coli* BL21 (DE3) with the glucose phosphate isomerase (*pgi*) gene-deleted mutant for excess availability of UDP-xylose along with 7-*O*-glycosyltransferase (ArGt-4) from *Arabidopsis thaliana*. This newly engineered strain was fed naringenin to produce naringenin 7-*O*-xyloside [58]. Similarly, the pools of TDP-L-rhamnose and TDP-D-allose sugars were developed separately by expressing the respective sugar biosynthetic pathway genes from different bacterial sources in the same *pgi*-deleted *E. coli* BL21 (DE3) along with *Arabidopsis thaliana* glycosyltransferase (ArGt-3). When these recombinants were fed quercetin and kaempferol, the respective sugar-producing strains produced 3-*O*-rhamosyl quercetin, 3-*O*-rhamnosyl kaempferol, and only 3-*O*-allosyl quercetin [59]. In another similar recent report, *E. coli* BL21 (DE3) carrying three genes, *ugd* (UDP-glucose dehydrogenase), *arnA* (UDP-L-Ara4N formyltransferase//UDP-GlcA C-4″-decarboxylase), and *arnB* (UDP-L-Ara4O C-4″ transaminase), along with glycosyltransferase AtUGT78D3 from *Arabidopsis thaliana*, produced quercetin-3-*O*-Ara4FN when fed quercetin [60]. This approach of producing flavonoid glycosides might be helpful to generate medically important novel/unnatural flavonoids in *E. coli*. The general scheme of this approach is shown in Fig. 53.9.

During the last decade, metabolic engineering approaches have been used to produce various flavonoid compounds in microbial systems by heterologously expressing plant structural genes. Though strain titers and yields have recently been improved significantly, this strategy of fermentation faces problems like the requirement for supplementation of expensive phenylpropanoic precursors into the media. To overcome this problem, a four-step heterologous pathway consisting of the enzymes TAL, 4CL, CHS, and CHI was assembled within an engineered L-tyrosine *E. coli* overproducer [61] in order to enable the production of the main flavonoid precursor naringenin directly from glucose. Extensive optimization of the enzyme sources and relative gene expression levels achieved high *p*-coumaric acid and naringenin production. Moreover, addition of cerulenin, a fatty acid enzyme inhibitor, increased the 29 mg l^{-1} naringenin production from glucose to 84 mg l^{-1} in minimal medium without additional precursor supplementation [21].

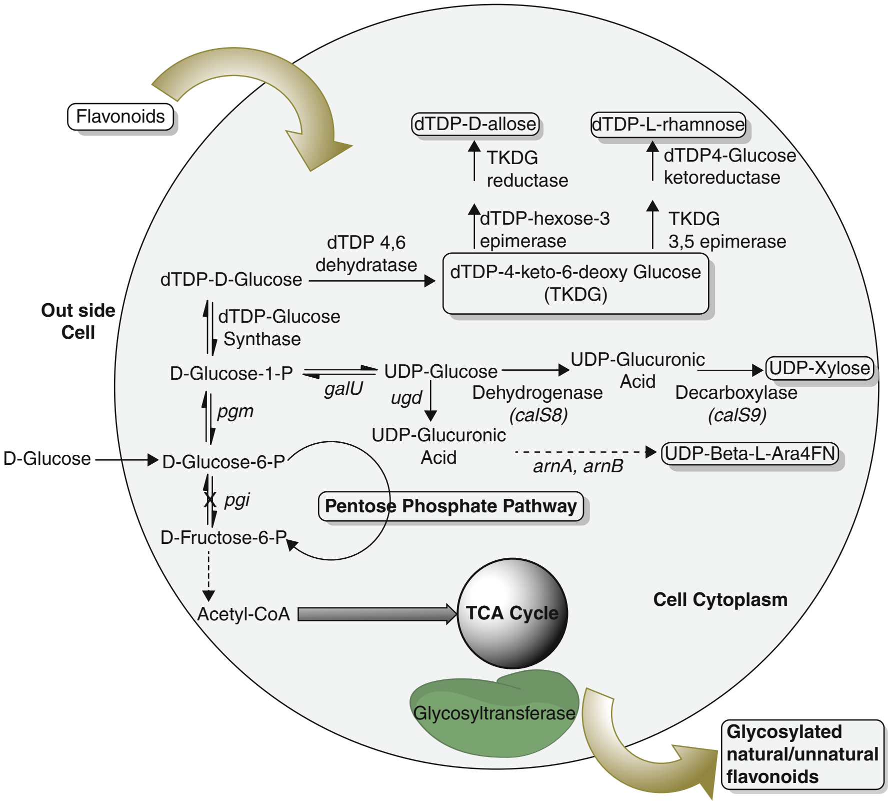

Flavonoids
Out side Cell
dTDP-D-allose
TKDG reductase
dTDP-L-rhamnose
dTDP4-Glucose ketoreductase
dTDP-hexose-3 epimerase
TKDG 3,5 epimerase
dTDP 4,6 dehydratase
dTDP-D-Glucose
dTDP-4-keto-6-deoxy Glucose (TKDG)
dTDP-Glucose Synthase
D-Glucose-1-P
galU
UDP-Glucose
UDP-Glucuronic Acid
UDP-Xylose
ugd
Dehydrogenase (calS8)
Decarboxylase (calS9)
pgm
UDP-Glucuronic Acid
D-Glucose
D-Glucose-6-P
arnA, arnB
UDP-Beta-L-Ara4FN
X pgi
Pentose Phosphate Pathway
D-Fructose-6-P
Acetyl-CoA
TCA Cycle
Cell Cytoplasm
Glycosyltransferase
Glycosylated natural/unnatural flavonoids

Fig. 53.9 General scheme of production of flavonoid glycosides by using sugar pathway engineered *E. coli*. dTDP-D-allose, dTDP-L-rhamnose, and UDP-xylose sugar biosynthetic pathways genes are overexpressed in *E. coli* BL21(DE3)/(Δ*pgi*) mutant and UDP-*β*-L-Ara4FN sugar biosynthetic pathways genes are overexpressed in nonmutant *E. coli*. The overexpressed genes are mentioned in the pathways

Besides flavonoids and other natural products, other unnatural compounds with significant titer are alternatively synthesized from glucose in *E. coli* through de novo design and development of novel metabolic pathway petrochemicals [62]. Thus, metabolic engineering and use of *E. coli* as a microbial factory has extensively attracted interest in the development of a novel system for modification and efficient production of highly useful compounds.

3.3 Production of Flavonoids from *Streptomyces* and Fungi

Streptomyces and fungi are potential sources of different antibiotics and other natural compounds. Many antibacterial, antiviral, anticancer, and other potential pharmaceutically applicable secondary metabolites have been isolated from these species. Only a few species can grow in defined medium in the laboratory. However, some of them have been developed as potential industrial organisms. For example, *Streptomyces avermitilis*, *S. ceolicolor*, *S. venezualae*, and *S. lividans* are widely used in industry for different purposes. Recently, the genome sequence of different *Streptomyces* became available, which led to the easy development of such microbial cells for industrial purposes. Unlike *E. coli* and other bacteria, *Streptomyces* and fungi are complex in their physiology and metabolism. They grow relatively slowly and require complex culture medium. However, the recent development of *Streptomyces* as a microbial factory has revolutionized the heterologous production of different antibiotics and natural compounds. Plenty of flavonoids and other natural product modification enzymes have been found in the genome of *Streptomyces*. Thus, these microbial cells convert compounds by different modifications by native enzymes, such as hydroxylation, methylation, glycosylation, alkylation, etc. Moreover, different modifying genes have been introduced for specific modification of the target compounds in *Streptomyces*. For example, *S. lividans* TK21 carrying the *bphA1* (*2072*) *A2A3A4* gene cluster, which codes for a shuffled biphenyl dioxygenase holoenzyme with broad substrate specificity, was used for the bioconversion of flavanone and flavone. Flavone was converted into two products, 2′, 3′-dihydroxyflavone (a major product) and 3′-hydroxyflavone (a minor product), whereas flavanone was converted into three products 2′, 3′-dihydroxyflavanone (a major product), 2′-hydroxyflavanone, and 3′-hydroxyflavanone (minor products). Similarly, 6-hydroxyflavone and 6-hydroxyflavanone were converted into 2′, 6-dihydroxyflavone and 3′,6-dihydroxyflavanone, respectively [63]. Few studies have reported the modification of flavonoids by whole cell biotransformation without any modification in the host *Streptomyces* species. For example, *S. avermitilis* MA-4680 showed high *ortho*-dihydroxylation activity to produce 3′,4′,7-trihydroxyisoflavone and 3′,4′,5,7-tetrahydroxyisoflavone from daidzein and genistein, respectively [64, 65]. Similarly, baicalein and hydroxylated phloretin were produced from exogenously fed chrysin and phloretin, respectively, in *S. avermitilis* and *S. ceolicolor*. Use of the cytochrome P450 inhibitor erythromycin in *S. ceolicolor* completely blocked the bioconversion of chrysin [66]. Besides whole cell biotransformation, in vitro bioconversion of flavonoids has been carried out using different modifying enzymes from *Streptomyces* and other sources [67].

In another separate study, native pikromycin polyketide synthase gene-deleted strain *S. venezuelae* DHS2001 was used for expression of flavanone and stilbene biosynthetic genes of different origin for the production of the respective compounds. 4-Coumarate/cinnamate: Coenzyme A ligase from *S. coelicolor* (ScCCL) and CHS from *Arabidopsis thaliana* (atCHS) introduced into a recombinant *Streptomyces* strain produced racemic naringenin and pinocembrin from 4-coumaric acid and cinnamic acid, respectively. When both genes were placed under the *ermE* promoter, production of both flavanones was increased significantly. Resveratrol and pinosylvin stilbenes were produced by overexpression of ScCCL and codon-optimized STS gene from *Arachis hypogaea* [68]. The same strain carrying the codon-optimized flavone synthase I (FS I) gene from *Petroselium crispum* produced flavones apigenin and chrysin from exogenous naringenin and pinocembrin, respectively, while expression of the codon-optimized F3H and FLS gene from *Citrus siensis* and *Citrus unshius*, respectively, produced flavonols, kaempferol, and galangin from flavanones [69]. The overexpression of malonate assimilation operon matB and matC encoding malonyl-CoA synthetase and the putative dicarboxylate carrier protein from *S. coelicolor* enhanced the production of flavanones and flavones, respectively, up to 40 and 30 mg l^{-1}in both biosynthetic gene-overexpressing strains [70].

Fungi, a separate group of microorganisms, are used in biotechnology industries for the production of valuable primary and secondary metabolites. Most fungi have pathogenic properties in plants and animals because of the production of fatal toxins and other secondary metabolites. Therefore, fungi are not widely explored for the biotransformation of natural products in contrast to yeast, *Streptomyces*, *E. coli*, and other bacteria. However, a few reports are available regarding the whole cell biotransformation of flavonoids using fungi. For example, among 138 screened soil fungi, only strain *Trichoderma harzianum*NJ01 was able to grow in media containing puerarin. Under optimized conditions, the mycelium of the fungus converted 41 % of supplemented puerarin into 3′-hydroxypuerarin [71]. Similarly, *Penicillium griseoroseum* isolated from *Coffeea arabica* seeds is grown in 5,7, 3′,4′,5′-pentamethoxyflavanone containing Czapeck's medium. Interestingly, the fungus incorporated a dimethylated tetraketide clavatol, atypical fungal secondary metabolite, into the flavanone structure at C-6, resulting in a novel benzylated flavonoid (**48**) [72].

Besides whole cell fungal biotransformation of flavonoids, aromatic peroxygenases from fungus *Agrocybeaegerita* (*Aae*APO) regioselectively catalyzes the H_2O_2-dependent monohydroxylation of diverse flavonoids [73].

3.4 Production of Flavonoids from Transgenic Plants and Plant Tissue Cultures

Plants are the natural sources of diverse kinds of flavonoids. Isoflavonoids are predominantly found in leguminous plants but rarely in other plants. Therefore, Liu et al. engineered nonleguminous transgenic tobacco, lettuce, and petunia with genes encoding key enzymes of the flavonoid/isoflavonoid pathway to produce isoflavonoids in nonleguminous plants. Upon introduction of soybean IFS into these plants, which do not naturally produce isoflavonoids, genistein was detected in petals and leaves. Likewise, suppression of F3H in tobacco by its antisense gene and overexpression of soybean IFS at the same time increased genistein yield. In addition, overexpression of PAL also led to enhanced genistein production in tobacco petals and lettuce leaves in the presence of IFS compared to that in plants that overexpressed only IFS [74].

Similarly, Sugiyama and coworkers recently used broad substrate-specific prenyltransferases, NphB, SCO7190, and NovQ, from *Streptomyces* and two plant genes, N8DT and G6DT, from *Sophora flavescens* to generate 624 transgenic

Lotus japonicus plants for the production of wide varieties of prenylated flavonoids. In fact, no prenylated products were detected from those transgenic plants. However, when leaves were incubated with naringenin and genistein, prenylated flavonoids 7-*O*-geranylgenistein, 6-dimethylallylnaringenin, 6-dimethylallylgenistein, 8-dimethylallynaringenin, and 6-dimethylallyl genistein were produced in the transgenic plants [75]. This study demonstrated the potential for the production of novel prenylated polyphenols in transgenic plants. A different concept of flavonoid diversification can be applied in plant tissue culture. Different natural product-modifying enzymes are naturally present in the plant cell. The use of such cell cultures may produce diverse types of target compounds in the laboratory. Shimoda et al. investigated the biotransformation of naringin and naringenin by using cultured cells of *Eucalyptus perriniana*. Altogether, ten different types of glycosylated compounds were detected in very small amounts from the culture extract treated with naringin and naringenin. Naringin was converted into diverse glycosides, such as naringenin 7-*O*-β-ᴅ-glucopyranoside, naringenin, naringenin 5,7-*O*-β-ᴅ-diglucopyranoside, naringenin 4′,7-*O*-β-ᴅ-diglucopyranoside, naringenin 7-*O*-[6-*O*-(β-ᴅ-glucopyranosyl)]-β-ᴅ-glucopyranoside, naringenin 7-*O*-[6-*O*-(α-ʟ-rhamnopyranosyl)]-β-ᴅ-glucopyranoside, and 7-*O*-β-ᴅ-gentiobiosyl-4′-*O*-β-ᴅ-glucopyranosylnaringenin. On the other hand, naringenin 7-O-β-ᴅ-glucopyranoside, naringenin 5,7-*O*-β-ᴅ-diglucopyranoside, naringenin 4′,7-*O*-β-ᴅ-diglucopyranoside, naringenin 7-*O*-[6-*O*-(β-ᴅ-glucopyranosyl)]-β-ᴅ-glucopyranoside, naringenin 7-*O*-[6-*O*-(α-ʟ-rhamnopyranosyl)]-β-ᴅ-glucopyranoside, naringenin 4′-*O*-β-ᴅ-glucopyranoside, naringenin 5-*O*-β-ᴅ-glucopyranoside, and naringenin 4′,5-*O*-β-ᴅ-diglucopyranoside were isolated from cultured *E. perriniana* cells that had been treated with naringenin. Among them, two products, 7-*O*-β-ᴅ-gentiobiosyl-4′-*O*-β-ᴅ-glucopyranosyl naringenin and naringenin 4′,5-*O*-β-ᴅ-diglucopyranoside, were novel compounds [76]. Although the compounds were converted into diverse glycosides, the production level was very low.

4 Conclusion

Flavonoids are ubiquitously found in plants and diverse in structure. The significance of flavonoids in human health and their functions in plant physiology and growth have attracted a variety of research into these molecules. Currently, flavonoids are being developed as molecules of choice for drug development. In plant science, the flavonoid biosynthetic pathways have been intensively studied to define the role and mechanism of each gene involved. The recent development of modern molecular tools has expanded the possibility of production of such compounds beyond the native producers, the plants. The incorporation of natural and hybrid biosynthetic pathways into microorganisms has created the potential for not only defined biosynthesis of a multitude of natural products, but also the opportunity to generate unnatural derivatives with potentially novel or refined pharmaceutical activities [77, 78]. Microbial cells like *E. coli*, *Saccharomyces cerevisiae*, and *Streptomyces* are emerging as enzyme bags through genetic engineering modifications. Such microbial factories have produced wide varieties of natural as well as unnatural flavonoids and are apparently expanding the market size for flavonoids along with other biotech-derived small molecules [79]. However, significantly higher production titer has not been achieved yet. Thus, the production of almost all flavonoids still depends on the plant sources. But, once the flavonoid backbones are produced in microbes, they can easily be modified through combinatorial biosynthesis by introducing different modifying genes to generate ranges of derivatives.

In the last decade, *E. coli* and *S. cerevisiae*, as well as some *Streptomyces* species, have been engineered as factories for the production and biotransformation of natural products. The development of microbial factories as a small molecule production platform requires identification of bottlenecks in the biosynthetic pathway and rewiring of native pathways via enhanced carbon flux toward precursors and intermediates to lead to higher production of target compounds. However, exploration of new microbial hosts and novel enzymes is still essential to expand the product range and to create a powerful system [25]. The recent achievement in synthesis of entire bacterial genome sequence has opened the doors for the development of bacterial cells of interest [80]. The combined application of different modern approaches could give birth to new useful microbes that are programmable, controlled, and highly robust for natural product production by a simple fermentation technique, which would also allow us to biosynthesize several other value-added flavonoid molecules in the near future.

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Isoflavonoid Production by Genetically Engineered Microorganisms

54

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Contents

1 Metabolic Engineering 1650
1.1 Background 1650
1.2 Metabolic Engineering Products 1651
1.3 Microorganisms as a Production Platform for Plant Natural Products 1651
2 Plant Phenylpropanoid Biosynthesis 1653
2.1 Background 1653
2.2 Plant Phenylpropanoid Biosynthetic Pathway 1654
2.3 Plant Flavonoid Pathways 1655
3 Plant Isoflavonoid Production in Microbes 1657
3.1 Construction of an Artificial Biosynthetic Pathway for Flavonoid Production in Microbes 1657
3.2 Engineering the Plant Isoflavonoid Pathway in Microbes 1658
4 Mutasynthesis and Protein Engineering for Non-natural Isoflavonoid Production in Microbes 1664
4.1 Mutasynthesis for Non-natural Isoflavonoid Production 1666
4.2 Protein Engineering for Non-natural Isoflavonoid Production 1667
4.3 Other Isoflavonoid Biotransformations 1670
5 Conclusions 1671
References 1672

Abstract

Isoflavonoids are a class of plant natural products gaining attention due to their pharmaceutical properties. These natural compounds constitute a subclass of flavonoids, which belong to a broader class of plant products known as phenylpropanoids.

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Flavonoids have been associated with medicinal properties, while isoflavonoids have shown anticancer, antioxidant, and cardioprotective properties due to their role as inhibitors of estrogen receptors. Isoflavonoids are naturally produced by legumes and, more specifically, organisms belonging to the pea family. Harvesting of these natural products through traditional extraction processes is limited due to the low levels of these phytochemicals in plants, so alternative production platforms are required to reduce cost of production and increase availability. Over the last decade, researchers have engineered artificial flavonoid biosynthesis pathways into *Escherichia coli* and *Saccharomyces cerevisiae* to convert simple, renewable sugars like glucose into flavonoids at high production levels. This chapter outlines the metabolic engineering research that has enabled microbial production of plant flavonoids and further details the ongoing work aimed at producing both natural and non-natural isoflavonoids in microorganisms.

Keywords

Metabolic engineering • mutasynthesis • non-natural isoflavonoids • protein engineering • strain improvement

Abbreviations

3GT	3-*O*-glucosyltransferase
4CL	4-Coumarate-CoA ligase
ACC	Acetyl-CoA carboxylase
Ala	Alanine
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
API	Active pharmaceutical ingredient
Arg	Arginine
BDO	Biphenyl dioxygenase
BMC	Bacterial microcompartment
C4H	Cinnamate 4-hydroxylase
CHI	Chalcone isomerase
CHS	Chalcone synthase
CPR	Cytochrome P450 reductase
CUS	Curcuminoid synthase
DFR	Dihydroflavonol reductase
DH	*Salmonella typhimurium* LT2 TDP-glucose 4,6-dehydratase
EPI	*Streptomyces antibioticus* Tu99 TDP-4-keto-6-deoxyglucose 3,5-epimerase
ER	Endoplasmic reticulum
F7GAT	Flavonoid 7-*O*-glucuronosyltransferase
FHT	Flavanone 3β-hydroxylase
FLS	Flavonol synthase

FSI	Soluble flavone synthase
FSII	Membrane-bound flavone synthase
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
GALU	Glucose-1-phosphate uridylyltransferase
GERF	*Streptomyces* sp. KCTC 0041BP TDP-hexose 3-epimerase
GERK	*Streptomyces* sp. KCTC 0041BP TDP-4-keto-6-deoxyglucose reductase
Glu	Glutamic acid
Gly	Glycine
HEK	Human embryonic kidney cells
hER	Human estrogen receptor
HI4′OMT	2,7,4′-Trihydroxyisoflavanone 4′-*O*-methyltransferase
HID	2-Hydroxyisoflavanone dehydratase
HIDH	2-Hydroxyisoflavanone dehydratase hydroxy type
HIDM	2-Hydroxyisoflavanone dehydratase methoxy type
IFR	Isoflavone reductase
IFS	Isoflavone synthase
Ile	Isoleucine
k_{cat}	Turnover number
K_m	Michaelis constant
KR	*Streptomyces antibioticus* Tu99 TDP-glucose 4-ketoreductase
LAR	Leucoanthocyanidin reductase
LB	Luria-Bertani medium
LDOX	Leucoanthocyanidin dioxygenase
NADPH	Nicotinamide adenine dinucleotide phosphate
NDK	Nucleoside diphosphate kinase
NDO	Naphthalene dioxygenase
PAL	Phenylalanine ammonia-lyase
PGI	Glucose-6-phosphate isomerase
PGM	Phosphoglucomutase
Phe	Phenylalanine
RCIFS	Red clover isoflavone synthase
RCPR	Rice cytochrome P450 reductase
SaOMT-2	*Streptomyces avermitilis* MA-4680 7-*O*-methyltransferase
ScCCL	*Streptomyces coelicolor* A3 cinnamate/coumarate:CoA ligase
Ser	Serine
SERM	Selective estrogen receptor modulator
STS	Stilbene synthase
TAL	Tyrosine ammonia-lyase
TB	Terrific broth
TDP	Thymidyldiphosphate

TGS	*Thermus caldophilus* GK24 thymidyldiphosphoglucose synthase
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UDG	Uridine diphosphoglucose dehydrogenase
UDP	Uridine diphosphate
UGT	Uridine diphosphate glycosyltransferase
UTP	Uridine triphosphate
UXS1	Uridine diphosphate glucuronic acid decarboxylase
Val	Valine
V_{max}	Maximum reaction rate

1 Metabolic Engineering

1.1 Background

Metabolic engineering involves the genetic manipulation of metabolism for a specific goal, often high-level production of a secondary metabolite. Secondary metabolites are those not critical to the survival of an organism in its normal environment, and they are thus typically found in far lower quantities than primary metabolites involved in energy maintenance and growth [1, 2]. As secondary metabolites have evolved to serve in important ecological roles – usually through interaction with other organisms – they possess unique properties and are thus the target of many metabolic engineering projects [3–5]. Although metabolic engineering has been a distinct discipline for over two decades, advancing technologies in areas such as DNA sequencing and synthesis, computational modeling and optimization, synthetic biology, and protein engineering are enabling metabolic engineers to create economically viable microbial production platforms for specialty chemicals like pharmaceuticals and biofuels [6].

Throughout the past decade, much work has focused on both plant and microbial metabolic engineering for production of pharmaceutically and nutraceutically important plant isoflavonoids [7–11]. This class of phytochemicals has been shown to possess a diverse array of pharmacological activities and demonstrates potential for treatment of certain cancers, cardiovascular diseases, and other conditions [12–17]. In particular, isoflavonoids have high affinity toward human estrogen receptors (hERs) and are therefore being investigated as estrogen receptor agonists and antagonists to modulate estrogen metabolism [18–20]. The relatively low abundance of these valuable compounds in plants makes microbial metabolic engineering an excellent alternative candidate for large-scale isoflavonoid production.

1.2 Metabolic Engineering Products

The majority of work in the field of metabolic engineering has focused on the production of commodity chemicals and biofuels from renewable, simple carbon sources such as glucose and glycerol, or the production of pharmaceutical chemicals and proteins [21–24]. In general, metabolic engineering can be viewed as the process by which scientists combine genes from different sources to construct a biosynthetic pathway in a host organism to convert an inexpensive feedstock into a valuable product. Classic metabolic engineering projects range from the microbial production of biofuels like ethanol and butanol to the production of commodity chemicals like xylitol. Although these efforts are important for ensuring long-term stability of commodity supply from renewable resources, microbial metabolic engineering of valuable plant natural products and other active pharmaceutical ingredients (APIs) with high overhead has the potential to make a much greater impact on society by lowering cost and ensuring availability and widespread access to medically important compounds [6].

1.3 Microorganisms as a Production Platform for Plant Natural Products

1.3.1 Advantages of Microbial Hosts

Microorganisms serve as excellent hosts for production of phytochemicals. The relatively lower genetic complexity of microbes compared to multicellular eukaryotes allows for more accurate prediction of the effects of genetic manipulations in microbes than in plants. Modulation of gene copy or expression level typically leads to an imbalance in reaction fluxes and, subsequently, the accumulation of pathway intermediates. If a genetic pathway is not decoupled from its native environment, accumulation of intermediates can become toxic or elicit unintended regulatory effects like feedback inhibition. Such uncharacterized genetic interactions in multicellular eukaryotic hosts are currently difficult to predict and can be largely avoided by transplanting genes from evolutionarily distinct organisms into an artificial pathway in a microbial host [25].

Perhaps the strongest argument for utilizing microorganisms for metabolic engineering of plant natural products is the high degree of genetic tractability that currently exists for microbial workhorses like *Escherichia coli*, *Saccharomyces cerevisiae*, and *Bacillus subtilis*. Thanks to decades of research, these hosts have innumerable data sets and molecular biology tools available for facile genetic manipulation, characterization, modeling, and scale-up. This genetic tractability reduces experimental unknowns and allows for faster, more predictable experimentation and data collection. Additionally, the high growth rates and simple media requirements associated with microorganisms enable culturing with limited resources [25, 26].

1.3.2 Alternative Production Platforms

Plant natural products have traditionally been harvested through extraction methods, as evidenced by the preparation of traditional medicines and the steeping of tea leaves and coffee beans for millenia. Since plant natural products are generally found at low levels in plant biomass, extraction is usually not a sustainable mass production avenue. Although extraction is still utilized to harvest APIs like the antimalarial drug artemisinin (from *Artemisia annua*, known as Sweet Wormwood) and the chemotherapeutic paclitaxel (from *Taxus brevifolia*, the Pacific yew tree) when chemical synthesis is difficult or expensive, there is a trend and growing necessity to shift toward alternative production platforms to lower cost and increase availability [27, 28].

Alternative production platforms include organic synthesis, plant cell culture, plant tissue culture, and even mammalian cell culture. The field of organic synthesis of complex plant natural products has advanced significantly but is limited as an industrial-scale flavonoid production platform by frequent use of toxic chemicals and extreme reaction conditions, a high number of required steps, exorbitant costs, relatively low overall yields, and nonspecific catalysts leading to by-products and often difficult-to-separate racemic mixtures of target compounds [29–35]. Semisynthesis, which combines organic synthesis steps with biosynthetic steps, is also limited by similar challenges. It is then reasonable to consider plant cell and tissue culture as a closely related alternative production platform since the metabolites of interest are endogenously produced in undifferentiated plant cells [36].

A well-known example of industrial-scale production in plant cell lines is the induction of paclitaxel production through methyl jasmonate elicitation, yielding 0.5 % of dry weight compared to 0.01 % of dry weight by extraction from the Pacific yew [37, 38]. By contrast, chemical synthesis of paclitaxel requires 35–51 steps, with a yield of only 0.4 % [39]. Plant tissue culture is another option, as many secondary metabolic biosynthetic pathways are only active in specific stages of development or in certain tissues [40, 41]. Thus, elicitation of differentiated plant cell tissues by small molecules or light can also be utilized to produce secondary metabolites. Despite progress in plant cell and tissue culture, the elucidation and characterization of all enzymes involved in plant secondary metabolite biosynthetic pathways are still challenging tasks; moreover, the difficulty in unequivocally discerning all sensitive, multilevel regulatory effects instigated by minimal variations in metabolite concentrations often makes the outcome of metabolic engineering in plant cell and tissue cultures unpredictable.

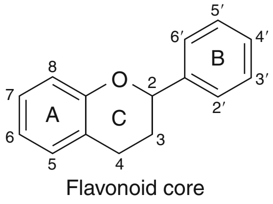
With advances in metabolic engineering of mammalian cells, it is foreseeable that plant natural products might one day be produced and derivatized using mammalian cell culture to take advantage of mammal-specific biotransformations and glycosylation patterns leading to improved pharmaceutical properties and applications. Therapeutic phytochemical production pathways might even be engineered into specific tissues to enable in situ biosynthesis for disease treatment or prophylaxis. To date this alternative remains relatively unexplored; however, engineering of a resveratrol artificial biosynthetic pathway into human embryonic kidney cells (HEK293) circumvented purported difficulties associated with yeast expression of tyrosine ammonia-lyase (TAL) and highlighted the opportunity to move plant pathways into mammalian cells for in situ production of phytochemical therapeutics in human tissue [42]. Just as predictable metabolic engineering of plant cell and tissue cultures is currently limited by cellular complexity, metabolic engineering of mammalian cells can be encumbered with the same difficulties.

2 Plant Phenylpropanoid Biosynthesis

2.1 Background

Isoflavonoids belong to a broad class of compounds known as phenolics. Any chemical containing one or more phenol group can be classified as a phenolic compound, although the plant phenolics with the most biotechnological relevance are flavonoids and other phenylpropanoids. Phenylpropanoids are secondary plant metabolites that are considered to be beneficial for human health [43]. In particular, a subclass of phenylpropanoids known as flavonoids is typified by bioactive compounds with antioxidant, antiviral and antibacterial, anticancer, antiobesity, and estrogenic properties [9]. The microbial production of flavonoids has attracted much attention due to the prospect of utilizing flavonoids for personal health applications [44]. Flavonoids are currently used as dietary supplements and are the subject of intense investigation as pharmaceutical precursors to treat chronic human pathological conditions like cancer and diabetes [45–51]. Anthocyanins **17**, another class of flavonoids, possess brilliant natural colors and are potential replacements for artificial dyes that have adverse health effects. The antioxidant properties of these glycosylated flavonoids may have a positive health influence and make anthocyanins **17** well suited as natural colorants for the food and beverage industry [52–54]. Anthocyanins **17** are good targets for metabolic engineering since glycosylations remain a challenge from a chemical synthesis perspective. Furthermore, plant extraction of phenolics seldom yields greater than 1 % of the dry weight. Metabolic engineering of flavonoid biosynthesis has already gained traction due to the long-standing interest in phenolic compounds and the corresponding detailed characterization of related genetic pathways and enzymes [43].

As a general classification, phenolics do not contain nitrogen and may contain multiple hydroxyl groups as well as heteroatom substituent groups. Phenolics with greater than 12 phenolic groups are generally considered as polyphenols, lignins, or tannins. Flavonoids are the most well characterized and largest class of natural phenolics, and they are biosynthesized from the aromatic amino acid phenylalanine **2** through the common precursor, chalcone **11**. Further classification draws a distinction between five types of flavonoids that are derived from the common flavanone **12** precursor: flavones **14**, flavonols **15**, isoflavones **13**, flavanols, and anthocyanins **17** [55]. Flavonoids are composed of a C_6-C_3-C_6 skeleton that serves as a 15-carbon phenylpropanoid core **1** for downstream decorations such as methylations, hydroxylations, reductions, oxidations, glycosylations, acylations, methoxylations, alkylations, and various rearrangements [44, 56–58]. The flavonoid core **1** consists of 3 rings, labeled A, B, and C.



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Other phenylpropanoids, so named due to their common phenylalanine **2** precursor, include hydroxycinnamic acids, cinnamic aldehydes and monolignols, coumarins, and stilbenoids **8**.

2.2 Plant Phenylpropanoid Biosynthetic Pathway

Phenylpropanoid biosynthesis is initiated by the conversion of phenylalanine **2** to cinnamic acid **5** as catalyzed by phenylalanine ammonia-lyase (PAL). Cinnamic acid **5** is then converted to flavanone **12** through a series of subsequent enzymatic reactions involving the following steps: the hydroxylation of cinnamic acid **5** to *p*-coumaric acid **6** through cinnamate 4-hydroxylase (C4H); the ligation of *p*-coumaric acid **6** to a CoA group using 4-coumarate-CoA ligase (4CL); the sequential decarboxylative condensation of three acetate units from malonyl-CoA **10** to 4-coumaroyl-CoA **19** by chalcone synthase (CHS), a type III polyketide synthase, to form chalcone **11** in a ring closing step; and the stereospecific isomerization of chalcone **11** to flavanone **12** catalyzed by chalcone isomerase (CHI). Downstream enzymes then catalyze the conversion of flavanones **12** into compounds belonging to the various flavonoid subclasses.

Type III polyketide synthases are particularly relevant to this chapter because they catalyze the formation of phenolic compounds. This group of polyketide synthases consists of CHSs, stilbene synthase (STS), and curcuminoid synthase (CUS), which perform decarboxylative condensations between a starter unit, either *p*-coumaroyl-CoA **19** or cinnamoyl-CoA **18**, and an extender unit, malonyl-CoA **10**. CHS, STS, and CUS convert the substrate molecules into flavonoids (C_6-C_3-C_6), stilbenoids **8** (C_6-C_2-C_6), and curcuminoids **9** (C_6-C_7-C_6), respectively [59]. Stilbenoids **8** and curcuminoids **9** are out of the scope of this chapter but possess medicinal properties as well; resveratrol is a well-known stilbenoid **8** associated with longevity, and curcumin is a common curcuminoid **9** that is responsible for the yellow color in turmeric and can be utilized as a natural pigment possessing antioxidant and anti-inflammatory properties [60–63]. For an in-depth treatment of plant polyketide production in microbes, the reader is directed to a recent comprehensive review by Boghigian et al. [64].

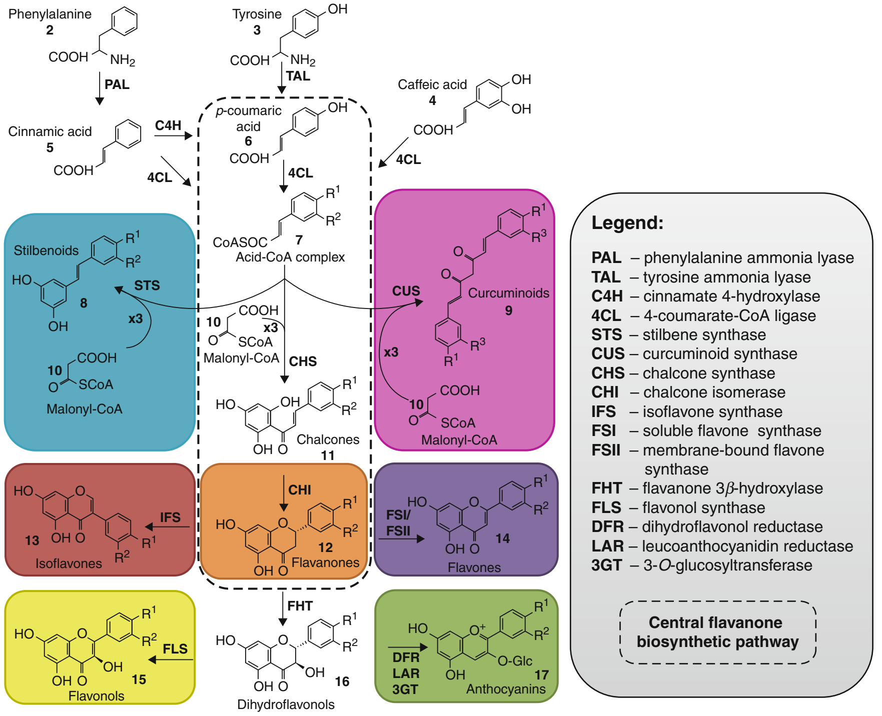
2.3 Plant Flavonoid Pathways

Plant flavanones **12** are enzymatically converted to five major subclasses of flavonoids. Flavanones **12** are oxidized to flavones **14** by the action of either a soluble flavone synthase (FSI) or, as in most cases, a membrane-bound cytochrome P450 monooxygenase flavone synthase (FSII) [65]. Flavone synthases belong to the oxidoreductase family of enzymes and effectively remove the stereocenter from flavanones **12** by oxidation of C3 and introduction of a double bond between C2 and C3. Apigenin, luteolin, and chrysin are common flavones **14** that contribute to human diet as glycosides and are found in large quantities in parsley and celery [66–68].

Alternatively, isoflavone synthase (IFS) catalyzes the 1,2-aryl ring B migration from C2 to C3 on ring C of the phenylpropanoid core **1** and the hydroxylation of C2, converting flavanones **12** to 2-hydroxyisoflavanones [69, 70]. Dehydration of 2-hydroxyisoflavanones into isoflavones **13** occurs spontaneously through the 1,2-elimination of water, but accelerated dehydration is catalyzed by one of two hydro-lyases known as 2-hydroxyisoflavanone dehydratases (HID hydroxy type, HIDH; HID methoxy type, HIDM), depending upon the occurrence of an intermediate 4′-*O*-methylation catalyzed by 2,7,4′-trihydroxyisoflavanone 4′-*O*-methyltransferase (HI4′OMT) [71]. Isoflavonoids are characterized by a 3-phenylchroman skeleton, in contrast to the 2-phenylchroman core **1** possessed by flavonoids, and are incredibly diverse in structure despite being limited to natural existence primarily in leguminous plants [72]. Soy beans and soy bean food products contain high concentrations of isoflavone **13** glycosides such as genistin **31** and daidzin **30** and relatively lower quantities of their respective aglycones, daidzein **27** and genistein **26** [74]. Isoflavones **13** are classified as phytoestrogens because of the structural similarity shared with estrogens, and they are among the most highly studied polyphenols due to their affinities for steroid receptors and demonstrated pharmacological properties [18–20, 74]. These characteristics make isoflavones **13** important metabolic engineering targets.

Flavanones **12** also serve as the substrate for flavanone 3*β*-hydroxylase (FHT), which catalyzes the hydroxylation of C3 on the flavanone core **1** into dihydroflavonol **16**, the common precursor to both flavonols **15** and anthocyanins **17**. Dihydroflavonols **16** are subsequently converted to flavonols **15** by reduction of C2 by the oxidoreductase enzyme flavonol synthase (FLS), again removing the stereocenter and introducing a double bond between C2 and C3 [75]. Flavonols **15** such as kaempferol and quercetin exist primarily as glycosides at appreciable levels in onions and kale [67, 68].

Initiating another branch of the flavonoid pathway, C4 of dihydroflavonol **16** can be reduced from a carbonyl group to a hydroxyl group by the oxidoreductase enzyme dihydroflavonol reductase (DFR), producing leucoanthocyanidins, or the colorless precursors to anthocyanins **17**. Leucoanthocyanidins are unstable and are quickly converted to anthocyanidins by anthocyanidin


Phenylalanine
2
COOH
NH2
PAL
Tyrosine
3
OH
TAL
Caffeic acid
4
p-coumaric acid
6
Cinnamic acid
5
C4H
4CL
CoASOC
7
Acid-CoA complex
R1
R2
R3
Stilbenoids
HO
STS
8
x3
10
COOH
SCoA
Malonyl-CoA
CUS
Curcuminoids
9
CHS
Chalcones
11
CHI
Flavanones
12
IFS
Isoflavones
13
FSI/
FSII
Flavones
14
FHT
FLS
Flavonols
15
Dihydroflavonols
16
DFR
LAR
3GT
O-Glc
Anthocyanins
17
Legend:
PAL – phenylalanine ammonia lyase
TAL – tyrosine ammonia lyase
C4H – cinnamate 4-hydroxylase
4CL – 4-coumarate-CoA ligase
STS – stilbene synthase
CUS – curcuminoid synthase
CHS – chalcone synthase
CHI – chalcone isomerase
IFS – isoflavone synthase
FSI – soluble flavone synthase
FSII – membrane-bound flavone synthase
FHT – flavanone 3β-hydroxylase
FLS – flavonol synthase
DFR – dihydroflavonol reductase
LAR – leucoanthocyanidin reductase
3GT – 3-O-glucosyltransferase
Central flavanone biosynthetic pathway

Flavonoid subclass	Phenylalanine (2) precursor (R^1 = H; R^2= H)	Tyrosine (3) precursor (R^1 = OH; R^2= H)	Caffeic Acid (4) precursor (R^1 = OH; R^2= OH; R^3 = OMe)
Flavanones (12)	(2*S*)-pinocembrin **(22)**	(2*S*)-naringenin **(23)**	(2*S*)-eriodictyol
Isoflavones (13)	5,7-dihydyisoflavone	Genistein (26)	Orobol
Flavones (14)	Apigenin	Luteolin	Chrysin
Flavonols (15)	Kaempferol	Quercetin	Myrecetin
Anthocyanin 3-*O*-glucosides (17)	Palargonidin 3-*O*-glucoside	Cyanidin 3-*O*-glucoside	Delphinidin 3-*O*-glucoside
Stilbenoids (8)	Pinosylvin	Resveratrol	Piceatannol
Curcuminoids (9)	Dicinnamoylmethane	Bisdemthoxycurcumin	Curcumin

Fig. 54.1 Plant phenylpropanoid and flavonoid biosynthetic pathways; representative compounds from each subclass are named

synthase (ANS), synonymously leucoanthocyanidin dioxygenase (LDOX), working jointly with DFR [76]. Anthocyanidins and leucoanthocyanidins can alternatively be reduced to their corresponding flavan-3-ols (proanthocyanidins, or condensed tannins) by anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR), respectively. A flavonoid glycosyltransferase then adds a sugar to the anthocyanidin, enabling pigment storage in the form of stable anthocyanins **17** [77]. Many brilliant red, blue, and purple plant hues arise from anthocyanin-mediated coloration. Figure 54.1 illustrates the alternative pathways for biosynthesis of various plant phenylpropanoid and flavonoid subclasses.

3 Plant Isoflavonoid Production in Microbes

3.1 Construction of an Artificial Biosynthetic Pathway for Flavonoid Production in Microbes

The first construction of an artificial plant flavonoid biosynthetic pathway in microbes involved the transformation of *E. coli* with four heterologous genes. These genes are required for the synthesis of flavanones **12** from phenylalanine **2** and tyrosine **3** (through a promiscuous PAL having the ability to accept both phenylalanine **2** and tyrosine **3** as substrates) [78–80]. This exercise provided a platform for the microbial biosynthesis of a plethora of natural and non-natural flavanone **12** derivatives. It should also be noted here that bacterial TAL catalyzes the conversion of tyrosine **3** to *p*-coumaric acid **6** in one step and can replace the two-step conversion of phenylalanine **2** to *p*-coumaric acid **6** by PAL and C4H in an artificial biosynthetic pathway if so desired [81]. Also, depending upon choice of aromatic amino acid precursor, two parallel biosynthetic paths exist for phenylalanine-based flavonoids in contrast to tyrosine-based flavonoids; other common natural and non-natural aromatic acrylic acids like caffeic acid **4** serve as substrates for 4CL in plants and microbes [82, 83]. The substrate flexibility of all enzymes involved allows for perpetuation of extra hydroxyl side groups throughout the entire pathway, affording flavanones **12** or other flavonoids with divergent hydroxylations. Another key distinction to note while reading this section is whether the project being described utilizes an entirely fermentative process to produce complex compounds from primary microbial metabolites or whether the project takes advantage of intermediate chemical supplementation. Although neither approach is absolutely superior to the other, distinctions can be drawn between them.

For instance, a fermentative approach often suffers from low production due to pathway complexity and increased number of steps, but it allows for production of complex compounds such as phytochemicals from simple, renewable carbon compounds like glucose. Conversely, intermediate supplementation is often utilized to simplify pathway construction and is associated with higher product yields. Although supplementing a microbial culture with an expensive precursor might be feasible for a small-scale experiment, it severely hinders industrial applicability. However, if an inexpensive, readily available intermediate can be utilized as a precursor, an entirely fermentative process with lower titers might not be justifiable. A metabolic engineer must then weigh the impact of generating a complex product entirely from primary metabolites versus the value associated with significantly higher production levels. As will be seen throughout this chapter, research efforts are often initiated with intermediate supplementation in order to limit confounding variables, and full fermentative pathways are constructed after significant breakthroughs are achieved and once distinct metabolic pathways can be connected in vivo.

The experiment described in the beginning of this section involved the incorporation of four heterologous genes: *S. cerevisiae* PAL, *Streptomyces coelicolor* A3 cinnamate/coumarate:CoA ligase (ScCCL) with substrate specificity toward both cinnamic acid **5** and *p*-coumaric acid **6**, licorice plant (*Glycyrrhiza echinata*) CHS,

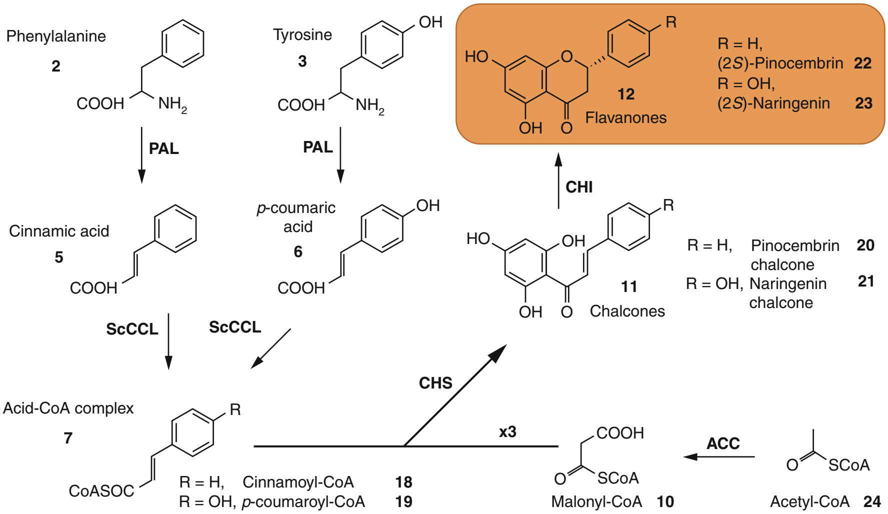

Phenylalanine
2
COOH
NH2
Tyrosine
3
OH
PAL
PAL
Cinnamic acid
5
p-coumaric acid
6
ScCCL
ScCCL
Acid-CoA complex
7
CoASOC
R
R = H, Cinnamoyl-CoA 18
R = OH, p-coumaroyl-CoA 19
CHS
x3
Malonyl-CoA 10
SCoA
ACC
Acetyl-CoA 24
HO
OH
O
11
Chalcones
R = H, Pinocembrin chalcone 20
R = OH, Naringenin chalcone 21
CHI
12
Flavanones
R = H, (2S)-Pinocembrin 22
R = OH, (2S)-Naringenin 23

Fig. 54.2 Artificial construction of plant flavanone **12** biosynthetic pathway in microbes

and *Pueraria lobata* CHI. Transformation of *E. coli* with a plasmid harboring these four heterologous genes coupled with overexpression of the *Corynebacterium glutamicum* gene encoding two acetyl-CoA carboxylase subunits, accBC and dtsR1, produced ~60 mg L^{-1} of the flavanones **12** (2*S*)-naringenin **23** and (2*S*)-pinocembrin **22**. The artificial biosynthetic pathway constructed for plant flavanone **12** biosynthesis in microbes is shown in Fig. 54.2. Acetyl-CoA carboxylase (ACC) was selected for overexpression to increase the intracellular pool of malonyl-CoA **10**, which is required for synthesis of flavanones **12** from either 4-coumaroyl-CoA **19** or cinnamoyl-CoA **18**. Further introduction of FSI from *Petroselinum crispum*, FHT from *Citrus sinensis*, and FLS from *Citrus unshiu* produced the flavones **14** apigenin and chrysin, as well as the flavonols **15** kaempferol and galangin in low concentrations [80, 84]. This seminal work has enabled the production in *E. coli* and *S. cerevisiae* of many valuable phenylpropanoid compounds, including natural and non-natural flavones **14**, flavonols **15**, anthocyanins **17**, stilbenoids **8**, and curcuminoids **9** [42, 60, 65, 82, 84–107]. As this chapter focuses on isoflavonoids, however, the reader is directed to a detailed review of microbial biosynthesis of other valuable plant phenylpropanoids by Limem et al. [43].

3.2 Engineering the Plant Isoflavonoid Pathway in Microbes

3.2.1 Production of Isoflavonoid Aglycones in Microbes

The successful construction of an artificial plant flavonoid biosynthetic pathway in microbes, combined with the first report of functional activity of IFS in yeast microsomes by Akashi and coworkers in 1999, paved the way for high-level isoflavonoid production [69]. However, a significant barrier to prokaryotic expression of IFS hampered progress and precluded taking advantage of the high growth rate of *E. coli* and the abundance of molecular biology tools available for the microbe. IFS is a membrane-bound cytochrome P450 that requires an electron transfer system that is not present in bacterial cells; thus, coexpression of functional IFS with the flavanone **12** pathway in recombinant *E. coli* required creative engineering solutions. Eukaryotic microbes like *S. cerevisiae* and other unicellular fungi possess the requisite machinery for cytochrome P450 enzyme expression; specifically, they constitutively express an endogenous cytochrome P450 reductase (CPR) that is an integral redox partner for IFS and other cytochrome P450s, and they possess an endoplasmic reticulum (ER) on which the N-terminal signal-anchor peptide sequences of cytochrome P450 enzymes can bind [108, 109].

Katsuyama et al. overcame this impediment by coculturing a flavanone-producing *E. coli* strain with recombinant *S. cerevisiae* transformed with a T7-inducible plasmid harboring IFS from *G. echinata*. To demonstrate the production of the isoflavone **13** genistein **26** and the feasibility of coincubation, the yeast strain was first transformed with a pESC vector containing the genes CHS from *G. echinata*, CHI from *P. lobata*, and IFS from *G. echinata*, all under the control of galactose-inducible *GAL* promoters. Growth under supplementation with the precursor, *N*-acetylcysteamine-attached *p*-coumarate (*p*-coumaroyl-NAC), yielded ~342 μg L^{-1} genistein **26**. To examine the possibility of production without precursor feeding, a naringenin-producing *E. coli* strain (57 mg L^{-1} of (2*S*)-naringenin **23**) was constructed as described in the previous section and cocultured with a recombinant yeast strain transformed with a vector containing *G. echinata* IFS under control of a galactose-inducible *GAL* promoter [80]. Simultaneous incubation of equal weights of engineered *E. coli* and *S. cerevisiae*, in addition to supplementation of the coculture media with 3 mM tyrosine **3** as a substrate for *E. coli*, yielded ~6 mg L^{-1} of genistein **26** [110]. This "one-pot synthesis" scheme for production of genistein **26** from tyrosine **3** represented the most valuable microbial isoflavonoid production platform at the time of its publication. Optimization of coculture conditions subsequently improved genistein **26** production up to 100 mg L^{-1} [111].

In order to produce isoflavonoids in a model plant, a native flavonoid pathway must be hijacked by diverting a common precursor away from its natural product and toward the desired isoflavonoid product. Tian and colleagues accomplished production of genistein **26** in the nonleguminous, model plant tobacco through protein engineering of a fusion between IFS and CHI [112]. The spatial proximity between CHI and IFS was engineered to increase the local concentration of the IFS substrate, naringenin **23**, such that the production of nonnative genistein **26** was favored over the dominant, endogenous pink anthocyanin **17** accumulation pathway. Localization of the protein chimera at the ER was maintained by constructing the fusion with IFS at the N-terminus so its innate, hydrophobic N-terminal membrane anchor was free to target the ER as usual [87, 113]. A flexible linker peptide composed of glycine-serine-glycine (Gly-Ser-Gly) residues connected the C-terminus of IFS with the N-terminus of CHI to ensure proper folding of the two independent catalytic domains. Expression of this engineered protein fusion in transgenic tobacco successfully shifted flavonoid accumulation toward isoflavonoids and enabled production of isoflavonoids in nonleguminous plants. Yeast expression of the protein fusion under precursor supplementation conditions also produced isoflavonoids and highlighted the possibility to utilize protein engineering to improve plant natural product titers in microbes [112].

Although *E. coli* and *S. cerevisiae* have both been utilized as model organisms for plant flavonoid production, it is often beneficial to express entire biosynthetic pathways in a single organism to avoid bidirectional metabolite transport limitations through the cell walls of two organisms simultaneously and to obviate media optimization for two different species at once. Functional expression of IFS in *E. coli* would eliminate the necessity for coculture with yeast. As such, Leonard and colleagues designed and expressed a set of artificial P450 enzymes that enabled robust biosynthesis of the isoflavones **13** genistein **26** and daidzein **27** from the flavanones **12** naringenin **23** and liquiritigenin in *E. coli* for the first time [114]. Two challenges to functional prokaryotic expression of eukaryotic cytochrome P450 enzymes were overcome in this research: the translational fusion of *Catharanthus roseus* CPR to *Glycine max* IFS spatially organized the redox partners for efficient electron shuttling from nicotinamide adenine dinucleotide phosphate (NADPH) to substrate, and rational design of several IFS N-terminal membrane signal sequences modulated activity of the protein fusion, enabling selection of a high-level isoflavone **13** producing chimera [114].

The protein engineering effort began with deletion of 71N-terminal amino acids from CPR to minimize membrane association without hindering catalytic activity. A glycine-serine-threonine (Gly-Ser-Thr) linker sequence was then designed to connect the CPR N-terminus with the IFS C-terminus while thwarting any secondary structure formation that could lead to incorrect folding of the two domains. The protein fusion was then truncated by a varying number of residues from the N-terminus of IFS, and two peptide leader sequences (one mammalian and one endogenous) were independently appended to these constructs in a semicombinatorial manner. Each chimera was separately expressed in *E. coli* and evaluated for production of isoflavone **13** from supplemented precursor. The most prominent fusion produced 10 and 18 mg g^{-1} (dry cell weight) of genistein **26** and daidzein **27**, respectively, and consisted of the deletion of 6 membrane-anchor residues and the addition of an 8 residue mammalian leader sequence to the N-terminus of IFS. To determine a baseline production level, plant IFS and CPR were coexpressed in *E. coli* and found to yield negligible isoflavonoid concentrations compared to the engineered strain. *S. cerevisiae* coexpressing plant IFS and CPR produced isoflavones **13** at low concentrations approaching those of the poorly performing protein fusion constructs expressed in *E. coli*. After accounting for the significantly higher biomass of yeast versus *E. coli* in minimal media, the specific production level of isoflavones **13** in *E. coli* represented approximately 20-fold increase over yeast [114]. The methodology implemented in this work provides an approach for soluble expression of other eukaryotic membrane-bound cytochrome P450s in prokaryotes. Although not performed in this set of experiments, this research facilitated the impending construction of a complete artificial biosynthetic pathway from aromatic amino acids to isoflavonoids in a single microorganism.

A later report of functional expression of IFS in prokaryotes involved construction of a protein fusion between red clover IFS (RCIFS) and rice CPR (RCPR) in *E. coli* [115]. This work built upon previous results demonstrating that coexpression in yeast of IFS with CPR from rice can convert 100 μM naringenin **23** to 77 μM genistein **26**, research predicated on the hypothesis that a plant CPR, as opposed to a constitutively expressed yeast CPR, would interact more efficiently with a plant IFS [103]. In this project RCIFS was truncated by deletion of the codons for the first 21 amino acids on the N-terminus, a sequence predicted to code for a helical region as indicated by computational secondary structure analysis. Changing the first remaining codon to a start codon (encoded by the nucleotide sequence ATG) enabled functional expression of RCIFS in *E. coli*, while removal of the IFS stop codon and addition of a Gly-Ser-Thr linker sequence followed by the RCPR coding sequence (also with the N-terminal membrane binding domain deleted) enabled expression and proper folding of the two fused domains. It should be noted here that this protein fusion design differs from that constructed previously by Leonard primarily because, in this case, the hydrophobic N-terminal membrane-associated domains were entirely removed from both enzyme constituents in the fusion to enhance solubility of the final construct. The functional expression and spatial proximity afforded by the soluble RCIFS-RCPR protein fusion enabled conversion of 80 μM naringenin **23** into 56 μM genistein **26** in *E. coli*. Difference in conversion between yeast and *E. coli* was not investigated but could be due to disparate expression and growth levels in the two distinct species. Again, it is likely that higher-efficiency electron transfer from NADPH to substrate occurred in *E. coli* due to the conjoined RCPR and RCIFS domains [115].

Coexpression in *S. cerevisiae* of all seven genes in the artificial isoflavone **13** pathway (PAL and CPR from a hybrid poplar, *Populus trichocarpa* × *Populus deltoides* and C4H, 4CL, CHS, CHI, and IFS from soybean, *G. max*), with phenylalanine **2** supplementation, was ultimately achieved by Trantas et al. and marked the first reported reconstitution of an entire isoflavonoid biosynthetic pathway in microbes. Although yeast contains a chromosomal copy of CPR, coexpression of a heterologous CPR from a the hybrid poplar increased *p*-coumaric acid **6** production fourfold, once again demonstrating the advantage of selecting a plant CPR to improve activity of the other enzymes in the cytochrome P450 metabolon [101, 116]. Only 0.1 mg L^{-1} genistein **26** was produced when the cultures were fed with phenylalanine **2** versus 7.7 mg L^{-1} when fed with naringenin **23**, suggesting the presence of at least one limiting enzyme or that cellular metabolism was burdened by the genes upstream of naringenin **23**. On average, the yeast strains in this work consumed 3.4 mmol L^{-1} phenylalanine **2**, while the wild-type strain consumed 2.8 mmol L^{-1}, a difference in phenylalanine **2** uptake of 0.8 mmol L^{-1} that can be attributed to flux through the heterologous flavonoid pathway. Stoichiometrically, this should lead to 0.8 mmol L^{-1} genistein **26**, but production of only 0.4 μmol L^{-1} indicated approximately 0.05 % efficiency of conversion of phenylalanine **2** to genistein **26** through the artificial biosynthetic pathway. Measurement of some upstream intermediates showed 83 % flux efficiency through PAL and C4H, efficient conversion through 4CL as deduced from the rapid depletion of *p*-coumaric acid **6**, and approximately 8 % efficiency to naringenin **23**, which suggests that CHS or CHI are rate limiting but could not be confirmed due to the inability to quantify concentrations of the intermediate compounds 4-coumaroyl-CoA **19** and naringenin chalcone **21** [101]. As described by Akashi, coexpression of an HIDH in this engineered *S. cerevisiae* strain could potentially accelerate the spontaneous conversion of naringenin **23** to genistein **26** but was not attempted in this work [71].

The first attempt to coexpress HIDH with IFS and CPR confirmed this speculation. Chemler and coworkers coexpressed IFS, CPR, and HIDH from five various plant sources in yeast in a combinatorial fashion to determine the impact of gene source on individual enzyme activity and coupled enzyme activities [117]. IFS-encoding genes from *G. max*, *Trifolium pratense*, *G. echinata*, *Pisum sativa*, and *Medicago truncatula* were individually cloned into a pYES2.1 vector under control of the *GAL1* promoter and transformed into *S. cerevisiae* strain INVSc1. After growth on minimal medium, the cultures were induced with galactose and supplemented with naringenin **23**. Genistein **26** production was monitored, and *T. pratense* IFS was selected as the best enzyme because it showed significantly higher in vivo activity than the IFS enzymes from other sources. Since it had previously been shown that plant IFS activity in yeast is improved upon coexpression of plant CPR, the researchers coexpressed CPR from *C. roseus* and *G. max* with IFS from either *G. max* or *T. pratense* to determine the enzyme pair with highest coupled activity. Upon comparing genistein **26** production in these engineered strains to yeast expressing IFS with endogenous CPR, the strain coexpressing *T. pratense* IFS with *G. max* CPR was found to be the highest producer at 15 mg L^{-1} day^{-1}. This illustrates the value in combining different gene sources to determine optimal protein pairing, particularly in the case of enzyme-mediated redox reactions. To assess whether expression of plant HIDH could increase genistein **26** production over its spontaneous generation from its 2-hydroxyisoflavanone precursor in yeast, coexpression of *G. max* or *G. echinata* HIDH was evaluated in the engineered strains. The best triple-enzyme combination was found to include *T. pratense* IFS, *G. max* CPR, and *G. max* HIDH, followed closely by the cognate combination of *G. max* IFS, CPR, and HIDH. Interestingly, *T. pratense* IFS holds some advantage over *G. max* IFS when coexpressed with *G. max* CPR and HIDH, despite presumption that the *G. max* enzymes evolved to work optimally together. Ultimately the three-enzyme combination showed greater than tenfold improvement in production rate over expression of IFS alone, but total production in all strains maximized at around 35 mg L^{-1} genistein **26**. After further experimentation, it was shown that isoflavones **13** like genistein **26** and biochanin A **29** strongly inhibit conversion of naringenin **23** by IFS in yeast. It was speculated that isoflavone **13** glycosylations, methylations, and other enzymatic biotransformations might ameliorate product inhibition and increase overall isoflavonoid production [117]. The basic artificial biosynthetic pathway for plant isoflavone **13** production from flavanones **12** in microbes is illustrated in Fig. 54.3.

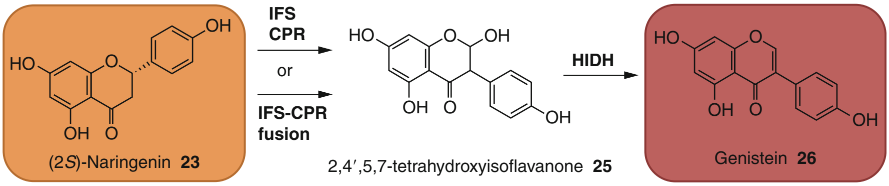

(2*S*)-Naringenin 23
IFS
CPR
or
IFS-CPR
fusion
2,4′,5,7-tetrahydroxyisoflavanone 25
HIDH
Genistein 26

Fig. 54.3 Aggregate artificial biosynthetic pathway for plant isoflavone **13** production from flavanones **12** in microorganisms

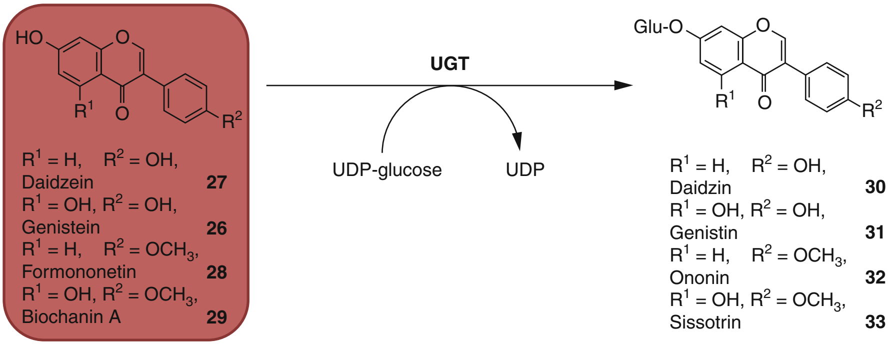

UGT
UDP-glucose
UDP
Glu-O
R1 = H, R2 = OH, Daidzein 27
R1 = OH, R2 = OH, Genistein 26
R1 = H, R2 = OCH3, Formononetin 28
R1 = OH, R2 = OCH3, Biochanin A 29
R1 = H, R2 = OH, Daidzin 30
R1 = OH, R2 = OH, Genistin 31
R1 = H, R2 = OCH3, Ononin 32
R1 = OH, R2 = OCH3, Sissotrin 33

Fig. 54.4 Microbial bioconversion of isoflavone **13** aglycones to isoflavone **13** glycosides

3.2.2 Production of Isoflavonoid Glycosides in Microbes

Many flavonoids and other secondary metabolites exist as glycosides in plants, and examples of engineered microbial glycosylation of various flavonoids like quercetin and anthocyanidins have been reported over the last decade [85, 96, 118, 119]. Glycosylation of flavonoid aglycones is important because it often increases mammalian bioavailability, solubility, and stability [68, 120–129]. In the first example of microbial isoflavonoid glycosylation, expression of UGT71G1, a uridine diphosphate glycosyltransferase (UGT) from the model legume *M. truncatula*, in heterologous *E. coli* with supplementation of genistein **26** and biochanin A **29** yielded mg quantities at greater than 70 % conversion to genistein **26** and biochanin A **29** 7-*O*-glucosides (genistin **31** and sissotrin **33**, respectively) after 24-h incubation (Fig. 54.4). Terrific broth (TB) culture medium supported higher growth than Luria-Bertani (LB) culture medium and thus provided 3.5-fold higher yield of 7-*O*-glucoside. Scale-up to 500 mL culture achieved conversion rates of 30–60 %, about 80 % efficient compared to small scale, yielding up to 20 mg L^{-1} of isoflavanone glycosides [98]. Of note, 90 % of the glycosylated products were secreted from the cell, enabling facile collection and suggesting that increased solubility or sugar moiety-related signaling affects efflux from the cell. As such, this work suggests that coexpression of *M. truncatula* UGT71G1 in Chemler's engineered yeast strain (*T. pratense* IFS, *G. max* CPR, *G. max* HIDH) could convert naringenin **23** to genistin **31**, the genistein **26** 7-*O*-glucoside, at much higher rates than previously reported because feedback inhibition would be minimized by the glycosylation and subsequent export from the cell. Whereas extraction of plant flavonoid glycosides is inefficient, and regioselective glycosylation of flavonoids through chemical synthesis methods requires intermittent blocking and deblocking of hydroxyl groups and yields only about 50 % conversion due to the occurrence of nonspecific glycosylations, glycosylation through biotransformation offers a highly efficient and cheap alternative [130–133]. A major barrier to high-level microbial production of any flavonoid glycoside, however, is intracellular supply of uridine diphosphate (UDP) glucose.

As seen in Fig. 54.4, nucleotide-activated sugars are required as donors for glycosylation. In previous work, Yan and colleagues engineered a four-step metabolic pathway for plant anthocyanin **17** biosynthesis in *E. coli*, which involved expression of four heterologous genes including *Malus domestica* FHT and ANS, *Anthurium andraeanum* DFR, and *Petunia hybrida* UDP-glucose:flavonoid 3-*O*-glucosyltransferase (3GT). Anthocyanidins were converted by 3GT to the first stable glycosidic anthocyanins **17** in the flavonoid biosynthetic pathway, pelargonidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside [85]. The researchers identified UDP-glucose as the rate-limiting step in anthocyanin **17** biosynthesis in *E. coli* and thereafter optimized UDP-glucose production by supplementing with orotic acid, a cheap uridine triphosphate (UTP) precursor, and performing a gene deletion and a set of gene overexpressions. As synthesis of UDP-glucose interfaces nucleotide biosynthesis, the pentose phosphate pathway, glycolysis, and energy production pathways, engineering its overproduction is a nontrivial task. Episomal overexpression of endogenous phosphoglucomutase (PGM) and glucose-1-phosphate uridylyltransferase (GALU), which convert glucose-6-phosphate (G6P) to glucose-1-phosphate (G1P) and produce UDP-glucose from G1P and UTP, respectively, shunted carbon flux from the pentose phosphate pathway toward UDP-glucose through the G6P branching point [57, 134]. These genetic modifications combined with the overexpression of endogenous nucleoside diphosphate kinase (NDK), the limiting step in the linear UTP synthesis pathway by orotic acid assimilation, and deletion of a gene encoding UDP-glucose dehydrogenase (UDG), which consumes UDP-glucose to form UDP-glucuronic acid, to yield increased UDP-glucose accumulation of 104 mg L^{-1} [96, 135, 136]. Due to the natural production of UDP-glucose in *E. coli* for cell wall synthesis and the ability to achieve increased production of UDP-glucose, microbial glycosylation of isoflavonoid aglycones with heterologous glycosyltransferases is an economically viable option. To the best of our knowledge, Table 54.1 summarizes the most representative studies of microbial production of plant natural isoflavonoids to date.

4 Mutasynthesis and Protein Engineering for Non-natural Isoflavonoid Production in Microbes

Mutasynthesis is a common semisynthetic tool that hijacks natural product biosynthesis through the feeding of non-natural substrate analogs to produce non-natural analogs to natural products. This methodology takes advantage of the natural allowable range of

Table 54.1 Reports demonstrating microbial production of plant natural isoflavonoids

Isoflavonoid target	Precursor	Host organism	Genes: Donors	Reference
Genistein **26**	*N*-acetylcysteamine-attached *p*-coumaric acid	*E. coli*	CHS: *G. echinata* IFS: *G. echinata* CHI: *P. lobata*	[110]
Genistein **26**	Tyrosine **3**	*E. coli* and *S. cerevisiae* coculture	PAL: R. rubra 4CL: *S. coelicolor* CHS: *G. echinata* CHI: *P. lobata* IFS: *G. echinata* ACC: *C. glutamicum*	[110]
Genistein **26**	Naringenin **23**	*S. cerevisiae*	IFS: *T. pratense* CPR: *O. sativa*	[103]
Genistein **26**	Phenylalanine **2**	*S. cerevisiae*	PAL: *P. trichocarpa* × *P. deltoides* CPR: *P. trichocarpa* × *P. deltoides* C4H: *G. max* 4CL: *G. max* CHS: *G. max* CHI: *G. max* IFS: *G. max*	[101]
Genistein **26**	*p*-coumaric acid **6**	*S. cerevisiae*	PAL: *P. trichocarpa* × *P. deltoides* CPR: *P. trichocarpa* × *P. deltoides* C4H: *G. max* 4CL: *G. max* CHS: *G. max* CHI: *G. max* IFS: *G. max*	[101]
Genistein **26**	Naringenin **23**	*S. cerevisiae*	PAL: *P. trichocarpa* × *P. deltoides* CPR: *P. trichocarpa* × *P. deltoides* C4H: *G. max* 4CL: *G. max* CHS: *G. max* CHI: *G. max* IFS: *G. max*	[101]
Genistein **26**, Daidzein **27**	Naringenin **23**, Isoliquiritigenin	*S. cerevisiae*	CHI[a]: *M. sativa* IFS[a]: *G. max*	[112]
Genistein **26**	Naringenin **23**	*S. cerevisiae*	IFS: *G. max*	[137]
Genistein **26**, Daidzein **27**	Naringenin **23**, Liquiritigenin	*S. cerevisiae*	IFS: *G. echinata*	[69]

(*continued*)

Table 54.1 (continued)

Isoflavonoid target	Precursor	Host organism	Genes: Donors	Reference
Genistein **26**, Daidzein **27**	Naringenin **23**, Liquiritigenin	*E. coli*	CPR[a]: *C. roseus* IFS[a]: *G. max*	[114]
Genistein **26**, Daidzein **27**	Naringenin **23**, Liquiritigenin	*S. cerevisiae*	CPR: *C. roseus* IFS: *G. max*	[114]
Genistein **26**	Naringenin **23**	*S. cerevisiae*	CPR[a]: *O. sativa* IFS[a]: *T. pratense*	[116]
Genistin **31**, Sissotrin **33**	Genistein **26**, Biochanin A **29**	*E. coli*	UGT: *M. truncatula*	[98]
See Table 54.2	See Table 54.2	*S. cerevisiae*	CPR: *G. max* IFS: *T. pratense* HIDH: *G. max*	[117]

[a]Protein fusion

enzyme-substrate specificity and favors highly promiscuous enzymes that can convert non-natural analogs of the natural substrate to novel products. Since many plant natural products possess valuable medicinal properties, it is of significant interest to explore the space of non-natural product analogs that has not yet been evolutionarily surveyed because of the lack of non-natural substrates in the environment. Presumably some of these non-natural analogs could have enhanced or even unique pharmaceutical properties. Production of flavonoids using mutasynthesis or substrate feeding has been accomplished by several groups as reported elsewhere [60, 82, 97, 100].

Structural studies often utilize protein engineering tools such as site-directed mutagenesis to evaluate the roles of various amino acid residues in catalytic mechanisms. While this can furnish indispensable insight on enzyme-substrate interaction, it is of significant interest to metabolic engineers because it also enables construction of tailor-made enzyme mutants with improved kinetic properties, with the ability to accept structurally related substrates, with reaction reversibility for substrate-product interconversion, and with altered substrate and product regiospecificity. Protein engineering tools such as site-directed mutagenesis and directed evolution have been applied to improve production of both natural and non-natural flavonoid, isoflavonoid, and other plant natural product derivatives [87, 138–147]. Plant natural products can also be microbially catalyzed by enzymes native to the microbe to form compounds not known to exist in plants [82, 100, 119, 148–153]. These phytochemical derivatives have the potential to be utilized as human therapeutics, as the microbes catalyzing these novel reactions have been isolated from the human gut and are purported to have beneficial health impacts on their human hosts [152–157].

4.1 Mutasynthesis for Non-natural Isoflavonoid Production

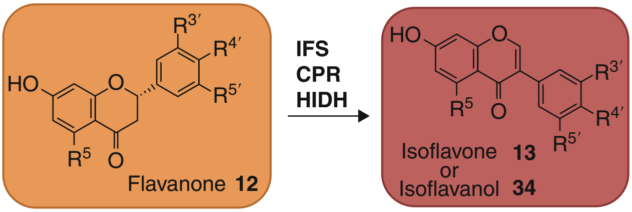
Mutasynthesis involves the chemical synthesis of non-natural substrates that are similar in structure to natural substrates. After a library of non-natural analogs are chemically synthesized, enzymatic conversion of the non-natural analogs is performed to isolate novel non-natural compounds, and the results can then be assessed to elucidate mechanisms of enzymatic catalysis and to determine substrate specificity requirements. This so-called semisynthetic approach, or the combination of chemical synthesis and biosynthesis, has also been utilized for production of non-natural isoflavonoids.

In a multiplex experiment, Chemler and colleagues evaluated the substrate specificity of IFS enzymes from five different plant species (*G. max, T. pratense, G. echinata, P. sativa, and M. truncatula*) [117]. Each enzyme was cloned into yeast and was supplemented with compounds from a library of natural and non-natural flavanones **12**. Non-natural flavanones **12** were synthesized to mimic natural flavanones **12** and isoflavones **13**; specifically, many library constituents were 7-monohydroxylated or 5,7-dihydroxylated. The library also consisted of flavanones **12** with B-ring substituents, such as single or multiple hydroxy, methoxy, ethoxy, and halide side groups. Ultimately 19 non-natural flavanones **12** and 7 natural flavanones **12** were utilized to assess IFS substrate flexibility, resulting in the biosynthesis of 4 natural isoflavones **13** and 14 non-natural isoflavone **13** analogs which are tabulated in Table 54.2. IFS substrate requirements were deduced from the rate of conversion of different flavanones **12**, including the necessity for hydroxylation at C7, the expendability of C5 hydroxylation, the incompatibility of C2′ or C6′ substitutions, the toleration of small side-group substitutions at C3′ or C5′, and the absolute requirement of C4′ hydroxylation for production of 2-hydroxyisoflavones. Due to the high affinity of genistein **26** for human estrogen receptors *α* (hER*α*) and *β* (hER*β*), isoflavones **13** are selective estrogen receptor modulator (SERM) drug candidates [158–161]. SERMs can be used to inhibit or stimulate estrogen receptors, thereby enabling their use as hormone replacements and decreasing the risk of diseases such as osteoporosis and breast cancer [17, 50, 53, 160]. In an effort to determine the therapeutic potential of the semisynthetic isoflavones **13** in the previously described library, the interaction of each compound with hER*α* and hER*β* was assessed using an in vitro competitive binding assay. As expected, the different isoflavones **13** were found to show variable activity against the human estrogen receptors. Of particular interest, both 3′-bromo-4′,5,7-trihydroxyflavone and the natural isoflavone **13** orobol displayed binding capabilities equal to genistein **26**. Structure-activity relationships between isoflavones **13** and hERs were then deduced to yield insight for future design of isoflavone **13** SERMs. Of note, the authors suggest that novel isoflavones **13** with small substituents at the C3′ position should elicit improved interactions with estrogen receptors [117].

4.2 Protein Engineering for Non-natural Isoflavonoid Production

Protein engineering has been utilized to study the mechanism by which isoflavonoid aglycones are converted to isoflavonoid glycosides by uridine diphosphate glycosyltransferases, a large protein class catalyzing the transfer of activated

Table 54.2 Mutasynthesis for natural and non-natural isoflavonoid production



	Side-group decoration				
Flavanone precursor	R^5	R$^{3'}$	R$^{4'}$	R$^{5'}$	Primary biotransformation product
Naringenin 23	OH	H	OH	H	Genistein 26
Liquiritigenin	H	H	OH	H	Daidzein 27
Eriodictyol	OH	OH	OH	H	Orobol
Butin	H	OH	OH	H	3′,4′,7-Trihydroxyisoflavone
Homoeriodictyol	OH	OCH_3	OH	H	3′-Methoxy-4′,5,7-trihydroxyisoflavone
4′,7-Dihydroxy-3′-methoxyflavanone	H	OCH_3	OH	H	4′,7-Dihydroxy-3′-methoxyisoflavone
3′,5′-Dimethoxy-4′,5,7-trihydroxyflavanone	OH	OCH_3	OH	OCH_3	3′,5′-Dimethoxy-4′,5,7-trihydroxyisoflavone
4′,7-Dihydroxy-3′,5′-dimethoxyflavanone	H	OCH_3	OH	OCH_3	4′,7-Dihydroxy-3′,5′-dimethoxyisoflavone
3′-Ethoxy-4′,5,7-trihydroxyflavanone	OH	OCH_2CH_3	OH	H	3′-Ethoxy-4′,5,7-trihydroxyflavanone
4′,7-Dihydroxy-3′-ethoxyflavanone	H	OCH_2CH_3	OH	H	4′,7-Dihydroxy-3′-ethoxyflavanone
3′-Methyl-4′5,7-trihydroxyflavanone	OH	CH_3	OH	H	3′-Methyl-4′,5,7-trihydroxyisoflavone
4′7-Dihydroxy-3′-methylflavanone	H	CH_3	OH	H	4′,7-Dihydroxy-3′-methylisoflavone
3′,5′-Dimethyl-4′,5,7-trihydroxyflavanone	OH	CH_3	OH	CH_3	3′,5′-Dimethyl-4′,5,7-trihydroxyisoflavone
4′,7-Dihydroxy-3′,5′-dimethylflavanone	H	CH_3	OH	CH_3	4′,7-Dihydroxy-3′,5′-dimethylisoflavone
3′-Chloro-4′,5,7-trihydroxyflavanone	OH	Cl	OH	H	3′-Chloro-4′,5,7-trihydroxyisoflavone
3′-Chloro-4′,7-dihydroxyflavanone	H	Cl	OH	H	3′-Chloro-4′,7-dihydroxyisoflavone
3′-Bromo-4′,5,7-trihydroxyflavanone	OH	Br	OH	H	3′-Bromo-4′,5,7-trihydroxyisoflavone
3′-Bromo-4′,7-dihydroxyflavanone	OH	Br	OH	H	3′-Bromo-4′,7-dihydroxyisoflavone

sugars to various substrates. These studies yield insight into the interactions between specific amino acid residues and substrate, enabling rational design of enzyme mutants for specific purposes. Structure-guided enzyme engineering is often directed at or around the active site or binding pocket region to alter substrate specificity, enzymatic activity, and product regioselectivity. In the case of UGT71G1 from *M. truncatula*, a point mutation in residue 202 from tyrosine (Tyr) **3** to alanine (Ala), Y202A, enables the conversion of genistein **26** to both 7-*O*-glucoside and 5-*O*-glucoside, whereas the native enzyme only enables conversion of genistein **26** to the 7-*O*-glucoside product. Residue 202 is located at one end of the acceptor (isoflavonoid aglycone) binding pocket, so this mutation from an amino acid with a large aromatic side group to one with a small side group presumably increases the volume of the pocket, providing the acceptor with an increased number of possible docking configurations [140].

Another protein engineering effort for isoflavonoid production focused on *M. truncatula* UGT85H2. A point mutation in residue 305 from isoleucine (Ile) to threonine (Thr), I305T, showed a 19-fold increase in enzyme activity with a 25-fold decrease in the Michaelis constant (K_m) for conversion of biochanin A **29** into sissotrin **33**. Additionally the mutation of residue 200 from valine (Val) to glutamic acid (Glu), V200E, imparted deglycosylation activity in the presence of UDP in the reaction mixture, enabling the removal of the glucose residue from sissotrin **33**, the biochanin A **29** 7-*O*-glucoside, to form biochanin A **29** aglycone. The mutation also decreased K_m by sevenfold, increased maximum velocity (V_{max}) and turnover number (k_{cat}) by sevenfold, and increased catalytic efficiency by 54-fold. Amino acid 200 resides on one end of the acceptor binding pocket, and docking studies indicate that the negatively charged glutamic acid side group might interact with the 7-OH of biochanin A **29**. This novel method utilizing mutagenesis to impart reversibility could be applied to deglycosylation of other flavonoids [141].

The aforementioned UGT mutagenesis studies involved variations in activity and regioselectivity. However, glycosylation of flavonoids with sugars other than glucose occurs in nature and should be possible to engineer in microbes. In addition to UDP-glucose, for instance, UDP-glucuronic acid, UDP-galactose, UDP-xylose, and UDP-rhamnose are all known to act as nucleotide-activated sugar donors in various plant species [162]. In *Bellis perennis* (red daisy) BpUGT94B1, the positively charged guanidinium side group of a single arginine (Arg) residue at position 25 is critical for UDP-glucuronic acid donor activity due to its interaction with the negatively charged carboxylate group on glucuronic acid [139]. Similarly, a family of UGTs known as flavonoid 7-*O*-glucuronosyltransferases (F7GATs) found in plants from the Lamiales order share a conserved arginine residue in the sugar donor binding pocket that is responsible for the specificity toward UDP-glucuronic acid. Site-directed mutagenesis of *Perilla frutescens* UGT88D7 residue 350 containing arginine (which corresponds to tryptophan (Trp) 360 in UGT71G1) to Trp abolished UDP-glucuronic acid specificity and instead invoked UDP-glucose sugar donor specificity. Once again the cationic guanidinium moiety on arginine is crucial for recognition and interaction with the anionic carboxylate group on UDP-glucuronic acid.

4.3 Other Isoflavonoid Biotransformations

In a series of recent reports, a G6P isomerase (PGI, catalyzing the isomerization of G6P to fructose-6-phosphate) knockout strain of *E. coli* was engineered to produce flavonoid glycosides from flavonoid aglycones. Specifically, the strain produced 7-*O*-xylosyl naringenin and 7-*O*-glucuronyl quercetin by overexpressing an *Arabidopsis thaliana* UGT and an artificial UDP-sugar biosynthetic gene cluster (containing *E. coli* K-12 GALU and *Micromonospora echinospora* spp. *calichensis* UDG and UDP-glucuronic acid decarboxylase, known as UXS1) in combination with naringenin **23** and quercetin feeding [163, 164]. Continuing their efforts, Simkhada and coworkers recently engineered *E. coli* for production of 3-*O*-rhamnosyl quercetin, 3-*O*-rhamnosyl kaempferol, and 3-*O*-allosyl quercetin by assembling artificial thymidyldiphosphate (TDP)-sugar biosynthetic pathways for TDP-L-rhamnose and TDP-6-deoxy-β-D-allose and feeding the strain with quercetin and kaempferol aglycones.

TDP-sugar production was enabled by the deletion of PGI to shunt flux toward G1P and overexpression of TDP-glucose synthase (TGS) from *Thermus caldophilus* GK24 to form the activated nucleotide sugar [165]. TDP-L-rhamnose was produced by overexpression of *Salmonella typhimurium* LT2 TDP-glucose 4,6-dehydratase (DH) and *Streptomyces antibioticus* Tu99 TDP-4-keto-6-deoxyglucose 3,5-epimerase (EPI) and TDP-glucose 4-ketoreductase (KR); TDP-6-deoxy-β-D-allose was produced by overexpression of *T. caldophilus* GK24 DH and *Streptomyces* sp. KCTC 0041BP TDP-hexose 3-epimerase (GERF) and TDP-4-keto-6-deoxyglucose reductase (GERK). Overexpression of a 3GT from *A. thaliana* completed the 3-*O*-glycosylation of the flavonoid aglycone precursors with the TDP-sugars [166]. These engineering efforts demonstrate the potential for regiospecific glycosylation of isoflavonoids with tailored sugar moieties that could one day enable design of therapeutics with altered activities and varying degrees of bioavailability; from a microbial production perspective, customizable glycosylations might also mitigate cellular toxicity while improving isoflavonoid solubility, stability, and transport from the cell, ultimately leading to higher product yields [126, 167].

Other flavonoid biotransformations catalyzed by microbial enzymes will also allow for production of novel, nonplant flavonoids from amino acid precursors. Two bacterial nonheme dioxygenases, biphenyl dioxygenase (BDO) and naphthalene dioxygenase (NDO), have recently been shown to regioselectively and stereoselectively convert flavonoids, including isoflavones **13** and isoflavanols **34**, to epoxides and dihydrodiols [151, 168–172]. BDO from *Pseudomonas pseudoalcaligenes* KF707 and NDO from *Pseudomonas* sp. strain NCIB9816-4 are able to accept various flavonoids as substrates due to the presence of biphenyl and naphthalene moieties within the flavonoid core structure **1** [168]. Additionally, expression of *Streptomyces avermitilis* MA-4680 7-*O*-methyltransferase (SaOMT-2) in *E. coli* shows substrate promiscuity and transfers a methyl group to flavones **14** and isoflavones **13** [173]. This is the first example of a methyltransferase known to act upon both flavones **14** and isoflavones **13**, opening up a route for biosynthesis of non-natural methylated isoflavones **13** by feeding of non-natural precursors. Another example of microbial isoflavonoid biotransformation is the reduction of daidzein **27** to equol. Although several microorganisms isolated from mammalian digestive tracts have been shown to catalyze the nonstereospecific transformation, a recently isolated gram-negative anaerobic species, MRG-1, shares high homology with *Coprobacillus* species and was shown to exhibit stereospecific reductase activity for conversion of several isoflavones **13** to the corresponding isoflavanones. Stereoselective reduction from the highly active MRG-1 isoflavone reductase (IFR) opens new biotechnological routes for production of enantiopure flavanones **12** [153].

5 Conclusions

Metabolic engineering of microbes for isoflavonoid biosynthesis showcases state-of-the-art methodologies for high-level production of pharmaceutically and nutraceutically relevant compounds. Decoupling production of plant secondary metabolites from their native, convoluted regulatory backgrounds enables predictable control and design, while transplanting biosynthetic pathways into fast-growing, well-characterized microorganisms allows utilization of advanced genetic and computational tools and an abundance of biological data. Genetically tractable microbes such as *E. coli* and *S. cerevisiae* provide an unmatched platform for combinatorial biosynthesis of complex plant natural products and their non-natural derivatives by transformation with heterologous genes from different organisms.

Though microbial production of plant natural products is a promising alternative to traditional methods, further research will continue to improve titers and assist in the discovery of novel isoflavonoid biotransformations. A significant challenge that has not yet been accomplished is the expression of the entire isoflavonoid metabolic pathway in *E. coli*, from aromatic amino acid precursors without supplementation of intermediates. Given the propensity for feedback inhibition and host toxicity of many flavonoid and isoflavonoid intermediates, protein engineering efforts will likely be required to enable high-level isoflavonoid production [174–176]. Furthermore, in vivo characterization of all enzymes in the isoflavonoid pathway will help determine rate-limiting steps that require higher relative promotion or expression level. Stoichiometric-based modeling and computational algorithms can also be utilized to predict genetic manipulations for maintaining high growth coupled with high specific production. Several thorough reviews have addressed the relative merits of various algorithms [177–182].

Feedback inhibition can be limited by optimizing both upstream and downstream enzyme expression such that the inhibitor does not significantly accumulate. In instances where a metabolite inhibits an enzyme in the isoflavonoid pathway, enzyme mutagenesis can alter the structural interaction between the enzyme and its inhibitor to block the inhibition mechanism. Recently, allosteric feedback inhibition of a tomato peel 4CL by naringenin **23**, a product several steps downstream, was significantly reduced through directed evolution in *E. coli* [181].

Cellular toxicity can also be ameliorated by various engineering strategies. Toxicity caused by intracellular accumulation of an intermediate can be limited by pathway optimization to ensure that the metabolite is utilized soon after it is produced. Pathway optimization can be achieved by accurate in vivo characterization of all enzymes in the pathway. Additionally, spatial localization of the enzymes catalyzing subsequent steps in a pathway serves as a "pipeline" to channel intermediate substrates to their respective catalyzing enzymes [184, 185]. This spatial proximity effectively leads to increased local substrate concentration and can be engineered by creating a protein fusion between adjacent enzymes, by docking multiple enzymes to a protein or RNA scaffold at minimal distance from each other, or by compartmentalizing all of the enzymes in a biosynthetic pathway in an isolated enclosure, such as a bacterial microcompartment (BMC) or an artificial organelle [186–191]. Such methodologies have enabled significant improvement in production levels of other microbial products and are outlined in great detail in a recent review by Agapakis and colleagues [185]. If the final product is toxic to the cell, one conceivable method for reducing the toxicity would be to engineer BMCs or artificial organelles for sequestration of the responsible metabolites. An alternative method is to engineer product transport. Overexpression of a library of efflux pumps and extracellular transporters can pinpoint proteins capable of selective export of a target product, while product glycosylation or deglycosylation could also improve export from the cell [192–194]. It is also important to consider if the product is natively transported into the cell from the extracellular environment; blocking transport of the toxic compound back into the cell can be accomplished by knocking out genes involved in product uptake.

Further work aimed at bioprospecting, culturing hard-to-culture microbes, searching for "unknown" and "orphan" enzymes that have not yet been characterized, and designing promiscuous enzymes capable of decorating and transforming flavonoids and their unnatural analogs will increase the range of isoflavonoid derivatives produced in microbes [195]. The search for enzymes capable of such manipulations should not be limited to plants, however, as many microbes endemic to mammalian guts have evolved to metabolize the plant phenylpropanoids ingested by their hosts. Current research efforts in these areas will lead to economically viable microbial platforms for production of isoflavonoids and products of high medicinal value.

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Bioproduction of *trans*-Resveratrol from Grapevine Cell Cultures

55

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Contents

1 Introduction 1685
2 Distribution and Localization of Stilbenes and *trans*-Resveratrol in Plants and Specifically in *Vitis* Species 1686
3 The Biosynthetic Pathway of *trans*-Resveratrol: Key Enzymes and its Regulation 1688
4 Biotechnological Approaches to Improve *trans*-Resveratrol and Stilbene-Related Production Using *Vitis* Cell Cultures Without Genetic Transformation 1691
4.1 Elicitation Strategies to Improve *trans*-Resveratrol and Stilbene-Related Production 1693
4.2 Large-Scale *trans*-Resveratrol and Stilbene-Related Production 1700
5 Biotechnological Approaches to Improve *trans*-Resveratrol and Stilbene-Related Production Using Genetic Engineering in Different Biological Systems 1703
5.1 Increasing *trans*-Resveratrol Content by Genetic Manipulations in Plants and Grapevine Cell Cultures 1703
5.2 Incorporating Genes from the *trans*-Resveratrol Biosynthetic Pathway in Microbial Organisms 1705
6 Conclusions 1706
References 1707

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Abstract

The use of grapevine cell cultures to increase *trans*-resveratrol and stilbene-related production under controlled conditions is viewed as a promising biotechnological alternative to their extraction from the whole plant or their chemical synthesis. At present, in vitro plant cultures are rarely used as a commercial method for the production of bioactive compounds. The major problems hindering the development of the large-scale culture of plant cells include low productivity, cell line instability, and the difficulties involved in scaling up. This chapter describes how the composition of the medium, culture conditions, elicitors, bioreactor design, and other critical parameters influence the behavior of grapevine cell cultures and how optimization of these factors could allow improvements in *trans*-resveratrol and stilbene-related production. For these reasons, this chapter describes how high-producing *Vitis* cell lines, nutrient and precursor feeding, elicitation with stress factors, and signaling molecules, as well as metabolic engineering can be used to increase the production of these secondary metabolites.

Keywords

Bioproduction • *Vitis vinifera* • grapevine • in vitro cultures • stilbenes • *trans*-Resveratrol • biosynthetic pathway

Abbreviations

4CL	4-coumarate-CoA ligase
araE	The low-affinity high-capacity bacterial *araE* transporter
C4H	Cinnamate 4-hydroxylase
CHS	Chalcone synthase
cv	Cultivar
DW	Dry cell weight
ET	Ethylene
FW	Fresh cell weight
JA	Jasmonic acid
Phe	Phenylalanine
MeJa	Methyl jasmonate
PAL	Phenylalanine ammonia-lyase
SA	Salicylic acid
STS	Stilbene synthase
trans-R	*trans*-Resveratrol
3,4′,5	Trihydroxystilbene
UV	Ultraviolet
Vsts	*Vitis* stilbene synthase

1 Introduction

Plants elaborate a vast array of natural products, which, as they evolve, confer selective advantages against environmental stresses. Among such products are the phenylpropanoids, a large family of secondary metabolites that are involved in plant responses to biotic and abiotic stresses. Many phenylpropanoids are antimicrobial compounds synthesized in response to pathogen or herbivore attack and classified as phytoalexins. However, other roles have been described for stress-induced phenylpropanoids, including the signaling of defense responses and protection against ultraviolet (UV) light damage [1].

Stilbenes are a small group of phenylpropanoids characterized by a 1,2-diphenylethylene backbone. Most plant stilbenes have phytoalexin activity and are mainly derivatives of the monomeric unit *trans*-resveratrol (*trans*-R, 3,4′,5-trihydroxystilbene) although other stilbene structures are found in plants.

Vitis is a genus of about 60 species of dicot angiosperm plants. The fruit, which is a berry normally ovoid in shape and juicy, is grown commercially in the case of several *Vitis* species for consumption as fresh grapes and for fermentation into wine. Worldwide, *Vitis vinifera* (Fig. 55.1) is one of the most important species since it is used for the production of table grapes, raisins, and wine. The berries and the products derived from them are one of the most important sources of stilbenes available in nature [2].

The formation of stilbenes is considered to be a part of the general defense mechanism since they display strong antifungal and antimicrobial activities [3, 4]. In fact, *trans*-R is found in both grapevine tissue and berries and in cell cultures as the result of both abiotic and biotic stress [5]. Since *trans*-R is postulated to be involved in the health benefits associated with a moderate consumption of red wine, it is one of the most extensively studied natural products.

Indeed, hundreds of studies have reported the beneficial effects of *trans*-R on neurological [6] and cardiovascular systems [7]. One of the most striking biological activities of *trans*-R is its anticancer activity, preventing carcinogenesis in the three stages of tumor development [8]. Further data provide interesting insights into the effect of this compound on the life span of different organisms, suggesting that *trans*-R might be regarded as a potential antiaging agent in treating age-related human diseases [9]. In addition, the effects described in mice subjected to a high-calorie diet [10] open up new approaches for treating not only age-related diseases but also obesity-related disorders [11].

trans-R was first detected in 1939 as a root constituent of the medicinal plant white hellebore lily (*Veratrum grandiflorum*). Years later, the presence of *trans*-R was described in a species native to Western Australia, *Eucalyptus wandoo*, by Hathway and Seakins [12] although the richest source of this compound is the roots of the weed *Polygonum cuspidatum* [13], an Asian medicinal plant whose root extracts are used in traditional Chinese and Japanese medicine (Ko-jo-kon) for their

Fig. 55.1 *V. vinifera* plant

high content of *trans*-R and derivatives. Therefore, the strategy commonly used to produce stilbenes and specifically *trans*-R involves their direct extraction from plants. However, in some cases, the production of stilbenes from plants is not always satisfactory since their presence is often restricted to a given species or genus and might be activated only during a particular development stage or under specific seasonal, stress, or nutrient availability conditions [14]. For this reason, the chemical synthesis is often considered to obtain these compounds, although the stereospecificity, the strict conditions of the reactions, and the high costs involved represent a disadvantage of this approach. As a consequence, much effort has been put into the use of in vitro cultures as one attractive biotechnological strategy for producing this natural compound of commercial interest. In fact, plant cell cultures offer a good alternative to whole plants because they allow *trans*-R and stilbene-related production in controlled and reproducible conditions. Likewise, several strategies including precursor feeding, in situ product removal, elicitation, and metabolic engineering have been described as useful for increasing the production of these secondary metabolites.

In this chapter, we describe these strategies and recent advances in our understanding of the *trans*-R biosynthetic pathway, its regulation, and how to gain further knowledge using biotechnological, genomic, and metabolic approaches to improve the production of these high value compounds.

2 Distribution and Localization of Stilbenes and *trans*-Resveratrol in Plants and Specifically in *Vitis* Species

Stilbenes and particularly *trans*-R are found in a large number of plant families. Indeed, these low molecular weight phenolics are found in gymnosperm plants belonging to the Pinaceae and Gnetaceae families; in monocot angiosperm

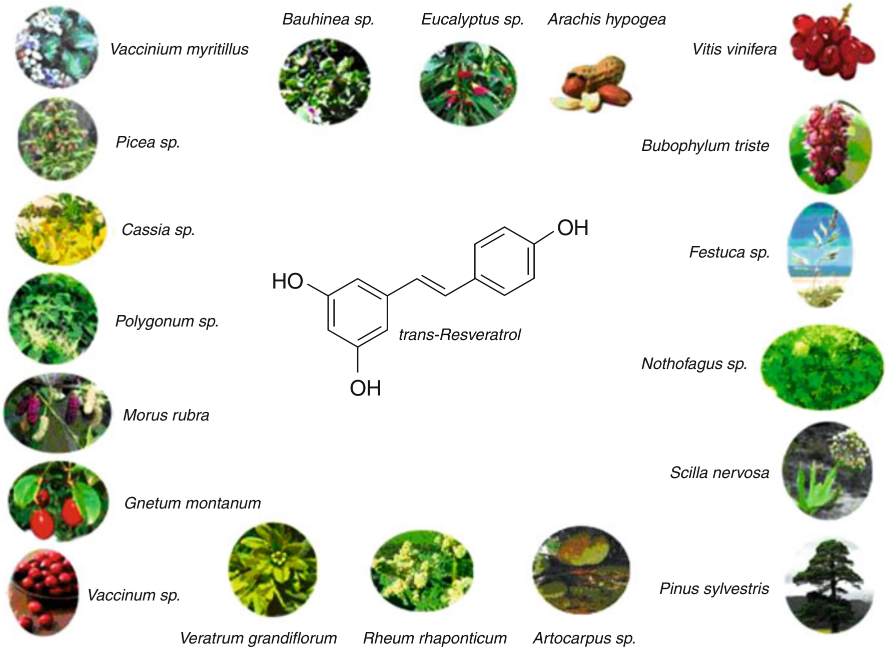

Vaccinium myritillus
Bauhinea sp.
Eucalyptus sp.
Arachis hypogea
Vitis vinifera
Picea sp.
Bubophylum triste
Cassia sp.
OH
HO
Festuca sp.
Polygonum sp.
trans-Resveratrol
OH
Nothofagus sp.
Morus rubra
Scilla nervosa
Gnetum montanum
Vaccinum sp.
Pinus sylvestris
Veratrum grandiflorum
Rheum rhaponticum
Artocarpus sp.

Fig. 55.2 Plants which are able to biosynthesize *trans*-R

plants belonging to the Poaceae, Cyperaceae, and Liliaceae families; and in dicot angiosperm plants from the family of Myrtaceae, Fabaceae, Moraceae, Fagaceae, Palmaceae, Polygonaceae, and Vitaceae (Fig. 55.2) [15].

Stilbenes and *trans*-R are synthesized by several species belonging to the genus *Vitis*. Most *Vitis* species are found in temperate regions of the Northern Hemisphere, where they occur in widely different geographical areas and show a great diversity of forms. The exact number of species is uncertain, with an estimates ranging from 40 to more than 60. Some of the more notable are *V. vinifera*, the European grapevine, native to the Mediterranean area and Central Asia; *V. labrusca* and *V. riparia*, native to the eastern part of the United States and Canada; *V. rotundifolia* and *V. aestivalis*, native to the southeastern part of the United States; *V. amurensis*, native to Asia, including parts of Siberia and China; and *V. coignetiae*, a species from East Asia. *V. vinifera* is a species distributed in the Mediterranean region, central Europe, and southwestern Asia, from Morocco and the north of Portugal to southern Germany and in northeastern Iran. There are many different grapevine cultivars (cv) and hybrids, such as the primary crosses between *V. vinifera* and one or more of the *V. labrusca*, *V. riparia*, and *V. aestivalis* [16].

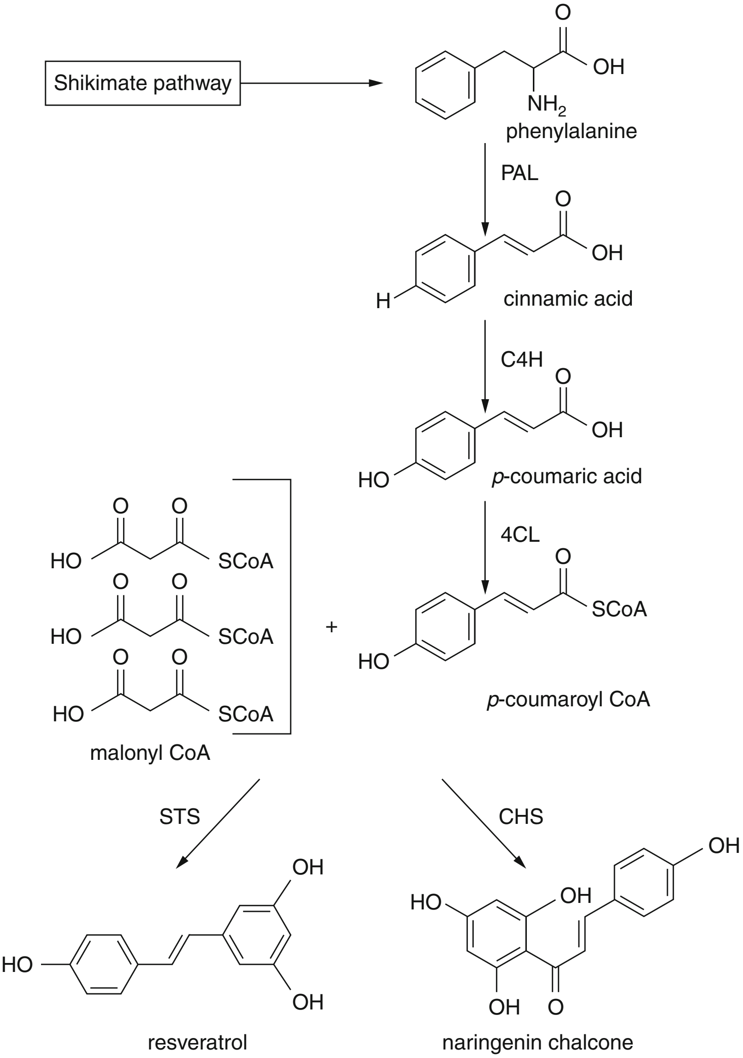
Stilbenes and *trans*-R are induced (as phytoalexins) in nonwoody plant organ/tissues [17], whereas they are constitutively synthesized in woody and hard plants [18]. Most of them are present in berries and wines produced from them. The production of stilbenes depends on the grapevine cv, and their levels can also vary from year to year. The level of these compounds can also be modified by nutritional conditions and stress factors associated with the vineyard such as weather, UV light, and fungal or bacterial infections. In grape berries, most *trans*-R is located in the skin, while the type of wine fermentation determines the final *trans*-R content in wines [17]. For example, the content of *trans*-R in Spanish white wines (0.05–1.80 mg L^{-1}) is lower than in Spanish red ones (1.92–12.59 mg L^{-1}) and red musts (1.14–8.69 mg L^{-1}), probably due to the very high concentration of *trans*-R found in the skin of red grapes (about 1.50–7.80 mg *trans*-R g^{-1} fresh weight (FW)). The ability of grape berries to synthesize stilbenes and *trans*-R varies during grape development, since the content of *trans*-R decreases during ripening, whereas the level of flavonoids increases.

3 The Biosynthetic Pathway of *trans*-Resveratrol: Key Enzymes and its Regulation

Flavonoids and stilbenes have a common origin as both derive from the phenylpropanoid metabolism. However, flavonoids are ubiquitous in plants, whereas stilbenes are specific to certain plant families. Phenylalanine (Phe), an end product of the shikimate pathway, is a key intermediate linking the primary metabolism with phenylpropanoid, flavonoid, and stilbenoid secondary metabolic pathways. Thus, the first step in the stilbene pathway consists in the transformation of Phe into cinnamic acid in a reaction catalyzed by phenylalanine ammonia-lyase (PAL). The consecutive action of cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL) transforms cinnamic acid into p-coumaroyl-CoA, which is the common precursor of most of the phenolic compounds found in plants: lignins, flavonoids, and stilbenoids (Scheme 55.1). Then, one molecule of p-coumaroyl-CoA is condensed with three C2 units from malonyl-CoA to produce *trans*-R, through the action of stilbene synthase (STS), or to produce naringenin chalcone by the action of chalcone synthase (CHS) [19]. However, *trans*-R is synthesized via cleavage of four carbon dioxide molecules, while naringenin chalcone is formed via cleavage of three carbon dioxide molecules. Thus, both STS and CHS catalyze three condensation reactions with malonyl-CoA molecules, giving the same long-chain polyketide intermediate, but STS also catalyzes the loss of the terminal carboxyl group, which leads to the production of the C14 molecule instead of the CHS-catalyzed C15 product [19] (Scheme 55.1).

Structure–function analysis suggests that STS uses an aldol-switch mechanism to carry out a region-specific C2–C7 cyclization, rather than the C1–C6 cyclization of CHS. CHS is responsible for the formation of chalcone in many higher plants, but STS, which shares 75–90 % amino acid sequence identity with CHS, is only found in species that accumulate stilbenes. Tropf et al. [20] showed evidence indicating that STS has evolved in a limited number of phylogenetically related plants by gene duplication and subsequent mechanistic divergence from CHS.

The STS protein was purified first from elicited cell cultures of peanut (*Arachis hypogaea*). The cloning of two peanut *sts* genes revealed extensive homology with


Shikimate pathway
phenylalanine
PAL
cinnamic acid
C4H
p-coumaric acid
4CL
p-coumaroyl CoA
malonyl CoA
+
STS
CHS
resveratrol
naringenin chalcone
OH
NH2
HO
H
SCoA
O

Scheme 55.1 The biosynthetic pathway of *trans*-R and naringenin chalcone

the peanut *chs* gene throughout the coding region, and the position of the single intron was conserved in both genes. Subsequently, the first gene coding a *Vitis* stilbene synthase (*Vsts*) was cloned by Melchior and Kindl [21]. Later, *sts* genes were also cloned from Scots pine (*Pinus sylvestris*) and other species. *sts* genes exist as a family of related genes in many plant species ([1] and references therein). STS proteins are classified into p-coumaroyl-CoA-specific types, such as resveratrol synthase, or cinnamoyl-CoA-specific types, such as pinosylvin synthase, depending on their preferred starter molecule. The former type occurs mostly in angiosperms such as peanut, grapevine, and sorghum, whereas the latter type is typical in gymnosperms and has been reported in several pine species such as *P. sylvestris*, *P. strobus*, and *P. densiflora*. Analysis of the grapevine genome sequence confirmed the large size of this multigene family, with an estimated number of around 20–40 *sts* genes. At least 20 different *sts* genes are expressed in grapevine following infection with *Plasmopara viticola*, which is consistent with this estimation ([1] and references therein).

On the other hand, stilbene biosynthesis is induced in response to a wide range of biotic and abiotic elicitors, resulting in increased gene expression of the stilbene biosynthetic pathway and accumulation of the corresponding enzymes. Regulation of stilbene biosynthesis by stress is particularly well documented in grapevine, where the expression of *sts* genes and the production of stilbenes are induced upon elicitation with different fungal pathogens, including powdery mildew (*Erysiphe necator*), downy mildew (*P. viticola*), and gray mold (*Botrytis cinerea*). Stilbene biosynthesis is also triggered by abiotic stimuli such as aluminum ions, methyl jasmonate (MeJa), ethylene, and UV light irradiation [22]. Cloning of different *sts* genes in various plant species revealed the differential regulation of some members of this gene family. In grapevine, expression patterns of two *sts* genes exhibited striking differences in elicited grapevine cell cultures, indicating they may be responsible for different responses to one inducer treatment [23]. Similarly, three *sts* genes of peanut were transcribed in response to various inducers, indicating that different members of this gene family respond differentially to these different types of stress [1]. However, Lijavetzky et al. [24] found the expression of two *sts* genes, strongly coordinated with the expression of *pal*, *c4h*, and *4cl* genes, increased when grapevine cell cultures were simultaneously treated with two different elicitors. Furthermore, the synergistic interaction of both elicitors on *trans*-R production was the result of the synergistic effect observed on gene expression of the biosynthetic pathway.

In recent years, a growing number of transcription factors involved in the regulation of flavonol, lignin, and anthocyanin metabolism have been characterized [1]. However, to date, no transcription factors responsible for regulating the stilbene biosynthetic pathway have been characterized.

Grapevine *sts* genes retain their inducibility to stress when they are used to transform other plant species that do not normally synthesize stilbenes. Thus, tobacco was transformed with a grapevine genomic DNA fragment containing the *Vsts1* and *Vsts2* genes, under the control of their own promoters. *sts* gene expression was sensitive to elicitor treatments in these transgenic tobacco lines, indicating that *sts* genes are regulated by stress response pathways, which are not specific to plants that naturally synthesize stilbenes [19].

As regards the localization of STS protein in grape berry, STS is found in berry exocarp tissues in all stages of fruit development, but the levels of this protein decrease gradually from exocarp to mesocarp, where the protein is only detected occasionally. STS localization is the same before and after berry veraison. Nevertheless, the amount of this protein decreased significantly in ripe berries, in agreement with the lower *trans*-R content measured in the same tissues.

At the subcellular level, STS is predominantly found within vesicles, along the plasma membrane and in the cell walls, suggesting a protein secretion pathway toward the apoplast compartment. Also, STS localization in exocarp cell walls is consistent with its role in synthesizing defense compounds and suggests that a differential localization of phenylpropanoid biosynthetic machinery regulates the deposition of specific secondary products at different action sites within the cells ([19] and references therein).

4 Biotechnological Approaches to Improve *trans*-Resveratrol and Stilbene-Related Production Using *Vitis* Cell Cultures Without Genetic Transformation

Much effort has been dedicated to the use of plant in vitro cultures as a biotechnological strategy to produce secondary metabolites of commercial interest. The advantages of these systems for the industrial production of these compounds include uniform product quality, independence from climate and seasonal changes, supply stability, and a closer relationship between supply and demand. In some cases, the production of secondary metabolites using large-scale in vitro cultures is technically feasible since the generation and maintenance of cell biomass are accompanied by a high yield of these metabolites.

In this sense, *Vitis* cell cultures could become an important system as cell factories for the production of stilbenes. The analysis of *trans*-R production in untreated plant cell cultures revealed low levels of *trans*-R accumulation, less than 0.1 mg g^{-1} dry weight (DW) or 2–3 mg L^{-1} [22]. Different approaches have been used to increase the production of *trans*-R, elicitation being the most important strategy in this respect, in some cases, combined with the use of highly productive *Vitis* cell lines, optimized culture conditions, and sugar and/or nutrient feeding. Also, genetic modification and metabolic engineering are promising biotechnological approaches that could improve the production of both stilbenes and *trans*-R.

Screening and selection for high-producing *Vitis* cell lines starts with the selection of the plant from which cell and tissue cultures will be initiated. Then, cell and tissue cultures have to retain the capacity to biosynthesize the secondary metabolite and maintain production stability during subsequent subcultures. Considering *trans*-R and stilbene-related biosynthesis as a result of the defense response (constitutive or inducible) and the variability of this response in *Vitis* genotypes, it is important to choose genotypes that have the ability to produce high *trans*-R levels. In addition, elicitors, which stimulate defense responses, have been used as a tool to select high-yielding cell lines. Likewise, the levels of *trans*-R and stilbene-related production that can be obtained in response to elicitors fluctuate widely according to the plant species and cultivars, the elicitors used, and culture conditions [25].

Zamboni et al. [26] studied the differential response of *Vitis* cell cultures for the production of *trans*-R, finding that elicited cell cultures of *V. amurensis* and those resulting from the crossing between *V. riparia* and *V. berlandieri* were able to produce more *trans*-R than cell cultures of *V. vinifera* cv Pinot Noir or Merzling.

Also, modification of the culture medium and physical factors is important for increasing the metabolite accumulation. The expression of many secondary metabolite pathways is easily altered by factors such as nutrients, light, and growth regulators. Many of the constituents of plant cell culture media are important determinants of growth and the accumulation of secondary metabolites. In the case of *Vitis* cell cultures, the medium and culture conditions are critical parameters that influence the improvement of *trans*-R and stilbene-related production. Waffo-Teguo et al. [27] observed that *trans*-R and stilbene glycosides accumulated when *V. vinifera* cv Gamay Fréaux was grown in a polyphenol-inducing medium in which $(NH_4)_2SO_4$, NaH_2PO_4, $MgSO_4$, and sucrose concentrations were changed. Indeed, the amount of the sugar is one of the key culture conditions since sugars represent the carbon source of the culture medium. In plants, sugars are not only energy sources and structural components but are also physiological signals regulating the expression of a variety of genes involved in both primary and secondary metabolism. The effect of sugars on plant cell cultures seems to be mainly associated with the coupling of two mechanisms: osmotic stress and a disturbed cellular metabolism [28]. In addition, sugars act as specific regulatory signals through an intracellular sugar-sensing pathway.

Since grapevine cell cultures are grown in heterotrophic conditions, usually adding sucrose as carbon source, the level of sucrose added to the culture medium has been seen to regulate the production of polyphenols in *V. vinifera* cv Gamay Fréaux cell cultures. In fact, the production of anthocyanins markedly increased in *V. vinifera* cells after adding 0.15 M (51.30 g L^{-1}) sucrose to the culture medium, while the content of piceids (glucosylated forms of both *cis*- and *trans*-R) was only slightly affected [29]. Also, the addition of 0.08 M sucrose (27.36 g L^{-1}) to the same *V. vinifera* cell cultures provoked a slight extracellular accumulation of *trans*-R which peaked after 18 h of treatment (52 nmol g^{-1} FW; i.e., 11.87 μg *trans*-R g^{-1} FW), whereas this sucrose concentration did not induce any accumulation of piceids in these cells [30]. In fact, the addition of sucrose to these cells did not affect the expression of *pal* or *sts* genes.

On the other hand, precursor feeding has been proved to increase the levels of *trans*-R in grapevine cell cultures. In particular, feeding with Phe has been extensively studied [31–33]. Kiselev et al. [31] demonstrated that feeding *V. amurensis* callus cultures with 0.1 mM Phe resulted in a slight increase of *trans*-R (0.50 mg g^{-1} DW) compared with control cells (0.43 mg g^{-1} DW), while the addition of higher concentrations of Phe did not cause any further increase of this compound. Therefore, the possibility that a low pool of *trans*-R precursors might be the cause of low *trans*-R production is unlikely since the addition of Phe to the culture medium was not very effective [31].

Saigne-Soulard et al. [32] analyzed the influence of Phe on stilbene biosynthesis in *V. vinifera* cv Gamay Fréaux cell cultures, observing that the treatment of the cells with 1 mM Phe resulted in an enrichment in stilbenes (62 mg L^{-1}), the maximal stilbene levels being reached when Phe was completely metabolized by the cells. Aumont et al. [33] also studied the effect of the administration of 3 mM Phe to *V. vinifera* cv Gamay Fréaux cell cultures. Their results pointed out an increase in the production of stilbenes (360 mg L^{-1}) after 14 days when *V. vinifera* cell cultures were fed with Phe using a high cell density (300 g FW L^{-1}) in a 2-L stirred-tank bioreactor.

In contrast to these results, Yue et al. [34] analyzed the effect of different Phe concentrations on the production of *trans*-R in *V. vinifera* cv Gamay Fréaux cell cultures showing that the addition of Phe (1–3 mM) did not result in a significant increase in the production of *trans*-R.

On the other hand, Chen et al. [35] analyzed the effect of adding alanine to *V. labrusca* cell cultures on *trans*-R production and gene expression involved in its biosynthetic pathway. They found that alanine feeding (50 mg L^{-1}) significantly increased the production of *trans*-R both in the culture medium (0.27 mg L^{-1}) and in the cells (0.09 mg L^{-1}). Moreover, the addition of this amino acid also induced *pal*, *c4h*, and *sts* gene expression.

4.1 Elicitation Strategies to Improve *trans*-Resveratrol and Stilbene-Related Production

The term *elicitor* refers to chemicals from various sources, biotic or abiotic, as well as physical factors, that can trigger a response in living organisms resulting in a high accumulation of secondary metabolites. Therefore, elicitors are useful tools for improving the production of plant valuable compounds ([36] and references therein). The effectiveness of elicitation as a tool to enhance the production of secondary metabolites depends on a complex interaction between the elicitor and the plant cell. There is evidence that the same elicitor can stimulate secondary metabolism in different cell cultures and that certain plant cultures are responsive to diverse elicitors. Treatment of a particular plant cell culture with different elicitors will result in the accumulation of a particular class of compounds, since these are specific of each plant culture. Although the class of metabolite depends on the plant species, the kinetics of induction or accumulation levels can vary with different elicitors.

As has been described previously, *V. vinifera* cell cultures have been used to the production of *trans*-R [22, 25]. The main advantage of these cultures is that genetic modification of cells is not necessary because grapevine cells produce *trans*-R constitutively, or in response to stress, so grapevine cell cultures can be directly exploited. Therefore, the use of biotic or abiotic elicitors could be considered one of the most effective strategies to increase the productivity of this compound.

4.1.1 The Use of Biotic Elicitors Enhances *trans*-Resveratrol and Stilbene-Related Production

When grapevine is under biotic stress, *trans*-R and derivatives are synthesized acting as phytoalexins (namely, viniferins in *Vitis*). Gray mold (*B. cinerea*), downy mildew (*P. viticola*), powdery mildew (*E. necator*), berry rot (*Rhizopus stolonifer*), and some *Aspergilli* sp. are some of the main fungi that attack grapevine and lead to *trans*-R and stilbene-related production in both grape berries and leaves [37]. Therefore, extracts from these fungi can be used as elicitors. In fact, elicitors resulting from the activity of fungal-degrading enzymes on host cell walls may induce *trans*-R and stilbene-related production by cells, as in the case of grapevine cells treated with cell fragments of *B. cinerea* [38] or with a cellulase extracted from the fungus *Trichoderma viride*, which also acts as a biofungicide to control other pathogenic fungi [39]. Commun et al. [40] studied *trans*-R and stilbene-related production in *Vitis* sp. protoplasts during their isolation and culture. Even using low concentrations of hydrolytic enzymes for cell wall removal, *trans*-R could be detected as early as the fourth hour of incubation, and it gradually accumulated during cell wall digestion. In addition, *trans*-R production during cell wall digestion was not specific to one *Vitis* genotype since this compound was detected in leaf protoplasts of different *Vitis* genotypes tested, and its production was due to the de novo activation of the *Vsts1* gene. Also, leaf protoplasts in culture, regardless of the genotype, continued to produce *trans*-R during the first week of the culture. For example, when *trans*-R produced from Chardonnay leaf protoplasts was quantified during the first 2 weeks of culture, maximal *trans*-R accumulation was observed between days 2 and 7 (over 15 μg *trans*-R 10^{-6} protoplasts, which were obtained from 0.03 g fresh leaves). These results suggest that cell wall enzymatic digestion with different fungal cellulases induces the expression of *Vsts1* gene, thus leading to the accumulation of *trans*-R.

Similarly, chitosan, a linear D-(1,4) glucosamine polymer produced by the deacetylation of chitin, which is an important structural component of the cell wall of several fungi, can also mimic a fungal pathogen attack, and thereby promote *trans*-R and stilbene-related production [41, 42]. In particular, the endogenous content of *trans*-R in *V. vinifera* cv Barbera cell cultures treated with 50 μg mL^{-1} chitosan was maximal 4 days after treatment (over 34.23 μg *trans*-R g^{-1} DW). However, chitosan decreased *trans*-R release into the culture medium by 62% (around 45.65 μg *trans*-R g^{-1} DW, at day 2, [43]). Proteomic analysis of chitosan-treated cells at day 4 demonstrated the specific effect of chitosan in increasing the amount of six different STS protein spots compared with to those observed in controls [43]. However, the response to one elicitor could be cultivar-dependent since the *trans*-R content in *V. vinifera* cv Italia cells elicited with 50 μg mL^{-1} chitosan was 141 μg *trans*-R g^{-1} DW, a very similar value to that found in control cells (98 μg *trans*-R g^{-1} DW, [42]). Also, this treatment led to a release of a very low amount of *trans*-R (0.12 mg L^{-1}) to the culture medium.

Grapevine cells also respond to the presence of polysaccharides. For example, when laminarin, which is a β-1,3 glucan derived from the brown algae *Laminaria digitata*, was used as elicitor in *V. vinifera* cv Gamay cells, 65 μg *trans*-R g^{-1} FW was accumulated in the culture medium after 8 h of treatment [44]. ε-viniferin was present predominantly inside the cells, where it peaked (130 μg g^{-1} FW) after 20 h, although a significant amount of ε-viniferin (62 μg g^{-1} FW) was also detected after 12 h in the extracellular medium [44].

Oligogalacturonides have also been used to produce *trans*-R in grapevine cells cv Gamay [45]. In fact, 2 mg oligogalacturonides g^{-1} FW induced an extracellular accumulation of 20 μg *trans*-R g^{-1} FW after 12 h of treatment, and similar *trans*-R levels were found when these Gamay cells were elicited with a purified glycoprotein from *B. cinerea* (namely, endopolygalacturonase 1). In cells treated with this glycoprotein, ε-viniferin was detected inside the cells reaching its highest levels between 24 and 60 h after treatment in comparison with the low levels of ε-viniferin detected in oligogalacturonide-treated cells [45].

Oligosaccharides appear to be efficient in the production of *trans*-R. In fact, the most significant success in increasing *trans*-R content from *Vitis* cell cultures has been reached using cyclodextrins (CDs). CDs are a family of compounds made up of sugar molecules bound together in a ring (cyclic oligosaccharides). CDs are produced from starch by enzymatic conversion. They are used in the food, pharmaceutical, and chemical industries, as well as in agriculture and environmental engineering, since they are able to solubilize nonpolar compounds in aqueous media. A typical CD contains several glucose monomers ranging from six to eight units in a ring, forming a cone shape. β-CDs, which are seven-sugar ring molecules, have often been used for increasing *trans*-R in *Vitis* cell cultures [4, 5, 46]. In fact, under CD elicitation, grapevine cell cultures produced high levels of *trans*-R, which is secreted and accumulated outside the cells. This effect is due to the fact that CDs chemically resemble the alkyl-derived pectic oligosaccharides released from the cell walls during fungal attack [5] and grapevine cells respond to their presence by synthesizing *trans*-R. Thus, when cell cultures of *V. vinifera* cv Gamay were treated with different types of β-CDs [5], these treatments resulted in high levels of *trans*-R (more than 3,000 mg L^{-1}), which were secreted to the culture medium, facilitating the extraction of this compound. Similarly, *V. vinifera* cv Monastrell cell cultures accumulated high amounts of *trans*-R (up to 4,000 mg L^{-1}) after treatment with different types of CDs [5]. In addition, the high levels of *trans*-R accumulated in the culture medium have no toxic effect on the cell lines, allowing successful subcultures and subsequent re-elicitations using the same cell biomass [47]. Therefore, CDs act not only as inducers of *trans*-R biosynthesis but also as promoters of complexes that remove *trans*-R from the medium, reducing both feedback inhibition and *trans*-R degradation, and allowing its accumulation in high concentrations [48].

4.1.2 The Addition of Signaling Molecules Induces *trans*-Resveratrol and Stilbene-Related Production

When plants are attacked by pathogens, they defend themselves against such invasion with an arsenal of defense mechanisms. Studies on early signal transduction pathways with elicitors revealed the mobilization or generation of diverse signaling molecules which regulate many processes, including the production of phytoalexins and pathogenesis-related proteins through transcriptional and metabolic changes [49]. In this sense, a sequential event in elicitor-induced defense responses involves the production of ethylene (ET), salicylic acid (SA), and jasmonate. These compounds coordinate the activation of a large set of defense responses, and when applied exogenously, they are able to induce resistance in both plants and cell cultures.

Studies with different plant–pathogen systems have shown that plants can activate different defense pathways which depend on the type of infection [50]. The ET- and jasmonic acid (Ja)-dependent defense responses seem to be activated by necrotrophic pathogens [51], whereas the SA-dependent response is triggered by biotrophic pathogens [52]. Some studies indicate that ET or Ja and SA responses inhibit each other, suggesting that cross talk exists between the pathways, enabling the plant to adapt the response depending on the type of pathogen [53].

These signaling molecules can be used in *Vitis* cell cultures to enhance *trans*-R production, the most commonly used being a Ja-active derivative, MeJa [30, 31, 54, 55], and SA [31, 43].

As described above, Ja and MeJa act as key compounds of the signal transduction pathway involved in the induction of the secondary metabolite biosynthesis which takes part in plant defense reactions [56, 57]. Thus, the production of secondary metabolites increases when plant cell cultures are elicited with jasmonates [36, 49, 58]. In *Vitis*, *trans*-R and stilbene-related production is increased by adding Ja or MeJa [30, 54]. In this respect, Tassoni et al. [54] showed that the addition of 10 μM MeJa was more effective in stimulating endogenous *trans*-R accumulation (24 μg g^{-1} DW) than 10 μM Ja (around 4.5 μg g^{-1} DW), although both elicitors promoted its release into the extracellular medium in *V. vinifera* cv Barbera cell cultures (around 8 μg *trans*-R g^{-1} DW after 2 days of elicitation). In the same way, Santamaria et al. [42] also investigated the ability of Ja (20 μM) and MeJa (20 μM) to increase the production of *trans*-R in *V. vinifera* cv Italia cell cultures. They detected a peak of *trans*-R inside cells (55 and 84 μg g^{-1} DW using Ja and MeJa, respectively) after 20 days of culture. In this case, Ja was most effective in inducing the release of *trans*-R into the medium (1.55 mg L^{-1}) than MeJa (0.27 mg L^{-1}). Belhadj et al. [30] working with *V. vinifera* cv Gamay Fréaux cell cultures elicited with 20 μM MeJa detected an extracellular accumulation of 11.87 μg *trans*-R g^{-1} FW after 18 h of treatment. Belchi-Navarro et al. [59] observed that the addition of 25 μM MeJa provoked an enhancement of *trans*-R in the culture medium of 1.02 mg g^{-1} DW (i.e., 51 μg g^{-1} FW) in *V. vinifera* cv Monastrell cell cultures. Moreover, these authors showed that in the presence of 50 or 100 μM MeJa, the *trans*-R levels increased to reach values of 3.74 mg g^{-1} DW (i.e., 187 μg g^{-1} FW).

Krisa et al. [60] showed that the amount of total stilbenes secreted to the culture medium when 25 μM MeJa was added to three *V. vinifera* cultivars (one of Gamay Fréaux and two strains of Cabernet-Sauvignon, CS4 and CS6) was negligible, although the accumulation of piceids inside cells of Cabernet-Sauvignon, strain CS4 was notably induced (6.30 mg piceids g^{-1} DW). In contrast to these results, when *V. vinifera* cell cultures cv Gamay Fréaux were elicited with 10 μM Ja, the total production of *trans*-piceid decreased compared with the control cells, and *trans*-R was not detected [34].

These results suggest that the production of *trans*-R is dependent on both MeJa dosage and grapevine cell line elicited, so that the use of both an optimal MeJa dose and highly productive cell lines is strongly advisable.

On the other hand, SA has long been known to play a central role in plant defense reactions. SA levels increase in plant tissue following pathogen infection, and exogenous application of SA enhances the resistance to a broad range of pathogens [61]. In fact, SA is involved in some signal transduction pathways in which enzymes catalyzing biosynthetic reactions are induced to form defense compounds of a phenolic nature [62]. In fact, the addition of 50 μM SA to *V. amurensis* callus cultures increased both *pal* and *sts* gene expressions while *trans*-R levels reached were 0.45 mg g^{-1} DW [63]. However, the addition of 100 μM SA or 1 mM ET to *V. vinifera* cv Monastrell cell cultures did not increase *trans*-R production [64]. Similarly, Yue et al. [34] observed that the content of *trans*-R was negligible when *V. vinifera* cv Gamay Fréaux cell cultures were treated with 500 μM SA for 10 days. Therefore, the levels of *trans*-R in *Vitis* cell cultures after treatment with MeJa or SA [31, 54, 59, 60] did not exceed 3.74 mg g^{-1} DW.

4.1.3 The Use of Abiotic Elicitors Enhances *trans*-Resveratrol and Stilbene-Related Production

It has also been shown that UV light acts as an abiotic factor which stimulates the biosynthesis of secondary metabolites [65]. The effect of UV light irradiation on the stilbene content of grapevine cell cultures is little known, and most of the research related with UV light has been directed at enhancing the stilbene content of grape berries [66, 67], leaves [68], and callus tissue [69, 70]. For example, Keller et al. [71] found that only actively growing calli of grapevine cv Cabernet-Sauvignon irradiated with UV-C light were capable of producing stilbenes, whereas old calli had lost this ability. Similar results were described by Keskin and Kunter [69] working with Cabernet-Sauvignon callus cultures irradiated with UV-C light. They found that the effect of UV-C light on *trans*-R production was dependent on callus age since the highest *trans*-R production was found in 12-day-old calli (62.66 μg g^{-1} FW), which was greater than those values obtained in 15-day-old calli (18.12 μg g^{-1} FW) for the same irradiation time (10 min). These authors also studied the effects of UV-C light on *trans*-R production in callus cultures of *V. vinifera* cv Öküzgözü. In this cultivar, the highest *trans*-R accumulation (62.23 μg g^{-1} FW) was determined after 48 h of treatment in callus irradiated for 15 min [70]. Similarly, the effect of UV-C irradiation for 10, 20, or 30 min on *trans*-R production in grapevine callus was studied by Liu et al. [72]. They observed an increase of *trans*-R accumulation to 19.60, 22.50, and 23.30 μg g^{-1} FW after 10, 20, and 30 min of treatment, respectively. In addition, they observed that the accumulation of *trans*-R peaked at about 32.50 μg g^{-1} FW at 48 h after 20 min UV-C irradiation. These authors also studied the effect of genotypic variation and the origin of tissue type on *trans*-R accumulation in callus at 48 h after 20 min UV-C irradiation [72]. The results showed significant differences in the levels of *trans*-R among the different grape genotypes and tissue types. The highest levels of *trans*-R were found in calli from "Zhi 168" and "Beta" (38.60 and 32.50 μg g^{-1} FW, respectively), whereas "Jingxu" and "Merlot" were the genotypes that accumulated the lowest levels of *trans*-R (16.0 and 12.0 μg g^{-1} FW, respectively). Likewise, they observed maximal values of *trans*-R in callus obtained from leaves 32.50 μg g^{-1} FW and exocarp 29.70 μg g^{-1} FW, whereas callus obtained from seed produced the lowest levels of *trans*-R (11.70 μg g^{-1} FW).

Recently, Almagro et al. [48] studied the effect of short or long exposures to UV-C or A light on *trans*-R production in *V. vinifera* cv Monastrell cell cultures, finding that the irradiation with UV light produced a negligible extracellular amount of *trans*-R.

On the other hand, phenolic compounds are recognized as being strongly affected targets of highly phytotoxic ozone [73]. Sgarbi et al. [74] analyzed two differentially ozone-sensitive cell cultures obtained from *V. vinifera* cv Lambrusco leaf explants, one rich in polyphenols and brown pigmented (Ph+) and other, phenol-poorer and pale (Ph-). An accumulation of *trans*-R (over 342.37 μg g^{-1} FW) was observed in Ph- callus 24 h after ozone treatment. In addition, they also observed a strong induction of STS activity in Ph- callus between 12 and 24 h after ozone treatment. These results suggest that the *trans*-R could be directly involved in the effective detoxifying response of Ph- grapevine callus to ozone, acting as an antioxidant compound.

On the other hand, Cai et al. [75] examined the effect of hydrostatic pressure on the extracellular production of stilbenes using *V. vinifera* cv Gamay Fréaux cell cultures. They observed that the pressure treatment of 40 MPa for 10 min provoked an increase in *cis*-resveratrol 3-*O*-β-glucoside (identified as *cis*-piceid by Waffo-Teguo et al. [27]) of 2.20 μg g^{-1} FW on the second day of the treatment.

4.1.4 The Combined Use of Elicitors Enhances *trans*-Resveratrol and Stilbene-Related Production

Biotic and abiotic elicitors are associated to different mechanisms of elicitation and, when used in combination, could enhance metabolite production in plant cell cultures. Indeed, the combined effect of elicitors is a widely used strategy to increase *trans*-R and stilbene-related production [48].

V. vinifera cv Gamay cell cultures responded to the combination of 20 μM MeJa and 0.08 M sucrose by increasing extracellular *trans*-R levels (27.39 μg g^{-1} FW, [30]) over those obtained with MeJa or sucrose alone (described above).

Similarly, Belchi-Navarro et al. [59] studied the effect of different sucrose concentrations (10, 20 or 30 g L^{-1}) in *V. vinifera* cv Monastrell cell cultures elicited with a fixed concentration of 50 mM CDs and different MeJa concentrations. Their results showed that when grapevine cell cultures were elicited in a culture medium with 10 g L^{-1} sucrose, maximal *trans*-R production (11.48 mg *trans*-R g^{-1} FW; i.e., 229.61 mg *trans*-R g^{-1} DW or 2,290 mg L^{-1}) was reached using the lowest MeJa dose (25 μM), whereas the highest degree of *trans*-R production was obtained using 30 g L^{-1} sucrose and 50 μM MeJa (13.80 mg *trans*-R g^{-1} FW; i.e., 276.18 mg *trans*-R g^{-1} DW or 2761.80 mg L^{-1}). In addition, the maximal levels of *trans*-R were observed using 20 g L^{-1} sucrose and 100 μM MeJa (over 304.7 mg g^{-1} DW; i.e., 3047.60 mg L^{-1}). In contrast, when the cell cultures were elicited only with CDs, the *trans*-R production values were lowest (over 73.7 mg g^{-1} DW; i.e., 737.0 mg L^{-1}), and no significant differences in *trans*-R production were seen at the different sucrose concentrations tested.

Yue et al. [34] found that the combination of Ja with adsorbents like HP2MGL (a non-aromatic resin, 200 g L^{-1}) added to *V. vinifera* cv Gamay Fréaux cell cultures provided a much higher *trans*-R yield (around 2,300 mg L^{-1}) than when used separately (0 and 1,800 mg L^{-1} using Ja and HP2MGL, respectively) so that the effect on *trans*-R production was synergistic. These authors also reported that the combination of SA and HP2MGL did not induce any increase in the levels of *trans*-R. Moreover, when SA, Ja, and HP2MGL were jointly added, the total production of *trans*-R was improved, reaching 2666.7 mg L^{-1}. Also, when these grapevine cells were fed with 1 mM L-Phe and elicited in the presence of 10 μM Ja and 500 μM SA, the production of *trans*-R increased gradually from day 5 (746.20 mg L^{-1}) to day 10 (1144.10 mg L^{-1}) of treatment.

Cai et al. [75] examined the joint effect of hydrostatic pressure and elicitors on the extracellular production of stilbenes using *V. vinifera* cv Gamay Fréaux cell cultures. They observed that the highest production of *cis*-piceid (0.56 mg L^{-1}) was observed when 28 mg L^{-1} ethephon and a hydrostatic pressure of 40 MPa were jointly used as elicitors, showing that hydrostatic pressure is an effective stimulant for the production of *cis*-piceid, and ethephon may enhance this production. However, *trans*-R was not detected in any of the conditions tested.

Saigne-Soulard et al. [32] analyzed the effect of the joint action of various inductive factors: 25 μM MeJa, 50 mg L^{-1} of hydrolyzed fungal cell wall isolated from the inner bark of *Taxus chinensis*, or 0.15 M (51.30 g L^{-1}) sucrose combined with 1 mM L-Phe on stilbene production in *V. vinifera* cv Gamay Fréaux cell cultures. They observed that the treatments of the cells with sucrose and L-Phe were not significantly different from the effect obtained with L-Phe alone (described above). However, the addition of fungal elicitor or MeJa in the presence of L-Phe induced an increase in the production of stilbenes.

The most significant success in increasing the *trans*-R content in *Vitis* cell cultures was reached using 50 mM CDs in combination with 100 μM MeJa [59, 76]. In fact, when grapevine cell cultures were simultaneously elicited with CDs and MeJa, the accumulation of *trans*-R increased (304.7 mg g^{-1} DW; i.e., 3047.60 mg L^{-1}) exponentially reaching a concentration plateau after 168 h. These same authors also used CDs separately or in combination with MeJa, plus SA and/or ET to elicit *V. vinifera* cv Monastrell cell cultures during 96 h [64]. The results showed that *trans*-R levels in cells elicited with CDs and ET (68 mg g^{-1} DW; i.e., 680 mg L^{-1}) were higher than those observed in the presence of CDs and SA (26 mg g^{-1} DW; i.e., 260 mg L^{-1}). Likewise, they observed that the presence of CDs, MeJa, and ET induced a greater increase in *trans*-R levels (154 mg g^{-1} DW, 1,540 mg L^{-1}) than when they used CDs, MeJa, and SA (104 mg g^{-1} DW, 1,040 mg L^{-1}). In addition, when cell cultures were elicited with CDs, MeJa, SA, and ET, the production of *trans*-R was 136 mg g^{-1} DW. These findings indicate that the presence of SA provokes a decrease in the production of *trans*-R [64].

Almagro et al. [48] carried out experiments with *V. vinifera* cv Monastrell cell cultures using 50 mM CDs and 100 μM MeJa, followed by short or long exposures to UV-C light. They provided lower *trans*-R levels (5 min and 30 min UV-exposed cells, 22.8 and 4 mg g^{-1} DW, respectively) than UV-unexposed cells (134.4 mg g^{-1} DW), so that UV-C light exposure was clearly detrimental to produce *trans*-R. In fact, prolonged exposure to UV-C light caused a drastic reduction in *trans*-R accumulation, although no cell browning was observed. However, when *V. vinifera* cv Monastrell cell cultures were jointly elicited with CDs and MeJa and exposed to UV-A light, the maximal level of *trans*-R was found after long exposures (30 min, 165.2 mg g^{-1} DW). The results suggested that the effect of UV light on *trans*-R production was dependent not only on exposition time (short or long) and UV light type (C or A) but also on the presence of other elicitors (CDs and MeJa).

4.2 Large-Scale *trans*-Resveratrol and Stilbene-Related Production

Taking the biotechnological production of a natural product by plant cell cultures from laboratory's scale in shaken flasks to a commercial scale relies on the cultivation of the cell cultures in bioreactors as an essential step of the process. Initially, the production is optimized in laboratory scale bioreactors, typically of 2-L capacity, and then the bioreactor geometry is kept while the capacity is increased steadily from some tens to several thousand times in a bioreactor cascade system. The world's largest plant cell culture facility, located in Ahrensburg, Germany, has a cascade of five stirred-tank bioreactors ranging from 75 to 75,000 L. The transfer from shake flasks to bioreactors raises several issues that in many cases are species- or even cell-line-dependent; thus, upscaling is not always straightforward and has to be resolved in a case-by-case manner. The major issues to tackle are linked to the bioreactor design, the operation mode, the culture environmental conditions in the bioreactor and biomass, and the target compound productivity in the bioreactor as compared to shaken flasks. In addition, when biomass and target compounds production are not coupled, a two-stage production system, for example, first biomass and then metabolite, has to be implemented in bioreactors.

The scale-up of natural compound bioproduction by grapevine cells in bioreactors has been attempted by different laboratories. In some cases, the major issue was the intracellular polyphenolics accumulation linked to biomass production [33, 77–79] in a one-stage culture system. Others addressed the advantageous extracellular accumulation of resveratrol mediated by the use of elicitors that either promotes the secretion of the metabolite [80] or the simultaneous overproduction and extracellular accumulation in a one-stage [55] or two-stage [81, 82] culture system. A further refinement of the bioproduction process involves the reuse of the producing cells for several cycles in a fed-batch production system [82].

In spite of the initial perception of the difficulty in culturing plant cell in suspension due to their high shear sensitivity, a wide variety of bioreactor designs have been successfully used for cultivating plant cell cultures. These designs include classical configurations such as stirred tank, airlift, and bubble column bioreactors but also new types of bioreactor specially designed for highly

Table 55.1 Grapevine cell biomass production in bioreactors

Bioreactor design	Working volume (L)	Grapevine cv/sucrose (g L^{-1})	Aeration (vvm)/ agitation (rpm)	Maximal biomass (g FW L^{-1})	Biomass productivity (g FW L^{-1} day^{-1})	References
Stirred tank	15	Gamay Fréaux/60	0.2/100	269	10	[77]
	2	Gamay Fréaux/50	0.075–0.15/ 75	518	32	[33]
	0.8	Barbera/30	0.2/100	40	2.8	[80]
	2	Rootstock 41B/30	0.025/50	546	18	[55]
	1.1	Gamay/30	2/80	521	23	[81]
Wave	1	No data		884	40	[79]
Bubble column V-shaped	1.1	Gamay/30	0.95/–	246	10	[81]
Bubble column cylindrical	5.8	Gamay/20	0.47–0.54/–	183	10.5	[82]

shear-sensitive plant cultures such as the centrifugal impeller bioreactor [83] and disposable concept bioreactors made of plastic bags [84] which can be gently agitated by a rocking platform that creates a wave motion of the culture [85]. For a comprehensive review on bioreactor designs suitable for plant cell and tissue cultures, see [78]. Most of the efforts carried out to grow up grapevine cell suspensions in bioreactors have used stirred-tank commercial designs [33, 55, 77, 80, 81] but also designs of low shear stress such as wave motion [78], and bubble column bioreactors [81, 82] have proved to be suitable for this purpose. Table 55.1 summarizes the grapevine biomass concentration and productivity achieved in the different works carried out in bioreactors. Productivity in terms of g FW L^{-1} day^{-1} or g DW L^{-1} day^{-1} is very much dependent on the initial concentrations of carbon source and biomass inoculums, and these vary among the different studies. Likewise, the operation mode in batch or fed batch greatly influences the final biomass yield. Unfortunately, growth in terms of growth rate constant (μ day^{-1}) in the exponential phase is not always provided. A comparison between two bioreactor designs, stirred tank and bubble column V-shaped, using the same cell line shows that μ may not vary significantly with the sucrose concentration, only slightly among the bioreactors, while the maximum biomass production and the productivity greatly depended on the initial sucrose concentration [81]. The highest reported productivity of 40 g FW L^{-1} day^{-1} has been achieved in a 1-L wave bioreactor [79], but also in a stirred-tank bioreactor biomass, productivities as high as 32 g FW L^{-1} day^{-1} have been reported [33]. In general, the different trials to grow up grapevine cells in bioreactors have been successful although there are differences that are in part due to the differential shear sensitivity of the cell lines. *V. vinifera* cv Gamay Fréaux cell cultures appear to be quite tolerant to shear stress [77] displaying a good biomass

Table 55.2 Stilbene and resveratrol (both *cis*- and *trans*-isomers) production by grapevine cell cultures grown in bioreactors

Bioreactor design	Process mode	Working volume (L)	Grapevine cv/sucrose (g L^{-1})	Elicitor	Stilbene and resveratrol yield (mg g^{-1} FW) and location	References
Stirred tank	Batch, one-stage	15	Gamay Fréaux/60	None	0.11 Intracellular (only piceid)	[77]
	Batch, one-stage	2	Gamay Fréaux/50	None	0.54–0.70 Intracellular (only piceid)	[33]
	Batch, two-stage	0.8	Barbera/30	Chitosan	1.2 Total (stilbene monomers)	[80]
	Batch, one-stage	2	Rootstock 41B/30	MeJa	1.0 Extracellular + 0.1 intracellular (in both cases, resveratrol) Bonus ε-viniferin extracellular	[55]
	Batch, one-stage	1.2	Gamay/20	CDs	3.1 Extracellular resveratrol	[81]
				CDs + MeJa	13.5 Extracellular resveratrol	[81]
Bubble column V-shaped	Batch, one-stage	1.2	Gamay/20	CDs	2.2 Extracellular resveratrol	[81]
				CDs + MeJa	13.5 Extracellular resveratrol	[81]
Bubble column cylindrical	Fed-batch three cycles, one-stage	5.8 × 3	Gamay/20	CDs + MeJa	11 × 3 Extracellular resveratrol	[82]
	Fed-batch three cycles, two-stage	5.8 × 3	Gamay/20	CDs + MeJa	11.2 × 3 Extracellular resveratrol	[82]

yield in different bioreactors and under high regimes of aeration even at moderate agitation speeds. However, rootstock 41B (*V. vinifera* cv Chasselas x *V. berlandieri*) cell cultures tolerated only very low aeration flows and agitation speeds [55], and *V. vinifera* cv Barbera cell cultures underwent a decrease of cell biomass ranging from 20% to 60% during the first 4 days of cultivation [80] probably due to cell lysis. Table 55.2 summarizes the results achieved in relation to stilbene and *trans*-R production in bioreactors using grapevine cell cultures. In one application, grapevine cells were used to produce ^{13}C bio-labeled polyphenols, including *trans*-R, by feeding the culture with [1-^{13}C] L-Phe, but *trans*-R was accumulated intracellularly only as the glycosylated derivative, piceid [33], as occurred in a previous work where labeled precursors were not added [77]. The rest of studies utilized elicitors, in particular chitosan, MeJa, and CDs, with the aim of enhancing *trans*-R production and promoting its extracellular accumulation. *trans*-R production is often reported as g L^{-1}, but the biomass concentration has a strong influence on overall production; thus, a more appropriate variable to compare across different studies is the specific production expressed in mg g^{-1} and referred to either FW or DW. Elicitation with chitosan or MeJa led to a similar yield slightly above 1 mg g^{-1} FW. However, while the addition of chitosan in *V. vinifera* cv Barbera cell cultures led to a mixture (*cis*-, *trans*- and free and glycosylated) of intra- and extracellular resveratrol monomers, the addition of MeJa in the rootstock 41B cell cultures led mainly to extracellular *trans*-R, and also the dimer ε-viniferin. In addition, the significantly higher biomass yield of the rootstock 41B cell cultures as compared to the *V. vinifera* cv Barbera cell cultures involves a much higher overall *trans*-R production.

In shaken flasks, *V. vinifera* cv Monastrell cell cultures elicited with CDs has proven to be the most efficient way to promote the accumulation of large amounts of *trans*-R in the extracellular medium [5], and the combination with MeJa leads to a synergistic effect that multiplies the accumulation for at least threefold [24]. Thus, the *trans*-R yield becomes higher than 3 mg g^{-1} FW for elicitation with CDs alone, and higher than 10 mg g^{-1} FW when combined with MeJa. As seen in Table 55.2, such yields are reached in all the bioreactors tested either in one-stage batch or in two-stage fed-batch modes. Thus, the scale-up of CDs and MeJa-mediated *trans*-R production in bioreactors is demonstrated as feasible.

5 Biotechnological Approaches to Improve *trans*-Resveratrol and Stilbene-Related Production Using Genetic Engineering in Different Biological Systems

5.1 Increasing *trans*-Resveratrol Content by Genetic Manipulations in Plants and Grapevine Cell Cultures

Metabolic engineering offers new perspectives for improving the production of compounds of interest. Metabolic engineering is generally defined as the redirection of one or more enzymatic reactions to produce new compounds in an organism, to improve the production of existing compounds, or to mediate the degradation of compounds. Although progress in pathway gene discovery and the ability to manipulate gene expression in transgenic plants has been impressive during recent decades, attempts to use these tools to engineer plant metabolism have met with limited success. Though there are notable exceptions, most attempts at metabolic engineering have focused on modifying (positively or negatively) the expression of single genes affecting pathways. This approach can be used for improved metabolite production in a plant or plant cell cultures, or even in other plant species or organisms.

Engineering of *trans*-R in plants and microbial organisms is such an approach, and one that has become feasible in the last two decades. In fact, many authors have carried out the overexpression of *sts* genes as a valuable strategy to increase *trans*-R production in plants ([22] and references therein). Unfortunately, there is abundant information about the *trans*-R content of transgenic plants; the same is not true for transformed cell cultures. However, the comparison of *trans*-R levels in different transgenic plant overexpressing *sts* genes is interesting. For example, the transformation of various plants including *Vitis* with the *Vsts* gene resulted in increased *trans*-R levels. In fact, the highest *trans*-R levels (around 350 μg g^{-1} DW) were obtained after transforming the genome of 41B rootstock with a chimeric gene which combines an alfalfa PR10 promoter, which is a DNA fragment of a pathogenesis-related class 10 proteins, and *Vsts1* [86]. In the same way, the transformation of *Nicotiana tabacum* with *Vsts1* and *Vsts2* under control of the Vsts1 promoter provoked an increase of *trans*-R levels (400 μg g^{-1} FW) [87]. In both cases, these transgenic plants showed increased resistance to *B. cinerea* [86, 87]. However, the transformation of other plants did not result in high *trans*-R levels. Thus, the transformation of *Solanum lycopersicum* with *Vsts* gene under control of CaMV 35 S promoter triggered only a small amount of *trans*-R (4 μg g^{-1} FW) [88]. In addition, using the same promoter but incorporating the *Vsts* gene in *Lactuca sativa*, the values of *trans*-R were 56.40 μg g^{-1} FW [89]. Therefore, it is possible that the enzymes encoded by these *Vsts* genes do not actively participate in *trans*-R biosynthesis or have a low specific activity [90].

On the other hand, it has been reported that *rol* genes located in R_i-plasmids of *Agrobacterium rhizogenes* may enhance the biosynthesis of certain groups of secondary metabolites in transformed plant in vitro cultures [91]. In fact, the introduction of *rolB* gene, which is essential for plant cell growth and development, in *N. tabacum* root cultures and *Rubia cordifolia* cell cultures has been shown to strongly induce the production of alkaloids and anthraquinones, respectively [92, 93]. Recently, Kiselev et al. [31] have shown that the *rolB* gene also stimulates *trans*-R biosynthesis in *V. amurensis* callus cultures. These authors established two *rolB* transgenic callus lines of *V. amurensis* with different levels of *rolB* expression [31] and found that the higher expression of *rolB*, the greater the production of *trans*-R (31.50 mg g DW^{-1}). They also observed that *trans*-R production in the transformed callus cultures was dependent on the presence of both 6-benzylaminopurine and 1-naphthaleneacetic acid. However, an increase in the concentration of indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid had a negative effect on *trans*-R biosynthesis. These same authors also demonstrated that *trans*-R production is enhanced by increasing gene expression levels of *pal* and *sts* in *V. amurensis* callus cultures transformed with the *rolB* gene.

Kiselev et al. [63] also showed that the addition of 50 μM SA enhanced both gene expression of *pal* and *sts* and the production of *trans*-R (0.4 mg g^{-1} DW) in *rolB* transgenic callus lines of *V. amurensis*. In same way, Dubrovina et al. [94] observed an increase in the production of *trans*-R (1.40 mg g^{-1} DW) as well as specific *pal* and *sts* gene expression in *rolC* transgenic callus lines of *V. amurensis*. Their results suggested that transformation of *V. amurensis* calli with the *rol*C gene induced *trans*-R production via selective enhancement of expression of certain *pal* and *sts* genes.

5.2 Incorporating Genes from the *trans*-Resveratrol Biosynthetic Pathway in Microbial Organisms

Microorganisms are widely used biological systems for the production of numerous valuable molecules. Engineering bacteria or yeasts for *trans*-R production might thus represent a valuable means for its large-scale production. The *trans*-R pathway needs to be tailored because they do not possess the genes encoding enzymes responsible of this pathway. One strategy consists in introducing specific genes from the phenylpropanoid and stilbene pathways, such as *pal*, *c4h, 4cl*, and *sts* genes, using p-coumaric acid as a starting block [25]. In this sense, Trantas et al. [95] were capable of transforming yeast (*Saccharomyces cerevisiae*) with *pal*, *c4h, 4cl*, and *sts* genes isolated from *Populus trichocarpa x P. deltoides, Glycine max, V. vinifera*, and *P. trichocarpa x P. deltoides*, respectively. This strain secreted *trans*-R into the culture medium when it was fed with 164 mg L^{-1} p-coumaric acid, reaching values of 0.31 mg L^{-1}. Similarly, Becker et al. [96] reported that the co-expression of *4cl* and *sts* genes (obtained from *P. trichocarpa x P. deltoides* and from *V. vinifera*, respectively) in yeast cells (*S. cerevisiae*) produced 1.50 mg L^{-1} of *trans*-R in the presence of 10 mg L^{-1} p-coumaric acid. In the same way, Zhang et al. [97] demonstrated that when *4cl* and *sts*, cloned from *A. thaliana* and *V. vinifera*, respectively, were incorporated to *S. cerevisiae* genome, the strain exhibited a slight increase of *trans*-R production (5.25 mg L^{-1}) in presence of 12 mg L^{-1} p-coumaric acid. These results suggest high p-coumaric acid concentrations did not result in an improvement of *trans*-R production. Moreover, it was reported that p-coumaric acid inhibited the growth of yeast cells at high concentrations (above 20 mg L^{-1}, [98]).

Wang et al. [98] working with yeasts (*S. cerevisiae*) were capable of overexpressing a resynthesized tyrosine ammonia-lyase gene (namely, *tal-made*), which is able to catalyze the formation of 4-coumaric acid from tyrosine directly, improving the availability of p-coumaric acid in the culture medium. Thus, yeast cultures that co-expressed *4cl* and *sts* together with *tal-made* were able to produce 1.06 mg *trans*-R L^{-1}. Moreover, the production of *trans*-R increased (1.90 mg L^{-1}) when yeast cells fed with tyrosine. Likewise, Wang et al. [98] also demonstrated that the low-affinity, high-capacity bacterial *araE* transporter enhanced the accumulation of *trans*-R when yeast cells were transformed with *araE* and *sts*, reaching the *trans*-R content of 3.1 mg L^{-1}. This result suggests that the arabinose-H^+ transport protein participates in transporting *trans*-R even though this transporter shows no affinity toward *trans*-R.

On the other hand, the production of *trans*-R in bacterial cells can be higher than when yeast cells are used. Beekwilder et al. [99] compared the production of *trans*-R in *S. cerevisiae* cells with those obtained from *Escherichia coli* cells. In both systems, *4cl* from *N. tabacum* and *sts* from *V. vinifera* were expressed. These authors observed that the addition of *p*-coumaric acid increased the production of *trans*-R, reaching levels of 16 mg L^{-1} in *E. coli* and 6 mg L^{-1} in yeast cells. Similarly, Watts et al. [100] used *E. coli* transformed with *4cl* from *A. thaliana* and *sts* cloned from *A. hypogea* to analyze the production of *trans*-R. The expression of these enzymes in *E. coli* fed with 4-coumaric acid provoked an increase of *trans*-R levels (over 100 mg L^{-1}). Moreover, the introduction of *4cl* gene from *Lithospermum erythrorhizon* and *sts* gene from *A. hypogaea* in *E. coli* and the subsequent feeding of this recombinant strain with p-coumaric acid produced very high levels of *trans*-R (171 mg L^{-1}) [101].

6 Conclusions

The use of grapevine cell cultures to increase *trans*-R and stilbene-related production under controlled conditions is viewed as a promising biotechnological alternative to their extraction from the whole plant or their chemical synthesis. At present, only few plant in vitro cultures are undertaken commercially for the production of bioactive compounds. The major problems hindering the development of the large-scale culture of plant cells include low productivity, cell line instability, and difficulties involved in scaling up. Medium composition, culture conditions, elicitors, bioreactor design, and other critical parameters influence the behavior of grapevine cell cultures. However, the optimization of medium composition and culture conditions does not improve substantially the production of these compounds.

Grapevine cell cultures enhance the levels of *trans*-R and stilbene-related production in response to biotic and abiotic elicitors so that elicitation of cells using different elicitors in combination might represent a powerful strategy for increasing *trans*-R and stilbene-related production. Moreover, when an adsorbent is added to elicited grapevine cell cultures, as *trans*-R is produced, this is removed from the culture medium, reducing both feedback inhibition and *trans*-R degradation, and allowing its accumulation in high concentrations. Thus, the combined addition of Ja and HP2MGL to *V. vinifera* cv Gamay Fréaux cell cultures provided a high *trans*-R yield (around 2,300 mg L^{-1}), which increased even more when SA, Ja, and HP2MGL were jointly added (2666.7 mg L^{-1}). However, other authors observed that the presence of CDs, MeJa, and ET induced a greater increase in *trans*-R levels (1,540 mg L^{-1}) than when they used CDs, MeJa, and SA (1,040 mg L^{-1}) to elicit *V. vinifera* cv Monastrell cell cultures for 96 h. These findings indicate that different high-producing *V. vinifera* cell lines respond differentially to the presence of signaling molecules.

One of the most significant successes in increasing the *trans*-R content in *Vitis* cell cultures was reached using CDs in combination with MeJa. In these conditions and using shaken flasks, the accumulation of *trans*-R increased exponentially reaching a concentration plateau (3047.60 mg L^{-1}) after 168 h. Indeed, the *trans*-R yield was 15.24 mg g^{-1} FW, and such yields can also be reached in all the bioreactors tested either in one-stage batch or in two-stage fed-batch modes. Thus, the scale-up of CDs- and MeJa-mediated *trans*-R production in bioreactors is clearly feasible.

Many efforts have been made to synthesize high levels of *trans*-R using metabolic engineering in plants and microbes. There is abundant information about the *trans*-R content of transgenic plants, although *trans*-R levels were seen to be not very high (around 350 μg g^{-1} DW). Only experiments with *rolB* transgenic callus lines of *V. amurensis* showed an enhanced production of *trans*-R (31.50 mg g DW^{-1}).

Efforts to increase the production of *trans*-R for commercial applications have focused on heterologous expression of the phenylpropanoid and stilbene pathways in *S. cerevisiae* and *E. coli*, although as the stilbene pathway does not exist in yeast or bacteria, the entire functional pathway needs to be introduced. Thus, when in both systems, *4cl* from *N. tabacum* and *sts* from *V. vinifera* were expressed, 16 and 6 mg L^{-1} *trans*-R were produced in *E. coli* and yeast cells, respectively. However, the highest levels of *trans*-R (171 mg L^{-1}) were reached using a *4cl* gene from *L. erythrorhizon* and a *sts* gene from *A. hypogaea* in recombinant *E. coli*.

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Functional Foods: Genetics, Metabolome, and Engineering Phytonutrient Levels

56

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Contents

1 Introduction 1717
2 Flavonoids/Phenolics 1719
2.1 Diet Source/Distribution/Occurrence 1719
3 Carotenoids 1729
3.1 Diet Source/Distribution/Occurrence 1729
4 Essential Fatty Acids 1736
4.1 Diet Source/Distribution/Occurrence 1736
5 Conclusions 1738
References 1740

Abstract

Phytonutrients in fruits and vegetables or their individual components (nutraceuticals) positively contribute to human health. Mostly, these nutrients

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have antioxidative property that impacts redox imbalance and can lead to prevention of cancer, cardiovascular diseases, diabetes, osteoporosis, and age-related disorders such as dementia. Over 7,000 flavonoids (and phenolic compounds) and 600 naturally occurring carotenoids seemingly with health benefits have been documented in plants. Fruits and vegetables are dietary sources of pro-health nutrients (nutraceuticals); however, the level of an individual antioxidant is low in the currently used germplasm, thus limiting them in meeting the recommended daily allowance (RDA). Nonetheless, the awareness about their health benefits has increased the global demand for and consumption of fruits and vegetables. Advanced molecular breeding and genetic engineering approaches are providing novel tools to greatly increase the levels of many desirable nutraceuticals, which is being made easier because their metabolic pathways are now known. The biotechnological interventions have already allowed severalfold increases in the content of flavonoids and carotenoids in fruit crops and essential fatty acids in oil crops. This chapter gives an overview of three classes of phytonutrients – flavonoids, carotenoids, and essential fatty acids, their dietary sources, metabolic pathways, and important genes/enzymes involved in their production. Several examples of the biotechnological intervention to boost endogenous levels of phytonutrients in various crop plants are highlighted.

Keywords

Biotechnology • genetic engineering • functional food • GM crops • nutrition • transgenic plants

Abbreviations

ABA	Abscisic acid
ACP	Acyl carrier protein
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid (C22:6n-3)
DW	Dry weight
EPA	Eicosapentaenoic acid (C20:5n-3)
FW	Fresh weight
GLA	ω-6 γ-linolenic acid
LDL	Low-density lipoprotein
PA	Proanthocyanidin
PUFA	Polyunsaturated fatty acids
RDA	Recommended daily allowance
SDA	Stearidonic acid (C18:4n-3)
UV-B	Ultraviolet-B radiation

1 Introduction

Functional foods is a designated term for foods that can provide health benefits in addition to basic nutrition. Functional foods include edible produce in which the concentration of one or more bioactive compounds (phytonutrients) – vitamins, antioxidants, micronutrients – has been increased through breeding, environmental manipulations or precision-based genetic engineering, and processed foods fortified with health-promoting additives. Fruits and vegetables are main sources of dietary phytonutrients especially antioxidants. Functional components, sources, and benefits of various phytonutrients are listed in Table 56.1. Numerous epidemiological studies and clinical trials have suggested that diets rich in fruits and vegetables can reduce the risk of chronic disease [1]. Thus, regular intake of phytonutrients could potentially prevent cancer, cardiovascular diseases, diabetes, osteoporosis, and age-related disorders such as dementia [2–4]. Likewise, carotenoids including lycopene, flavonoids, vitamins C and E, and isothiocyanates/glucosinolates have the potential to prevent chronic diseases, including epithelial cancers, cardiovascular diseases, digestive disorders, and immune deficiency [2, 3, 5]. In this regard, carotenoids have attracted significant attention, particularly because β-carotene as a provitamin A is important for retinal health: preserving eyesight, preventing night blindness, and protection against cataract [6] as also lutein in preventing age-related macular degeneration of eye [7]. Carotenoids have also been implicated in reducing low-density lipoprotein (LDL) improving immune system and preventing neurodegenerative diseases such as Alzheimer’s, Parkinson’s, and vascular dementia [4]. Flavonoids also have antioxidant properties that reduce the risks of age-related diseases, heart ailments, and cancers [8]. The potential of phytonutrients in protecting against or delaying the incidence of fatal diseases is believed largely to occur via their antioxidative and free radical-scavenging properties, by protecting cellular macromolecules from oxidative damage induced by prooxidants, especially during aging [9].

Fruits and vegetables contain thousands of biologically active phytochemicals some of which likely interact in a number of ways to prevent disease and promote health [9, 10]. The nature of such multiple interactions among nutrients that promote health and prevent disease is unclear [11, 12]. Evidence that very high doses of isolated individual micronutrients or phytochemicals, grouped as nutraceuticals, can be as effective as eating fruits and vegetables is inconsistent and relatively weak. The awareness of their health-promoting properties has increased the worldwide consumption of fruits and vegetables and globally increased their demand. Enhancement of nutritional quality of staple foods in developing and underdeveloped countries is of paramount importance in reducing several diseases and disorders due to nutrition-deficient foods. However, the available level of a specific phytonutrient in the currently used germplasm is not sufficient to meet the recommended daily allowance within a reasonable daily intake of edible portions. Thus, a significant focus of scientists in recent years

Table 56.1 Functional components, sources, and health benefits of phytonutrients[a]

Functional component(s)	Source	Potential benefits
Phenolics		
Anthocyanidins	Fruits	Neutralize free radicals; reduce risk of cancer
Catechins	Tea	-do-
Flavanones	Citrus	-do-
Flavones	Fruits/vegetables	-do-
Lignins	Flax, rye, vegetables	Prevention of cancer, renal failure
Tannins (proanthocyanidins)	Cranberries, cranberry products, cocoa, chocolate	Improve urinary tract health. Reduce risk of cardiovascular disease
Carotenoids		
α-Carotene/β-carotene	Carrots, fruits, vegetables	Neutralize damaging free radicals
Lutein	Green vegetables	Reduces the risk of macular degeneration
Lycopene	Tomato products (ketchup, sauces)	Reduces the risk of prostate cancer
Fatty Acids		
Long-chain ω-3 fatty acids – DHA/EPA	Salmon and other fish oils	Reduce risk of cardiovascular disease; improve mental and visual functions
Conjugated linoleic acid (CLA)	Cheese, meat products	Improves body composition; decreases risk of certain cancers

[a] Adapted from http://lpi.oregonstate.edu/infocenter/phytochemicals/flavonoids/index.html#intro

has been to elevate cellular levels of known health-promoting nutrients in fruits and vegetables using novel approaches.

Genetic engineering has been increasingly applied to secure nutritional enhancement of the crops once the need of nutritional food security became apparent as a major health risk in the world [9]. Many desirable crop traits are multigenic in nature, the final outcome being a function of a group of genes. However, most metabolic pathways leading to the formation of important nutrients have a rate-limiting step(s), which calls for innovation in selecting the right kind of genes and using specific promoters to drive their expression. Three basic strategies have been used to accomplish the objective of enhancing a multigenic trait. These include the co-expression of multiple genes and engineering transcription factors that control a number of downstream genes. Simultaneous introduction of several genes helped develop high-β-carotene rice, now commercially known as Golden Rice [13]. The latter study also demonstrated that the source of gene(s) plays a significant role in increasing a preferred molecule/nutrient. Noticeably, the use of carotene desaturase from *Erwinia uredovora* resulted in a 23-fold increase in total carotenoids in rice [13]. Other phytonutrients whose content has been enhanced by genetic engineering include α-tocopherol [14–19], vitamin C corn [20–22], and folate [23–25].

Molecular breeding based on genetic analysis has provided another tool that has made “candidate” gene approach a reality to test phenotypic role of a particular gene by altering its expression during plant growth and development [26]. A major lesson learned from such studies is that a clear understanding of the complex gene expression, selection of the promoter, and regulation of the production of a desired metabolite are critical in enabling targeted expression of a transgene at a desired stage of development of a specific tissue. The gain of function by using ectopic overexpression or loss of function by antisense RNA repression or RNAi approach has made it possible to characterize the phenotypes associated with a single gene and its potential to regulate desirable phenotype in crop plants. This chapter focuses on and summarizes examples in the literature on the genetic engineering of fruits and vegetables, in particular the studies on enhancing the content of flavonoids, carotenoids, and essential fatty acids.

2 Flavonoids/Phenolics

2.1 Diet Source/Distribution/Occurrence

Flavonoids are aromatic, low-molecular-weight secondary metabolites [27]. Their hydrophilic properties [28] complement the hydrophobic nature of carotenoids. This group of antioxidants includes secondary metabolites constituting more than 7,000 chemical structures [29–31]. Flavonoids are classified, based on degree of unsaturation and oxidation of a three-carbon bridge in flavone skeleton between phenyl groups of flavonoids, and divided into subclasses (Table 56.2) such as flavanols (catechin, epicatechin, proanthocyanidins), flavanones (hesperetin, naringenin, eriodictyol), flavonols (quercetin, kaempferol, myricetin, isorhamnetin), flavones (apigenin, luteolin), and anthocyanidins (cyanidin, delphinidin, malvidin). Flavonoids are present at different levels in fruits, vegetables, legumes, tea, and red wine. Flavonoid content of some flavonoid-rich foods (Table 56.3) are, in fact, approximate values considering that a number of factors affect their levels, including agricultural practices used, environmental factors, ripening process, processing, storage, and cooking. Green and black tea, apple, and apricot are rich in flavanols; parsley, thyme, and green celery in flavones; the veggies – onions, kale, leek, broccoli, green chili pepper, and celery in flavonols; and fresh orange and grapefruit in flavanone. Fruit juices containing high levels of flavanones are a major source of flavonoids in human diet. The total fruit juice consumption may account for 20–30 % of dietary intake of flavonoids [27]. The berries (blackberry, blueberry) and red grapes are considered super sources of flavonoids including anthocyanins and proanthocyanidins (PAs) and have high order of antioxidative capacity (Table 56.3) [32–34]. A multitude of phenolic compounds (proanthocyanidins, anthocyanin, flavonol, and phenols) have been detected in the berries and leaves of sea buckthorn; their contents are variable depending upon the species and cultivar type, cultivation site, and the ecological and environmental conditions [35–37]. There is only minimal information on the temporal and spatial accumulation patterns of these compounds during fruit development.

Table 56.2 Subclasses of common dietary flavonoids[a]

Flavonoid subclass	Dietary flavonoids	Some common food sources
Flavanols	Monomers (catechins): Catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate Dimers and polymers: Theaflavins, thearubigins, proanthocyanidins	Catechins: teas (particularly green and white), chocolate, grapes, berries, apples. Theaflavins, thearubigins: teas (particularly black and oolong). Proanthocyanidins: chocolate, apples, berries, red grapes, red wine
Flavanones	Hesperetin, naringenin, eriodictyol	Citrus fruits and juices, e.g., oranges, grapefruits, lemons
Flavonols	Quercetin, kaempferol, myricetin, isorhamnetin	Widely distributed: yellow onions, scallions, kale, broccoli, apples, berries, teas
Flavones	Apigenin, luteolin	Parsley, thyme, celery, hot peppers
Isoflavones	Daidzein, genistein, glycitein	Soybeans, soy foods, legumes
Anthocyanidins	Cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	Red, blue, and purple berries; red and purple grapes; red wine

[a] Adapted from http://lpi.oregonstate.edu/infocenter/phytochemicals/flavonoids/index.html#intro

Polyphenolic compounds are derived via the shikimate-phenylpropanoids-flavonoids pathways. They have been shown to protect plants against UV-B light and microbes [38, 39]. The antimicrobial property is important for fruits to maintain their resistance against fungi during storage. Though these flavonoids play significant role in the interaction of plants to the environment functions, for example, as pigments, signaling molecules, UV-B protectants, phytoalexins, and auxin transport regulators [40–43], the specific relationship between structure and function of flavonoids is not fully understood. Flavonoids also contribute toward various fruit quality attributes including color (red, violet, blue), flavor, and texture. The undesirable brown pigmentation (bruises) on fruit surface has been attributed to oxidation of phenols to quinones, which then polymerize into brown pigments, for example, flavan-3-ols in apples [27, 44, 45]. The flavonols are perceived to protect fruit skin against UV-B light [46]. They also accumulate in the seed coat [47]. The proanthocyanidins (PAs) are condensed tannins, oligomers, and polymers of flavan-3-ols. They are mostly common in woody plants but are also present in herbaceous species, as factors that contribute to defense and stress resistance. In seeds, PAs are often present in the seed coat [48]. The red and blue pigments of ripe fruits are anthocyanins that attract frugivores, fruit-eating birds that assist in pollination and seed dispersal [49]. However, in the developing fruits, the astringency and bitterness of PAs provide a defense mechanism against frugivores [50]. Interestingly, while the anthocyanins and PAs share a common biosynthetic pathway, their biological functions are typically opposite, one attracting and the other repelling herbivores, respectively.

Table 56.3 Anthocyanin, flavonol, and proanthocyanidin contents of selected sources (mg/100 g or 100 ml$^{\copyright}$)

Flavonoid-rich foods	Anthocyanins	Pro-anthocyanidins	Flavanols	Flavones	Flavonols	Flavanones
Green tea	–	–	24–216	0–1	3–9	–
Black tea	–	4	5–158	0	1–7	–
Chocolate, dark	–	90–322	43–63	–	–	–
Apple, red delicious with peel	1–4	89–148	2–12	0	2–6	–
Apricot	–	8–13	10–25	0	2–5	–
Flavone-rich foods						
Parsley, fresh	–	–	–	24–634	8–10	–
Thyme, fresh	–	–	–	56	0	–
Celery hearts, green	–	–	–	23	–	–
Celery	–	–	–	0–15	4	–
Oregano, fresh	–	–	–	2–7	0	–
Chili peppers, green	–	–	–	5	13–21	–
Flavanone-rich foods						
Lemon juice, fresh	–	–	–	0	0–2	2–175
Grapefruit juice, fresh	–	–	–	0	0	10–104
Orange juice, fresh	–	–	–	0–1	0	5–47
Grapefruit, fresh	–	–	–	–	1	55
Orange, fresh	–	–	–	–	–	42–53
Flavonol-rich foods						
Onion, yellow	–	–	0	0	3–120	–
Kale	–	–	–	0	30–60	–
Leek	–	–	0	0	3–22	–
Broccoli	–	–	0	0	4–13	–
Anthocyanin-rich foods						
Blackberry	89–211	6–47	13–19	–	0–2	–
Blueberry	67–183	88–261	1	–	2–16	–
Grapes, red	25–92	44–76	2	–	3–4	–
Raspberries (red)	10–84	5–59	9	–	1	–
Strawberry	15–75	97–183	–	–	1–4	–
Red wine	1–35	24–70	1–55	0	2–30	–
Plum	2–25	106–334	1–6	0	1–2	–
Red cabbage	25	–	0	0–1	0–1	–
Red onion	13–25	–	–	0	4–100	–

(*continued*)

Table 56.3 (continued)

Flavonoid-rich foods	Anthocyanins	Pro-anthocyanidins	Flavanols	Flavones	Flavonols	Flavanones
Blood orange juice	3–10	–	–	–	–	10–22

©Per 100 g (fresh weight) or 100 ml (liquids); 100 g is equivalent to about 3.5 oz; 100 ml is equivalent to about 3.5 fluid ounces [32–34]

Adapted from http://lpi.oregonstate.edu/infocenter/phytochemicals/flavonoids/flavonoidrefs.html

2.1.1 Biosynthetic Pathway, Genes, and Enzymes Involved

Basic flavonoid structures and their development from the standpoint of molecular biological and natural products chemistry aspects have been well characterized. Thirty-five flavonoid biosynthetic genes, including genes encoding 12 transcription factors, 10 structural enzymes, and 10 modification enzymes, have been characterized in the model plant *Arabidopsis* [47, 51–56]. Flavonoids are mainly synthesized from phenylalanine via the phenylpropanoid pathway. Following cinnamate hydroxylation by cinnamate 4-hydroxylase and 4-coumarate-CoA ligase step, flavonoid biosynthesis pathway branches out into phenolics (chlorogenic acid) and favonols (naringenin, quercetin, and their derivatives) [57, 58]. Condensation of p-coumaroyl-CoA (C6-C3) with three malonyl-CoA (C3) molecules results in naringenin chalcone with a diphenylpropane (C6-C3-C6) unit, which is converted to naringenin with the flavone (2-phenylchromen-4-one) backbone by conjugate ring closure. These and further modifications yield a variety of structural forms including chalcones, flavanones, dihyroflavonols, flavans, anthocyanins, flavones and flavonols, and isoflavonoids.

Flavonoid biosynthesis was originally discovered and characterized in Arabidopsis (*Arabidopsis thaliana*), maize (*Zea mays*), and petunia (*Petunia hybrida*) [47]. Entry into the flavonoid pathway from general phenylpropanoid metabolism is controlled by chalcone synthase (CHS), which condenses p-coumaroyl-CoA and three malonyl-CoAs into a chalcone, followed by isomerization by chalcone isomerase (CHI) to form a flavanone (Fig. 56.1). This intermediate is subsequently hydroxylated by flavanone-3 β-hydroxylase (FHT) to dihydroflavonol. It is further converted to a flavonol via flavonol synthase or reduced by dihydroflavonol reductase (DFR) to leucoanthocyanidin, a key intermediate for PAs and anthocyanins. Hydroxylation at the C-3′ and C-5′ positions of the β-ring occurs in some species via the activity of cytochrome P450-dependent flavonoid hydroxylases (F3′H and F3′5′H). The anthocyanidin flavylium ion is produced by anthocyanidin synthase (ANS) and then glycosylated by UDP-Glc-flavonoid-3-O-glycosyltransferases (UFGT). Methylation of the 3′ and 5′ hydroxyl groups of anthocyanins gives rise to peonidin, malvidin, and petunidin (Fig. 56.1). Anthocyanidins can be diverted into PA synthesis via anthocyanidin reductase (ANR), which produces epicatechin-type flavan-3-ols (Fig. 56.1). Catechin-type flavan-3-ols are thought to be produced from leucocyanidins by leucoanthocyanidin

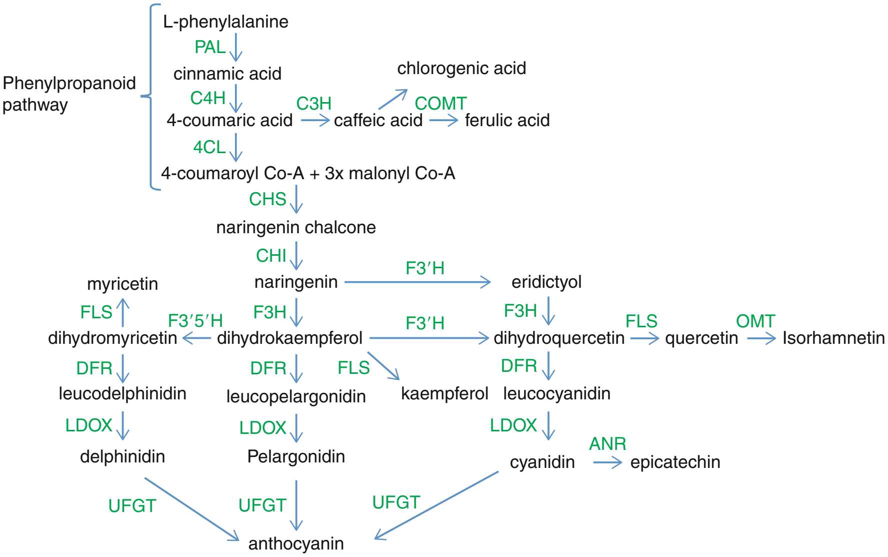
Phenylpropanoid pathway
L-phenylalanine
PAL
cinnamic acid
C4H
4-coumaric acid
C3H
caffeic acid
chlorogenic acid
COMT
ferulic acid
4CL
4-coumaroyl Co-A + 3x malonyl Co-A
CHS
naringenin chalcone
CHI
naringenin
F3′H
eridictyol
myricetin
FLS
F3′5′H
F3H
dihydromyricetin
dihydrokaempferol
F3′H
F3H
dihydroquercetin
FLS
quercetin
OMT
Isorhamnetin
DFR
DFR
FLS
DFR
leucodelphinidin
leucopelargonidin
kaempferol
leucocyanidin
LDOX
LDOX
LDOX
delphinidin
Pelargonidin
ANR
cyanidin
epicatechin
UFGT
UFGT
UFGT
anthocyanin

Fig. 56.1 A schematic presentation of the flavonoid biosynthetic pathway in plants. Enzymes (with abbreviations) indicated are phenylalanine ammonia-lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*), 4-coumarate CoA ligase (*4CL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3 beta-hydroxylase (*F3H*), flavonoid 3′-hydroxylase (*F3′H*), flavonoid 3′ 5′-hydroxylase (*F3′5′H*), flavonol synthase (*FLS*), dihydroflavonol 4-reductase (*DFR*), leucoanthocyanidin dioxygenase (*LDOX*), UDP glucose-flavonoid 3-o-glucosyl transferase (*UFGT*), O-methyltransferase (*OMT*), leucoanthocyanidin reductase (*LAR*), and anthocyanidin reductase (*ANR*) (Source http://pmn.plantcyc.org. MetaCyc (MetaCyc.org) database was used to draw the pathway [59])

reductase (LAR) [60]. It was recently demonstrated that PA precursors are actively moved into the vacuole by multidrug and toxin extrusion transport proteins, where they are polymerized.

Various regulatory and biosynthetic genes involved in the flavonol and anthocyanidin pathways have been isolated and characterized from various plants [30, 61]. However, sequential glycosylation, acylation, and methylation modification in the flavonoid pathways are still comparatively less understood. In earlier investigations, the flavonoid biosynthesis pathway was studied by genetic analyses of inheritance pattern of flower colors, to which radiolabeling and improved analytical methods were later added. In recent years, the candidate gene approach using genetic engineering technology has played a major role in expanding our knowledge about biosynthetic enzymes, substrates and their diversity among various plant species (Table 56.4), and added to our understanding of flavonoids and their biosynthesis pathways. Mutants and transgenic plants have provided direct evidence on the function of various genes involved in flavonoid biosynthesis pathway [83].

Table 56.4 Important food crops transformed with biosynthetic and regulatory genes for flavonoid metabolism

Transgenzic crop	Gene origin/ GE	Promoter/gene	Enzyme	Metabolic product	Metabolite (higher/lower)	References
Tomato	Tomato/RNAi	CaMVd35S/ *CHS*	Chalcone synthase	Chalcones	Lower total flavonoid and parthenocarpic fruit	[62]
Tomato	(Petunia) (alfalfa)/ overexpression	CaMV35S/ *CHS1* + *CHR*	Chalcone synthase + chalcone reductase	Chalcones + deoxychalcones	Higher butein, isoliquiritigenin, naringenin, chalcone, and rutin	[63]
Strawberry	Strawberry/ antisense	CaMV35S/*CHS*	Chalcone synthase	Chalcones	Higher phenylpropanoids (100 versus 1 % control), cinnamoyl glucose caffeoyl glucose, feruloyl glucose, p-coumaryl alcohol and p-coumaryl-1-acetate	[64]
Flax	Petunia hybrid/ overexpression	CaMV35S/CHS + CHI + DFR	Chalcone synthase, chalcone isomerase, and dihydroflavonol 4-reductase	Chalcones, flavanones, chlorogenic acid	Higher in flavonoids (kaempferol), phenolic acids (coumaric, ferulic, synaptic acids) in seedcake extracts	[65]
Tomato	Grape/ overexpression	TomLoxB/ *StSy*	Stilbene synthase	Resveratrol	Higher resveratrol, *trans*-resveratrol and piceid	[66]
Tomato	Grape/ overexpression	CaMV35S/ *StSy*	Stilbene synthase	Resveratrol	High trans-resveratrol (48.48 mg kg^{-1} FW) and trans-piceid (126.58 mg kg^{-1} FW)/ decrease in rutin (2-fold) and naringenin (2.4-fold) Seedless fruit	[67]
Tomato	Grape/ overexpression	CaMVd35S/ *STS*	Stilbene synthase	Resveratrol	High stilbenes (resveratrol and piceid), naringenin chalcone, rutin	[63]

Apple	Grape/ overexpression	Vst1/ *Vst1*	Stilbene synthase	Flavanol/*trans*-piceid	Higher flavanol (20–80 μg g^{-1} fw) and piceid	[68]
Strawberry	Frost grape/ overexpression	CaMV 35S and fil1/Vst3	Stilbene synthase	Resveratrol	Higher cinnamate, coumarate, and ferulate derivatives/ lower flavonols	[69]
Tomato	Soybean/ overexpression	CaMV35S/ *IFS2*	Isoflavone synthase	Genistein	Higher genistin in leaves but marginal increase in fruit peel, higher naringenin chalcone in fruit peel	[70]
Tomato	Petunia/ overexpression	CaMVd35S/ *chi-a*	Chalcone isomerase	Flavanones	Higher flavonols mainly rutin in peel (78-fold)	[71]
Tomato	Petunia gerbera/ overexpression	CaMV35S/ *CHI* + *FNS-II*	Chalcone isomerase + Flavone synthase	Flavanones + flavones	Higher rutin (16-fold), luteolin-7-glucoside, luteolin aglycon, quercetin glycosides, naringenin chalcone	[63]
Potato	Petunia hybrida/ overexpression	CaMV 35S/ *DFR*	Dihydroflavonol 4-reductase	Chlorogenic acid	Higher phenolic acid and anthocyanin; chlorogenic acid (2.5 vs.1.5 mg g^{-1}dw; pelargonidin:0.4 vs. 0.1 mg g^{-1}dw; petunidin: 3.0 vs. 0.5 mg g^{-1}dw)	[72]
Apple	Maize/ overexpression	Lc	Lc	Leaf color regulatory	Higher levels of anthocyanin idaein (12-fold), flavan 3-ol epicatechin (14-fold), isomeric catechin (41-fold), and dimeric proanthocyanidins (7–134-fold) in leaves	[73]

(*continued*)

Table 56.4 (continued)

Transgenzic crop	Gene origin/ GE	Promoter/gene	Enzyme	Metabolic product	Metabolite (higher/lower)	References
Rice	Rice/ overexpression	*CaMV35S*/(Rc-MYB1, Rc-MYB2 and Rc-bHLH)	*bHLH, DFR*	MYC-type regulatory proteins	Higher proanthocyanidins/red-colored rice	[74]
Tomato	Sesame/ overexpression	CaMV35S/ *Myc-rp*	RP	Myc-like TFs regulate anthocyanin biosynthesis	Higher anthocyanin in vegetative tissues and flowers	[75]
Tomato	Antirrhinum/ overexpression	CaMV35S/*Del*	Delila	Myc TFs that activate biosynthesis of anthocyanin	Higher anthocyanins in mature leaves (23-fold), corolla (40-fold), and stamen (50-fold) but no change in fruit	[76]
Tomato	Snap dragon/ overexpression	E8/ *Ros1* + *Del*	Rosea1 + Delila	TFs that activate biosynthesis of anthocyanin	Higher anthocyanin in pericarp comparable to blackberries and blueberries	[77]
Tomato	Arabidopsis/ overexpression	CaMV35S/ *MYB12*	MYB12	R2R3-MYB TF mediates the accumulation of flavonoids in tomato peel	Higher chlorogenic acid (27-fold), dicaffeoylquinic acid (26-fold), tricaffeoylquinic acid (42-fold), quercetin rutinoside (67-fold), kaempferol rutinoside (593-fold)	[54]
Tomato	Tomato/RNAi	CaMV35S/ *MYB12*	MYB12	R2R3-MYB TF mediates the accumulation of flavonoids in tomato peel	Lower flavonoid pigment naringenin chalcone Exhibited a *y*-like phenotype	[78]
Tomato	Maize/ overexpression	E8 or CaMVd35S/*LC* + *C1*	C1 + LC	MYB-type C1 and MYC-type LC are TFs required for production of anthocyanin in plants	Induced flavonoid synthesis in fruit flesh Higher total flavonoids (10-fold) and total flavonol (20-fold) mainly kaempferol	[79]

Tomato	Tomato/ overexpression	CaMV35S/ *MYB12*	MYB12	R2R3-MYB TF mediates the accumulation of flavonoids in tomato peel	Rescued colorless-peel "y" tomato mutant phenotype	[78]
Tomato	Tomato/ overexpression	CVM/ *ANT1*	ANTHOCYANIN 1	Flavonoid-related R2R3-MYB TF	Higher anthocyanin (500-fold)	[80]
Tomato	Tomato (*Solanum chilense*)/ overexpression	CaMV35S/*ANT1*	ANTHOCYANIN 1	Flavonoid-related R2R3-MYB TF	Higher anthocyanidins (petunidin, malvidin, delphinidin) in fruit	[77]
Tomato	Tomato/ overexpression	E8/ *TAGL1* (*TAGL1-SRDX*)	TOMATO AGAMOUS-LIKE 1	MADS-box TF	Lower lycopene and isoprenoids	[81]
Tomato	Tomato/ overexpression	CaMV35S/ *TAGL1*	TOMATO AGAMOUS-LIKE 1	MADS-box TF	Higher lycopene and naringenin chalcone	[81]
Tomato	Tomato/RNAi	CaMV35S/ *CUL4*	Cullin 4	DDB1a and DET1 form a complex with CUL4, a ubiquitin-conjugating E3 ligase, and target proteins for proteolysis	Pleiotropic phenotype higher anthocyanins and carotenoids and also higher lycopene (2-fold)	[82]
Tomato	Tomato/RNAi	E8/ *DDB1*	UV-DAMAGED DNA BINDING PROTEIN 1	TF negatively regulates photomorphogenic responses	Higher pigment accumulation due to increase in plastid compartment space	[82]

2.1.2 Biotechnological Interventions

Crops successfully enhanced for higher accumulation of flavonoids using genetic engineering are summarized in Table 56.4. Genetic transformation of flax (*Linum usitatissimum* L.), in which a group of genes of the pathway – chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) – were simultaneously expressed, resulted in a significant increase of antioxidant capacity of the transgenics [65]. A strong correlation was found in the phenolic acid content and antioxidant capacity. Field tests of these plants revealed that seedcake extracts of transgenic flax were significantly higher in flavonoids (kaempferol), phenolic acids (coumaric, ferulic, synaptic acids), and lignan content compared to non-transformed control plants. Fibers, another product of flax plant, were enriched in catechine and acetylvanillone but were decreased in phenolic acids [84]. Another feature of transformed flax plants was their higher yield and resistance to *Fusarium culmorum* and *F. oxysporum* as compared to the control plants [65].

In tomato fruit peels, the main flavonoids that accumulate are naringenin chalcone, flavonol rutin, and kaempferol 3-*O*-rutinoside [71, 79, 85]. The ectopic expression of petunia chalcone isomerase (*CHI*) in tomato resulted in a substantial, 78-fold increase in peel flavonols, which was mainly due to accumulation of a quercetin glycoside, rutin. Even after fruits from this line were processed, the remaining paste still contained 65 % of the total flavonols found in fresh fruit, supporting the potential of transgenic plants for food-based industry [79]. Tomato fruit flesh is richer in carotenoids than flavonoids. Therefore, genetic engineering was used to increase flavonoid content in fruit flesh, including the production of new flavonoids by introducing stilbene synthase, chalcone synthase, chalcone reductase, chalcone isomerase, and flavone synthase genes [63]. This strategy caused a threefold higher content of novel flavones and flavonols in tomato fruit that also resulted in higher total antioxidant capacity.

Tomato fruit does not normally produce resveratrol, a stilbenoid flavonoid, because it lacks stilbene synthase (*StSy*) activity. Nonetheless, expression of a grape *StSy* not only induced the production of resveratrol in transgenic tomato fruits but also led to accumulation of *trans*-resveratrol and *trans*-resveratrol-glucopyranosides (piceid), further elevating the antioxidant capacity of the tomato fruit [66, 67]. The combined expression of Petunia chalcone synthase and alfalfa chalcone reductase was found to induce higher levels of butein and isoliquiritigenin (deoxychalcones). Constitutive expression of hydroxycinnamoyl transferase *HQT* gene [86] and that of cryptochrome *cry-2* gene [87] caused severalfold accumulation of chlorogenic acid and other flavonoids. The combined expression of Petunia chalcone isomerase and Gerbera flavone synthase elevated production of luteolin-7-glucoside, luteolin aglycon (flavones), and quercetin glycosides (flavonols) in transformed tomato. The constitutive overexpression of *StSy* that led to a tenfold higher resveratrol content resulted in male sterility mainly due to deficiency of coumaric and ferulic acid [88]. However, it is noted here that seedless parthenocarpic fruit phenotype associated with male sterility is desired by both the consumer and food industry [89–91]. Isoflavones are legume-specific flavonoids. Tomato plants engineered to constitutively overexpress soybean isoflavone synthase (*35S:GmIFS2*) showed only a marginal increase of isoflavones in fruit peel but increased naringenin chalcone, indicating that naringenin is a rate-limiting substrate for isoflavone biosynthesis in tomato peel [70]. In another study where β-carotene content was increased using RNAi suppression of the *DET1* gene, the authors also found severalfold increase in flavonoid content [92]. Another co-stimulation of carotenoids with flavonoids was reported in tomato by upregulating *TOMATO AGAMOUS-LIKE 1* (*TAGL1*), a MADS-box transcription factor, which resulted in higher accumulation of lycopene and naringenin chalcone [81].

Various transcription factors from different plant sources have also been employed to enhance flavonoid content. Expression in tomato of maize transcription factors implicated in anthocyanin production, MYB-typeC1 and MYC-type LC, led to higher flavonoid content in fruit flesh [79]. Main increase seen in the ripe tomato fruit was that of kaempferol [79, 93]. Employing other anthocyanin-related transcription factors, *Roseal* and *Delila*, fused to ripening-specific E8 promoter enhanced anthocyanin content in fruit pericarp whose concentrations were comparable to normally anthocyanin-rich blackberries and blueberries [77]. Similarly, anthocyanidins including petunidin, malvidin, and delphinidin were enhanced in transgenic tomatoes constitutively transformed with flavonoid-related R2R3-MYB transcription factor, *ANTHOCYANIN1* (*ANT1*) [94].

In strawberry, antisensing chalcone synthase (*CHS*) caused the substrates to be utilized by the pathway making other phenolics such as cinnamoyl glucose, caffeoyl glucose, feruloyl glucose, *p*-coumaryl alcohol, and *p*-coumaryl-1-acetate at levels ranging from 363 % to 1,092 % of the control plants. These phenolics occur otherwise only in trace amounts in the control fruit [64]. These results highlighted the step of CHS as a key point of substrate flow between the flavonoid and the phenylpropanoid pathways. An increase in such phenolics at the cost of flavonoids may compromise resistance of strawberry (and other fruits) against disease [69].

In apple (*Malus domestica* Borkh.), flavonoid biosynthetic pathway was engineered by introducing maize leaf color (Lc) regulatory gene [73]. Leaf tissue of Lc-transgenic lines had higher levels of anthocyanin idaein (12-fold), flavan 3-ol epicatechin (14-fold), isomeric catechin (41-fold), and dimeric proanthocyanidins (7- to 134-fold).

Rice pericarp colors are varied and attempts to modify them have also been successful. The dihydroflavonol-4-reductase (DFR) gene is associated with red-color (*Rd*) locus. Its introduction into a brown-colored rice mutant (*Rcrd*) resulted in red-colored rice [74].

3 Carotenoids

3.1 Diet Source/Distribution/Occurrence

Carotenoids are an important class of over 600 naturally occurring molecules synthesized by plants, algae, and photosynthetic bacteria. Dependent upon which

Table 56.5 Food rich in carotenoids[a]

Alpha-carotene	Beta-carotene	Beta-cryptoxanthin	Lycopene	Lutein + zeaxanthin
Carrots	Carrots	Papayas	Watermelon	Kale
Plantains	Cantaloupe	Sweet red peppers	Tomatoes	Spinach
Tomatoes	Spinach	Tangerines	Pink grapefruit	Collard Greens
Tangerines	Lettuce	Watermelon	Peppers, sweet, red	Turnip, greens
Avocados	Apricots	Oranges	Cabbage, red	Squash

[a] Adapted from
http://lpi.oregonstate.edu/infocenter/phytochemicals/carotenoids/carotenoidrefs.html

of them is preponderant can impart yellow, orange, or red pigmentation to an organism [95]. They are also highly antioxidative. Fruits and vegetables are the source of dietary carotenoids: α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, and zeaxanthin [96]. Broadly, carotenoids fall into two classes: carotenes (α-carotene, β-carotene, and lycopene) and xanthophylls (β-cryptoxanthin, lutein, and zeaxanthin). α-Carotene, β-carotene, and β-cryptoxanthin are provitamin A carotenoids, converted by the body to retinol. Lutein, lycopene, and zeaxanthin are not converted to retinol. The foods rich in various carotenoids per serving are listed in Table 56.5. Fruits – raw tangerines, cantaloupe, watermelon, oranges, and nectarines – serve as good sources of carotenoids. Tomato and tomato products (paste, puree, soup, and juice) are concentrated in lycopene as also vegetable juice cocktail, raw watermelon, and pink grapefruit (Table 56.5). Vegetables (spinach, kale, turnip, collards, and green dandelion) contain lutein, zeaxanthin, α-carotene, β-carotene, and β-cryptoxanthin. Carotenoids in foods are mainly in the all-*trans* form. Carotenoids form complexes with proteins and therefore to make them bioavailable plants need to be chopped, homogenized, and cooked [97, 98]. Lycopene becomes more bioavailable from tomatoes by boiling or heating them in oil [99, 100].

3.1.1 Biosynthetic Pathway, Genes, and Enzymes Involved

Carotenoids are essential for diverse processes in plant biology, ranging from photosynthesis, photomorphogenesis, free radical detoxification, lipid peroxidation, to synthesis of plant hormone abscisic acid [4]. The carotenoid biosynthetic pathway (Fig. 56.2) is well known, enzymes involved have been identified, and genes encoding the enzymes have been cloned. Nevertheless, the processes that regulate their biosynthesis and accumulation are complex and poorly understood [102]. The first dedicated step in carotenoid biosynthesis is the ubiquitous C20 isoprenoid precursor geranylgeranyl pyrophosphate (GGPP) [103]. Phytoene synthase (PSY) condenses two GGPP molecules in a head to tail manner to form phytoene (C40). Phytoene contains three conjugated double bonds as a 15-cis geometric isomer. Desaturation reactions extend the series of conjugated double bonds. Phytoene desaturase (PDS) introduces a double bond at the 9′ of the phytoene molecule to create 15, 9′-di cis-phytofluene, and another double bond at the 9 position forms 9, 15, 9′-tricis-ζ-carotene. Seven conjugated

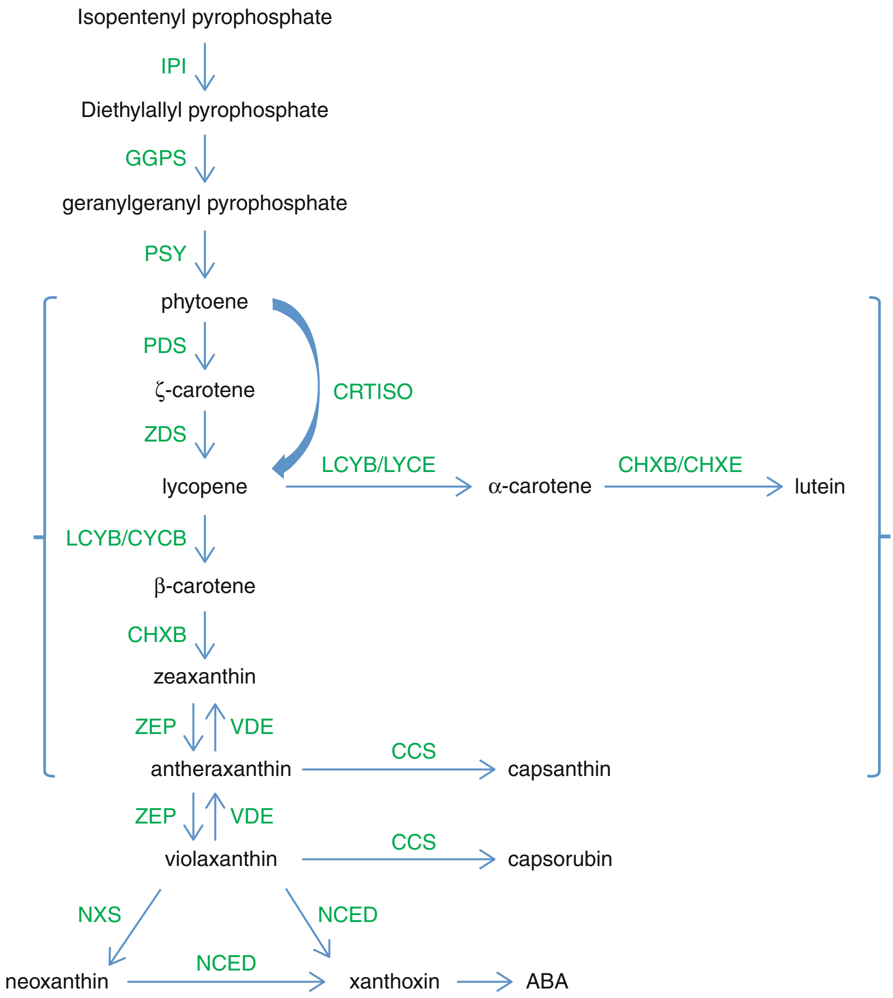
Isopentenyl pyrophosphate
IPI
Diethylallyl pyrophosphate
GGPS
geranylgeranyl pyrophosphate
PSY
phytoene
PDS
ζ-carotene
ZDS
CRTISO
lycopene
LCYB/LYCE
α-carotene
CHXB/CHXE
lutein
LCYB/CYCB
β-carotene
CHXB
zeaxanthin
ZEP
VDE
antheraxanthin
CCS
capsanthin
ZEP
VDE
violaxanthin
CCS
capsorubin
NXS
NCED
neoxanthin
NCED
xanthoxin
ABA

Fig. 56.2 Carotenoid biosynthesis pathway in plants. Enzymes (with abbreviations) indicated are isopentenyl pyrophosphate isomerase (*IPI*), geranylgeranyl pyrophosphate synthase (*GGPS*), phytoene synthase (*PSY*), phytoene desaturase (*PDS*), zeta-carotene desaturase (*ZDS*), carotenoid isomerase (*CRTISO*), lycopene beta-cyclase (*LCYB*), lycopene epsilon-cyclase (*LCYE*), beta-ring carotene hydroxylase (*CHXB*), epsilon-ring carotene hydroxylase (*CHXE*), zeaxanthin epoxidase (*ZEP*), violaxanthin de-epoxidase (*VDE*), capsorubin-capsanthin synthase (*CCS*), neoxanthin synthase (*NXS*), 9-cis epoxycarotenoid dioxygenase (*NCED*), and carotenoid cleavage dioxygenase (*CCD*). (Source: [101], drawn using KeGG pathway)

double bonds give this molecule a characteristic yellow/green color. Lycopene with eleven conjugated double bonds forms the chromophore that imparts red color to ripe tomato fruit. Lycopene is then stepwise cyclized to β-carotene and α-carotene by β-cyclase (LCY-B, CYC-B) and ε-cyclase (LCY-E) enzymes, respectively.

Cyclization of lycopene proceeds only after an all-*trans* lycopene is formed by the action of carotene isomerase (CRTISO) in nongreen tissue. In the photosynthetic tissues, this conversion is catalyzed by light and chlorophyll (acting as a sensitizer). Oxygenation of the cyclic carotenoids yields xanthophylls. Introduction of hydroxyl groups at positions 3 and 3′ in β-carotene produces zeaxanthin. Zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) act in tandem to regulate the formation of violaxanthin. Violaxanthin is next converted to 9-cis-neoxanthin, ABA precursor, by neoxanthin synthase. Lutein is mainly present in photosynthetic tissues, biosynthesized from α-carotene via catalysis by β- and ε-hydroxylases.

Carotenoid accumulation in fruits is coordinated with fruit ripening and cellular changes that transform chloroplast to chromoplast. The chloroplast-chromoplast differentiation involves quantitative and qualitative change in the carotenoid profiles [104].

3.1.2 Biotechnological Interventions

Carotenoids have attracted attention because of their potent antioxidant property and their potential in preventing malnutrition and disease. Fruits and vegetables (to name some, tomato and carrots) are good sources of carotenoids. However, the levels present are below the RDA [105, 106]. Molecular approaches used to enhance the carotenoid levels in edible produce to levels that are in sync with the RDA have achieved reasonable success. Some of these advances include the production of β-carotene in rice endosperm [13] and maize [22] and lycopene in tomatoes [92, 107]. A major regulatory step in carotenoid biosynthesis is *Psy* gene, which encodes phytoene synthase. The *Psy* cDNA from different sources was used along with the bacterial phytoene desaturase (*Crt1*) gene under the endosperm-specific promoter to transform rice. Using maize *psy*, the total carotenoid level of 37 μg/g achieved in Golden Rice 2 [13] was 23-fold higher than previous reports [108, 109]. In addition to β-carotene, lutein and zeaxanthin also accumulated in the transgenic rice. Four cDNAs encoding enzymes in β-carotene, ascorbate, and folate pathways were used to develop transgenic maize [22]. This study showed that maize endosperm of the transformed plants accumulated β-carotene (59.32 μg/gDW), lycopene (22.78 μg/gDW), vitamin C (ascorbate) (106.94 μg/gDW), and zeaxanthin (35.76 μg/gDW). Using a different strategy, yeast SAM decarboxylase (*ySAMdc*) gene was expressed in a fruit ripening-specific manner (using E8 promoter) in tomato, which led to the accumulation of biogenic amines (polyamines), spermidine, and spermine, at the cost of their precursor diamine putrescine [107]. Fruits of these transgenic tomato lines accumulate 120–175 μg/gFW lycopene as well as the micronutrient choline in addition to other attributes including higher processing quality [107, 110].

A number of other studies have focused on increasing the levels of β-carotene in tomato (Table 56.6). Ectopic expression of a bacterial phytoene synthase (crtB) in a fruit-specific manner increased phytoene (2.4-fold), lycopene (1.8-fold), β-carotene (2.2-fold), and lutein levels in tomato fruit [113]. Suppression RNAi was used to downregulate *DE-ETIOLATED1* (*DET1*), photomorphogenesis

Table 56.6 Important food crops transformed with biosynthetic and regulatory genes for carotenoid metabolism

Transgenic crop	Gene origin/GE	Promoter/ gene	Metabolite (higher/lower)	References
Tomato	Arabidopsis/ overexpression	*CaMV35S/ HMGR-1*	Higher phytosterol (2.4-fold). No change in lycopene or β-carotene	[111]
Tomato	Bacteria/ overexpression	Fibrillin or CaMV35S/ *DXS*	Higher carotenoids (1.6-fold), phytoene (2.4-fold), and β-carotene (2.2-fold)	[111]
Potato	*E. coli*/ overexpression	*Patatin/DXS*	Higher 3.0 versus 0.4 μg g^{-1}fw in control	[112]
Tomato	Bacteria/ overexpression	*PG/crtB*	Higher phytoene (2.4-fold), lycopene (1.8-fold), and β-carotene (2.2-fold)	[113]
Tomato	Tomato/ overexpression	CaMV35S/ *Psy-1*	Higher total carotenoids (1.2-fold), β-carotene (1.3- fold), phytoene (2.3-fold), and phytofluene (1.8-fold)	[53]
Tomato	Bacteria/ overexpression	CaMV35S/ *crtI*	Higher β-carotene (3-fold)/lower lycopene and phytoene/no effect on total carotenoids on plant growth and development	[114]
Rice	Daffodil/ overexpression	CaMV35 and Gt1 PSY	Higher phytoene with both promoters; 0.74 μg g^{-1}dw (with pGt1); 0.32 μg g^{-1}dw(with CaMV35S)	[115]
Rice	Daffodil and bacteria/ overexpression	Gt1 and CaMV35S/ *Psy* and *Crt1*	Higher accumulation of β-carotene and lutein and zeaxanthin	[108]
Rice	Daffodil and bacteria/ overexpression	Gt1 and CaMV35S/ *Psy* + *Crt1* and *Lcy*	Higher accumulation of β-carotene (1.6 μg/g) in heterozygous lines and variable total carotenoids	[108]
Rice	Maize and daffodil/ overexpression	Glu1/psy + crtI	Higher total carotenoid (37 μg g^{-1}dw) and β-carotene, 84 μg g^{-1}dw	[13]
Potato	Bacteria/ overexpression	Patatin/*CrtB*	35 versus 5.6 μg g^{-1}fw	[116]
Potato	Potato/antisense	Pat1/*CrtI*, *CrtB*, and *CrtY*	Higher total carotenoid, 114 versus 5.8 μg g^{-1}dw; β-carotene, 47 vs. 0.013 μg g^{-1}dw;	[117]
Maize	Bacteria/ overexpression	LMW glutelin/ *Psy1and crt1*	β-carotene, 4.79 versus 0.09 μg g^{-1} dw; lycopene, 22.78 versus 0 μg g^{-1}dw	[22]
Maize	Bacteria/ overexpression	γ-Zein/*crtB* + *crt1*	Provitamin A, 7 versus 0.39 μg g^{-1}dw; total carotenoids, 33 versus 1.01 μg g^{-1}dw	[118]

(*continued*)

Table 56.6 (continued)

Transgenic crop	Gene origin/GE	Promoter/ gene	Metabolite (higher/lower)	References
Potato	*Cyanobacterium*/ co-transformation	CaMV 35S/ *crtO*	Accumulation of ketocarotenoids; up to ∼ 10–12 % of total carotenoids	[119]
Brassica	*B. napus* (RNAi)	P35S /ε-CYC	Total carotenoid: versus 5.34 µg g^{-1}fw; β-carotene, 90.76 versus 0.49 µg g^{-1}fw; lutein, 76.22 vs.3.30 µg g^{-1}fw	[120]
Flaxseeds	Bacteria/ overexpression	CaMV35S FAE1/*crtB*	Higher carotenoid (7.8- to18.6-fold, 156.3 versus 8.4 µg g^{-1}dw	[121]
Canola	Bacteria/ overexpression	Napin/*crtB*	Higher (50-fold, 1,000–1,500 µg/ gFW) total carotenoid/orange-colored seed	[122]
Canola	Bacteria/ overexpression	*crtI* + *crtb*	Higher carotenoid 857 versus 5 µg g^{-1}fw	[123]
Canola	Bacteria/ overexpression	*crtI* + *crtb*	44 versus 33 µg g^{-1}fw	[123]
Canola	Bacteria/ overexpression	*crtI* + *crtb*	44 versus 33 µg g^{-1}fw	[123]
Canola	RNAi	CaMV35S/ *DET1*	7 µg versus 0.5 µg g^{-1}fw	[124]
Carrot	Algae (Haematococcus pluvialis)/ overexpression	Ubi, CaMV35S and RolD/ *CrtO*	Higher amounts of total β-carotene (70 %) converted to ketocarotenoids (2.400 µg g^{-1}dw)	[125]
Tomato	Tomato/antisense	CaMV35S/*B*	Higher lycopene but not significant/lower levels of β-carotene (>6- folds)	[126]
Tomato	Tomato/ overexpression	CaMV35S/ *Lycb-1*	Higher β-carotene (31.7-fold)/ lower lycopene/normal plants	[127]
Tomato	Tomato/antisense	Tomato Pds/ *β-Lcy*	Higher lycopene (1.3-fold), lutein (1.7-fold), and lower β-Lcy expression (1.7-fold)	[128]
Tomato	Citrus/ overexpression	CaMV35S/ *Lycb-1*	Higher total carotenoids (30 %) and β-carotene (4.1-fold)	[129]
Tomato	Arabidopsis/ overexpression	Tomato Pds/ *β-Lcy*	Higher β-carotene (6-fold) but no effect on lycopene	[128]
Tomato	Bacteria/ overexpression	atpI/*crtY*	Higher β-carotene (4-fold)/lower total carotenoids and lycopene	[130]
Tomato	Arabidopsis and pepper/ overexpression	Pds/*b-Lcy and b-Chy*	Higher total xanthophyll (10-fold) and β-carotene (12-fold)	[131]
Potato	Potato/antisense	Patatin B33/ *LCY-e/*	Higher total carotenoid, 12,272 versus 4,672 ng g^{-1}dw; β-carotene, 43 versus 3 ng g^{-1}dw; zeaxanthin, 990 versus 262 ng g^{-1}dw	[118]

(*continued*)

Table 56.6 (continued)

Transgenic crop	Gene origin/GE	Promoter/ gene	Metabolite (higher/lower)	References
Tomato	Tomato/ RNAi	E8/*NCED3*	Higher β-carotene and lycopene/ lower ABA (20–50 %)	[132]
Potato	Cauliflower/ overexpression	GBSS/*Or*	Higher total carotenoid (6-fold, 31 versus 5.5 μg gdw^{-1})	[133, 134]
Tomato	Tomato/RNAi	P119 2A11 TFM7/*DET1*	Higher lycopene (2-fold), β-carotene (4-fold), flavonoids (3.5-fold)	[92]
Tomato	Tomato/ overexpression	CaMV35S/ *CRY2*	Higher lutein (1.5-fold), carotenoids (1.7-fold) and flavonoids (2.9-fold)	[87]
Tomato	Tomato/RNAi	CaMV35S/ *HY5*	Lowered carotenoid level	[10]
Tomato	Tomato/RNAi	CaMV35S/ *COP1-like*	Higher carotenoids (2-fold)↑	[10]
Tomato	Yeast/ overexpression		Higher lycopene (40 %)	[135]
Tomato	Apple/ overexpression	CaMV35S/ *SPDS1*	↑ *PSY* and *PDS* and ↓ *CRTL-B* and *CRTL-E* transcripts 1.3- to 2.2-fold ↑ lycopene	[136]
Tomato	Yeast/ overexpression	E8/*SPE2*	Higher lycopene (2- to 3-fold)	[107]
Wheat	*Oat*	Ubi-1/*ADC*	Higher polyamines in seeds putrescine (7-fold), spermidine (2.5-fold), and spermine (1.8-fold)	[137]

regulatory protein gene that negatively regulates light-mediated responses [92]. The transformed tomato accumulated high carotenoid levels. Fruit-specific [128, 131] or constitutive [127] expression of lycopene β-cyclase, and constitutive expression of phytoene desaturase [114], all led to high levels of β-carotene in tomato fruit.

Among other studies, canola was transformed with *E. uredovora* phytoene synthase (*crtB*) gene driven by seed-specific napin promoter [122]. Orange-colored canola seeds were thus obtained which accumulated a 50-fold higher (1.0–1.5 mg/gFW) total carotenoid level than the wild type (0.033 mg/gFW). Later, canola that coexpressed *crtI* and *crtB* genes accumulated lycopene (29 μg/gFW) and β-carotene (857 μg/gFW) [123]. Expression of bacterial *crtB* gene in a seed-specific manner in flaxseeds led to 7.8- to18.6-fold increase in the carotenoid levels [138]. Transgenic potatoes expressing the *crtB* gene in a tuber-specific manner accumulated violaxanthin, lutein, and β-carotene in the tuber [116]. The constitutive expression of citrus lycopene β-cyclase (*CRTL-B*) in tomato increased β-carotene 4.1-fold with a 30 % increase in total carotenoids while suppressing fluxes downstream into β-carotene pathway and concomitant increase in α-carotene [129]. However, phytoene, lycopene, and lutein decreased by about 26 %, 10 %, and 44 %, respectively, in the transgenic lines [129].

Constitutive RNAi suppression of a component of Cul4-based E3 ligases, CUL4, also resulted in increased carotenoid accumulation in tomato [82]. Tuber-specific expression of the cauliflower *Or* gene, which causes β-carotene accumulation in cauliflower, led to a sixfold higher level of total carotenoids in transgenic potato versus the controls [133, 134].

Other strong antioxidants in plants comprise ketocarotenoids. They are used as dietary supplements and pigments in aquaculture and nutraceutical industries. Constitutive expression of algal *Haematococcus pluvialis* β-carotene ketolase gene in transgenic carrot converted β-carotene to ketocarotenoid that reached a level of 2.4 mg/g root dry weight [125]. Such transgenic carrots are well suited for biopharming ketocarotenoids as functional food and in nutraceutical and aquaculture industries. A mutation in zeaxanthin epoxidase (*zep1*) creates abscisic acid (ABA) deficiency in tomato plants with concomitant accumulation of 30 % more carotenoids in mature red tomato fruit [139]. Similarly, suppression of ABA synthesis led to accumulation of upstream carotenoids, β-carotene and lycopene, in transgenic tomato fruits in which 9-cis-epoxycarotenoid dioxygenase 3 (*NCED3*), an enzyme that catalyzes first step of ABA biosynthesis, was silenced in a fruit-specific manner using RNAi [132]. Likewise, expression of cryptochrome 2 (35S:CRY2) resulted in 1.7-fold increase in carotenoids and 2.9-fold increase in flavonoids [87]. Repression of *ELONGATED HYPOCOTYL 5* (*HY5*) by RNAi reduced carotenoid accumulation, while suppression of *CONSTITUTIVELY PHOTOMORPHOGENIC 1* (*COP1*)-like gene elevated carotenoid levels in tomato fruit, suggesting involvement of light signaling factors in carotenoid biosynthesis [10].

4 Essential Fatty Acids

4.1 Diet Source/Distribution/Occurrence

The “omega” fatty acids are polyunsaturated fatty acids (PUFA), considered as essential nutrients since mammals including humans are unable to synthesize unsaturated fatty acids with a double bond at the n-6 (ω6) or the n-3 (ω3) position in the *cis* orientation [140]. The substrate for ω-6 series is linoleic acid (18:2n-6), while α-linolenic acid serves as the substrate for 18:3n-3 series. The “fatty” diet of humans in the Western world has changed from 1:1 ratio of ω-6 to ω-3 fatty acids [97] to close to a 10:1 (ω 6/ω 3) ratio because of the increased use of oils rich in LA [141].

Linoleic acid is found in soybean, safflower and corn oil, nuts, seeds, and some vegetables. α-Linolenic acid is found in flaxseeds, walnuts, and their oils; canola oil is also a good source of α-linolenic acid. Edible oils rich in monounsaturated fatty acids are stable, flavorful, and nutritious for humans and animals. Oleic acid-rich soybean oil is more resistant to degradation by heat and oxidation and requires little or no postrefining processing (hydrogenation). In addition, α-linolenic acid is a substrate for the synthesis of longer chain ω-3 fatty acids found in fish, eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3). Stearidonic acid (SDA; C18:4n-3), EPA, and DHA possess anticancer properties and have been implicated in the regulation of inflammatory immune reactions and blood pressure, brain development, and the development of cognitive function [142–144]. Research indicates that the ratio of n-3 to n-6 fatty acids may be as important to health and nutrition as the absolute amounts present in the diet or in the body tissues. EPA and DHA in the diet are generally provided by fish, a source that is not a possibility for vegetarians and also appears unable to cope up with the increasing global demand [145].

4.1.1 Biosynthetic Pathway, Genes, and Enzymes Involved

Fatty acid synthase (FAS) constitutes a multisubunit complex in the plastid where it catalyzes ordered synthesis of fatty acids, initiated from acetyl CoA and malonyl CoA [146, 147]. Stepwise FAS activity generates the products 16:0-acyl carrier protein (ACP) and 18:0-ACP. Most of the 18:0-ACP is desaturated by a soluble stearoyl-ACP desaturase, yielding 18:1 D9-ACP [148]. Acyl-ACP thioesterases release ACP from 16:0-ACP and 18:0-ACP; the deacylated fatty acids exit the plastid and are then esterified with coenzyme A (CoA) to form respective acyl-CoAs [148]. These acyl moieties are then esterified to phosphatidylcholine (PC) and then undergo desaturation by D12- and D15-desaturases to yield the essential fatty acids, linoleic acid, and α-linolenic acid [148–150]. All higher plants have the enzymes for synthesizing the C18 PUFAs linoleic acid and α-linolenic acid. The primary genes involved in PUFA biosynthesis have been reviewed [151].

4.1.2 Biotechnological Interventions

Traditional breeding, induced selection, and modern biotechnology have eased modifying the fatty acid composition of oilseeds to enhance their nutritional value and food oil applications. Because of the nature of its use on a daily basis, modifying fatty acid composition in vegetables, and thereby in vegetable oil, is a convenient means of delivering a healthier product to consumers, requiring no significant change in the daily diet. Marketed modified oils include low- and zero-saturated fat soybean and canola oils; medium-chain fatty acid canola oil; high-stearic acid canola oil (for trans-fatty acid-free products); high-oleic acid (monounsaturated) soybean oil; and canola oil containing the PUFA γ-linolenic acid (18:3 n-6) and stearidonic acid (18:4 n-3), long chain fatty acids [152], and ω-3 fatty acids [153] (Fig. 56.3; Table 56.7).

Oleate desaturase expression by antisense RNA in soybean led to an oil containing over 80 % oleic acid as compared to a normal 23 %, with significant decrease in polyunsaturated fatty acids [155]. Canola does not normally accumulate capric and caprylic acids. But the introduction of the *Cuphea hookeriana* acyl-ACP thioesterase cDNA in canola resulted in the accumulation of these two medium-chain fatty acids in the seeds [156]. In plants, ω-6 desaturase-catalyzed pathway in the microsomes is a major source of polyunsaturated lipids. Introduction of the

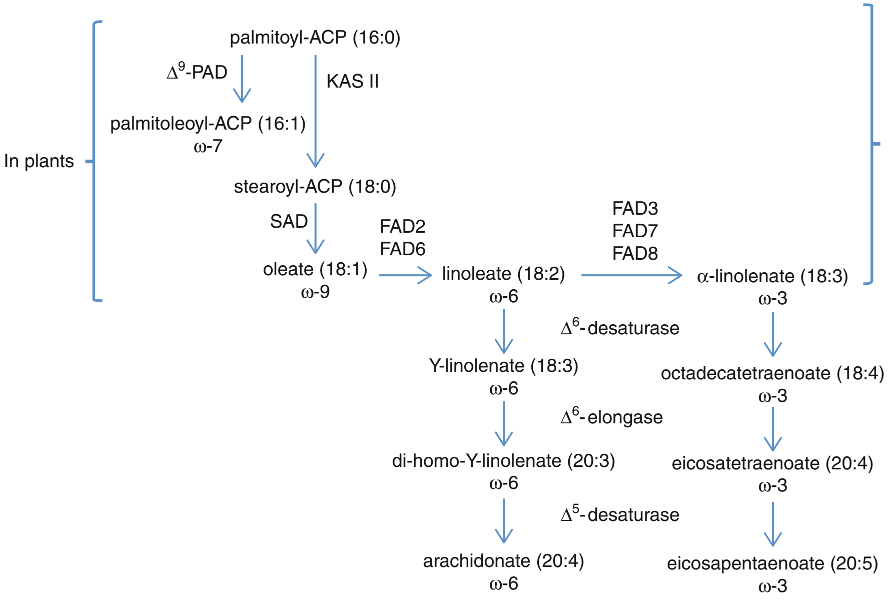
In plants
palmitoyl-ACP (16:0)
Δ9-PAD
KAS II
palmitoleoyl-ACP (16:1)
ω-7
stearoyl-ACP (18:0)
SAD
oleate (18:1)
ω-9
FAD2
FAD6
linoleate (18:2)
ω-6
FAD3
FAD7
FAD8
α-linolenate (18:3)
ω-3
Δ6- desaturase
Υ-linolenate (18:3)
ω-6
octadecatetraenoate (18:4)
ω-3
Δ6- elongase
di-homo-Υ-linolenate (20:3)
ω-6
eicosatetraenoate (20:4)
ω-3
Δ5- desaturase
arachidonate (20:4)
ω-6
eicosapentaenoate (20:5)
ω-3

Fig. 56.3 Generalized pathway for the biosynthesis of long-chain PUFA. The conventional Δ^6-desaturase/elongase pathway for the synthesis of arachidonic acid and EPA from the essential fatty acids linoleic acid and α-linolenic acid in plants (Source: [154])

fungal (Mortierella) Δ^6- desaturase gene in canola produced ω-3 fatty acids [157]. ω-6 and γ-linolenic acid (GLA) content was engineered in safflower oil to amounts as high as 40 %, which is four times higher than levels found in evening primrose (*Oenothera biennis*) and borage (*Borago officinalis*) [158]. Transformation of safflower by introducing *Saprolegnia diclina* Δ^6-desaturase produced GLA to levels higher than 70 % (v/v) [159]. Ruiz-Lopez et al. [151] consider the production of a plant equivalent of marine oil as a major challenge and that transgenic technology is critical for enhancing the levels of PUFAs for food and nutritional security.

5 Conclusions

Functional foods with the bioactive natural phytonutrients are emerging as the next frontier for the biologists and nutritionists alike. The revised US dietary guidelines have laid the emphasis on plant-based fiber and five to nine daily servings of fruits and vegetables with significant reduction in animal fat consumption. Increased understanding of how diet(s) influences whole body health, lesser susceptibility to diseases, high quality of life, and individual's (personal) genetic potential for longevity is highly desired and needed. Such an understanding will be directly linked to efforts for developing crop plants that have the capacity to accumulate

Table 56.7 Sources of omega-6 and omega-3 fatty acids[a]

Linoleic acid (18:2n-6)	Alpha-linolenic acid (18:3n-3)
Safflower oil	Flaxseed oil
Sunflower seeds, oil roasted	Walnuts, English
Pine nuts	Flaxseeds, ground
Sunflower oil	Walnut oil
Corn oil	Canola oil
Soybean oil	Soybean oil
Pecans, oil roasted	Mustard oil
Brazil nuts	Tofu, firm
Sesame oil	Walnuts, black

[a] Adapted from http://lpi.oregonstate.edu/infocenter/othernuts/omega3fa/

Other source: US Department of Agriculture, Agricultural Research Service. USDA National Nutrient Database for Standard Reference, Release 21. 2008

Available at http://www.nal.usda.gov/fnic/foodcomp/search/ to access this information

enhanced levels of beneficial bioactive nutrients. Enhancement of bioactive phytonutrient content in crop plants requires that a clear understanding be in place also of metabolic pathways and regulatory genes that control fluxes of primary and secondary metabolites. Fortuitously, a multitude of technologies are emerging that should help in accomplishing these charges. Thus, deep sequencing, proteomics, and metabolomics are collectively helping unravel “systems biology” of crops by elucidating networks between genes, protein (enzymes) and metabolites, and processes that regulate them. Based on such information, novel strategies of gene engineering are being coupled with transformation of important crops to develop designer crops, functional foods, with the desired enhancement(s) in the levels of bioactive compound(s). Significant progress has already been made in developing such genetically modified plants as is evident from the literature included in this chapter. Undoubtedly, rapid progress is assured in this field in the coming decades. One goal of these developments is to enable dietary intervention in human health to combat monogenic or polygenic diseases.

The recognition of phytonutrients as important determinants of human health has catalyzed investigations into broader aspects of plant-based nutraceuticals. These include elucidating biochemical pathways and identifying the rate-limiting steps; engineering metabolic pathways to direct the intermediary metabolism flux toward a particular nutrient (nutraceutical); testing efficacy of either an isolated nutraceutical or a crop engineered with an enhanced nutrient level in animal and human models; testing crops silenced for a health-detrimental factor including allergins; and comparing bioavailability of an individual nutrient (nutraceutical) fed either as a food supplement or in the form of a fortified food. However, the “functional foods” will have a limited success in advancing public health until their benefits are widely confirmed and effectively communicated to the consumer. In this regard, proactive efforts have been made by the Harvard School of Public Health (Boston, MA, USA) and the International Food Information Council

Table 56.8 Important food crops transformed with biosynthetic and regulatory genes for EFA metabolism

Transgenic	Gene origin/ GE	Promoter/ gene	Enzyme	Metabolic product	Metabolic effect	References
Canola	Bacteria	Napin/ *CrtB*	Phytoene synthase	Fatty acyl composition	Higher 18:1 (oleic acid) component	[122]
Sweet Potato	Tobacco	E12Ω (improved CaMV 35S)/ *NtFAD3*	ω-3 fatty acid desaturase	Linolenic acid	Higher linolenic acid (18:3) 22.1 % versus 10 % control	[160]
Tomato	Rapeseed/ overexpression	CaMV35S/ *FAD3, FAD7*	ω-3 fatty acid desaturase	Linolenic acid (18:3)	Higher 18:3/ 18:2 ratio	[161]
Tomato	Pepper/OE	*Native*/ *FIB1, FIB2*	Involved in synthesis of lipoproteins in certain chromoplast types		2-fold ↑ carotenoids, i.e., 118 % ↑ lycopene 64 % ↑ β-carotene 36 % ↑ β-ionone 74 % ↑ β-cyclocitral 50 % ↑ citral 122 % ↑ 6-methyl-5-hepten-2-one 223 % ↑ geranyl acetone	[162]

Foundation (Washington, D.C.) who released a set of communication guidelines aimed at diverse sections of the society to improve public understanding of emerging science and to help ensure that research results about nutrition, food safety, and health are communicated in a clear, balanced, and non-misleading manner [163].

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Part VI

Phenolics: Classes - Occurrence, Biosynthesis, Structure and Chemistry, Distribution

Flavanols: Catechins and Proanthocyanidins

57

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Contents

1 Introduction 1755
2 Structural Features 1755
2.1 Flavanol Monomers 1755
2.2 Proanthocyanidins 1756
3 Biosynthesis 1761
4 Occurrence 1764
5 Proanthocyanidin-Protein Interaction 1766
6 Copigmentation 1781
7 Chemical Transformations 1782
8 Conclusion 1787
References 1788

Abstract

Flavanols are a wide group of polyphenols that include flavan-3-ols (e.g., catechin and proanthocyanidins), flavan-4-ols, and flavan-3,4-diols. They arise from plant secondary metabolism through condensation of phenylalanine derived from the shikimate pathway with malonyl-CoA obtained from citrate that is produced by the tricarboxylic acid cycle, leading to the formation of the key precursor in the flavonoids biosynthesis: the naringenin chalcone. The exact nature of the molecular species that undergo polymerization and the mechanism of assembly in proanthocyanidins are still unknown. From a structural point of view, flavanols

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comprise a C15 (C6-C3-C6) general structure composed by a benzopyran moiety (A and C rings) with an additional aromatic ring (B ring) linked to carbon C-2 of C ring. Flavanols are present in nature in monomeric, oligomeric, and polymeric forms and differ from each other essentially in the configuration of carbon C-2, the hydroxylation/methoxylation pattern of the rings, the type of linkage between each unit, and the degree of galloylation. Flavanols in foods are described to present several beneficial effects such as antioxidant and anticarcinogenic properties and also contribute to the sensory properties of some food products, such as astringency and color. Some of these aspects are discussed herein.

Keywords
Biosynthesis • Chemical reactions • Flavan-3,4-diols • Flavan-3-ols • Flavan-4-ols • Flavanols • Occurrence • Proanthocyanidins • Structural features

Abbreviations

4CL	4-Coumarate:CoA ligase
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
C	Catechin
C3G	Catechin-3-*O*-gallate
C4H	Cinnamate 4-hidroxylase
CGCC	Catechin-gallocatechin-catechin
CHI	Chalcone isomerase
CHS	Chalcone synthase
DFR	Dihydroxyflavonol 4-reductase
DP	Degree of polymerization
E3G	Epicatechin-3-*O*-glucoside
EC	Epicatechin
EC3G	Epicatechin-3-*O*-gallate
EGC	Epigallocatechin
EEC	Epicatechin-epicatechin-catechin
EGC3G	Epigallocatechin-3-*O*-gallate
F3′5′H	Flavonoid 3′,5′-hydroxylase
F3H	Flavanone 3β-hydroxylase
F3′H	Flavonoid 3′-hydroxylase
GC	Gallocatechin
GC3G	Gallocatechin-3-*O*-gallate
GCCC	Gallocatechin-catechin-catechin
GCGCC	Gallocatechin-gallocatechin-catechin
HCA-CoA	Hydroxycinnamic acid-CoA
HMF	Hydroxymethylfurfural
HPLC	High-performance liquid chromatography
LAR	Leucoanthocyanidin reductase
MW	Molecular weight
PAL	Phenylalanine ammonia lyase

PAs	Proanthocyanidins
PCs	Procyanidins
PDs	Prodelphinidins
PPO	Polyphenol oxidase
PRPs	Proline-rich proteins

1 Introduction

Flavanol monomers and proanthocyanidins (*syn.* condensed tannins) are polyphenolic compounds derived from plant secondary metabolism being present in a wide variety of plants and plant-derived foods such as fruits, cereals, seeds, wines, ciders, teas, beers, and cocoa [1–7].

Flavanols are involved in the protection against the abiotic (e.g., sunlight) and the biotic stress (e.g., predation, pathogen attack) of plants [8, 9]. Proanthocyanidins (PAs) have the capacity to interact and precipitate alkaloids and proteins [10, 11]. Their ability to precipitate salivary proteins in the oral cavity is described to be at the origin of the astringency character that is generally associated to tannin-rich foods [11–13]. Flavanols can also contribute to the color of some food products such as red wines, in one hand through their association with anthocyanins (copigmentation phenomenon) enhancing the color of red wines [14–19] and on the other hand by their chemical reaction with anthocyanins leading to the formation of new colored compounds with different spectroscopic features [20–26]. Furthermore, flavanols may form stable complexes with metal ions [27–30] influencing the bioavailability of several minerals [31, 32]. Like other polyphenols, flavanols are good reducing agents showing important antioxidant and radical-scavenging properties [33–36]. Based on these properties, numerous studies have been published evidencing PAs health benefits over the last years. Proanthocyanidins have been shown to prevent low-density lipoproteins [37–40] and lipid peroxidation [41–44] and also to inhibit platelet aggregation [45, 46], which are two major mechanisms described to be at the origin of arteriosclerosis and cardiovascular diseases [47, 48]. Several in vitro studies have also suggested a protective role of PAs against several types of cancers [49–51]. More recently, PAs have been shown to present some antinutritional effects as they were found to inhibit the three main classes of digestive enzymes: lipases [52], glycosidases [53], and proteases [54, 55].

2 Structural Features

2.1 Flavanol Monomers

Flavan-3-ols, flavan-4-ols, and flavan-3,4-diols are different classes of flavonoid compounds comprising a C15 (C6-C3-C6) general structure of a benzopyran moiety (A and C rings) that presents an aromatic ring (B ring) linked to carbon C-2 of

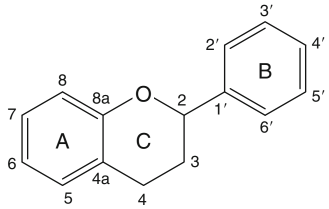


Fig. 57.1 General structure of the flavanic core

pyranic ring C (Fig. 57.1). The difference between each of these classes is in the hydroxylation pattern of the pyran ring. On the other hand, in the case of flavan-3-ols, flavan-4-ols, and flavan-3,4-diols, there is a hydroxyl group present at carbon C-3, C-4 or C-3, and C-4, respectively. In the case of flavans, there is no hydroxyl group in the pyranic ring.

However, these latter are more rarely found in nature [56–58]. Flavan-4-ols and flavan-3,4-diols are also unlikely to be detected in nature due to their high reactivity as electrophiles in weakly acidic conditions [59, 60].

On the other hand, flavan-3-ols are very abundant in nature (Table 57.1), and their structures differ from each other in the stereochemistry of the asymmetric carbons (C-2 and C-3) of the pyranic ring C and in the hydroxylation pattern of rings A and B. The most common flavan-3-ols in plant kingdom are hydroxylated at carbons C-5 and C-7 in ring A, differing only in the hydroxylation pattern of ring B and in the stereochemistry of carbon C-3 from ring C [62, 63] (Fig. 57.2). Carbon C-2 in the naturally occurring flavan-3-ols is almost exclusively present in the 2*R* configuration. The less common flavan-3-ols presenting a carbon C-2 with a 2*S* configuration are named with the prefix *ent*, as in *ent*-catechin ((−)-catechin) that has a 2*S*,3*R* absolute configuration [63, 64]. The carbon C-3 can be found in the 3*S* or 3*R* configuration. For example, in flavan-3-ols with an *ortho*-dihydroxylated ring B (C-3′,C-4′), two situations may be observed: carbons C-2 and C-3 present a 2*R*,3*S* absolute configuration (*trans* conformation) like in (+)-catechin or present a 2*R*,3*R* configuration (*cis* conformation) as in (−)-epicatechin (Fig. 57.2). Flavan-3-ols with a 3*R* absolute configuration in carbon C-3 present the prefix epi. Furthermore, ring B monohydroxylated and trihydroxylated give rise to (+)-afzelechin or (−)-epiafzelechin, (+)-gallocatechin or (−)-epigallocatechin, respectively (Fig. 57.2).

Flavan-3-ols can also be esterified with gallic acid or glucosylated in the hydroxyl group of carbon C-3 of the pyranic ring C [65, 66], although the glucosylated forms are scarce in the plant kingdom [63, 67, 68] (Fig. 57.3).

2.2 Proanthocyanidins

Proanthocyanidins are oligomeric or polymeric chains of flavan-3-ols that are present in nature in a great diversity of structures. This is due to structural features of the monomeric units and also to the type of interflavanic bond, the degree of polymerization, and esterification with gallic acid [69, 70].

Table 57.1 Distribution and degree of polymerization of the proanthocyanidins in food products [61]

	Degree of polymerization							
	1	2	3	4–6	7–10	>10	Total	Type[a]
Product	(mg/100 g fresh weight or mg/L for drinks)							
Blueberry	4.0 ± 1.5	7.2 ± 1.8	5.4 ± 1.2	19.6 ± 3.4	14.5 ± 2	129.0 ± 47.3	179.8 ± 50.8	PC
Black currant	0.9 ± 0.2	2.9 ± 0.4	3.0 ± 0.3	10.6 ± 1.7	9.9 ± 1.4	122.4 ± 28	147.8 ± 33	PC. PD
Cranberry	7.3 ± 1.5	25.9 ± 6.1	18.9 ± 3.4	70.3 ± 13.1	62.9 ± 14.7	233.5 ± 49.1	418.8 ± 75.3	A. PC
Strawberry	4.2 ± 0.7	6.5 ± 1.3	6.5 ± 1.2	28.1 ± 6.5	23.9 ± 3.5	75.8 ± 13.4	145.0 ± 24.9	PP. PC
Apple[b]	9.6 ± 0.9	13.8 ± 0.6	9.3 ± 0.4	30.2 ± 1.2	25.4 ± 1.2	37.6 ± 2.6	125.8 ± 6.8	PC
Apple juice[b]	1 ± 0	2 ± 0	1 ± 0	4 ± 0	1 ± 0	ND	9 ± 0	PC
Pear	2.7 ± 1.5	2.8 ± 1.3	2.3 ± 0.9	6.5 ± 1.9	4.6 ± 1	13.1 ± 11.3	31.9 ± 7.8	PC
Prune	11.4 ± 3.4	31.5 ± 7.4	23.9 ± 5.1	58.0 ± 12.5	33.8 ± 11.9	57.3 ± 24.4	215.9 ± 50.7	A. PC
Peach	4.7 ± 1.4	7.0 ± 2.2	5.0 ± 1.4	17.7 ± 5.5	10.9 ± 3.7	22.0 ± 7.7	67.3 ± 20.9	PC
Sorghum	27.8 ± 1.2	78.2 ± 3.4	99.2 ± 7.7	585.5 ± 50	734.3 ± 69.3	2440.4 ± 271	3965.4 ± 402.5	PC
Barley	11.0 ± 0.3	21.4 ± 1.1	14.6 ± 1	27.2 ± 0.6	ND	ND	74.2 ± 3	PC
Hazelnut	9.8 ± 1.6	12.5 ± 3.8	13.6 ± 3.9	67.7 ± 20.3	74.6 ± 21.9	322.4 ± 102.5	500.7 ± 152	PC. PD
Pistachio	10.9 ± 4.3	13.3 ± 1.8	10.5 ± 1.2	42.2 ± 5.2	37.9 ± 4.9	122.5 ± 37.1	237.3 ± 52	PC. PD
Almond	7.8 ± 0.9	9.5 ± 1.6	8.8 ± 1.7	40.0 ± 8.5	37.7 ± 8.4	80.3 ± 28.1	184.0 ± 48.2	PP. PC
Walnut	6.9 ± 3.4	5.6 ± 0.9	7.2 ± 1.2	22.1 ± 3.3	5.4 ± 0.8	20.0 ± 9.3	67.3 ± 14.7	PC
Peanut (butter)	2.0 ± 0.9	3.0 ± 0.7	8.1 ± 3.5	ND	ND	ND	13.2 ± 5.2	A. PC
Dark chocolate	31.4 ± 0.2	31.2 ± 0.9	21.1 ± 0.8	55.5 ± 3.5	38.5 ± 3	68.2 ± 8.8	246.0 ± 0.3	PC
Milk chocolate	26.9 ± 3	26.2 ± 2.5	19.3 ± 2.6	51.4 ± 9.8	35.3 ± 7.2	32.8 ± 9.2	192.0 ± 28.8	PC
Bear	4 ± 0	11 ± 1	3 ± 0	4 ± 0	ND	ND	23 ± 2	PC. PD
Red wine	20 ± 1	40 ± 1	27 ± 1	67 ± 2	50 ± 1	110 ± 2	313 ± 5	PC. PD
Grape (juice)	18 ± 0	34 ± 0	19 ± 0	80 ± 0	69 ± 0	303 ± 2	524 ± 2	PC. PD
Grape (dry seed)	660.3 ± 8.3	417.3 ± 4.8	290.2 ± 4.5	664.0 ± 8.2	400.3 ± 31.3	1100.1 ± 86.3	3532.3 ± 105.8	PC

[a] Type: *PP* propelargonidins, *PC* procyanidins; *PD* prodelphinidins; A-polymers with A-type linkage

[b] Apple Red Delicious; Apple juice Red Delicious peeled

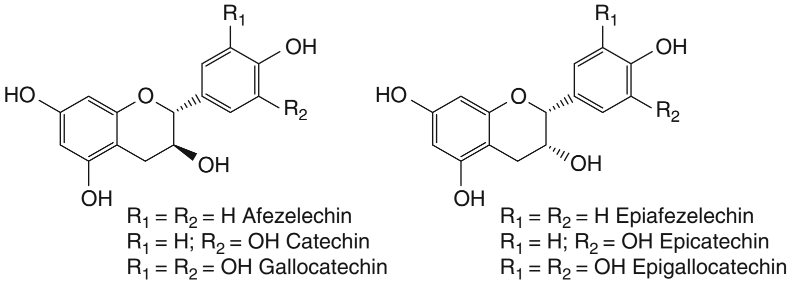
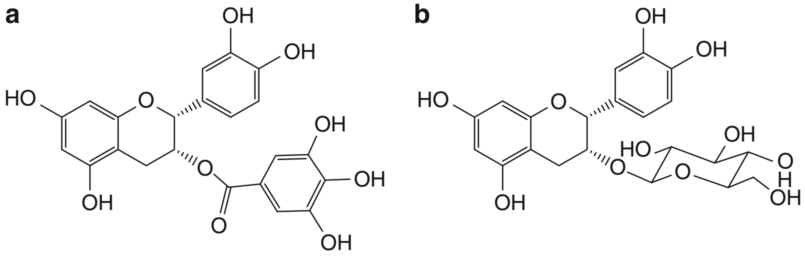

R1
OH
HO
O
R2
OH
OH
R1 = R2 = H Afezelechin
R1 = H; R2 = OH Catechin
R1 = R2 = OH Gallocatechin
R1
OH
HO
O
R2
OH
OH
R1 = R2 = H Epiafezelechin
R1 = H; R2 = OH Epicatechin
R1 = R2 = OH Epigallocatechin

Fig. 57.2 Structure of the flavan-3-ols monomeric units (afezelechins, catechins and gallocatechins)


a
OH
OH
HO
O
OH
O
OH
OH
OH
O
b
OH
OH
HO
O
OH
HO
O
O
O
H
OH
OH

(-)-Epicatechin-3-*O*-gallate

(-)-Epicatechin-3-*O*-glucoside

Fig. 57.3 Structure of the 3-*O*-subtituted (-)-epicatechin with (**a**) a galloyl and (**b**) a glucosyl group

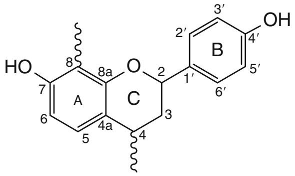
PAs can be divided in two main groups according to their ring A type: phloroglucinol or resorcinol derivatives (Fig. 57.4) [71, 72]. A-type phloroglucinol ring proanthocyanidins are much more abundant in nature. Among these, procyanidins (PCs) and prodelphinidins (PDs) represent the most common classes of proanthocyanidins with 3′,4′-dihydroxylated (catechins) and 3′,4′,5′-trihydroxylated (gallocatechins) ring B, respectively (Fig. 57.5) [10]. The names procyanidin and prodelphinidin result from the fact that these compounds are decomposed by heat in a strong mineral acidic medium, leading to the formation of cyanidin and delphinidin, respectively, according to the mechanism proposed by Bate-Smith [13].

Dimeric and trimeric PAs are divided in different groups according to the kind of interflavanic bond that is present between each monomeric unit. Each group is identified with a letter A, B, C, or D and a number that identifies the different isomers between each group with same type of interflavanic bond [73, 74].

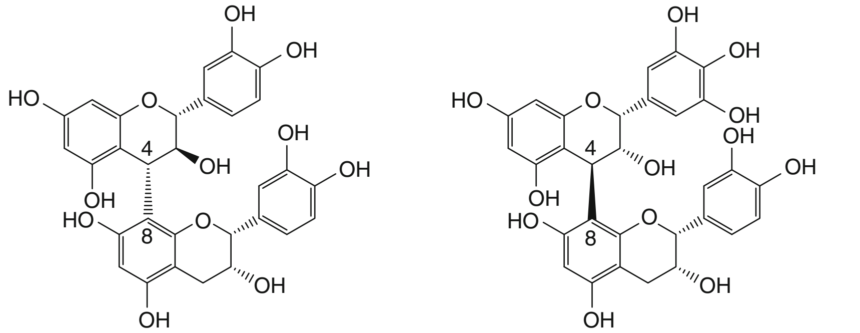
B-type dimeric PAs (Fig. 57.6) result from the condensation of two flavan-3-ol molecules where the upper unit is linked through carbon C-4 to the carbon C-8 or C6 of the lower unit [74]. In PCs and PDs, the C-4 → C-8 interflavanic bond is *trans* regarding to the hydroxyl group at carbon C3 [75].

In A-type dimeric PAs, in addition to the C-4 → C-8 or C-4 → C-6 linkage, there is an additional ether linkage between the carbon C2 of the upper unit and the hydroxyl group in carbon C-5 or C-7 of the lower unit (Fig. 57.7).

Fig. 57.4 Proanthocyanidins types



A ring type	Proanthocyanidin type	C-3	C-5	C-3′	C-5′
Resorcinol	Procassinidin	H	H	H	H
	Probutinidin	H	H	OH	H
	Proguibourtinidin	OH	H	H	H
	Profisetinidin	OH	H	OH	H
	Prorobinetinidin	OH	H	OH	OH
Phloroglucinol	Proapigenidin	H	OH	H	H
	Proluteolinidin	H	OH	OH	H
	Protricetinidin	H	OH	OH	OH
	Propelargonidin	OH	OH	H	H
	Procyanidin	OH	OH	OH	H
	Prodelphinidin	OH	OH	OH	OH



Procyanidin dimer B4
((+)-catechin-(4α→8)-(–)-epicatechin)

Prodelphinidin dimer B2
((–)-epigallocatechin-(4β→8)-(–)-epicatechin)

Fig. 57.5 Structures of B-type dimeric proanthocyanidins

Trimeric PAs can also be divided in two groups: C-type PAs that present two B-type linkages and D-type PAs that present one B-type and one A-type linkage. Higher polymerized structures may have a very high number of isomeric forms due to the variety of the bond linkage types and also to the stereochemistry of the monomeric units. Oligomeric PAs have up to six monomeric units, and polymeric ones have more than six condensed units (Fig. 57.8) [71].

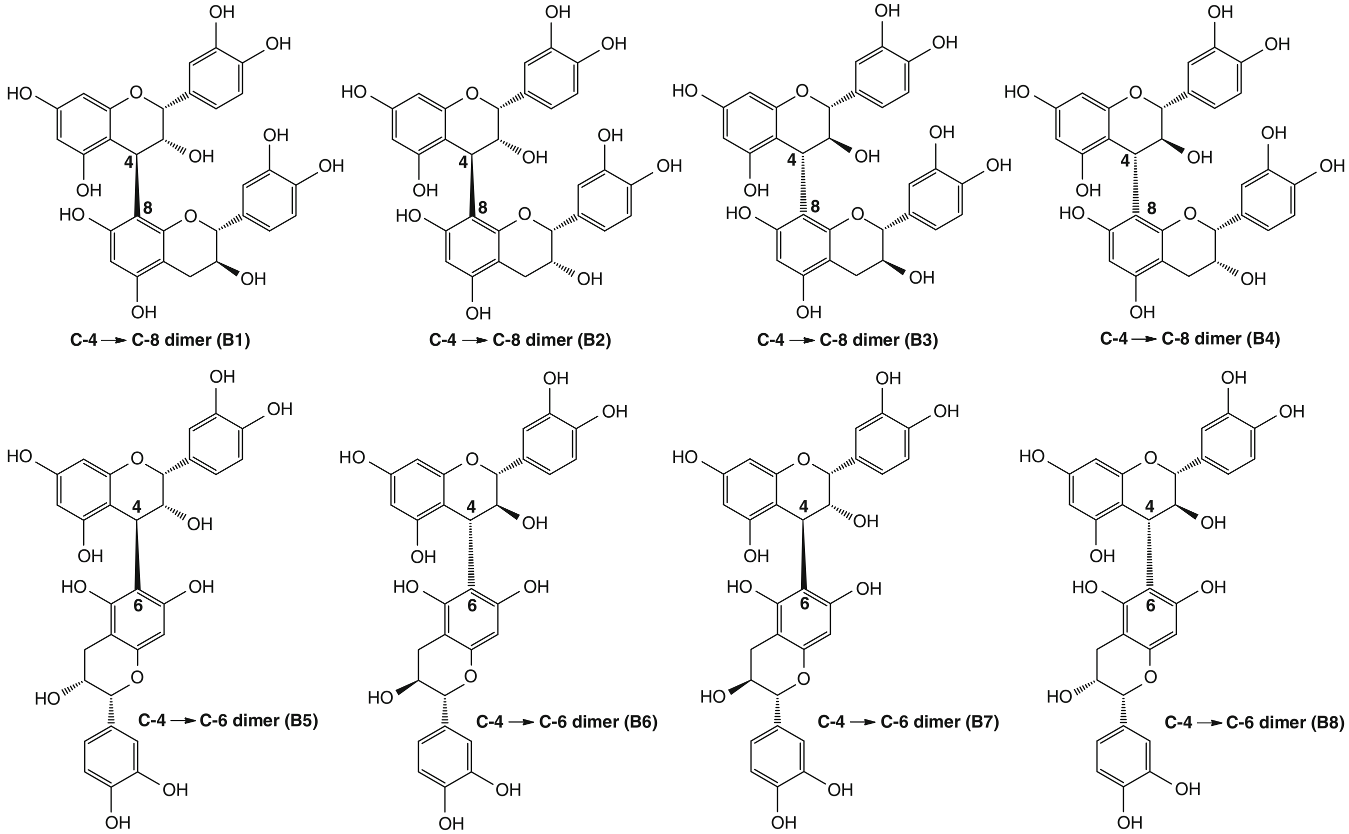

C-4 → C-8 dimer (B1)
C-4 → C-8 dimer (B2)
C-4 → C-8 dimer (B3)
C-4 → C-8 dimer (B4)
C-4 → C-6 dimer (B5)
C-4 → C-6 dimer (B6)
C-4 → C-6 dimer (B7)
C-4 → C-6 dimer (B8)
HO
OH
O
4
8
6

Fig. 57.6 B-type procyanidins with C-4→C-8 (B1-B4) and C-4→C-6 (B5-B8) interflavanic linkages

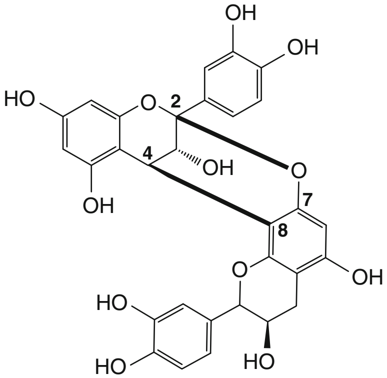

OH
OH
HO
O
2
4
OH
O
7
OH
8
O
OH
HO
HO
HO

Fig. 57.7 Structure of a A-type proanthocyanidin (procyanidin A2)

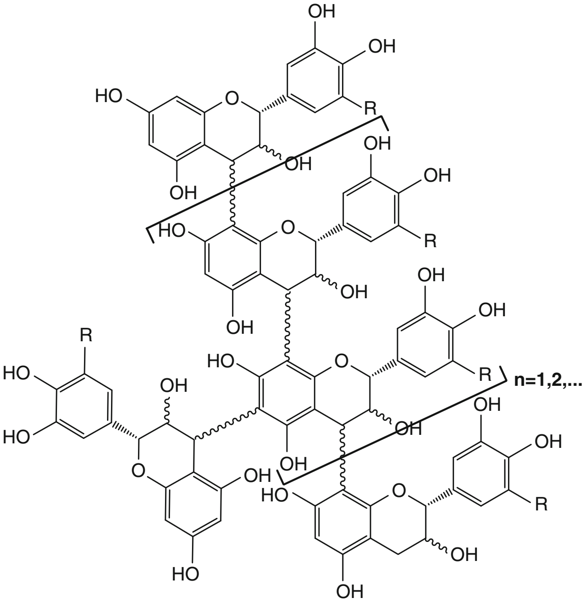

OH
OH
HO
O
R
OH
OH
OH
OH
HO
O
R
OH
OH
OH
OH
R
HO
O
R
n=1,2,...
HO
OH
HO
OH
OH
OH
OH
OH
OH
HO
O
R
O
OH
HO
OH
OH

Fig. 57.8 General structure of polymerized proanthocyanidins [71]

A nomenclature may also be adopted where the location of the interflavanic bond is represented within parentheses, and its orientation at C-4 is represented as α or β as in the IUPAC rules. For example, procyanidin B1 can also be represented as (−)-epicatechin-(4β → 8)-(+)-catechin (Fig. 57.5).

3 Biosynthesis

The two key flavonoid biosynthetic precursors are phenylalanine derived from the shikimate pathway and malonyl-CoA obtained from citrate that is produced by the tricarboxylic acid cycle (Fig. 57.9). Phenylalanine is converted into 4-coumaroyl-CoA through the action of several enzymes such as PAL (phenylalanine ammonia lyase) [76], C4H (cinnamate 4-hydroxylase) [77], and 4CL (4-coumarate:CoA ligase) [78]. Then, the first precursor in the PAs biosynthesis and in all flavonoids biosynthesis – naringenin chalcone – is formed from the condensation and subsequent intramolecular cyclization of three malonyl-CoA molecules with one hydroxycinnamic acid-CoA (HCA-CoA) ester, usually 4-coumaroyl-CoA catalyzed by the enzyme CHS (chalcone synthase) [79]. Then, naringenin chalcone is isomerized to naringenin flavone. Although this isomerization can occur spontaneously, it was demonstrated that the presence of CHI (chalcone isomerase) stereospecifically directs and accelerates the intramolecular cyclization of the chalcone to form the (2*S*)-flavanone naringenin through an acid catalysis mechanism [80, 81]. F3′H (flavonoid 3′-hydroxylase) and F3′,5′H (flavonoid 3′,5′-hydroxylase) catalyze the formation of the 3′-hydroxyl (eriodictyol) and 3′,5′-hydroxyl (pentahydroxyflavanone) groups in ring B of naringenin, respectively [82, 83]. By this way, these enzymes determine the B-ring hydroxylation pattern of flavan-3-ols and consequently of PA molecules. Additionally, (2*S*)-flavanones are stereospecifically converted to (2*R*,3*R*)-dihydroflavonols (dihydrokaempferol, dihydroquercetin, and dihydromyricetin) by the enzyme flavanone 3β-hydroxylase (F3H) [84, 85]. The dihydrokaempferol (dihydroflavonol) can also be substrate for the enzymes F3′H and F3′,5′H, leading to the formation of dihydroquercetin and dihydromyricetin, respectively [82, 83]. After this, (2*R*,3*R*)-dihydroflavonols are stereospecifically reduced by DFR (dihydroflavonol 4-reductase), giving rise to the respective leucoanthocyanidins ((2*R*,3*S*,4*S*)-flavan-2,3-*trans*-3,4-*cis*-diols) or flavan-3,4-diols (leucopelargonidin, leucocyanidin, and leucodelphinidin) [85, 86]. Anthocyanin synthase (ANS), also known as leucoanthocyanidin dioxygenase (LDOX), plays an important role not only in the anthocyanins biosynthesis but also in that of PAs [87]. Its function is to catalyze the oxidation of leucoanthocyanidins to anthocyanidins (pelargonidin, cyanidin, and delphinidin) [88]. The final step in the formation of the 2*R*,3*R*-*cis*-flavan-3-ols ((−)-epiafzelechin, (−)-epicatechin, and (−)-epigallocatechin) involves the reduction of anthocyanidins (or the pseudobase form) catalyzed by ANR (anthocyanidin reductase) enzyme [89]. Through another pathway, leucoanthocyanidins can also be substrate for the enzyme LAR (leucoanthocyanidin reductase) that removes the 4-hydroxyl group from leucoanthocyanidins to produce the respective 2*R*,3*S*-*trans*-flavan-3-ols such as (+)-afzelechin, (+)-catechin, and (+)-gallocatechin [90, 91].

The shikimate pathway is also important in the formation of other molecules such as gallic acid [92] that is essential for the synthesis of galloylated PAs, which are the major PAs found in numerous plants, for instance, in tea (*Camellia sinensis*) and grapes (*Vitis vinifera*) [93, 94]. Nevertheless, the galloylation mechanism in PAs remains unknown.

Flavanol biosynthesis occurs in the cytoplasm where all the enzymatic machinery is mostly present [95]. Although PAs are mainly found in the cell vacuoles [96–100], they can also be found bound to proteins of the internal face of the tonoplast or bound to cell wall polysaccharides [100–103]. So, it is important to understand the

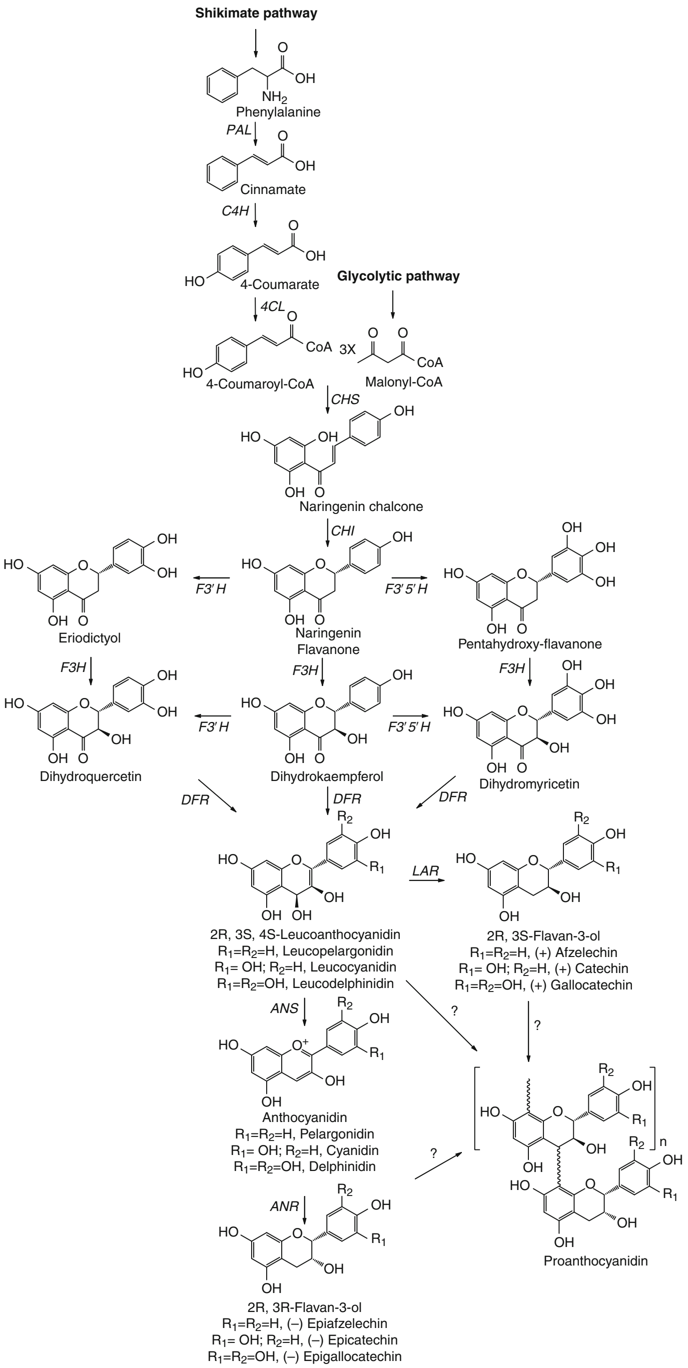

Shikimate pathway
Phenylalanine
PAL
Cinnamate
C4H
4-Coumarate
Glycolytic pathway
4CL
4-Coumaroyl-CoA
3X
Malonyl-CoA
CHS
Naringenin chalcone
CHI
Eriodictyol
F3'H
Naringenin
Flavanone
F3'5'H
Pentahydroxy-flavanone
F3H
Dihydroquercetin
Dihydrokaempferol
Dihydromyricetin
DFR
2R, 3S, 4S-Leucoanthocyanidin
R1=R2=H, Leucopelargonidin
R1= OH; R2=H, Leucocyanidin
R1=R2=OH, Leucodelphinidin
LAR
2R, 3S-Flavan-3-ol
R1=R2=H, (+) Afzelechin
R1= OH; R2=H, (+) Catechin
R1=R2=OH, (+) Gallocatechin
ANS
Anthocyanidin
R1=R2=H, Pelargonidin
R1= OH; R2=H, Cyanidin
R1=R2=OH, Delphinidin
ANR
2R, 3R-Flavan-3-ol
R1=R2=H, (–) Epiafzelechin
R1= OH; R2=H, (–) Epicatechin
R1=R2=OH, (–) Epigallocatechin
?
Proanthocyanidin
n

Fig. 57.9 General flavonoid biosynthetic pathway leading to the formation of flavan-3-ols and proanthocyanindins

flavan-3-ol polymerization that seems to occur also in the vacuole and the transport of the PA precursors from the cytoplasm to the vacuole [104–106].

Pang et al. [107] demonstrated that in the early stages of seed development in *M. truncatula*, it is possible to detect the presence of epicatechin-3-*O*-glucoside (E3G) that is thought to be the vacuolar transport of epicatechin. However, its fate inside the vacuole is still not known. The levels of E3G decrease to zero with the increase of the PA content, which suggests that hydrolysis occurs and that epicatechin is incorporated in the PA polymer. The exact nature of the molecular species that undergo polymerization and the mechanism of assembly in PAs is still unknown [94]. However, there are a few hypotheses trying to explain how this may occur, but all the models stated until now seem to fail at some point.

Most studies point to a nonenzymatic pathway for the polymerization of PAs involving the condensation of the molecules through the electrophilic attack of the carbon C-4 from the pyranic ring C of an extension unit (leucoanthocyanidin) to a nucleophilic carbon C-8 or C-6 of a terminal unit (flavan-3-ol) [76, 90, 91, 108, 109]. Polymerization continues with the electrophilic attack of carbon C-4 of a flavan-3,4-diol to the carbon C-8 or C-6 from the dimer previously formed. However, this hypothesis fails in the fact that the majority of extension units in PAs found in *planta* are 2,3-*cis* and leucoanthocyanidins are 2,3-*trans* [86]. Thus, it was proposed that a leucoanthocyanidin derivative must be involved in the polymerization mechanism, such as quinone methides and carbocation products. Another proposal comprises the conversion of the 2*R*,3*S*,4*S*-leucoanthocyanidins into 2*R*,3*S*-quinone methides that can be directly used as extension units. These compounds can also be converted in 2*R*,3*R*-quinone methides via flavan-3-en-3-ol that can be further used as extension units [110]. In another pathway, the 2*R*,3*S* and 2*R*,3*R*-quinone methides can be transformed in the respective carbocations and attack (+)-catechin or (−)-epicatechin, leading to the formation of the diverse PA molecules (Fig. 57.10).

An enzymatic approach for the polymerization of PAs includes the conversion of flavan-3-ols ((+)-catechin and (−)-epicatechin) to the corresponding quinone methide catalyzed by the enzyme PPO (polyphenol oxidase). These quinone methide could be converted in the corresponding carbocations with flavan-3-en-3-ol as intermediate or reduced to the carbocation through coupled nonenzymatic oxidation. These carbocation products might be used as direct extension units. Since PPO is mainly present in chloroplasts and PAs synthesis occur in the vacuoles [111], another form of PPO has to exist with an alternative localization [112] in order to the enzymatic pathway for the polymerization of PAs be possible. Nevertheless, polymerization of flavan-3-ols mediated by PPO did not give rise to PAs with a natural structure but oligomers composed by oxidized flavan-3-ols with specific configurations [113].

4 Occurrence

Flavanols are present in nature in a great diversity of plants. However, the data available in the literature about their concentrations in plants and foodstuffs regard

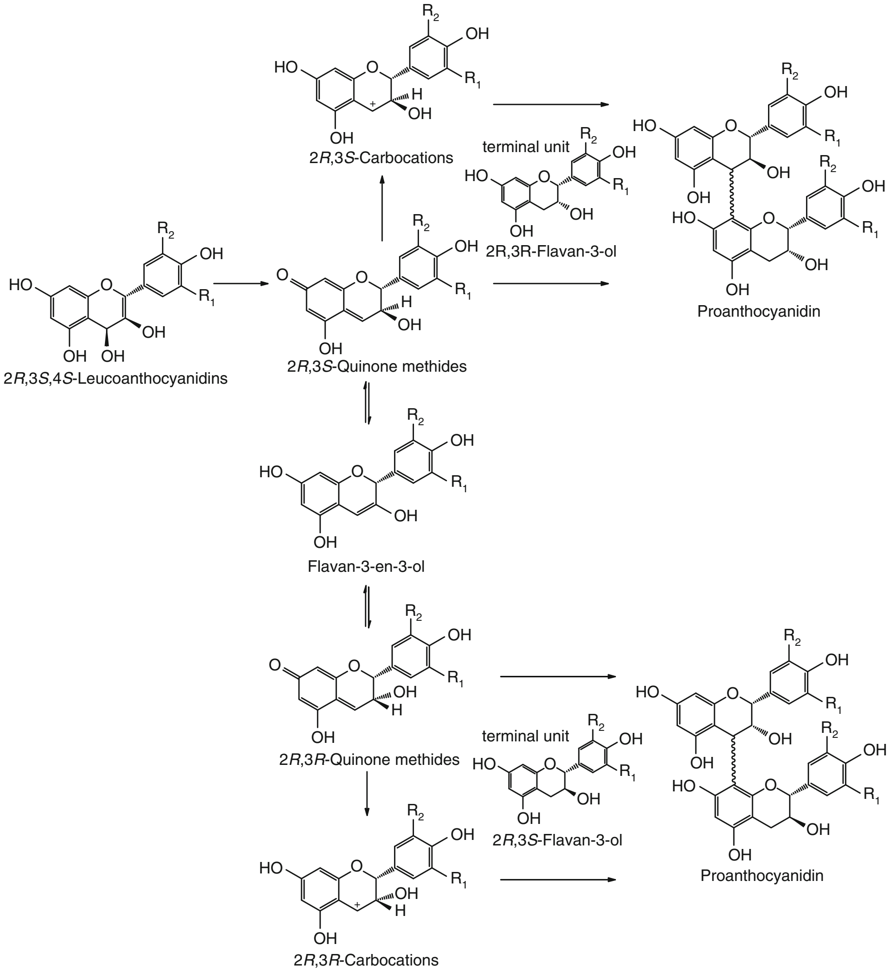

2R,3S,4S-Leucoanthocyanidins
2R,3S-Quinone methides
2R,3S-Carbocations
terminal unit
2R,3R-Flavan-3-ol
Proanthocyanidin
Flavan-3-en-3-ol
2R,3R-Quinone methides
2R,3R-Carbocations
terminal unit
2R,3S-Flavan-3-ol
Proanthocyanidin
HO
OH
R1
R2
O
H

Fig. 57.10 Putative formation of quinone methides and carbocations leading to the formation of proanthocyanidins

mainly flavanols in general and not specially to the PAs as these are difficult to extract and hence hard to quantify. PA oligomers and polymers are present in food extracts as complex mixtures, which makes their separation very difficult. By using reverse phase HPLC, the presence of many isomers yields in overlapping retention times of different PAs, being the separation of higher oligomers (DP > 4) and polymers not possible [114, 115]. The acid-catalyzed depolymerization of PAs in the presence of a nucleophilic agent such as phloroglucinol or benzyl mercaptan converts the flavan-3-ol extension units into the respective phloroglucinol adducts or thioesters, respectively, and the flavan-3-ol terminal units are released [116]. This allows the determination of the proportion of the constitutive units of the PAs present and also the estimation of the mean degree of polymerization (mDP). However, this method does not allow the determination of the proportion of individual PAs. Using normal-phase HPLC, it is possible to separate PA oligomers according to their degree of polymerization up to 10 [117, 118]. It is also possible to obtain a single group of PAs with DP $>$ 10 using a sharp gradient elution at the end of the HPLC run [117]. The extraction of PAs in their native form is most of the times very difficult as they have the tendency to form strong complexes with the insoluble polymeric plant material such as cell wall polysaccharides [115, 119–121]. Hence, the total PA content is most of the times underestimated if only the extractable PAs are quantified. There are a few studies combining normal phase and reverse phase HPLC to determine the extractable and unextractable PAs, respectively [122, 123]. Table 57.1 shows the content of PAs of several food products according to the degree of polymerization [61]. According to this data, in almost all food products, the main fraction of PAs refers to the most polymerized one (DP $>$ 10).

During fruit ripening, the amount of flavan-3-ols and proanthocyanidins of some fruits was described to decrease sharply from the early stages of development where they are present at the highest concentration until the harvest period where they stay constant. This drop in flavanol concentration affects more flavan-3-ol monomers than the proanthocyanidin dimers [124–130]. On the other hand, it appears that the mean degree of polymerization changes during fruit ripening, but the increase or decrease depends on the type of fruit [124, 127, 131–134]. For example, in grape seeds, the mDP seems to decrease and in grape skins to increase during ripening [131].

Tables 57.2 and 57.3 show the contents in flavan-3-ol monomers, as well as dimeric and trimeric PAs of several different sources of plants and foodstuffs determined by chromatographic techniques. There is a great divergence between the values obtained for a given product by the different authors, and this may be due to the method used in the extraction and quantification or may be owned to the intrinsic variability of the sample that includes the variety, stage of ripeness, and processing into foodstuff. From Table 57.2, it is possible to observe that (+)-catechin and (−)-epicatechin are the flavan-3-ol monomeric units more abundant in all the food products and that the 3-*O*-galloylated forms such as (+)-catechin-3-*O*-gallate, (+)-gallocatechin-3-*O*-gallate, (−)-epicatechin-3-*O*-gallate, and (−)-epigallocatechin-3-*O*-gallate are mainly present in green, oolong, and black teas. With respect to dimeric PAs (Table 57.3), it seems that B1, B2, B3, and B4 are the most abundant dimers in all the food products analyzed.

5 Proanthocyanidin-Protein Interaction

The term “tannins” also attributed to proanthocyanidins (condensed tannins) was defined by Bate-Smith and Swain in 1962 as “water soluble phenolic compounds having molecular weights between 500 and 3,000 Da and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatine and other proteins.” Tannins have been widely employed in the tanning process converting hide collagen in leather [292].

Table 57.2 Distribution of flavan-3-ol monomers in food products

		Flavan-3-ols								
		C	C3G	GC	GC3G	EC	EC3G	EGC	EGCG	References
Alcoholic beverages (mg/100 mL)										
Beers		0–1.01				0–0.38				[5, 135–145]
Ciders						0.03				
Wines	White	0–4.60		0–0.01		0–6.0				[137, 140, 141, 146–153]
	Red	1.38–39.0		0–0.42		0–16.50	0–0.93	0–0.28		[140, 141, 146–158]
	Rose	0.35–1.06		0.18		0.33–0.65	0–0.13	0.07		[146, 147, 149]
Cereals and cereal products (mg/100 g)										
Cereals		1.00–1.40								[142]
Cocoa										
Chocolate	Dark	10.75–50.00				32.74–125.00				[140, 159]
	Milk	2.69–12.0				12.49–24.00				[140, 159]
Cocoa	Powder	61.00–202.00				63.00–330.00				[159, 160]

(*continued*)

Table 57.2 (continued)

		Flavan-3-ols								References
		C	C3G	GC	GC3G	EC	EC3G	EGC	EGCG	
Fruits and fruit products (mg/100 g)										
Fruit berries	Red raspberry	0–0.97				0.30–8.26				[140, 161]
	Strawberry	1.57–18.74		0–0.12		0–0.02	0–0.66	0–0.15		[20, 140, 147, 161, 162]
	Black currant	0.70				0.47		0–0.36		[140]
	Blackberry	0.66–0.79				2.67–18.08				[140, 147]
	Red currant	1.22–1.33		1.22–1.35		0–0.19		0–0.36		[140, 147]
	White currant	0.30		0.70						[140]
	Gooseberry	1.67		0.44						[140]
	Cloudberry	0.50								[161]
	Red grape	0.82–8.94				0.70–8.64	0.17–2.81	0–0.08		[140, 147]
	White grape	0–2.47		0–0.03		0–1.02	0–0.43	0–0.04		[140, 147, 163]
Fruits – drupes	Peach, whole	2.33								[140]
	Peach, peeled	0.53–19.67				0.65–16.48	0.01			[147, 164, 165]
	Sweet cherry	0.61–2.17				5.45–9.53	0–0.20	0–0.11		[140, 147]
	Plum, fresh	3.35–5.40				0–4.35				[140, 147, 166]
	Apricot	0.31–6.62				0.02–8.28				[140, 147, 166, 167]
	Nectarine, whole	1.09–11.29								[140, 168]
	Nectarine, peeled	0–23.60				1.32–5.64				[165, 168]
	Apricot, puree	1.25–2.02				2.11–4.57				[167]

Fruits – pomes	Apple (dessert), whole	0–3.40			1.80–19.16				[140, 167, 169–171]
	Apple (dessert), peeled	0–5.52			0–19.80	0–0.19			[140, 147, 172–176]
	Apple (cider), peeled	0–58.04			0–141.00				[175–178]
	Pear, whole	0–0.96			0.16–7.54				[140, 179]
	Pear, peeled	0–2.10			0.64–3.85				[140, 147, 180]
	Quince, peeled	0.75			0.67				[147]
	Medlar	0.02			0.53	0.23	0.01		[147]
	Apple (dessert), puree	2.02			4.57				[167]
Fruits – tropical fruits	Mango	1.72							[140]
	Banana	0–10.29			0–0.20		0–0.01		[140, 147, 181, 182]
	Pomegranate	0.4		0.17	0.08		0.16		[147]
	Custard apple	0.58			5.63				[147]
	Persimmon, fresh	0.63		0.17					[147]
	Kiwi		0–0.10		0.10–0.60			0–0.20	[181]
Jams	Strawberry, jam	0.90							[140]
	Apricot, jam	0.15–0.66			0–1.37				[140, 167]
Nonalcoholic beverages (mg/100 mL)									
Cocoa beverage – chocolate	Chocolate, milk, beverage	1.20–16.10			0.60–11.14				[140]

(*continued*)

Table 57.2 (continued)

		Flavan-3-ols								
		C	C3G	GC	GC3G	EC	EC3G	EGC	EGCG	References
Fruit juices – berry juices	Sea-buckthorn berry, pure juice	1.90–2.60				0.28–0.52				[183]
	Fox grape (green), pure juice	1.30–1.70				1.70–3.10				[184]
	Grape (green), pure juice	0–5.80				0.11–6.30				[184, 185]
Fruit juices – drupe juices	Plum, prune pure juice	8.40–41.00								[186]
	Plum, prune juice from concentrate	17.90								[186]
	Apricot, nectar	0–0.66				0–1.22				[167]
Fruit juices – pome juices	Apple (dessert), pure juice	0–6.95				0.54–22.56				[179, 187–190]
	Apple (dessert), juice from concentrate	0–0.09				0–0.07				[191]
	Apple (cider), pure juice	0–40.70				0–82.20				[177, 178, 189, 192, 193]
	Apple (cider), juice from concentrate	0.05–0.19								[193]
Fruit juices – tropical fruit juices	Pomegranate, pure juice	0.01–0.84								[194]
	Kiwi, pure juice	0–0.11				0.26–0.37				[195]
Herb infusions	Peppermint, tea	0–0.60	0–0.90				3.72–14.76	0–0.48		[196]

Tea infusions	Tea (green), infusion	0–4.50		0–15.69	0–9.15	0–73.89	0.10–64.29	0.01–100.00	0.57–271.43	[147, 197–207]
	Tea (black), infusion	0–17.08	0.65–10.26	0–59.20	0–3.56	0–16.80	0–31.70	0.006–50.90	0–67.90	[140, 147, 197–205, 207–209]
	Tea (oolong), infusion	0–2.37			0.004–0.01	0.01–13.19	0.06–17.50	0.01–40.00	0.36–85.70	[200–202]
	Tea (black), bottled	0.20–2.45	0.03–0.80	0–1.90	0.33–3.31	0.23–1.14	0.26–1.14	0–2.85	0.45–4.58	[210]
	Tea (green), bottled	0.68–4.49	0.35–2.97	2.24–10.16	1.87–18.20	0.30–2.04	0.39–3.81	0.53–4.40	1.71–18.27	[210]
	Tea (oolong), bottled	0.93–1.59	0.69–0.95	2.50–5.43	3.67–7.77	0.43–1.47	0.72–1.80	1.02–2.63	3.83–6.39	[210]
Seasonings										
Herbs (mg/100 g)	Rosemary, dried	2.70								[211]
Other seasonings (mg/100 mL)	Vinegar	0–8.26				0–1.79				[212–214]
Seeds (mg/100 g)										
Nuts	Chestnut, raw	0.01		0.01			0.20			[147]
	Pecan nut	7.20			0.80	0.80		5.60	2.30	[181]
	Hazelnut	1.20			0.40	0.20		2.80	1.10	[181]
	Pistachio	3.50			0.50	0.80		2.10		[181]
	Almond	0.10–3.85			0.46	0.30–1.27		2.6		[181, 215]

(*continued*)

Table 57.2 (continued)

		Flavan-3-ols								
		C	C3G	GC	GC3G	EC	EC3G	EGC	EGCG	References
Pulses – beans – common bean	Common bean (white), whole, raw	0.01				0.09		14.03		[147]
	Common bean (others), whole, raw	5.07				0.14		0.05		[147]
Pulses – beans – other beans	Broad bean seed, whole, raw	12.83				22.51				[140]
Pulses – lentils	Lentils, whole, raw	0.07–0.35		0.14		0–0.41				[67, 147]
	Lentils, de-hulled, raw	0.02–0.28								[67]
Vegetables (mg/100 g)										
Pod vegetables	Green bean, raw	0–2.67				0–6.06				[140, 147, 180]
	Broad bean pod, raw	16.23		9.68		37.55		17.38		[147]
Pulse vegetables	Fresh pea, raw	0.01				0.01				[147]

Table 57.3 Distribution of proanthocyanidin dimers and trimers in food products

		PCs										PDs				
		B1	B2	B3	B4	B5	B7	C1	C2	EEC	T2	B3	CGCC	GCCC	GCGCC	References
Alcoholic beverages (mg/100 mL)																
Beers				0–0.36					0.03			0–0.45	0.02	0.01	0.04	[5, 139, 142–144]
Ciders																
Wines	White	0–0.05	0–0.03	0–0.02	0–0.05		0.06									[146, 147, 151]
	Red	2.15–14.00	0.43–9.00	0–11.96	0.08–11.30		0.27	0.22–2.63			6.71	0.11				[146, 147, 151, 156]
	Rose	0–0.57	0–0.21		0–0.02			0–0.01								[146, 147]
Cereals and cereal products (mg/100 g)																
Cereals				8.80–14.20								22.90–23.40				[142]
Cocoa																
Chocolate	Dark		21.00–54.00					13.00–44.00								[216]
	Milk															
Cocoa	Powder	112.00	13.00–262.00					5.0–36.00								[160, 216]

(*continued*)

Table 57.3 (continued)

		PCs										PDs				
		B1	B2	B3	B4	B5	B7	C1	C2	EEC	T2	B3	CGCC	GCCC	GCGCC	References
Fruits and fruit products (mg/100 g)																
Fruit berries	Red raspberry		0–0.30													[161]
	Strawberry	0.62	0–0.04	1.10	0.13					0.50						[147, 161]
	Black currant															
	Blackberry	0.23	0.83	0.33						0.06		0.07				[147]
	Red currant			0.2								1.7				[147]
	White currant															
	Gooseberry															
	Cloudberry		0.40													[161]
	Red grape	0.43	0.36	0.12	0.33			0.38								[147]
	White grape	0–1.79	0–0.19	0–0.32	0–1.73			0.07		0.13		0.12				[147, 163]
Fruits – drupes	Peach, whole															
	Peach, peeled	0.71–68.74	2.32	0.07–8.50	0.13	0.04	1.30	2.53		0.34						[147, 165]
	Sweet cherry	0.23	2.10	0.08	0.18	0.20	1.01	1.85								[147]
	Plum, fresh	8.84	5.20	1.00	1.02	1.59	4.69	10.01		7.73						[147]
	Apricot	0.09		0.05			0.01			0.01						[147]
	Nectarine, whole	2.80–17.70														[168]
	Nectarine, peeled	0–62.27														[168]
	Apricot, puree	0.09														[165, 168]

Fruits – pomes	Apple (dessert), whole		0.90–38.46										[169, 171, 217]
	Apple (dessert), peeled	0–17.28	0–21.26	0.10–0.35		0.18–1.97	0.57–5.75	2.45–9.72		0.43–2.44			[147, 176]
	Apple (cider), peeled	6.42	5.67–87.55										[176]
	Pear, whole												
	Pear, peeled	0–0.02	0.59–0.73	0–0.01		0–0.01	0–0.09	0.04–0.44		0–0.03			[147]
	Quince, peeled	0.73	1.34	0.10	0.24	0.12		0.94		0.28		0.08	[147]
	Medlar	0.14	0.78	0.05	0.13	0.03	0.17	0.52		0.11			[147]
	Apple (dessert), puree												
Fruits – tropical fruits	Mango												
	Banana											0.10	[147]
	Pomegranate	0.13		0.16									[147]
	Custard apple	1.27	7.01	0.31	2.48	0.82	2.31	3.52		0.97			[147]
	Persimmon, fresh	0.13								0.04		0.30	[147]
	Kiwi		0.14					0.11					[147]

(*continued*)

Table 57.3 (continued)

		PCs										PDs				
		B1	B2	B3	B4	B5	B7	C1	C2	EEC	T2	B3	CGCC	GCCC	GCGCC	References
Jams	Strawberry, jam															
	Apricot, jam															
Nonalcoholic beverages (mg/100 mL)																
Cocoa beverage – chocolate	Chocolate, milk, beverage		6.10													[160]
Fruit juices – berry juices	Sea-buckthorn berry, pure juice															
	Fox grape (green), pure juice	0.20–0.60	0.50–0.70	0.80–1.20												[184]
	Grape (green), pure juice	0–0.70	0.10–1.20	0.24–6.40												[184]
Fruit juices – drupe juices	Plum, prune pure juice															
	Plum, prune juice from concentrate															
	Apricot, nectar															

Fruit juices – pome juices	Apple (dessert), pure juice	0–2.75	0.06–22.94				19.93–44.00						[179, 189, 190]
	Apple (dessert), juice from concentrate	0–0.05	0–0.03		0–0.04								[191]
	Apple (cider), pure juice	0.24–2.04	0–55.00				0.51–4.96						[189]
	Apple (cider), juice from concentrate												
Fruit juices – tropical fruit juices	Pomegranate, pure juice												
	Kiwi, pure juice												
Herb infusions	Peppermint, tea												

(*continued*)

Table 57.3 (continued)

		PCs										PDs				
		B1	B2	B3	B4	B5	B7	C1	C2	EEC	T2	B3	CGCC	GCCC	GCGCC	References
Tea infusions	Tea (green), infusion	0.56	0.75	0.37	1.83		0.63	1.07				0.27				[147]
	Tea (black), infusion	0.21–6.65	0.20–5.68	0.49	1.80	0.01	0.46	0.76				1.65				[147, 208]
	Tea (oolong), infusion															
	Tea (black) bottled															
	Tea (green), bottled															
	Tea (oolong), bottled															
Seasonings																
Herbs (mg/100 g)	Rosemary, dried															
Other seasonings (mg/100 mL)	Vinegar															
Seeds (mg/100 g)																
Nuts	Chestnut, raw			0.01				0.02								[147]
	Pecan nut															
	Hazelnut															
	Pistachio															
	Almond															

Pulses – beans – common bean	Common bean (white), whole, raw			0.03				
	Common bean (others), whole, raw	1.22	0.12	0.82				[147]
Pulses – beans – other beans	Broad bean seed, whole, raw							
Pulses – lentils	Lentils, whole, raw		0–1.38	0.59–1.06			0.45	[67, 147]
	Lentils, de-hulled, raw							
Vegetables (mg/100 g)								
Pod vegetables	Green bean, raw	0–1.24	0–1.81	0–1.45				[147, 180]
	Broad bean pod, raw	11.26	12.08	8.17	18.47	0.13	23.50	[147]
Pulse vegetables	Fresh pea, raw							

The interaction between tannins and proteins has been described by many authors as a result from the cross-linking between the tannins and the proteins involving different kinds of bonds that include (1) hydrogen bonds between the hydroxyl groups of the tannin and the carbonyl group and amine groups of the protein [11, 134, 218–221], (2) Van der Waals interactions between the hydrophobic benzene ring of the phenolic compounds and the apolar amino acid side chains [222–227], (3) ionic bonds between the phenolate anions and cationic sites of proteins [218, 228], and (4) covalent bonds resulting from the reaction of the nucleophilic groups of proteins such as $-NH_2$ and $-SH$ and quinone groups resulting from phenolic oxidation [218, 229, 230] or carbocations resulting from acid-catalyzed condensed tannins depolymerization [231].

In a food context, the astringency sensation is one of the main characteristics associated to tannin-rich foods and is thought to result from the interaction between tannins and salivary proteins leading to the formation of protein-tannin aggregates in the mouth [232–234]. This feature leads to the diminution of the oral lubrication that is usually described as dryness, puckering, and rough mouthfeel [13]. In the case of astringency, only mechanisms (1) and (2) described above are thought to be involved in the tannin-protein interactions at slightly acid or neutral pH (Fig. 57.11).

All the referred interactions between the phenolic compounds and proteins are strongly influenced by the phenolic structural features including the degree of hydroxylation, size, and conformation. Generally, the affinity of tannins for proteins increases with the molecular weight (MW) and degree of galloylation due to the increase of the number of sites of interaction. It was demonstrated that grape seed PCs with high MW (mDP $\sim$ 6) interacted and precipitated human salivary proteins more readily than smaller PCs [235]. Similar studies with BSA (bovine serum albumin) and α-amylase showed that the binding affinity increases with the size of PCs up to MW $\sim$ 2,000 Da (mDP $\sim$ 6) [236]. However, the affinity to different model proteins of higher MW PCs (>3,400 Da) from grape seeds has shown to decrease, probably due to their lack of flexibility [237]. It has also been demonstrated that small differences in the PCs structure such as the configuration of the carbon C-3 or the hydroxylation pattern of the ring B could also result in different affinities with proteins. Epicatechin has shown a lower tannin specific activity for human proline-rich proteins (PRPs) than catechin [238]. Also, flavan-3-ols with at least one 1,2,3-trihydroxylated ring ((+)-gallocatechin, (−)-catechin-3-*O*-gallate, (−)-gallocatechin-3-*O*-gallate, and (−)-epigallocatechin-3-*O*-gallate) revealed a higher affinity for proteins when compared with (+)-catechin and B2 dimer [239]. Studies performed in model solutions with C-4 → C-8 dimers B3 and B4 have shown that they present a greater affinity to human PRPs when compared with their C-4 → C-6 dimers (B6 and B8) counterparts, which can be due to conformational constraints presented by the C-4 → C-6 interflavonoid linkage [240]. Different results were obtained by Ricardo da Silva and colleagues when measuring the PC dimers concentrations in wine model solutions of grape seed procyanidins after fining

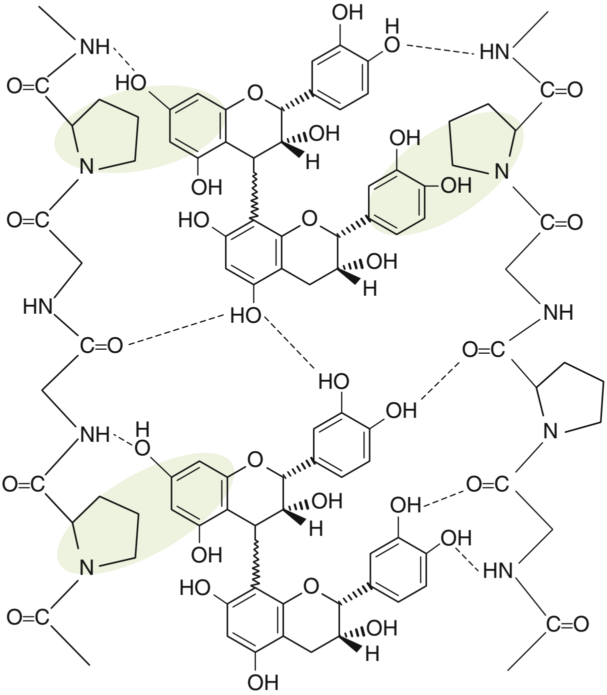


Fig. 57.11 Proanthocyanidin-protein interaction showing the hydrogen bonds and Van der Waals hydrophobic interaction between the benzene ring of the phenolic compounds and the apolar amino acid side chains

treatment with different proteins. In that study, the C-4 → C-6 dimers were found to bind more efficiently with proteins than the C-4 → C-8 dimers [241].

6 Copigmentation

Copigmentation is a phenomenon that occurs in solution when colored compounds (anthocyanins) form molecular associations or complexes with noncolored compounds, such as catechins and proanthocyanidins, phenolic acids, flavonols among others. These kinds of associations usually lead to the increase of the solution absorbance intensity (hyperchromism) and a positive displacement in the visible wavelength (λ_{max}) of the pigment (bathochromism). Intermolecular copigmentation generally occurs in berries and berry-derived products. Copigmentation occurs due to the formation of a vertical hydrophobic stacking between the aromatic core of the anthocyanin and the phenolic copigment strengthened by hydrogen bonds [242, 243] (Fig. 57.12). In aqueous solution, this complex prevent the nucleophilic attack of water to the colored flavylium structure of anthocyanin and therefore reduce the

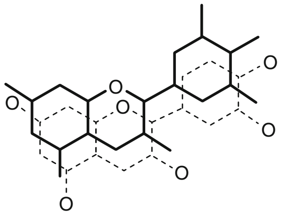


Fig. 57.12 Schematic representation of the intermolecular copigmentation between anthocyanins and flavonoids. (—), anthocyanin; (--), flavonoids [170]

color fade [71]. The extent of the copigmentation is directly related to the anthocyanidin and copigment structures.

Hydrophobic effects are thought to position the anthocyanin chromophore and the copigment to form a π-π complex [244], and by this way, the most efficient overlap would occur with planar flavonols when compared with hydroxycinnamoyl, galloyl esters, or with the nonplanar flavan-3-ols [245]. Usually, it is thought that the flavylium ion is the major colored species that contributes to the copigmentation phenomenon [244, 246]. However, some authors have suggested that the neutral quinoidal base is the main species involved [247, 248].

Although copigmentation is very important in nature, being responsible for the extraordinary colors observed in many flowers [71, 247, 249–251], the role of proanthocyanidins as copigments is almost exclusive of red wines [14, 15, 18, 252, 253].

7 Chemical Transformations

During food processing and conservation, flavanols can undergo several chemical reactions. These chemical transformations have been deeply studied in wine and tea [254–258].

According to the procedure of the manufacture of teas, it is possible to obtain three different kinds of teas: green tea that is unfermented, oolong tea that is partially fermented, and black tea that is fully fermented. Young green leaves of the tea plant (*Camellia sinensis* var. *sinensis*, *Camellia sinensis* var. *assamica*) contain high concentrations of flavanol monomers such as (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate [259]. Throughout the fermentation process, browning reactions occur induced by oxidative enzymes. Through oxidative transformation by polyphenol oxidase (PPO), flavanols originally present in green tea give rise mainly to dimeric theaflavins that present an orange-red color and red-brown oligo/polymeric thearubigins that are found in black tea [254, 255, 257, 258] (Fig. 57.13). Minor compounds such as the dimeric theasinensins were also found to occur [260, 261] (Fig. 57.13). Although thearubigins account for about 60% of the solids in a black tea infusion, their chemical structure is the least well understood as well as their formation mechanism. Figure 57.13 shows a hypothetic structure for thearubigins [254].

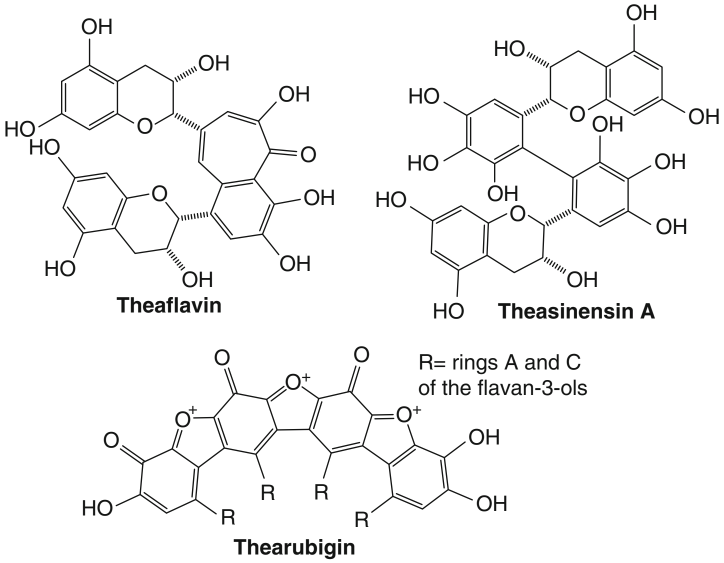


Fig. 57.13 Structures of the main tannin-like compounds found in black tea. The structure of thearubigin is hypothetic [180]

In wine, flavanols may also be transformed by oxidation through two different mechanisms: the enzymatic and the nonenzymatic browning [256]. The enzymatic browning occurs almost entirely in grape must. On the other hand, nonenzymatic browning happens both in must and wine. Phenolic compounds are considered the major substances at the origin of wine browning [262, 263], and the oxidation of phenols is dependent on the phenolic composition and their levels, being the *o*-diphenols the most oxidizable ones [264, 265].

In enzymatic browning, flavanols (*o*-diphenols) are firstly oxidized by PPO in the presence of oxygen to *o*-quinones. Then, a rapid coupled oxidation of the quinones with other phenolic molecules occur, leading to the formation of dimers or the regeneration of the original phenols that are catalyzed by acid. The quinones produced can also be polymerized with many other compounds (phenolic and non-phenolic molecules) forming brown pigments [113, 266] (Fig. 57.14). Like in enzymatic browning, in the nonenzymatic browning, also called chemical oxidation, *o*-diphenols such as caffeic acid, catechin, epicatechin, or cyanidin are considered the most susceptible compounds toward oxidation. Moreover, the levels of flavanols have been well correlated with the browning degree of white wines [267, 268]. In the chemical oxidation, the presence of iron and copper can catalyze the oxidation of phenols, leading to the formation of quinones that are further polymerized in a similar way to what happens in the enzymatic browning [269, 270].

Other routes for the polymerization of flavan-3-ols involve the presence of acetaldehyde and glyoxylic acid [25, 271–275]. Acetaldehyde and glyoxylic acid are present in wine due to the oxidation catalyzed by metals of ethanol and tartaric

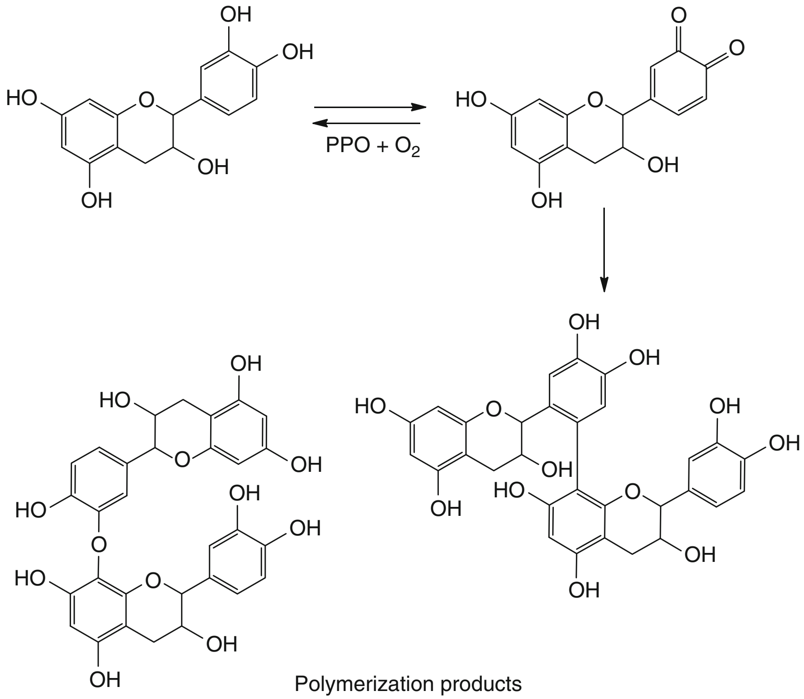


Fig. 57.14 Enzymatic browning products obtained in the oxidation of flavan-3-ols by PPO [113]

acid, respectively. Acetaldehyde can also be derived from the metabolism of yeast during fermentation. Flavanols in acidic medium can undergo electrophilic substitution by acetaldehyde on the electronegative positions C-6 or C-8 of ring A, leading to the formation of dimers and trimers (Fig. 57.15) according to the mechanism first proposed by Timberlake and Bridle [25] and later confirmed by other authors [272, 276].

It was demonstrated by Fulcrand et al. [277] that the glyoxylic acid formed in wine through the oxidation of tartaric acid could react with flavanols in a similar mechanism than that described for acetaldehyde, forming a colorless catechin-8,8-carboxymethine-catechin compound. Further transformation gives rise to the formation of a yellow xanthylium cation (Fig. 57.16) [259]. Also, a lactone structure was proposed from the cyclization of the carboxymethine group with the hydroxyl group of the phloroglucinol ring of the upper flavanol unit (Fig. 57.12) [278]. Similar xanthylium compounds were also identified from the reaction of flavanols with furfural and hydroxymethylfurfural (HMF) [279, 280].

Flavanols were also shown to react with cinnamic aldehydes like coniferaldehyde or sinapaldehyde, leading to the formation of colored (orange color) compounds with a catechinpyrylium structure (Fig. 57.17) [281, 282]. These compounds were named oaklins since they were found in wines aged in oak from which cinnamic aldehydes were extracted.

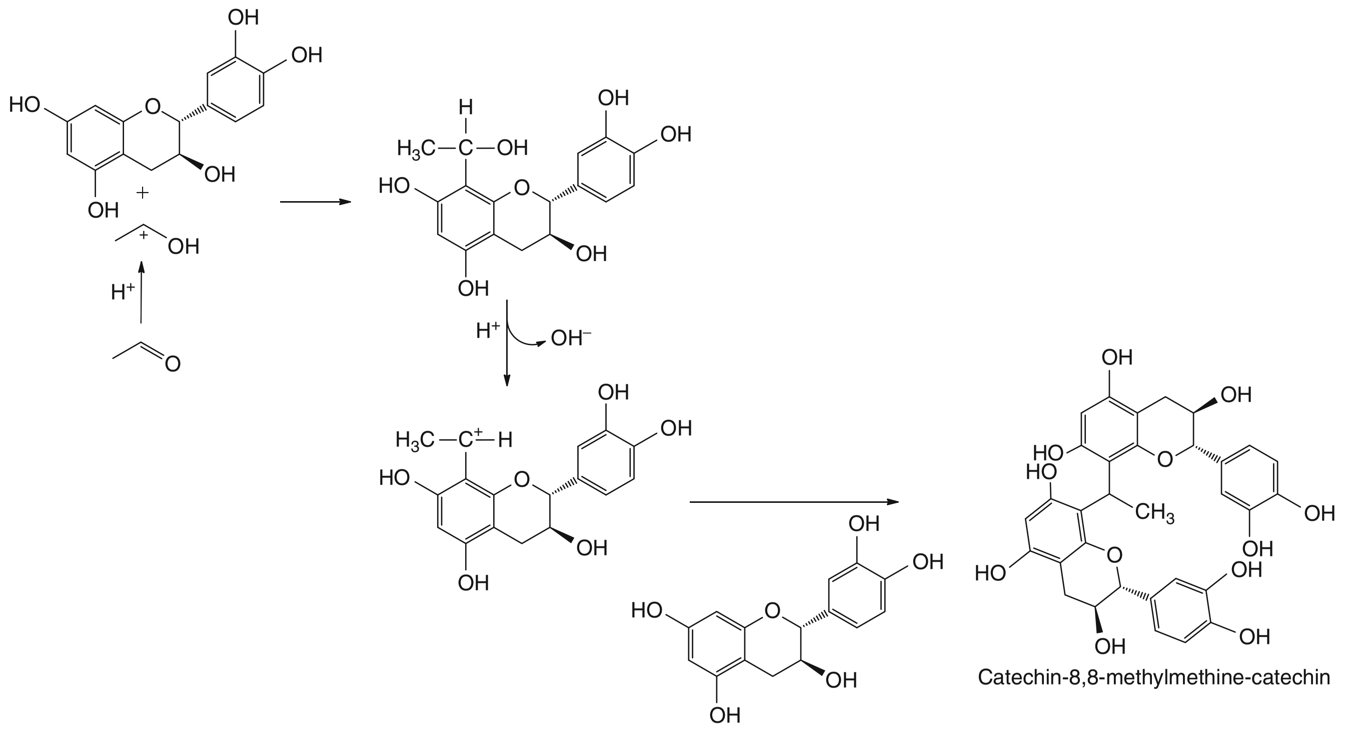

H+
OH−
Catechin-8,8-methylmethine-catechin

Fig. 57.15 Mechanism of formation of flavanol-8,8-methylmethine-flavanol adducts from the reaction of flavanols with acetaldehyde

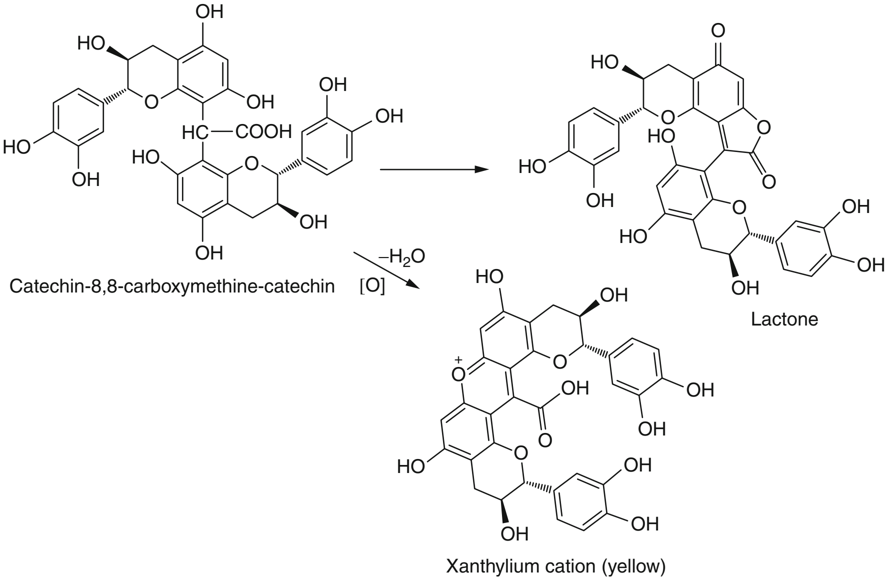

Catechin-8,8-carboxymethine-catechin
$-H_2O$
[O]
Lactone
Xanthylium cation (yellow)

Fig. 57.16 Products proposed for the condensation of flavanols with glyoxylic acid, lactone [204] and xanthylium structures [200]

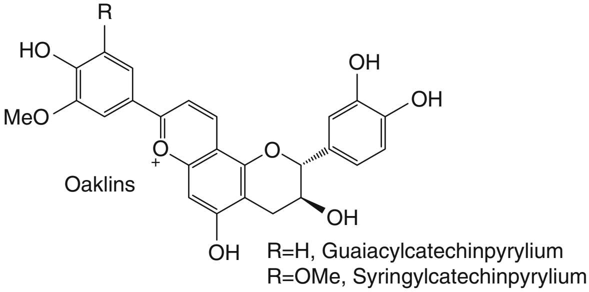

Oaklins
R=H, Guaiacylcatechinpyrylium
R=OMe, Syringylcatechinpyrylium

Fig. 57.17 Structure of the catechin-pyrylium pigments (oaklins) [207, 208]

In red wines, flavanols may also react directly with anthocyanins, leading to the formation of anthocyanin-flavanol and flavanol-anthocyanin adducts [283–285]. The condensation may also occur mediated by aldehydes, such as acetaldehyde, furfural, HMF, benzaldehyde, and others, leading to the formation of alkyl-methine bridged adducts. These latter display the same λ_{max} in the visible region at around 540 nm, which is bathochromically shifted when compared with the original anthocyanin ($\lambda_{max} \sim 525$ nm) [279, 280, 286–289], thereby contributing to the color change in red wine.

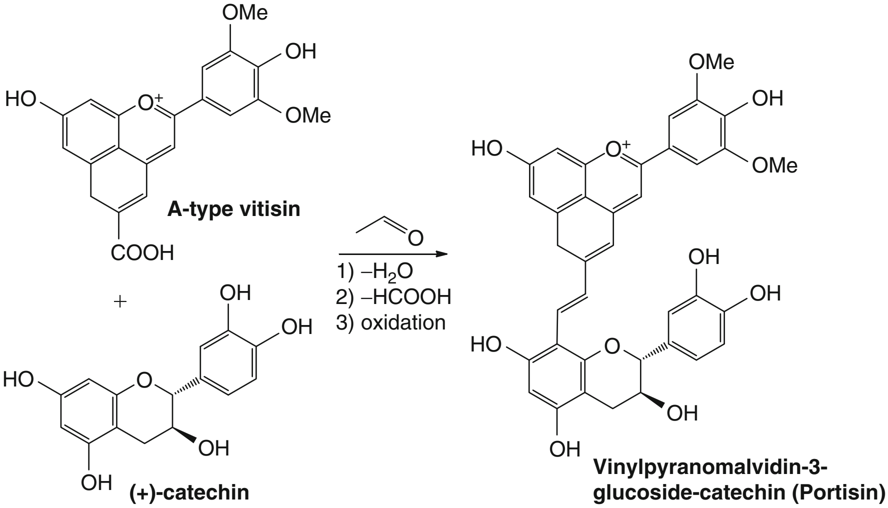


Fig. 57.18 Formation of vinylpyranomalvidin-3-glucoside-catechin (portisin) from the reaction of carboxypyranomalvidin-3-glucoside (A-type vitisin) and (+)-catechin in the presence of acetaldehyde

More recently, a new family of anthocyanin-derived compounds (named portisins) has been detected in Port wine and found to display unusual spectroscopic features, presenting a bluish color at acidic conditions [290] (Fig. 57.18). Studies performed in wine model solution revealed that these pigments are formed from the reaction of carboxypyranoanthocyanins with flavanols mediated by acetaldehyde [290, 291].

8 Conclusion

In the literature, there are a large number of different flavanols found in many different plants worldwide. Their structures differ from each other in the stereochemistry of the asymmetric carbons, the hydroxylation pattern of the aromatic rings, type and number of ester-linked substituents (e.g., methyl, glucosyl derivatives) or ester-linked substituents (e.g., phenolic acid derivatives), degree of polymerization, etc.

These natural compounds are of particular relevance due to their biological activities that contribute to the therapeutic properties attributed to some plants, especially those highlighted since many years ago by the traditional Chinese medicine. These features make flavanols a particular target of the pharmaceutical industry. Besides this, flavanols are also responsible for some antioxidant and sensorial properties presented in fruits and fruit-derived products. The astringency sensation is one of those properties resulting from the interaction between flavanols and salivary proteins in the oral cavity. On the other hand, flavanols can also contribute to the color of anthocyanin-rich foodstuffs, through their association with anthocyanins (copigmentation phenomenon) enhancing the overall color and by their chemical reaction with anthocyanins leading to the formation of new colored compounds with different spectroscopic features. Although there are already several works demonstrating the importance of flavanols in different fields, more research involving their structural characterization and food sensorial and bioactivity properties is expected in the forthcoming years.

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Anthocyanins

58

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Contents

1 Introduction 1804
2 Occurrence 1806
3 Phytochemistry 1807
4 Biosynthesis 1808
5 Biological Activities 1809
5.1 Prevention of Cardiovascular Disease 1811
5.2 Anticancer, Antitumor, and Antimutagenic Activity 1812
5.3 Beneficial Effects in Diabetes 1813
5.4 Ocular Effects 1813
5.5 Antimicrobial and Antiviral Activity 1813
5.6 Effects on Neurodegenerative Processes 1814
6 Biotechnological Approaches 1814
7 Conclusion/Prospects 1816
References 1816

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Abstract

Anthocyanins are a group of plant phenolics characterized by their blue-orange-red color. They have a double interest as plant constituents, one technological, since they can not only be used as natural colorants in the food industry but also can interact with other plant components influencing the final characteristics of the processed product, and the other is related to their implications on human health as they can be considered functional ingredients and have shown themselves as potential targets for the pharmacological industry.

This chapter summarizes the existing literature in this area: from anthocyanin occurrence and phytochemistry to their biosynthesis, biological activities, and an overview of the biotechnological approaches that are in use at present.

Keywords

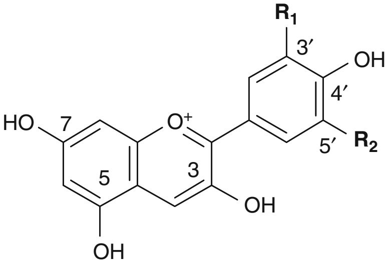
Anthocyanins • occurrence • biological activity

Abbreviations

AATs	Anthocyanin acyltransferases
ACE	Angiotensin-converting enzyme
CHS	Chalcone synthase
DFR	Dihydroflavonol 4-reductase
F3′H	Flavonoid 3′-hydroxylase
FLS	Flavonol synthase
MCP-1	Monocyte chemoattractant protein 1
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor-α
TRAP	Thrombin receptor-activating peptide
VEGF	Vascular endothelial growth factor

1 Introduction

The term anthocyanin is derived from the Greek words *anthos*, meaning flower, and *kyanos*, meaning blue. Anthocyanins, the largest group of water-soluble natural pigments, are widely distributed among plants. They are responsible for the attractive colors of flowers, fruits (particularly berries), and vegetables and thereby add to the aesthetic quality of products derived from many plant sources. These polyphenolic substances are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts. Glycosylation and acylation of the aglycone moieties (mainly six anthocyanidins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin) by different sugars and acids, at different positions, account for the broad structural diversity of these pigments (Fig. 58.1). The sugars most commonly bonded to anthocyanins are glucose, galactose, rhamnose, and arabidose. Di- and



R_1	R_2	Anthocyanidin
H	H	Pelargonidin
OH	H	Cyanidin
OH	OH	Delphinidin
OH	OCH_3	Petunidin
OCH_3	H	Peonidin
OCH_3	OCH_3	Malvidin

Fig. 58.1 General structure of anthocyanidins and substitution of the most commonly found in foods

trisaccharides, formed by combination of these four monosaccharides can also glycosylate anthocyanins. The most common acids, bonded to the sugar residue, are *p*-coumaric, caffeic, ferulic, synaptic, *p*-hydroxybenzoic, malonic, oxalic, malic, succinic, and citric acid [1].

In plants, anthocyanins play a role in attracting animals in pollination and seed dispersal; they may also enhance plant resistance to insect attack [2], act as endogenous plant antioxidants and as photoprotectors [3]. Available evidence also suggests that this group of phytochemicals, besides being nontoxic and nonmutagenic, could exhibit multiple biological effects, such as antioxidant-antiradical activity, anti-inflammatory action, inhibition of blood platelet aggregation, and antimicrobial activity, and could be used in the treatment of diabetic retinopathy and prevention of cholesterol-induced atherosclerosis [1, 4–6].

Anthocyanins are rather unstable compounds, and this has to be considered when trying to isolate or use them as part of more complex formulations. A number of factors influence anthocyanin stability, including pH, heat-humidity, light, oxygen, enzymes as well as the presence of ascorbic acid, sugars, sulfur dioxide or sulfite salts, metal ions, and copigments [7, 8]. A major impediment to the use of anthocyanins as food colorants is their inherent instability, whether in cellular juice or in aqueous model solutions and especially in complex systems such as foods. In actual food products, anthocyanins show greater stability under acidic conditions, but are generally unstable under normal processing and storage conditions, and easily degrade to form first colorless derivatives and subsequently insoluble brown pigments.

It is known that hydroxylation of the basic nucleus at position 5 or 7 tends to stabilize the molecule, whereas hydroxylation at position 3 renders it more labile. Similarly, hydroxy substitution in the B ring leads to low stability but this is overcome if the groups are methoxyl rather than hydroxyl. The substitution of hydroxyl groups by sugars or acyl groups also increases stability. Timberlake and Bridle [9] found that substitution of the anthocyanin at position 4 (not normally substituted) with methyl or phenyl groups conferred stability to the molecule.

Copigmentation is a phenomenon widely seen in plant tissues and their extracts, which influences the color and functional properties of this group of phenolics.

Molecules acting as copigments, such as flavonoids, alkaloids, and organic acids, usually have no color by themselves, but when added to an anthocyanin solution, they greatly enhance the color of the solution [10]. Results obtained by Maccarone et al. [11, 12] and Teh and Francis [13], working with model systems and fruit juices, supported the hypothesis that the phenomena of copigmentation and self-association can influence color, intensity, and stability of anthocyanins. Several studies which have dealt with encapsulated anthocyanins [14, 15] led to the conclusion that encapsulation significantly reduces the degradation rate of the core material and thereby improves the shelf life of the colorant at different humidity environments.

2 Occurrence

Anthocyanins are present in different plant organs, such as fruits, flowers, stems, leaves, and roots [16]. These pigments are normally found dissolved uniformly in the vacuolar solution of epidermal cells. However, in certain species, the anthocyanins are localized in discrete regions of the cell vacuole, called anthocyanoplasts [17]. The main sources of anthocyanin (Table 58.1) are red fruits, mainly berries and red grapes; cereals, principally purple maize and red-purple; and vegetables such as eggplant [6, 18].

In general, cyanidin and its glycosides are the anthocyanins most frequently found in fruits. This is the case of cherry, apple, figs, or peach. Grapes, for example, are characterized by the presence of, in some varieties, derivatives of the six most frequent anthocyanins. However, in most winemaking varieties, malvidin-3-glucoside is the most abundant one. On the other hand, delphinidin is characteristic of pomegranate or eggplant.

Anthocyanin content may vary from fruit to fruit of the same type due to different external and internal factors, such as genetic and agronomic factors, intensity and type of light, temperature, processing, and storage. For example, anthocyanin concentrations in red grapes are specially variable, being able to reach values of up to 250 mg/100g, while in red wines, the concentrations vary in accordance with the varieties of grapes employed as well as with the type of vinification and, specially, with aging. However, a medium value could be established of around 500 mg/L anthocyanins in young wines. Moreover, it is

Table 58.1 Classification of plant products based on their anthocyanin contents

High anthocyanin content products (>100 mg in 100 g)	Bilberry, black currant, black olives, black rice, blackberry, blueberry, cherry, chokeberry, elderberry, red grapes, pomegranate, purple corn, raspberry, red cabbage
Medium anthocyanin content products (>10 mg <100 mg)	Apple (red varieties), black bean, cranberry, eggplant, gooseberry, plum, red currant, red onion, red radish, rhubarb, strawberry
Low anthocyanin content products (<10 mg)	Grapefruit, lettuce, nectarine, peach, pear, strawberry

important to note that anthocyanins are used as food additives in beverages, fruit fillings, snacks, and dairy products.

The glycosides of the three non-methylated anthocyanidins (delphinidin, cyanidin, and pelargonidin) are the most abundant in nature, which represent 80% of leaf pigments, 69% in fruits, and 50% in flowers. The distribution of the six most common anthocyanidins in the edible parts of plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%). The most widespread anthocyanin in most fruit is cyanidin-3-glucoside [19]. However, malvidin glycosides are the most characteristic anthocyanins in red grape and derived products (wine, juice, etc.) [1].

3 Phytochemistry

The basic structure of anthocyanins can be differentially substituted to produce a variety of structures with different colors and properties. Differences between individual anthocyanins are the number of hydroxyl groups in the molecule, the degree of methylation of the hydroxyl groups, the nature and number of sugars attached to the molecule and the position of the attachment, and the nature and number of aliphatic or aromatic acids attached to the sugars in the molecule.

As mentioned before, the most common anthocyanins are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (Fig. 58.1). In nature, they are found mostly in their glycosylated forms, even if they have been also found in low amounts as their aglycons. One of the possible ways of classifying anthocyanins is based on the number of sugars linked to the basic anthocyanidin. In this way, we can talk of monosides, biosides, triosides, etc.

Since each anthocyanidin (aglycone) may be glycosylated and acylated by different sugars and acids, at different positions, the number of anthocyanins is 15–20 times greater than the number of anthocyanidins. The sugars most commonly bonded to the aglycones are glucose, galactose, rhamnose, and arabinose. The following four classes of anthocyanidin glycosides are the most common: 3-monosides, 3-biosides, 3,5-diglycosides, and 3,7-diglycosides. However, glycosylation of the 3′-, 4′-, and 5′-OH group has also been demonstrated. In many cases, the sugar residues are acylated by *p*-coumaric, caffeic, ferulic, synaptic, *p*-hydroxybenzoic, malonic, oxalic, malic, succinic or acetic acid. Methoxyl substituents are found at the 3′ and 5′ position and less frequently at positions 7 and 5.

However, all these changes are not sufficient to account for the large color variations attributed to anthocyanins in plants. These are in fact due to two types of mechanisms, namely, (1) conversion of the flavylium ions to secondary anthocyanins forms in aqueous media and (2) molecular interactions.

In aqueous media, most of the natural anthocyanins behave like pH indicators, being red at low pH, bluish at intermediate pH and colorless at high pH. It has been demonstrated that in acidic or neutral media, four anthocyanin structures exist in equilibrium: the flavylium cation (AH+), the quinoidal base, the carbinol pseudo base and the chalcone. In general, anthocyanins are present in the flavylium form at pH below 2, being the major form of anthocyanins the hemiacetal in equilibrium with the chalcone in the pH range 2–7, while the quinoidal base appears to be very unstable. But we can see the red color due to the presence of low amounts of this form at that pH.

At the plants' pH values (3–7), anthocyanins are mostly present as colorless structures. But a stabilizing mechanism takes place. Such effects are provided by complexation of the color form with other species present in the solution, which can be either an identical anthocyanin molecule (self-association), one of its aromatic acyl groups substituents (intramolecular copigmentation) or another molecule (intermolecular copigmentation).

Color of anthocyanins-containing media depends on the structure and concentration, of the pigment, pH, temperature, presence of ascorbic acid, copigments, metallic ions, enzymes, oxygen, sugars and their degradation products, sulfur dioxide, and other factors.

Anthocyanins are considered to be poor substrates for the enzymes responsible of other flavonoids degradation, such as polyphenol oxidase, peroxidases, glycolases, and esterases. Nevertheless, some papers in the literature confirm their direct degradation by some of them. But we cannot forget that even if the enzymes do not react directly with the anthocyanin, they can react with the quinones formed during anthocyanin degradation in a food process, and this could give a change in color.

Antioxidants, such as ascorbic acid or sulfites, often added to prevent enzymatic degradation, have been shown to decolorize anthocyanins.

Reactions of anthocyanins have been especially investigated in red wines and related model systems. In fact, the progressive change from the red-purple to tawny as the wine ages is believed to be due to conversion of grape anthocyanins to more stable polymeric forms, where a reaction with, for example, acetaldehyde is the first step leading the reaction.

Nevertheless, it has also been demonstrated that the anthocyanins with a methyl or phenyl group in C4 are much more stable, even more than some synthetic colorants.

However, some anthocyanin extracts (such as elderberry extract) are frequently used by the industry for coloring jams, juices, ice lollies, or candies.

Anthocyanin analysis generally involves extraction with weakly acidified alcoholic solvent, followed by concentration under vacuum and purification and separation of the pigments, while the whole extract is used for coloring foodstuffs.

4 Biosynthesis

The amino acid phenylalanine is derived from gallic acid, being this compound biosynthesized in the shikimic acid metabolic route. Most of the phenolic compounds from higher plants are also derived from this amino acid, formed in the phenylpropanoid metabolic route, in the cell cytoplasm, being various enzymes involved in this metabolism. Phenylalanine ammonia lyase interacts with phenylalanine forming cinnamic acid, that is, hydrolyzed by cinnamate-4-hydroxylase, rendering *p*-coumaric acid. Different hydroxylations and/or methoxylations, of this acid, would end in hydroxycinnamic acids or *p*-coumaroyl CoA, if the third key enzyme of this route, CoA ligase, is involved. This is a very complex biosynthetic route, in which very similar compounds are synthesized in different types of cells and in different development stages, implying very complex regulation mechanisms. Nevertheless, this route key regulation control point is located in those steps where the enzymes phenylalanine ammonia-lyase, cinnamate-4-hydroxylase, and CoA ligase interact. These three enzymes are well characterized due to their importance in the secondary metabolism, and their biosynthesis seems to be regulated by a multigenic form [20]. Stilbenes, hydroxycinnamic acids, and flavonoids, including anthocyanins, derive from *p*-coumaroyl CoA. By the action of chalcone synthase (CHS), three acetate units from malonyl-CoA and one *p*-coumaroyl-CoA molecule can condense to produce naringenin chalcone. Chalcone isomerase (CHI) then catalyzes the stereospecific isomerization of the yellow-colored naringenin chalcone to the colorless naringenin which is converted to dihydrokaempferol by flavanone 3-hydroxylase (F3H). Dihydrokaempferol is also the potential substrate for flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) to produce the corresponding colorless dihydroflavonols, dihydroquercetin, and dihydromyricetin, respectively. F3′,5′H can also convert dihydroquercetin to dihydromyricetin. Thereafter, dihydroflavonol 4-reductase (DFR) can reduce these dihydroflavonols to their corresponding leucoanthocyanidins. Anthocyanidin synthase (ANS), a 2-oxoglutarate iron-dependent oxygenase, catalyzes, with the help of ferrous iron, the oxidation of the colorless leucoanthocyanidins to their corresponding colored anthocyanidins (Fig. 58.2).

Anthocyanin modification typically involves *O*-glycosylation, *O*-acylation, and *O*-methylation. *O*-Glycosylation is the first modification to occur, and it is catalyzed by family 1 glycosyltransferases (UGTs) [21]. Glycosylation is an important modification for increasing the hydrophilicity and stability of anthocyanins and is also essential for their color stability. *O*-methylation occurs late in anthocyanin biosynthesis pathways to follow *O*-glycosylation of anthocyanidins. *O*-Acylation is an important modification because it plays an important role in the formation of pigment-stabilizing tertiary structures. Anthocyanin acyltransferases (AATs) catalyze transfer of either aromatic or aliphatic acyl groups from a CoA-donor molecule to hydroxyl residues of anthocyanin sugar moieties.

The phenylpropanoid biosynthetic route is induced by stress. In this way, the enzymes of phenylalanine ammonia-lyase and cinnamate-4-hydroxylase are induced at transcriptional level as a defense mechanism against different damages [22]. Specifically, anthocyanins are induced as a response to high levels of UV light [23]. Nutrient deficiencies, especially of phosphorus (P) and nitrogen (N), may also induce the accumulation of anthocyanin in many plant species [24].

5 Biological Activities

Many beneficial activities have been attributed to anthocyanins. Most of the first studies carried out in this sense in past were related to the antioxidant characteristics

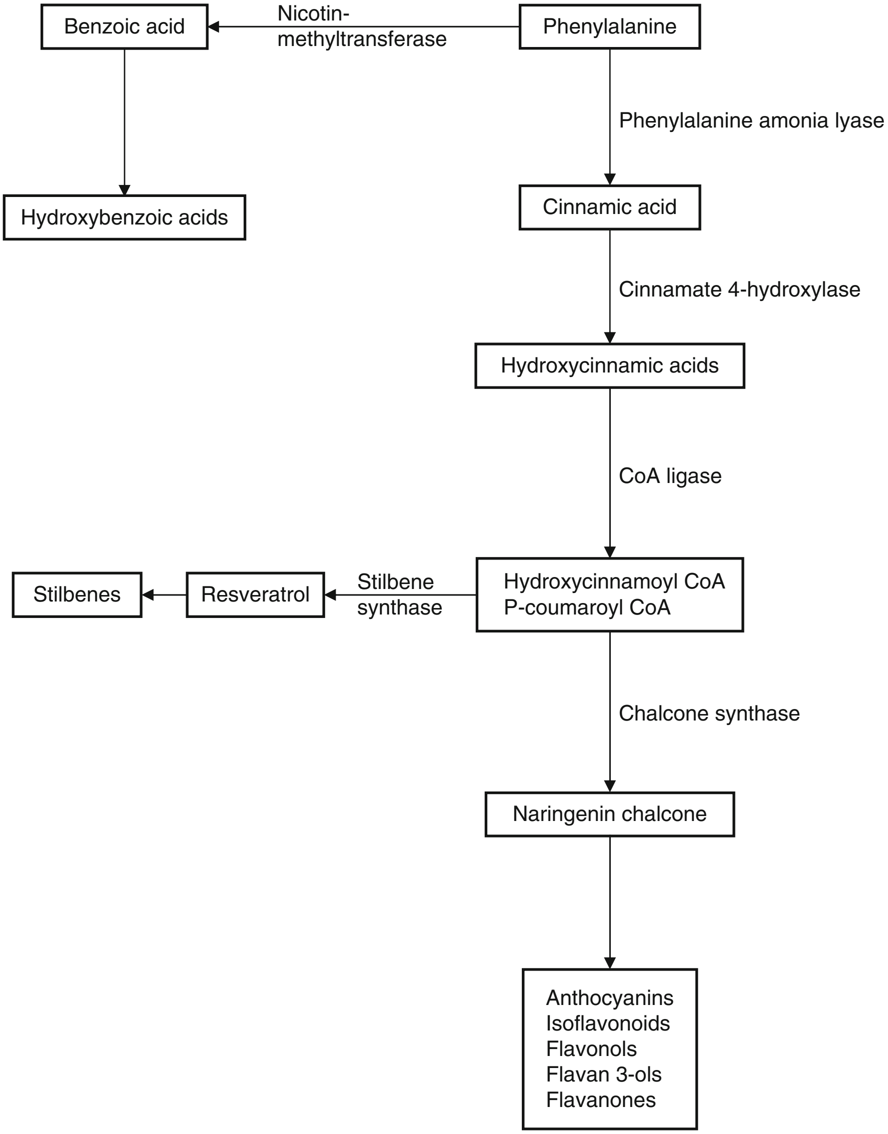

Benzoic acid
Nicotin-methyltransferase
Phenylalanine
Phenylalanine amonia lyase
Hydroxybenzoic acids
Cinnamic acid
Cinnamate 4-hydroxylase
Hydroxycinnamic acids
CoA ligase
Stilbenes
Resveratrol
Stilbene synthase
Hydroxycinnamoyl CoA
P-coumaroyl CoA
Chalcone synthase
Naringenin chalcone
Anthocyanins
Isoflavonoids
Flavonols
Flavan 3-ols
Flavanones

Fig. 58.2 Anthocyanin biosynthesis pathway

of anthocyanin-rich diets and the enhancement of cardiovascular health. However, most of them were in vitro studies, and a few of them were carried out in vivo in general by measuring total antioxidant capacity in plasma after consumption of a source rich in anthocyanins.

Anthocyanins are able to prevent oxidative damage to DNA, proteins, lipids, and other macromolecules caused by reactive oxygen species (ROS). Anthocyanins have a systemic action, since they are absorbed and circulate in the blood, and it is in this circulating form that they act upon different target tissues in the human body. Anthocyanins may also act locally, in the gut, if they are not absorbed by intestinal mucosa. Finally, they may also act as topical agents, for example, by protecting the skin from UV radiation.

In the case of the gut, it is not yet clear if these local effects are caused by the original anthocyanin or by the metabolites formed by the colonic microflora. The formation of these degradation products may be involved in the protective action of anthocyanins against colon and rectum cancer. This is an area of research that is growing very recently due to the potential of this kind of the interaction bacteria-anthocyanin as an explanation for the final health effect encountered for this group of polyphenols.

So far, anthocyanins have been found in biological samples, either urine, feces, plasma, or different animal tissues, in different forms. They may be found in their original form, either as glycosides or as acylated compounds, but also as their corresponding glucuronides, sulfate derivatives, or methylated in different positions. They can also be found as their breakdown products such as gallic acid, protocatechuic acid, or other phenolic acids. This complicated scenario makes it difficult to discern between the effects encountered for anthocyanins and those derived from the ingestion of other groups of flavonoids or polyphenols since they may have common metabolites and thus common biological actions.

The biological activity of isolated anthocyanins and anthocyanidins or of anthocyanin-rich foods can be manifested at different levels.

5.1 Prevention of Cardiovascular Disease

This is possibly the most studied effect and the one for which a great quantity of epidemiological evidence exists. Anthocyanins are capable of acting on different cells involved in the development of atherosclerosis, one of the leading causes to cardiovascular dysfunction. The chemokine monocyte chemotactic protein-1 (MCP-1) is known to mediate in the recruitment of macrophages to sites of infection or inflammation, and direct involvement of MCP-1 on atherogenesis has been established. Anthocyanins have been shown to have a protective effect against TNF-α-induced MCP-1 secretion in primary human endothelial cells [25]. Vascular endothelial growth factor (VEGF) is a major pro-angiogenic and pro-atherosclerotic factor, but anthocyanins have been shown to prevent the expression of VEGF in vascular smooth muscle cells [26]. In macrophages, blackberry anthocyanins inhibit LPS-induced nitric oxide biosynthesis [27]. Anthocyanins also inhibit TRAP-induced platelet aggregation but do not influence platelet reactivity when faced with strong agonists such as collagen and ADP [28]. Anthocyanin extracts from chokeberry, bilberry, and elderberry have shown endothelium-dependent relaxation capacity in porcine coronary arteries [29]. Moreover, anthocyanins have an effect on cholesterol distribution, protecting endothelial cells from CD40-induced proinflammatory signaling [30].

A key factor in cardiovascular disease prevention is vasodilation. It has been shown that anthocyanins act at this level by inhibiting angiotensin-converting enzyme (ACE) activity [31]. Anthocyanins may also induce endothelium-dependent vasorelaxation by activating endothelial nitric oxide synthase and increasing the production of the vasorelaxant agent nitric oxide, and by decreasing endothelin-1 production [32]. Finally, a possible implication of estrogenic receptor agonist/antagonist effect of anthocyanins has been described as a possible mechanism for their antiatherogenic activity [31].

5.2 Anticancer, Antitumor, and Antimutagenic Activity

It has been suggested that anthocyanins possess anti-inflammatory as well as chemopreventive properties. Distinct cell growth- and cell cycle-related pathways have already been identified as targets for anthocyanins in in vitro and laboratory animal studies. It has been shown that the anthocyanin-containing fraction of red wines is capable of suppressing proliferation of HCT-15 human colon cancer cells and gastric adenocarcinoma (AGS) cells [33, 34]. Administering purple corn, with a high anthocyanin content, to rats decreases the incidence and multiplicity of colorectal adenomas and carcinomas induced by 1,2-dimethylhydrazine (DMH) and 2-amino-1-methyl-6-phenylimidazo(4,5-β)pyridine (PhIP) [35]. Liu et al. [36] have shown that the proliferation of human hepatocellular liver carcinoma cells (HepG2) is inhibited by raspberry extracts, containing among other flavonoids anthocyanins. Marko et al. [37] have reported an inhibitory effect of the anthocyanidins, delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin on cell proliferation in human vulva carcinoma cells (A431) and human colon carcinoma cells (HT29). Moreover, the authors showed that depending on the substitution pattern of their B ring, anthocyanidins may interfere with different signaling cascades involved in the regulation of cell growth. It has been shown that cyanidin-3-glucoside as well as different anthocyanin-rich extracts induce apoptosis in different human leukemia cell lines [38].

Angiogenesis plays a critical role in the development of cancer. Liu et al. [39] have found that anthocyanins from black raspberry can reduce tumor development by inhibiting angiogenesis.

Mutagenicity is closely related to carcinogenicity. Juices from anthocyanin-rich fruits such as strawberry, blueberry, and raspberry significantly inhibited mutagenesis caused by the direct-acting mutagen methyl methanesulfonate and the metabolically activated carcinogen benzo(a)pyrene [40].

Another possible via for the protection against cancer is the inhibition of DNA damage. Sarma and Sharma [41] have observed that copigmentation between cyanidin derivatives and DNA has a protective effect on both molecules against the action of hydroxyl radicals generated by the Fenton reaction.

5.3 Beneficial Effects in Diabetes

Basically, anthocyanins may have a beneficial effect on diabetes at different levels. On one hand, they can interfere with glucose absorption and insulin secretion; on the other hand, they may have a protective effect on pancreatic cells. It has been shown that the simultaneous and daily administration to rats of anthocyanins from red wine together with streptozotocin, a diabetogenic compound with beta-cytotoxic effects, decreases glucose concentration in urine and serum. Additionally, anthocyanins from red wine prevent the generation of free oxygen radicals and decrease lipid peroxidation [19]. Jayaprakasam et al. [42] have shown that anthocyanins are also able to stimulate insulin secretion from rodent pancreatic beta-cells. It has been shown that cyanidin-3-glucoside and protocatechuic acid exert insulin-like activities by activation of peroxisome proliferator-activated receptor-γ (PPAR-γ), reveling a causal relationship between this transcription factor and adiponectin and GLUT4 upregulation [43]. Cyanidin-3-rutinoside has shown to retard the absorption of carbohydrates by inhibition of α-glucosidase [44].

5.4 Ocular Effects

Consumption of black currant-based extracts or concentrates of anthocyanins has a positive effect on night vision, i.e., dark adaptation, though the positive effect on human vision of *Vaccinium myrtillus* (bilberry) extracts is controversial and still needs to be clarified [45]. However, recent studies in animal models suggest that long-term supplementation with bilberry extracts is effective in the prevention of macular degeneration and cataract [46].

In subjects with myopia, it has been shown that the administration of anthocyanins for 4 weeks improved subjective symptoms and objective contrast sensitivity [47]. So far, several authors agree in the protective effect of anthocyanins against retinal damage via different mechanisms and using different experimental models. For instance, Liu et al. [48] have shown that the suppression of retinal damage by anthocyanins is mediated by protecting rod photoreceptor cells and improving the blood circulation of the retina under strong light conditions.

5.5 Antimicrobial and Antiviral Activity

Even though many studies have shown that isolated anthocyanins or plant extracts rich in this group of flavonoids have an effect on microbial growth, results are not yet conclusive. Puupponen-Pimia et al. [49] showed that extracts from different berries had an inhibitory effect on gram-negative bacterial growth, but had no effect on gram-negative bacteria. Moreover, they showed that black currant extracts stimulated the growth of different strains of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* but inhibited those of *Escherichia coli*. Recent studies have demonstrated that black currant concentrates inhibit the growth of different strains of *Staphylococcus aureus*, *Escherichia coli*, and *Enterococcus faecium* but stimulate the growth of *Saccharomyces cerevisiae*.

The effect of anthocyanins on microbial pathogens has not been studied in depth up to now. Some few studies exist, however, on the effect of berry extracts on pathogens. Among the berries and bacteria assayed by Puupponen-Pimia et al. [49], cloudberry and raspberry were the best inhibitors, and *Staphylococcus* and *Salmonella* the most sensitive bacteria. In another study by Nohynek et al. [50], they showed that of all the pathogens assayed, the most sensitive bacteria to berry phenolics were *Helicobacter pylori* and *Bacillus cereus*.

Different cyanidin glycosides present in extracts of *Ribes nigrum* L. have shown antiviral properties toward influenza A and B viruses and herpes-1 virus [51, 52].

5.6 Effects on Neurodegenerative Processes

Joseph et al. have shown that anthocyanin-rich fruits may be beneficial in reversing the course of neuronal and behavioral aging. Moreover, by using transgenic mice as a model for Alzheimer's disease, Joseph et al. [53] have reported on the beneficial effect of blueberry extracts on the outcome of this neurodegenerative illness. It has been shown that cyanidin-3-glucoside may ameliorate ethanol-induced neuronal death in the developing brain reducing the ethanol-meditated caspase-3 activation, neurodegeneration, and microglial activation in the cerebral cortex [54].

6 Biotechnological Approaches

Biotechnological approaches, by introduction of genes encoding novel anthocyanin enzyme activities and transcription factors or inactivation of endogenous genes, have been used to modify flower color and plant coloration as well as to analyze the potential of the anthocyanin pathway in plant defense response. Several important structural genes and regulatory elements required for anthocyanin biosynthesis have been cloned and characterized in a variety of plant species over the past few years, including some with direct practical applications. With the generation of transgenic petunia plants using a DFR gene from maize, a novel flower color was created by genetic engineering for the first time [55]. DFR enables the production of pelargonidin in petunia and leads to a change in flower color from pale pink to brick red. Similar results have been observed in petunia by overexpressing heterologous DFR genes from rose [56]. An approach involving the co-suppression of the CHS gene has been used to prevent the formation of flower pigments in different plant species. By contrast, overexpression of the CHS gene has been used to reduce flower pigments in petunia and chrysanthemum. White and blue-white transgenic varieties of Torenia hybrida taken from the blue variety cv. Summer wave were obtained by co-suppressing the expression of both the CHS and DFR genes [57].

F3′H and F3′5′H, members of the cytochrome P450 family, play a key role in determining the pattern of anthocyanin; while F3′H is necessary for the synthesis of

3′-hydroxylated anthocyanins (e.g., cyanidin), F3′5′H participates in the synthesis of 3′5′-hydroxylated anthocyanins (e.g., delphinidin). Overexpression of F3′5′H produced purple to violet transgenic flower colors due to the induction of the synthesis of delphinidin derivatives [56], and a dramatic change of flower color from pink to magenta with a high content of 3,5′-hydroxylated anthocyanins was observed in petunia. However, transgenic torenia expressing F3′H cDNA exhibited redder flower color due to the increase in 3′-hydroxylated anthocyanins [58].

Another useful strategy for introducing or increasing anthocyanin production in target ornamental species is the alteration of the balance between flavonoid enzyme activities by genetic modification. The dihydroflavonols are the direct substrates for colorless flavonol and colored anthocyanin biosynthesis. Thus, the introduction of a DFR sense transgene in white-flowered lines of petunia, which accumulates flavonol, resulted in a pink-flowered phenotype. Furthermore, inhibition of flavonol biosynthesis by co-suppression of the flavonol synthase (FLS) also led to anthocyanin production and a pink-flowered phenotype [59]. In torenia, the inactivation of the DFR gene by genetic transformation caused the accumulation of flavones, possible copigments, resulting in a copigmentation likely to make the torenia flowers bluer [60].

Advances are also being made in understanding the action mechanisms of regulators in anthocyanin biosynthesis. A general role for the basic helix-loop-helix (bHLH) MYB- and WD40-repeat proteins as regulators of the anthocyanin pathway has been conserved in a wide variety of species [61]. The overexpression of the R gene, encoding a bHLH, from maize enhanced the expression of anthocyanins in *Arabidopsis*, petunia, tobacco, and *Caladium* bicolor [62]. Overexpression of bHLH from *Antirrhinum* and *Perilla frutescens* also increased pigmentation in the whole tomato plant and in the tobacco flower [63]. A recent study shows that mutation of PH4, which encodes a MYB domain protein, results in a bluer petunia flower color, increased pH in petal extracts, and, in certain genetic backgrounds, the disappearance of anthocyanins and fading of the flower color [64].

Transgenic approaches uncovered a strong relationship between structural and/or regulatory anthocyanin gene expression and plant defense response levels. It has been suggested that anthocyanin functions as photoprotective pigments and that its accumulation can reportedly be induced in many plants by biotic and abiotic stresses, such as UV-B radiation, pathogen infection, or low temperature [65]. Anthocyanin accumulation appears to be a general response to cold stress. However, evidence suggests that anthocyanin itself is not essential for freezing tolerance, although some responses essential to freezing tolerance may be governed by a regulatory pathway or biochemical level that is also involved in the anthocyanin response [66].

The ectopic expression of anthocyanin 5-*O*-glucosyltransferase (5-UGT) cDNA in potato increased anthocyanin diglucoside levels and improved the resistance of transgenic lines to Erwinia carotovora subsp. Carotovora. Likewise, simultaneous expression of genes encoding CHS, CHI, and DFR in potato resulted in a significant increase in trisaccharide derivatives of pelargonidin and petunidin. However, transgenic lines showed a decrease in starch levels, accompanied by an increase in sucrose levels [67], suggesting that the limitation of flavonoid synthesis might depend on the availability of carbohydrates for phenolic compound synthesis.

Tomato fruits (*Lycopersicum esculentum*) are not usually reported to contain anthocyanin. However, tomato accession LA1996 with the anthocyanin fruit (Aft) gene has elevated anthocyanin levels in the skin and outer pericarp tissues of the fruit produced by the glycosylated anthocyanidins of petunidin, malvidin, and delphinidin [68]. These authors proposed that the introduction of the Anthocyanin fruit characteristic into carotenoid-rich tomatoes would provide the opportunity for developing new cultivars rich in water- and lipid-soluble antioxidants.

7 Conclusion/Prospects

Anthocyanins are excellent targets for biotechnological and health-related research lines. There is a great interest on increasing their directed biosynthesis, by increasing not only their total concentration in vegetable raw materials, but also the concentration of specific compounds with enhanced properties, either for their use as food colorants or for their health-related properties. Further studies are needed in the study of the biochemical pathways implicated in their glycosylation and acylation. The selective activation of those metabolic pathways conducting the synthesis of more stable or bioactive final products will open a promising perspective for the design of functional foods or new foods with health-related claims.

Regarding their biological activity, a lot is yet missed. Mechanistic studies that help to understand their implications in the prevention of cardiovascular disease, cancer, ocular health, and aging are still needed. Studies on absorption and distribution are still needed, especially those targeted to study the effect of intestinal microflora on anthocyanin structure and to identify gut flora metabolites. The effect of long-term exposure to anthocyanins is still largely unknown. The existing literature on biological activities gives enough evidences to think that anthocyanin-rich products, such as berries or red wine, may have a protective effect on human health, especially by preventing cardiovascular diseases and some types of cancer. However, more studies, preferably in vivo, are needed in order to establish the real implications of anthocyanins in these health-promoting properties.

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Flavones and Flavonols: Phytochemistry and Biochemistry 59

Qiang Zhang, Xinhuai Zhao, and Hongbin Qiu

Contents

1 Introduction 1822
2 Occurrence 1823
3 Biosynthesis 1825
3.1 Biosynthesis of Flavone and Flavonol Aglycones 1825
3.2 Flavone and Flavonol Modifications 1826
4 Biological Activity 1830
4.1 Role of Flavones and Flavonols in Plants 1830
4.2 Biological Activities of Flavones and Flavonols in Animals 1832
5 Genetic Engineering 1837
6 Conclusion 1839
References 1839

Abstract

Flavonoids are natural products widely distributed in the plant kingdom and currently consumed in large amounts in the daily diet. These are categorized according to their molecular structures into flavonols, flavones, flavanones, catechins, anthocyanidins, dihydroflavonols, isoflavones and chalcones. Among them, flavones and flavonols define the largest subgroups. The diverse functions of flavones and flavonols in plants as well as their various roles in the interaction with other organisms offer many potential applications, not only in plant breeding but also in ecology, agriculture, and human nutrition and

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pharmacology. In this chapter, we focus mainly on the occurrence and biosynthesis of flavones and flavonols, their biological functions in plants and animals, and metabolic engineering strategies of flavone and flavonol pathway in plants.

Keywords
Bioactivity • biotechnology • flavones • flavonols • phytochemicals

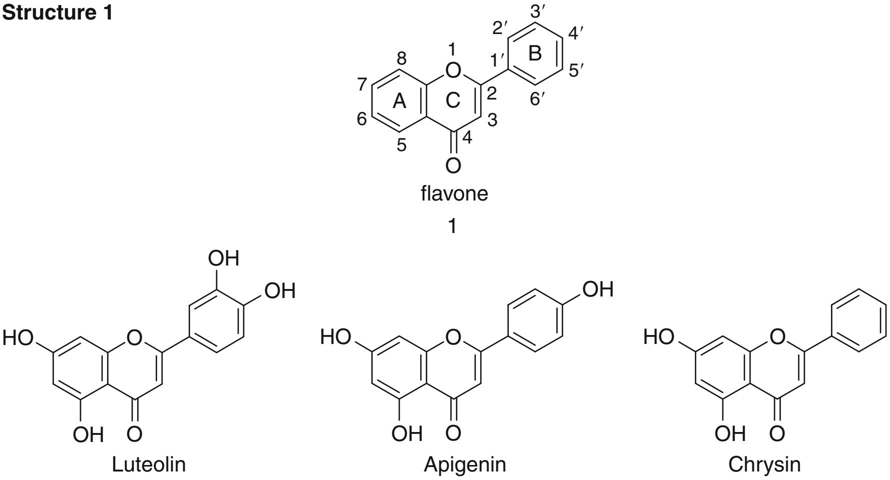
Abbreviations

2OGD	2-oxoglutarate-dependent dioxygenases
4CL	4-coumarate: CoA ligase
ACC	Acetyl-CoA carboxylase
C4H	Cinnamate 4-hydroxylase
CHI	Chalcone isomerase
CHS	Chalcone synthase
DHFs	Dihydroflavonols
F3H	Flavanone 3β-hydroxylase
FNS	Flavone synthases
IAA	Indole-3-acetic acid
NPA	Naphthylphthalamic acid
OMT	*O*-methyl transferase
PAL	Phenylalanine ammonia-lyase
PAPS	3′-phosphoadenosine 5′-phosphosulfate
ROS	Reactive oxygen species
STs	Sulfotransferases

1 Introduction

Within the secondary metabolite class of flavonoids which consist of more than 9,000 known structures [1], flavones and flavonols define the largest subgroups. Their natural distribution is demonstrated for almost all plant tissues. The basic structural features of flavones and flavonols are 2-phenyl-chromones and 2-phenyl-3-hydroxy-chromones, respectively (**1**–**2**) [2]. It is already well established that flavones and flavonols have a significant impact on various aspects of plant biology. They exhibit a wide range of functions in physiology, biochemistry, and ecology, for example, in UV protection, flower coloration, interspecies interaction, and plant defense. Moreover, flavones and flavonols have been utilized in the chemotaxonomy of some plants as useful markers. Other highly remarkable properties of certain flavones and flavonols are their nutritional values and medicinal benefits to humans, represented among others by antioxidant or putative anticancer activities [3]. In this chapter, we first describe the distribution and biosynthesis of flavones and flavonols in plants. Moreover, the various roles of flavones and flavonols in the plants' physiology and ecology and also their meaning for human health will be presented. At last, metabolic engineering strategies of flavone and flavonol pathways will be discussed.

Structure 1


3′
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1
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HO
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O
Luteolin
OH
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Apigenin
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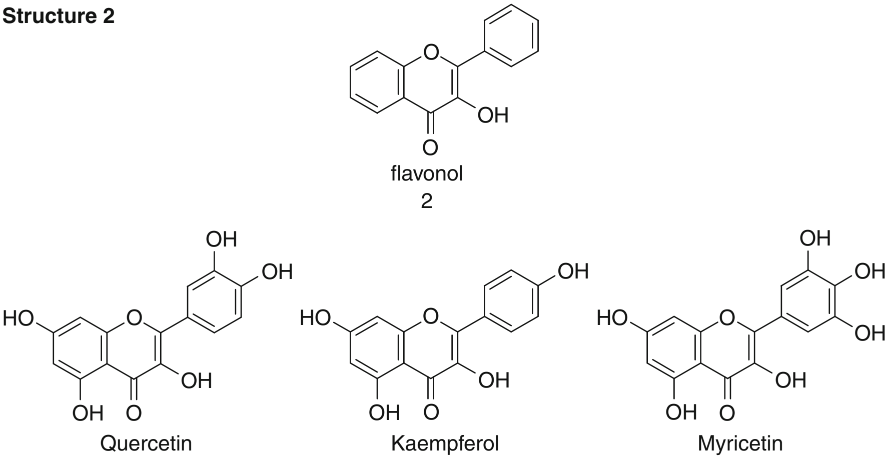
OH
O
Chrysin

Structure 2


O
OH
O
flavonol
2
OH
OH
HO
O
OH
OH
O
Quercetin
OH
HO
O
OH
OH
O
Kaempferol
OH
OH
HO
O
OH
OH
OH
O
Myricetin

2 Occurrence

Commonly, flavones are present in vacuoles of cells as *O*- and/or *C*-glycosides, but some compounds, especially simple and polymethylated flavones, occur in heartwood of *Prunus* species; farinose exudates of *Primula* spp. and ferns;

Table 59.1 The sorts and distribution of naturally occurring flavones

Sorts	Distribution
Flavone aglycones: more than 400 kinds	*Prunus* (Rosaceae) (heartwoods), *Alnus*, *Betula, Ostrya* (Betulaceae) (bud wax), *Primula* (Primulaceae) (farinose exudates), *Pityrogramma*, *Cheilanthes*, *Notholaena* (pteridophytes) (farinose exudates), etc.
Flavone *O*-glycosides: about 500 kinds (as commonly 7-, 3′-, 4′-glycosides and their various combinations), 5-*O*-glycosides (rare)	Widespread from bryophytes to higher plants
Flavone *C*-glycosides: about 300 kinds (as 6-, 8- or 6, 8-glycosides)	Widespread from Chlorophyta (*Nitella*) to bryophytes and higher plants

Pityrogramma, *Cheilanthes*, and *Notholaena* species; bud wax of *Alnus*, *Betula*, and *Ostrya* species (Betulaceae); and so on as aglycones [4, 5]. Up to now, more than 400 kinds of flavone aglycones have been identified and about 500 kinds of *O*-glycosides have widely been reported in plants as 7-, 3-, 4′-glycosides and in various combinations in most cases, but 5-*O*-glycoside is rare, since the 5-hydroxyl group forms hydrogen bonding with the adjacent 4-carbonyl group. Flavone *C*-glycosides (ca. 300 kinds) are also distributed from green algae to higher plants. The flavonoid compounds, which have been found in the most primitive plants, green algae, *Nitella hookeri*, liverworts, *Takakia lepidozioides*, and *T. ceratophylla*, are *C*-glycosyl flavones [6, 7] (Table 59.1).

The majority of flavonols are present as *O*-glycosides and very rarely as *C*-glycosides. There are about 900 flavonol *O*-glycosides identified as commonly 3-, 7-, 3-, 4′-glycosides and their various combinations. 5-*O*-Glycosides are rare as in the cases of the flavone glycosides. The glycosidic sugars are generally glucose, frequently galactose and rhamnose, and sometimes xylose, arabinose, and glucuronic acid. Very rare allose was found in *Glaucidium palmatum* Sieb. et Zucc. (Glaucidiaceae) as kaempferol, quercetin, and rhamnocitrin (kaempferol 7-methyl ether) 3-*O*-allosides [8]. Apiose, mannose, and galacturonic acid are also rare as glycosidic sugars. Flavonol glycosides are widely distributed in higher plants from Bryophytes. In a liverwort, *Takakia lepidozioides*, two flavonol glycosides, kaempferol 3-*O*-glucoside-7-*O*-xyloside and quercetin 3-*O*-glucoside, occur with some flavone *C*- and *O*-glycosides [7]. As the cases of flavones, free flavonols, particularly simple and polymethylated flavonols, also occur as farinose, bud, and leaf exudates of ferns, *Betula* (Betulaceae), *Cheilanthes* and *Notholaena*, *Artemisia* (Compositae), etc. [9, 10]. More exceptionally, two flavonols, 5, 2-dihydroxy-3, 7, 8- trimethoxyflavone and chlorflavononin (3′-chloro-5, 2′-dihydroxy- 3, 7, 8-trimethoxyflavone), were isolated from a fungus, *Aspergillus candidus* [11]. About 450 kinds of aglycones have been reported as naturally occurring substances (Table 59.2).

Table 59.2 The sorts and distribution of naturally occurring flavonols

Sorts	Distribution
Flavonol aglycones: about 450 kinds	*Artemisia* (Compositae) (leaf exudates), *Betula* (Betulaceae) (bud exudates), *Cheilanthes* (pteridophytes), etc.
Flavonol glycosides: about 900 kinds (as 3-, 7-, 3′-, 4′-glycosides and their various combinations), 6-*O*-glycosides (rare)	Widespread to higher plants from bryophytes

3 Biosynthesis

3.1 Biosynthesis of Flavone and Flavonol Aglycones

Almost all green plant cells are capable of synthesizing flavonoids. The biosynthesis invariably begins with the ubiquitous amino acid phenylalanine [12]. In the early steps of flavonoid biosynthesis, phenylalanine is converted into trans-cinnamate by phenylalanine ammonia-lyase (PAL) through the trans-elimination of ammonia and the pro-3S proton [13]. Then, cinnamate 4-hydroxylase (C4H) catalyzes the hydroxylation of trans-cinnamate to trans-4-coumarate in the initial oxygenation step of phenylpropanoid biosynthesis, which introduces the 4′-hydroxyl that is common to most flavonoids [14]. 4-Coumarate, the product of C4H, is key for flavonoid biosynthesis. It is activated by 4-coumarate: CoA ligase (4CL) for entry into the later branches of phenylpropanoid biosynthesis through formation of the corresponding CoA thiol esters.

Using 4-courmaroyl-CoA (in most species) and three molecules of malonyl-CoA, chalcone synthase (CHS) carries out a series of sequential decarboxylation and condensation reactions, to produce a polyketide intermediate that then undergoes cyclization and aromatization reactions that form the A-ring and the resultant chalcone structure. The chalcone formed from 4-courmaroyl-CoA is naringenin chalcone. In a few species, caffeoyl-CoA and feruloyl-CoA may also be used as substrates for chalcone formation. Malonyl-CoA is formed from acetyl-CoA by acetyl-CoA carboxylase (ACC). Acetyl-CoA may be produced in mitochondria, plastids, peroxisomes, and the cytosol by a variety of routes. It is the cytosolic acetyl-CoA that is used for flavonoid biosynthesis, and it is produced by the multiple subunit enzyme ATP-citrate lyase that converts citrate, ATP, and CoA to acetyl-CoA, oxaloacetate, ADP, and inorganic phosphate [15].

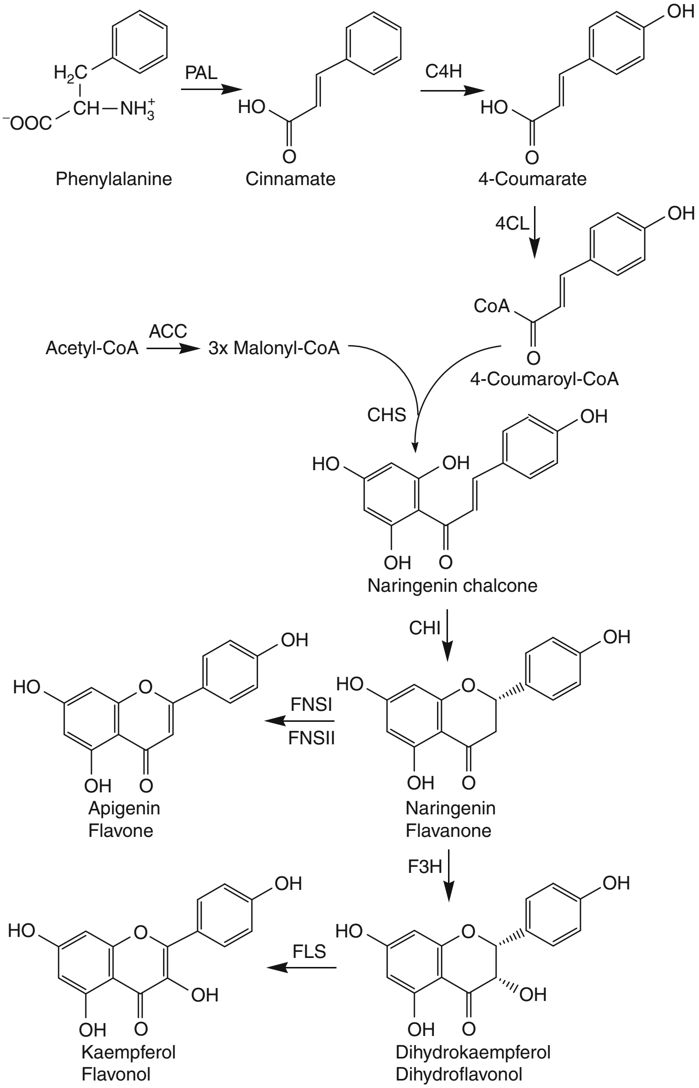
In a reaction that establishes the flavonoid heterocyclic C-ring, chalcone isomerase (CHI) catalyzes the stereospecific isomerization of chalcones to their corresponding (2*S*)-flavanones via an acid–base catalysis mechanism [16]. (2*S*)-Flavanones are converted stereospecifically to the respective (2*R*, 3*R*)-dihydroflavonols (DHFs) by flavanone 3β-hydroxylase (F3H). In the formation of both flavones and flavonols, a desaturation reaction forming a double bond between C-2 and C-3 of the C-ring is involved, and the respective substrates involved, (2*S*)-flavanones and (2*R*, 3*R*)-DHFs, differ only in the presence or absence of the 3-hydroxyl. Two distinct flavone synthases (FNS), FNS I and FNS II, have been characterized that convert flavanones to flavones. In most plants, FNS is a P450 enzyme (FNS II), but species in the Apiaceae have been found to contain the 2-oxoglutarate-dependent dioxygenases (2OGD) FNS I. Flavonols are formed from DHFs by the FLS. A similar reaction mechanism has been proposed for F3H, FLS, and FNS I, involving *cis*-hydroxylation at C-3 followed by dehydration, with (2*R*, 3*S*)-*cis*-DHFs as possible intermediates [17, 18]. Akashi et al. have suggested that the FNS II reaction likewise involves a C-2 hydroxylation step followed by dehydration [19]. However, the studies of Martens et al. [20, 21] suggest direct 2, 3-desaturation of flavanones by FNS I and FNS II, as previously proposed from biochemical studies of FNS I [22] (Scheme 59.1).

3.2 Flavone and Flavonol Modifications

The tremendous structural and functional diversity of flavonoids found in nature results from the modification by many enzymes that perform the substitution reactions. This is driven in part by opportunities for metabolic engineering of flavonoid biosynthesis for agronomic and nutritional improvement of plants. Glycosyltransferase, methylases, and sulfotransferases confer many of the ultimate chemical and bioactive properties of flavones and flavonols, for example, modulating flower color or enhancing the activity of these compounds for use in defense against herbivores and pathogens. These modifications also may play an important part in controlling the distribution of flux across branch pathways by altering intermediates to favor utilization by one or more competing enzymes at critical branch points. It is noteworthy that many of the substitution reactions are quite species-specific and most are still poorly understood both in terms of enzymology and their biochemical or physiological significance.

3.2.1 Glycosylation of Flavones and Flavonols

The addition of sugar groups has been well documented to enhance the solubility of flavonoids, as well as many other metabolites, and is likely to be critical for the transport and storage of these compounds at the final destinations in the vacuole or cell wall. Most flavonoid end products exhibit complex glycosylation patterns involving the addition of one or more glucose, rhamnose, or other sugars. Recent progress in understanding how this occurs includes elucidation of the terminal steps in the biosynthesis of maysin, a flavone produced in maize that provides resistance against the corn earworm, *Helicoverpa zea*. Quantitative trait locus and metabolite analysis of the maize *salmon silk* (sm) phenotype by McMullen and colleagues has shown that the *sm1* and *sm2* genes encode or control a glucose modification enzyme and a rhamnosyltransferase activity, respectively, in the maysin pathway [23]. This analysis has made


PAL
C4H
4CL
ACC
Acetyl-CoA
3x Malonyl-CoA
CHS
CHI
FNSI
FNSII
F3H
FLS
Phenylalanine
Cinnamate
4-Coumarate
4-Coumaroyl-CoA
Naringenin chalcone
Apigenin
Flavone
Naringenin
Flavanone
Kaempferol
Flavonol
Dihydrokaempferol
Dihydroflavonol

Scheme 59.1 Schematic of the major branch pathways of flavone and flavonol biosynthesis

possible the ordering of the final intermediates in the pathway from the flavone, luteolin, to isoorientin, to rhamnosylisoorientin, and then to the bioactive product, maysin.

Progress also has been made in the identification of genes encoding UDP-rhamnose: flavonol-3-*O*-rhamnosyltransferase and UDP-glucose: flavonol-3-*O*-glycoside-7-*O*-glucosyltransferase in *Arabidopsis* based on homology to other known flavonoid glycosyltransferases and combined genetic and biochemical analyses. The UGT78D1 gene encodes a specific UDP-rhamnose: flavonol-3-*O*-rhamnosyltransferase with activity only on kaempferol and quercetin, out of various flavonoids tested [24]. The recombinant protein from the UGT73C6 gene transfers glucose to the 7-hydroxyl of a range of flavonols and flavones, as well as the 6-hydroxyl of the unnatural 6-hydroxyflavone substrate. However, its in vivo activity is likely as a UDP-glucose: flavonol-3-*O*-glycoside-7-*O*-glucosyltransferase. The recombinant *Allium cepa* (onion) UGT73G1 protein also showed wide regiospecificity, adding glucose to the 3-, 7-, and 4′-hydroxyls of a wide range of flavonoids, including chalcones, flavanones, flavones, flavonols, and isoflavones, producing both mono- and diglucosides [25]. In contrast to these activities, recombinant protein from *A. cepa* UGT73J1 showed both high regiospecificity and tight substrate specificity, adding glucose at the 7-hydroxyl of only quercetin 3-*O*-glucoside and genistein out of many flavonoid substrates tested. A cDNA from *Vigna mungo* (black gram) seedlings encodes a protein with UDP-galactose: flavonoid 3-*O*-galactosyltransferase activity [26]. A 20-fold preference for UDP-galactose over UDP-glucose was found with kaempferol as a substrate.

P. hybrida pollen accumulates kaempferol and quercetin 3-*O*-(2″-*O*-glucopyranosyl)-galactopyranosides, which are not prevalent elsewhere in the plant, by the action of flavonol 3-*O*-galactosyltransferase (PhF3GalT) and flavonol 3-*O*-galactoside-2″-*O*-glucosyltransferase. Miller et al. [27] isolated a cDNA for a pollen-specific gene from *P. hybrida* whose recombinant protein showed the same activity profile as the previously characterized PhF3GalT. Unlike most of the GTs discussed previously, the PhF3GalT showed strong preference and high catalytic efficiency to kaempferol and quercetin, with other lower activities being limited to a range of flavonol aglycones. Notably, the PhF3GalT also catalyzed the reverse reaction, a deglycosylation. The enzyme, therefore, could be involved in modulating the abundance of a biologically active aglycone.

3.2.2 Methylation of Flavones and Flavonols

A variety of *O*-methylated flavones and flavonols have been described, involving substitutions at the 3, 5, 6, 7, 8, 2′, 3′, 4′, and 5′ positions. Many native *O*-methyl transferase (OMT) and their recombinant proteins are responsible for the formation of these compounds. A recombinant protein from semiaquatic freshwater plant *Chrysosplenium americanum* had an activity that methylated the 3′ hydroxyls of luteolin and quercetin [28]. Another recombinant protein from AtOMT1 (an *A. thaliana* cDNA) showed flavonol 3′-OMT activity, using quercetin and myricetin (flavonol aglycones) efficiently; however, it had much lower activity with luteolin [29]. A flavonoid 3′, 5′-OMT that has been identified from *C. roseus* could sequentially methylate the 3′- and 5′-hydroxyls of both myricetin and dihydromyricetin and showed weaker activity against the 3′-hydroxyl of dihydroquercetin [30]. A wide range of flavonols, including 6-hydroxykaempferol, quercetin, 6-hydroxyquercetin (quercetagetin), 8-hydroxyquercetin (gossypetin), myricetin, and quercetin 3-*O*-glucoside, were accepted as substrates by the recombinant protein from PFOMT (a *Mesembryanthemum crystallinum* cDNA), as were some flavones, flavanones, and HCA-CoA esters and glucosides. The reaction product for quercetin was shown to be isorhamnetin (3′-methoxyquercetin), while with quercetagetin five different products with 5-*O*-, 6-*O*-, 3′-*O*-, 5, 3′-*O*-, or 6, 3′-*O*-methylation were generated. This range of substrate choice and products with the recombinant protein is wider than for the purified native enzyme, the major products of which are only the 6-*O*- and 6, 3′-*O*-methyl ethers. This difference has been shown to be due to the N-terminal region of the protein, as a recombinant protein with the first 11N-terminal amino acids removed shows the same enzyme characteristics as the native enzyme [31]. The dual methylation reaction suggests a large and flexible active site, which is rare for the OMTs characterized to date.

3.2.3 Sulfurization of Flavones and Flavonols

Flavonoids esterified with sulfate groups have been reported to occur in many plant species, in particular mono- to tetrasulfate esters of flavonols and flavones and their methylated or glycosylated derivatives. These are likely generated by soluble sulfotransferases (STs), which transfer a sulfonate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS). Two subgroups of STs have been reported. The first contains enzymes with generally wide substrate acceptance that are typically involved in detoxification of small metabolites. Enzymes of the second subgroup, which includes the flavonoid STs, show high specificity, and in animals are involved in processes such as steroid transport and inactivation. The first plant STs characterized at the molecular level were the flavonol 3-*O*- and 4′-*O*-STs (F3ST and F4′ST) from *Flaveria chloraefolia* [32], followed by a second F3ST, BFST3, from *Flaveria bidentis* [33]. These enzymes form part of a group of flavonol STs that act sequentially to generate the range of flavonol polysulfates found in this genus. Strict specificities are shown to the 3-hydroxyl of flavonol aglycones (F3ST), or the 3′-, 4′- (F4′ST), or 7-hydroxyls of flavonol 3, 3′ or 3, 4′-disulfates [34]. Analysis of the recombinant proteins found the *F. chloraefolia* F3ST used only flavonol aglycones as substrates (both kaempferol and quercetin, and the methylated rhamnetin and isorhamnetin), and the F4′ST used only the flavonol 3-*O*-sulfates. BFST3 recombinant protein showed similar activity to the *F. chloraefolia* F3ST, except that kaempferol was not accepted as a substrate. A range of ST cDNAs has been identified from functional genomics studies of *A. thaliana*, including one (AtST3) that has been shown to encode a flavonoid 7-ST. Unlike BFST3, AtST3 recombinant protein accepts a number of flavonol and flavone aglycones, as well as their 3-*O*-monosulfated derivatives. However, strict specificity to the 7-hydroxyl was found. The plant soluble STs have around 25–30% amino acid identity with mammalian soluble STs, and are of a similar size [32]. Comparisons between *F. chloraefolia* F3ST and F4′ST, combined with mutational analysis and data from the crystal structure of mouse estrogen ST, have defined amino acid residues important for PAPS binding, substrate binding and catalysis, and the mechanism of sulfonate transfer [35, 36].

4 Biological Activity

4.1 Role of Flavones and Flavonols in Plants

The flavonoids are essential constituents of the cells of all higher plants. As two members of flavonoid family, flavones and flavonols have roles in many facets of plant physiology. One of their most important roles is to influence the transport of the phytohormone auxin, or indole-3-acetic acid (IAA) [37]. Flavonoids, particularly the flavone kaempferol and the flavonol quercetin could compete with naphthylphthalamic acid (NPA) to perturb auxin transport [38]. They are present in the root cap and columella of *Arabidopsis* seedlings [39, 40]. As kaempferol inhibits mammalian monoamine oxidases that are similar to characterized IAA oxidases [41] and flavonoid-deficient *Arabidopsis* mutants exhibit increased leakage of radiolabeled IAA or oxidized IAA from the root tip [39, 40], kaempferol may function in limiting the oxidation of auxin destined for basipetal redirection at the root tip. Quercetin accumulation in response to increased IAA levels may serve to scavenge reactive oxygen species that accumulate during IAA catabolism [42–44].

An obvious function of flavones and flavonols is as a filter for UV radiation [45]. UV-B radiation (280–315 nm) induces oxidative damage to DNA and proteins and degradation of the photosystem II reaction center [46]. These compounds filter UV-B radiation, thereby protecting plants by acting as a “sunscreen.” UV-B radiation also induces the synthesis of flavonols with higher hydroxylation levels. Because hydroxylation does not affect the UV-absorbing properties of these compounds but does affect their antioxidant capacity, it was suggested that flavonols may play as yet uncharacterized roles in the UV stress response.

Another important feature of flavones and flavonols that could have driven evolution is their allelochemical character. They are involved in various interactions with other organisms, microbes, as well as insects or other plants. For the host plant, these interactions can be both beneficial or harmful. Arbuscular mycorrhizae form mutualistic or symbiotic associations with plants. Quercetin, acacetin, and rhamnetin accumulated in roots of clover inoculated with mycorrhizae but not in noninoculated plants [47], suggesting that flavonols may mediate colonization. In addition, the root and shoot flavonoid composition was altered between colonized and noncolonized plants, which may be a direct or indirect effect of colonization [47]. Under low phosphate conditions, melons synthesized a *C*-glycosylflavone, isovotexin 2″-*O*-β-glucoside, which increased mycorrhizal colonization [48], thereby enhancing phosphate uptake. Apigenin, coumestrol, and daidzein increased mycorrhizal root colonization in soybean [49]. Although the signal that initiates colonization is unknown, flavonoids modulate the development of the association. Siqueira et al. [50] showed that among other flavonoids the flavone, chrysin can increase both mycorrhiza root colonization and root growth of *Trifolium repens*. Certain flavones have also been demonstrated to promote *Glomus* hyphal growth and spore germination [51].

A defined role for flavones as signaling molecules has been realized in the symbiosis between legumes and nitrogen-fixing rhizobia. Among other flavonoids, flavones from the host roots are exuded and selectively recognized by the respective bacterial symbiont. Subsequently, the synthesis of bacterial nod factor signals is initiated, which in turn are recognized by the plant host [52]. The first flavone identified to participate in such a communication was luteolin (5, 7, 3′, 4′-tetrahydroxyflavone) [53], but much more are described up to now [54]. Recent evidence has shown that flavones and flavonols are not only required to signal symbiotic bacteria in the legume-bacterium symbiosis but also play important direct roles in root nodule organogenesis [55, 56]. Flavonoids also accumulate in the progenitor cells for different legume organs [57, 58] and influence the development of in vitro root formation [59].

These elegant in vivo studies first confirmed that specific flavones, such as 7, 4′-dihydroxyflavone, were required to stimulate the production of nod factor synthesis in Sinorhizobia in the rhizosphere and in planta. Second, these findings suggested that certain flavonols such as kaempferol play direct roles in orchestrating plant organogenesis. This most likely occurs via the ability for kaempferol to induce localized inhibition of auxin transport, thus establishing more favorable hormonal gradients to enable root nodule formation from differentiated root cells [56].

In plant–insect interactions, flavones can be employed as copigments of delphinidin derivatives in blue-flowered plants, the preferred flower color of bees, thus contributing to the attraction of pollinators [60]. Again, in combination with a second compound that must be present, flavones (e.g., luteolin 7-*O*-(6″-*O*-malonyl)-β-D-glucoside together with *trans*-chlorogenic acid) are described to stimulate oviposition of certain insects on host plants [61]. Another aspect of flavones in plant–insect interaction is their impact on feeding of herbivores. As reviewed by Simmonds [62], flavones can affect insects in various ways, for instance they inhibit larvae feeding or act as feeding deterrent. Other flavones, such as 4″-hydroxy-maysin (5, 7, 3′, 4′-tetrahydroxyflavone 2″-*O*-α-L-rhamnosyl-6-*C*-[6-deoxy-*xylo*-hexos- 4-ulosyl]) can repress the development of corn earworm moth, *Heliothis zea*. An inheritance study with groundnut showed good evidence that the flavonols quercetin and its glycoside rutin are related to larval mortality of the tobacco armyworm *Spodoptera liture* [63]. The species *Arachis hypogaea* is a nonresistant host, whereas the wild species A. kempffmercadoi is resistant due to its flavonols. Feeding experiments with interspecific hybrids revealed a positive correlation between the flavonol content of the plants and larval mortality.

Besides their effectiveness against insects, certain flavones also exhibit activities against a variety of different organisms, for example, other plants [64, 65], nematodes [66], molluscs [67], fungi [68–70], oomycetes [69, 70], and bacteria [71]. These activities are often but not always and necessarily directed against attacking or phytopathogenic organisms. In such cases, the flavones are frequently inducible and have to be classified as phytoalexins. Mostly, these compounds are constitutively present in the particular plants. Unfortunately, no evident structure-function relationship has been identified that suggests why one flavone is involved in symbiotic communication, while others act as deterrents to herbivores or can be active as phytoalexins. However, a study by Picman et al. [72] on the inhibition of mycelial growth of the fungus *Verticillium albo-atrum* as indicator of biological activities showed that totally unsubstituted flavone was more effective (in the range of

1–5 ppm) than substituted derivatives, suggesting that at least the toxic effects might be due to hydrophobicity and their ability to interact with membrane structures.

4.2 Biological Activities of Flavones and Flavonols in Animals

Apart from being important components in plants, many biological activities of flavones and flavonols are known in animal systems, such as antioxidative activity. Flavones and flavonols are the powerful antioxidants for protecting the body against reactive oxygen species (ROS). ROS collectively denotes oxygen-centered radicals such as superoxide ($O_2^{\bullet -}$) and hydroxyl ($^{\bullet}OH$) as well as nonradical species derived from oxygen, such as hydrogen peroxide, singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl). Data obtained from many studies showed that flavones and flavonols, such as quercetin, kaempferol, morin, myricetin and rutin, by acting as antioxidants, exhibited beneficial effects such as anti-inflammatory, antiallergic, antiviral, as well as anticancer activity. They have also been suggested to play a protective role in liver diseases, cataracts, and cardiovascular diseases [73, 74]. The radical-scavenging activity of flavones and flavonols depends on the structure and the substituents of the heterocyclic and B rings, as suggested by Bors et al. [75]. The presence of a 3-hydroxyl group in the heterocyclic ring also increases the radical-scavenging activity, while additional hydroxyl or methoxyl groups at positions 3, 5, and 7 of rings A and C seem to be less important. These structural features contribute to increase the stability of the aroxyl radical, that is, the antioxidant capacity of the parent flavonoid. Thus, flavonols and flavones containing a catechol group in ring B are highly active, with flavonols more potent than the corresponding flavones because of the presence of the 3-hydroxyl group. Glycosylation of this group, as in rutin, reduces greatly the radical-scavenging capacity. An additional hydroxyl group in ring B (pyrogallol group) enhances further the antioxidant capacity, as exemplified by myricetin.

Because of their antioxidative properties, flavones and flavonols are likely to have a major influence on the vascular system. Oxygen radicals can oxidize LDL, which injures the endothelial wall and thereby promotes atherosclerotic changes. Flavones and flavonols may directly scavenge some radical species by acting as a chain-braking antioxidant [76]. The ability of quercetin and the quercetin glycosides to protect LDL against oxidative modification has shown a significant protective effect. Furthermore, flavonols are particularly antithrombotic because they directly scavenge free radicals, thereby maintaining proper concentration of endothelial prostacyclin and nitric oxide [77].

Numerous enzymes are inhibited by flavones and flavonols. They include hydrolases, oxidoreductases, DNA synthetases, RNA polymerases, phosphatases, protein phosphokinases, oxygenase, and amino acid oxidases. In some cases, the type of inhibition is competitive, but more often it is allosteric. The stunning variety of the types of enzymes, the activities of which are influenced by flavonoids, spans across almost all enzyme classes. The enzymes summarized in Table 59.3 are not exhaustive and aims to familiarize the reader with the extent of enzyme modulatory activities recorded.

Table 59.3 Effects of flavones and flavonols on activity of enzymes

Enzymes	Flavones or flavonols	References
Protein kinase C (PKC)	Fisetin, quercetin, and luteolin	[78, 79]
Mitogen-activated protein (MAP) kinase	Quercetin	[80]
Myosin light chain kinase (MLCK)	Kaempferol	[81]
Protein tyrosine kinases (PTK)	Quercetin, fisetin, robinetin, myricetin, and apigenin	[82–84]
Phospholipase A_2 (PLA2)	Quercetin, quercetagetin, kaempferol-3-O-galactoside, and scutellarein	[85, 86]
ATPases	Quercetin	[87–89]
Lipoxygenases	Baicalein, cirsiliol, quercetin	[90–95]
Cyclooxygenases	Luteolin, morin, galangin,	[96]
	Flavone, chrysin, apigenin, and phloretin	[97]
	Silymarin	[98]
	Oroxinidin, quercetagetin-7-glucoside, and tambuletin	[93]
Phospholipase C	Quercetin	[99]
Adenylate cyclase	Chrysin and apigenin	[97]
Reverse transcriptase	Amentoflavone, scutellarein, and quercetin	[100]
	Baicalein	[101]
	Quercetagetin, and myricetin	[102]
	Fisetin	[103]
HIV-1 proteinase	Myricetin, morin, quercetin, and fisetin	[104]
HIV-1 integrase	Quercetin	[105]
Ornithine decarboxylase (ODC)	Quercetin	[106]
	Apigenin	[107]
Topoisomerase	Fisetin and quercetin	[108]
	Baicalein, quercetin, quercetagetin, and myricetin	[109]
Glutathione *S*-transferase (GST)	Quercetin	[110]
Epoxide hydrolase	Flavone and 7,8-benzoflavone	[111]
Glyoxalase	Quercetin, fisetin, and myricetin	[112]
Xanthine oxidase	Quercetin	[113, 114]
	Baicalein	[115]
Aromatase	Quercetin, chrysin, apigenin, and kaempferol	[116]
		[117]
Aldose reductase	3′,4′-dihydroxyflavones	[118]
	3′,4′-dihydroxy-5,6,7,8-tetramethoxyflavone	[119]
Carbonyl reductase	Quercitrin	[120]
Hyaluronidases	Kaempferol and silybin	[121]
Malate dehydrogenase	Quercetin	[122]
Lactate dehydrogenase	Quercetin	[123]
Pyruvate kinase	Quercetin	[123]
Amylase	Quercetin	[124]

(*continued*)

Table 59.3 (continued)

Enzymes	Flavones or flavonols	References
RNA and DNA polymerases	Quercetin, kaempferol, and fisetin	[125]
	Quercetagetin, myricetin, and baicalein	[102]
Sialidase	5,7,4′-trihydroxy-8-methoxyflavone	[126, 127]
Nitric-oxide synthase	Quercetin, morin, apigenin, taxifolin, and fisetin	[128]
Monoamine oxidase	Coumarins	[129]

Flavones and flavonols can exert the anti-inflammatory activity in many animal models and are useful in allograft rejection and rheumatic diseases as an immunosuppressive agent. A number of reports have been published which demonstrate that flavones and flavonols, such as quercetin, myricetin, and fisetin, can modulate arachidonic acid metabolism via the inhibition of cyclooxygenase and lipoxygenase activity [130, 131]. Quercetin prevents immune cells and inhibits both the production and release of histamine and is useful in allergic conditions like asthma, hay fever, etc. [132].

Flavones and flavonols, especially quercetin, have been reported to possess antidiabetic activity. A cohort study that predicted the effects of flavonoids on chronic diseases showed that a trend toward a reduction in risk of type 2 diabetes was associated with higher quercetin and myricetin intakes. Vessal et al. reported that quercetin brings about the regeneration of pancreatic islets and increases insulin release in streptozotocin-induced diabetic rats [133]. Also in another study, Hif and Howell reported that quercetin stimulates insulin release and enhances Ca^{2+} uptake from isolated islet cells which suggest a place for flavonoids in non-insulin-dependent diabetes [134, 135].

It has become evident that flavones and flavonols are able to exert neuroprotective actions even at low concentration via their interactions with critical neuronal intracellular signaling pathways pivotal in controlling neuronal survival and differentiation, long-term potentiation, and memory. Early indications regarding the ability of flavonoids to impact upon brain function were reported in the 1950s, with flavones reported to act as novel brain-stem stimulants [136]. Jung et al. showed that quercetin at a dose of 10, 20, 40 mg/kg p.o. impairs cognitive function by suppressing pAkt and pCaMKII thus decreasing pCREB expression in hippocampus [137]. Maher et al. reported fisetin to facilitate memory by activating ERK and inducing cAMP response element-binding protein phosphorylation [138].

Some flavone derivatives were found to be ligands for the GABAA (γ-aminobutyric acid type A) receptors in the central nervous system, and thus, they bind to the benzodiazepine binding site with resulting depressant actions in mice [139]. These were also found to possess sedative action. Considering the sedative, the spontaneous locomotor activity and thiopental induce sleeping time effects obtained with the flavonoid glycosides. Position of the sugar on the flavonoid nucleus seems relevant as well, and position-7 is the most effective, but the presence of a double bond between carbons 2 and 3, resulting in flavone derivatives with planar configuration (i.e., linarin), does not appear to be critical for activity.

Flavonoid glycosides form the newest group within the growing family of flavonoids with activity on the central nervous system [139].

Flavones and flavonols have also been found to possess hepatoprotective activity. In a study carried out to investigate the flavonoid derivatives silymarin, apigenin, and quercetin, as putative therapeutic agents against microcrystin LR-induced hepatotoxicity, silymarin was found to be the most effective one [140]. Gulati et al. studied hepatoprotective studies on *Phyllanthus emblica* and quercetin and found that if the extract is producing hepatoprotection at a dose of 100 mg/100 g p.o., then quercetin is producing hepatoprotection at a dose of 15 mg/100 g p.o., thus concluding that quercetin is a potent hepatoprotective agent [141]. Oh et al. reported that among various flavonoids, that is, apigenin, luteolin, kaempherol-3-*O*-glucoside, and quercetin-3-*O*-glucoside isolated from *Equisetum arvense*, onitin and luteolin exhibited hepatoprotective activity against tacrine-induced cytotoxicity in human liver-derived Hep G2 cells [142].

Flavonoids have been used extensively since centuries for the treatment of various diseases. Galangin is a flavonol commonly found in propolis. Propolis has been used referred even in the Old Testament for its healing properties. The antimicrobial activity of propolis has been attributed to its high flavonoid content. It has been reported to possess inhibitory actions against *Aspergillus tamarii*, *Aspergillus flavus*, *Cladosporium sphaerospermum*, *Penicillium digitatum*, *Penicillium italicum* [143]. Rattanachaikunsopon et al. isolated morin-3-*O*-lyxoside, morin-3-*O*-arabinoside, quercetin, and quercetin-3-*O*-arabinoside from *Psidium guajava* leaves and reported that these four possess bacteriostatic action against all food-borne pathogenic bacteria including *Bacillus stearothermophilus*, *Brochothrix thermosphacta*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Salmonella enteric*, *Staphylococcus aureus*, *Vibrio cholerae* [144] (Table 59.4).

There has been mounting concern about the anticancer activity of flavones and flavonols. The weight of the epidemiological evidence for a protective effect of flavones and flavonols against cancer is impressive. A growing number of epidemiological studies suggest that high flavonoid intake may be correlated with a decreased risk of cancer [145]. In a cohort study of 25-year follow-up on 9,959 Finnish men and women aged 15–99 years and initially cancer-free, dietary intake of flavones and flavonols was inversely associated with the incidence of cancer at all sites combined [146]. Knekt and coworkers [147] also estimated flavonoid intakes of 10,054 men and women mainly on the basis of the flavonoid concentrations in Finnish foods with a dietary history method. They found that men with higher quercetin intakes had a lower lung cancer incidence, and men with higher myricetin intakes had a lower prostate cancer risk. A population-based case–control study in Hawaii further investigated the association between intake of flavone- and flavonol-powerful dietary and lung cancer risk. This study involved 582 patients with incident lung cancer and 582 age-, sex-, and ethnicity-matched control subjects [148]. These results agree well with a former case–control study involving 541 cases of lung cancer and 540 hospitalized controls in Uruguay [149]. Another case–control study in Spain, including 354 cases of gastric cancer and 354 hospitalized

Table 59.4 Antimicrobial activities of flavones and flavonols

Activity	Organism	Flavones or flavonols
Antibacterial activity	*Staphylococcus aureus*	Quercetin, Baicalin, Fisetin
	Staphylococcus albus	Fisetin
	Streptococcus pyogenes	Apigenin
	Streptococcus viridians	Apigenin
	Streptococcus jaccalis	Chrysin
	Streptococcus baris	Chrysin
	Streptococcus pneumonia	Chrysin
	Pseudomonas aeruginosa	Rutin, Baicalin, Hydroxyethylrutoside
	Escherichia coli	Quercetin
	Bacillus subtilis	Quercetin
	Bacillus anthracis	Rutin
	Proteus vulgaris	Datisetin
	Clostridium perferingens	Hydroxyethylrutoside
Antiviral activity	*Rabies virus*	Quercetin, Rutin
	Herpes virus	Quercetin
	Para influenza virus	Quercetin, Rutin
	Herpes simplex virus	Galangin, Quercetin, Kaempferol, Apigenin
	Respiratory syncytial virus	Quercetin
	Immunodeficiency virus	Apigenin
	Auzesky virus	Quercetin, Morin, Apigenin
	Polio virus	Quercetin
	Mengo virus	Quercetin
	Pseudorabies virus	Quercetin
Antifungal activity	*Candida tropicalis*	Quercetin
	Fusarium solani	Chrysoeriol
	Botrytis cinerea	Chrysoeriol
	Verticillum dahliae	Chrysoeriol
	Azotobacter vinelandii	Quercetin, Rutin
	Alternacia tennisima	Apigenin

controls, suggests that flavonoids such as quercetin and kaempferol may have protective effects against gastric cancer while specific carotenoids (alpha-carotene, beta-carotene, lutein, and lycopene) may not [150].

Many researchers have conducted *in vitro* studies on the potential anticancer activity of flavones and flavonols in diverse cell systems. An array of 55 flavones having a variety of substituents was evaluated by Cushman and Nagarathnam for cytotoxicity in five cancer cell cultures, A-549 lung carcinoma, MCF-7 breast carcinoma, HT-29 colon adenocarcinoma, SKMEL-5 melanoma, and MLM melanoma [151]. Other reports also revealed that flavones and flavonols exhibited cytotoxicity in vitro to many human cell lines, including esophageal cancer cells [152, 153], colon cancer cells [154, 155], hepatoma cells [156, 157], prostate carcinoma cells [158, 159], and cervical carcinoma cells [160, 161].

Animal studies and investigations using different cellular models suggested that certain flavones and flavonols could inhibit tumor initiation as well as tumor progression [162–164]. Quercetin inhibited DMBA-induced carcinogenesis in hamster buccal pouch [165] and in rat mammary gland [166]. When given during the initiation stage, quercetin also inhibited DEN-induced lung tumorigenesis in mice [167]. In a medium-term multiorgan carcinogenesis model in rats, quercetin (1 % in the diet) inhibited tumor promotion in the small intestine [168]. Feeding rats with quercetin (0.05 % in the diet), during initiation or promotion stage, inhibited 4-NQO-induced carcinoma formation in the tongue.

The plethora of effects of flavones and flavonols on the metabolism of cancer cells is difficult to rationalize to a few basic, specific mechanisms. These compounds interfere with a large number of regulatory pathways, for example, those of growth, energy metabolism, apoptosis, cell division, transcription, gene repair, neuronal transmission, inflammation, and stress response [169–176]. They can act as antioxidants, free-radical scavengers, enzyme inhibitors, hormones (including neurotransmitters), antihormones, or inducers of gene expression.

These effects can be divided into two classes, the electronic and the steric. The high mobility of the electrons in the benzenoid nucleus of flavones and flavonols accounts for both their antioxidant and free-radical-scavenging properties, whereas the structural resemblance between the flavone and flavonol aglycone and many substances inherent to the biochemistry of normal biological cells, for example, nucleic acid bases, coenzymes, steroid hormones, and neurotransmitters, explains their inhibition of enzymes, cytoplasmic/nuclear hormone receptors, and neurotransmitters, as well as gene induction. The high affinity of flavones and flavonols for heavy metal ions provides additional opportunities for interference with the action of enzymes and Zn^{2+} fingers in DNA-binding proteins. The biochemical pathways, which are influenced by flavones and flavonols, can roughly be classified according to their sensitivities. Generally, the actions mediated by the family of cytoplasmic/nuclear hormone receptors are highly sensitive to flavonoids, whereas enzyme inhibition and similar processes, for example, at synaptic membranes, require higher concentrations of flavonoids [177].

5 Genetic Engineering

Flavones and flavonols become more and more commercially important for the floricultural, agricultural, food, and pharmaceutical industry, respectively. Consequently, genetic engineering can provide a valuable tool to expand the plant gene pool and thus promoting the generation of new commercial plant varieties or plant-derived products (e.g., food supplements, functional foods, herbal drugs). Generally, flavones and also flavonols contribute as common copigments to the color appearance of cyanic pigments in plant tissues. Therefore, by metabolic engineering of this step the copigment level can be modified. However, as flavones and anthocyanins share common precursors, the levels of copigments and anthocyanin are generally negatively correlated. Practically, that means that a reduction on flavone level, for example, by antisense suppression, will likely cause in increase of anthocyanin level due to precursor flow in only one direction. However, downregulation of the FNS II gene in blue torenia *(Torenia hybrida)* using antisense technique decreased the level of flavones as expected. Those of its precursor, the flavanones, are increased. Unexpectedly, the levels of anthocyanins were reduced and the resultant flower color was pale blue [178]. The strong copigment effect of flavones was documented in transgenic carnation *(Dianthus caryophyllus)*. FLORIGENE Moonshadow^{TM}, the first blue carnation, was analyzed regarding the flavonoid pattern. Besides delphinidin derivatives, the transgenic petals also contained an apigenin derivative that is proposed to have a strong copigment effect on the anthocyanin [179].

Flavonol formation requires the expression of CHS, CHI, F3H, and FLS, this last gene encoding flavonol synthase, which converts dihydrokaempferol or dihydroquercetin to the corresponding flavonols, kaempferol, and quercetin, respectively. Little continues to be known about how this branch of the flavonoid pathway is regulated. The regulators of anthocyanin biosynthesis can induce the accumulation of flavonols, as recently shown by the comprehensive analysis of the metabolic changes associated with the expression of the R2R3-MYB transcription factor PAP1 gene in Arabidopsis [180]. Interestingly, however, while the overexpression of PAP1 resulted in a significant increase in the expression of many flavonoid biosynthetic genes, FLS was not among the upregulated genes, suggesting that the increase in flavonol accumulation is likely a consequence of the presence of a constitutively expressed FLS, together with an increased flux through the flavonoid pathway.

In tomato, which produces naturally only low amounts of kaempferol and quercetin in the fruit peel, the introduction and overexpression of the regulatory genes *Lc* and *C1* of maize led to an increase in kaempferol formation of up to 60 %, mainly in the flesh of the fruits. Moreover, introducing the *CHI* gene of *Petunia* resulted in an increase of up to 70 % in quercetin formation in the peels. Expressing *Lc* and *C1* in potatoes also caused a marked accumulation of kaempferol in the tubers [181]. In the future, the availability of the *FLS* gene from several sources may allow a more directed engineering of flavonol synthesis. Moreover, the recent cloning of the gene encoding FNS II [19, 182] provides the means for manipulating flavone formation for agronomic and nutritional purposes.

AtMYB12, encoding a R2R3-MYB transcription factor with high identity to the maize P1 regulator of the phlobaphene pigments [183], recently has been identified as a regulator of flavonol biosynthesis in Arabidopsis [184]. AtMYB12 regulates the expression of CHS, CHI, F3H, and FLS but not of the anthocyanin biosynthetic genes. A mutation in AtMYB12 results in a significant decrease in flavonol accumulation, suggesting that other regulators may contribute to the control of this branch of the pathway. These studies also showed that P1 can control the expression of the flavonol biosynthetic genes in Arabidopsis, suggesting that P1 may regulate flavonol biosynthesis in maize. However, in maize, P1 is unable to activate F3H, and no flavonols were detected among the several flavonoids induced by P1 expression [185]. Similar to P1, AtMYB12 functions independently of the known bHLH coactivators. The studies on AtMYB12 make this gene the only bona fide regulator of the flavonol pathway that so far has been described.

6 Conclusion

The diversity, ubiquity, and bioactivity of flavones and flavonols make these compounds of interest to a wide variety of research. It is still unknown exactly how these compounds exert their influence on growth, auxin transport, and other biologically relevant interactions in the plant kingdom. It is obvious that considerably more research is required to dissect out the complexities involved with flavone and flavonol functions in the animal kingdoms. There is a need to improve research on mechanisms of bioactivity in animals and plants. The advances in genomics, proteomics, and metabolomics provide new approaches to define the role of flavones and flavonols in plant development and to explore their potential application in agriculture and medicine.

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Isoflavonoids

60

Varsha Sharma and Kishan G. Ramawat

Contents

1 Introduction 1850
2 Occurrence 1852
3 Phytochemistry 1854
4 Biosynthetic Pathway 1856
5 Biological Activities 1858
5.1 Cancer Prevention 1859
5.2 Cardiovascular Diseases 1860
5.3 Antioxidant Activity 1860
5.4 Estrogenic Activity 1861
6 Biotechnological Approaches 1861
7 Conclusion 1863
References 1863

Abstract

Isoflavonoids are subclass of flavonoids and have been isolated from a wide variety of leguminous and non-leguminous plants. Isoflavones are characterized by having the B-ring attached at C_3 rather than C_2 position and undergo various modifications; these modifications lead to formation of simple isoflavonoids such as isoflavanones, isoflavans, and isoflavanols, as well as more complex structures including rotenoids, pterocarpans, and coumestans. Isoflavones are present in berries, wine, grains, nuts, soybeans, and other legumes including kudzu root (*Pueraria lobata*), peanuts (*Apios americana*), and chickpeas (*Cicer arietinum*). Isoflavonoids are derived from the phenylpropanoid pathway which is established both enzymatically and genetically. There are many biological

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activities associated with the isoflavones, including reduction in osteoporosis, cardiovascular disease, and prevention of cancer and for the treatment of menopause symptoms. Recent data indicate that the protective effect of isoflavonoids may extend beyond their antioxidant activity on molecular and cellular levels and modulating activity of many other enzymes. Biotechnological approaches have been used to produce isoflavonoids through cell cultures of different species grown in shake flasks and bioreactor using normal and transformed cells. Currently, isoflavonoids are an area of active and advancing research with huge potential for the benefit of human health.

Key Words

Glycine max • Isoflavonoids • polyphenolics • *Pueraria*

Abbreviations

CHS	Chalcone synthase
CHI	Chalcone isomerase
CHR	Chalcone reductase
HID	2-Hydroxyisoflavanone dehydratase
HDL	High density lipoprotein
IFS	Isoflavone synthase
LDL	Low density lipoprotein
OMT	*o*-Malonyltransferases
OMTs	*o*-Methyltransferases
UGTs	UDP-Glycosyltransferases
USDA	United States Department of Agriculture

1 Introduction

In recent years, the role of phenolic compounds and flavonoids as protective dietary constituents has become an increasingly important area of human nutrition research. Flavonoids are polyphenolic compounds comprising of 15 carbons with two aromatic rings connected by a three-carbon bridge. Isoflavonoids form a distinct class among flavonoids and have a characteristic structure with very limited distribution in nature (Fig. 60.1). Isoflavonoids are derived from the phenylpropanoid pathway and are synthesized predominantly in leguminous plants [1]. Simple isoflavonoids are dietary phytoestrogens and their glycosides, although coumestrol (a more complex isoflavonoid derivative) is also a phytoestrogen. The number of known isoflavone glycosides [e.g., genistin (genistein7-*O*-β-D-glucopyranoside)] is small when compared with the vast range of known flavonoid glycosides, and in this *O*-glycosides are in majority [2]. Up to now, the isoflavanoid compounds have been studied intensively and about 1,600 isoflavonoids have been identified. Much of this molecular diversity is generated through the biosynthetic modification of core isoflavonoid chemical scaffolds. In general, isoflavonoid

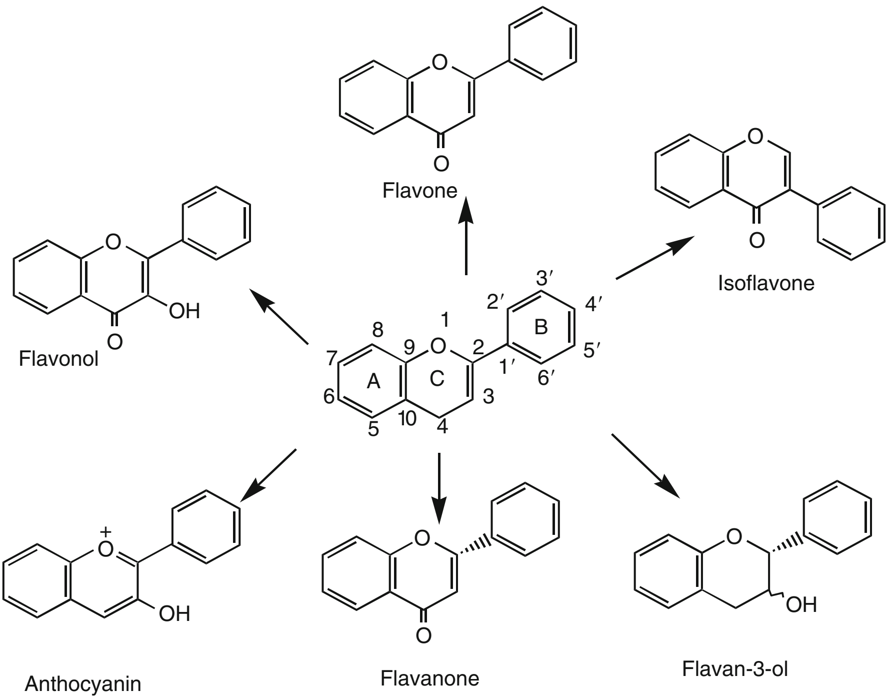

Fig. 60.1 Generic structures of the major flavonoids

compounds primarily occur in legumes, but have also been found in a considerable number of non-legumes. To date, at least 225 isoflavonoids have been isolated from about 59 non-leguminous families [1].

Isoflavone molecules do not exist at high levels in their biologically active form in natural food products, but rather are at high abundance in precursor form. For example, daidzin, the precursor of daidzein, is the glycosidic form that contains a carbohydrate moiety (portion) of the molecule. Daidzin is metabolized in the gastrointestinal tract by intestinal bacteria, which hydrolyzes the carbohydrate moiety, to the biologically active isoflavone, daidzein. Daidzein is then further metabolized in the intestine to equal at relatively low or high levels dependent upon several biological, dietary, and, presumably, environmental factors [3]. Isoflavones are inactive molecules when in the glycosidic form (genistin, daidzin), but as aglycones (without sugar moiety; genistein, daidzein) intestinal absorption is possible. Intestinal absorption varies greatly between individuals, which may be related to the content of dietary fiber and the state of intestinal microflora. After absorption, isoflavones are reconjugated to glucuronides and excreted unchanged in the urine [4]. Soy is recognized as the major dietary source of phytoestrogens, and soy-based products have been shown to contain significant quantities of total isoflavones. Isoflavones are known for their estrogenic activity due to their ability to bind to estrogen receptor and have received much attention due to their putative role as potential treatments for many disorders including cardiovascular disease, osteoporosis, age-related diseases, and hormone-dependent cancers [3].

Historically, legumes have played an important role in the diets of most cultural groups, and in many Asian countries, the most commonly consumed legume is the soybean (Table 60.1). Human consumption of isoflavones has the largest impact due to its availability and variety in food products. As expected, high levels of genistein and daidzein, as well as substantial amounts of coumestrol (Fig. 60.2), were found in traditional soy-based foods, as well as soy protein isolate, soy concentrate, or soy flour added to foods. Pulse grains contain a large number of bioactive compounds which have a metabolic benefit when consumed on a regular basis. The world production of soybean in 2009 is presented in Fig. 60.3. Demand for pulses has increased in recent years for human consumption either to extract a functional compound (e.g., starch, protein, or fiber), to incorporate this into cereal-based products, or to extract bioactive compounds which can be used as nutraceutical products [5].

2 Occurrence

Isoflavones are present in berries, wine, grains, and nuts, but are most abundant in soybeans and other legumes including kudzu root (*Pueraria lobata*), peanuts (*Apios americana*), and chickpeas (*Cicer arietinum*) [7]. Different isoflavone contents have also been reported in soybeans and soy-derived foods (soy milk, tofu, and tempeh), and other legumes such as adzuki beans, fenugreek, tepary beans, fava beans, soybeans, alfalfa, red clover, cowpeas, black gram, lentils, chickpeas, and licorice (Table 60.2). Daidzein and genistein are the two most well-characterized isoflavones [8]. Isoflavones are closely related to the antioxidant flavonoids found in other plants, vegetables, and flowers. Isoflavones, such as genistein and daidzein, are found in only some plant families, because most plants do not have the enzyme, CHI, which converts a flavone precursor into an isoflavone. *Pueraria lobata* (kudzu) and other species of *Pueraria* are also major source of isoflavonoids. Various parts of *Pueraria* plant like root, stem, and flowers accumulate isoflavonoids [9]. Isoflavonoid, puerarin (7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-(β-D-glucopyranoside), is the major isoflavonoid present in root tubers of *Pueraria* species.

Some of isoflavone molecules have also been identified in 23 other angiosperm families [10]. Several leguminous and non-leguminous plant species contain different isoflavonoids with estrogenic activity (Table 60.3). Major active compounds in several nutraceuticals are the soy phytoestrogens, the isoflavonoid puerarin from Kudzu vine (*Pueraria lobata*), and formononetin from clovers and fenugreek. Another example is the root of licorice (*Glycyrrhiza echinata*), which contains an estrogenic isoflavan (glabridin) and isoflavene (glabrene) [2]. Seven isoflavones were also isolated from sprouted chickpea seeds, namely, biochanin A, calycosin, formononetin, genistein, trifolirhizin, ononin, and sissotrin [11].

Table 60.1 World production and consumption of isoflavones containing major legume crops (Modified from [6])

Edible legumes Latin name	Common name	World crop production (metric tons)	Main region of consumption
Pulses		3,819	
Dry beans (*Phaseolus* spp.)		1,162	Worldwide Americas, Africa
Phaseolus vulgaris	Kidney bean		Asia, Japan
Vigna lunatus	Butter bean		
Vigna radiata	Green gram		
Vigna mungo	Black gram		
Phaseolus coccineus	Scarlet runner bean		
Dry peas (*Pisum* spp.)		892	
Pisum sativum var. *sativum*	Garden pea		Temperate region
Pisum sativum var. *arvense*	Protein pea		
Cicer arietinum	Chickpea	478	Asian and Middle East
Vigna unguiculata	Dry cowpea	350	Africa, Asia, South Americas
Cajanus cajan	Pigeon pea	103	Asia, Africa
Lens culinaris	Lentil	199	Worldwide

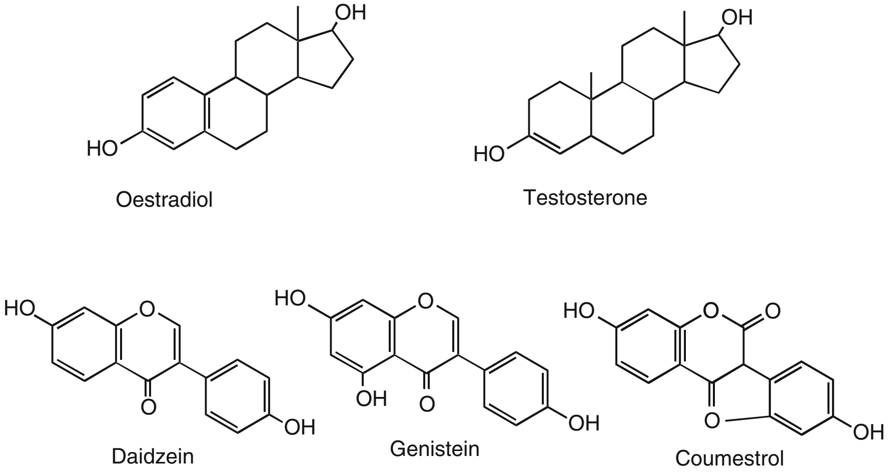

Fig. 60.2 Structures of the estrogen estradiol, the androgen testosterone, and the Isoflavonoids – daidzein, genistein, and coumestrol

Fig. 60.3 The world production of soybean in 2009

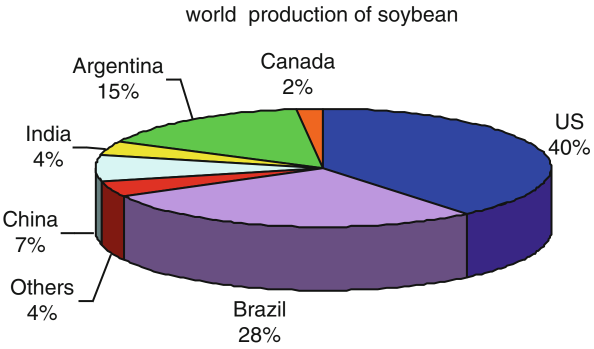

Table 60.2 Isoflavonoids content in different plant species

Plant name	Isoflavonoid content µg/100 g dry mass	
	Daidzein	Genistein
Glycine max ("Santa rosa"*)*	56,000	84,100
Glycine max ("Chapman")	41,300	46,400
Phaseolus vulgaris	28.2	158.0
Cajanus cajan	14.6	737.0
Cicer arietinum	34.2	69.3
Pisum sativum	7.9	22.8
Trigonella foenum-graecum	10.2	9.8
Vigna mungo	6.9	Traces
Arachis hypogea	49.7	82.7
Pueraria lobata (root)	185,000	12,600
Trifolium pratense	12,200	4,010
Psoralea obtusifolia	6,07,200	Not detectable
Genista sagittalis	16,33,600	3,100
Maackia amurensis	Traces	1,920
Pueraria tuberosa	6,042	8,682

3 Phytochemistry

Flavonoids are polyphenolic compounds containing 15 carbons with two aromatic rings connected by a three-carbon bridge, hence C_6-C_3-C_6 arrangements [12]. Flavonoids (about 5,000 structures known) are grouped into eight different subclasses, according to the oxidative status of the C-ring. These subclasses are flavanol, flavandiol, flavanone, dihydroflavanol, flavones, flavonol, isoflavone (about 1,000 structures known), and anthocyanidin. Isoflavonoids are a large subclass of flavonoids, consist of a phenyl ring fused with the six-membered heterocyclic C-ring and another phenyl ring (the B-ring) at the C_3 position, whereas for flavonoids, the B-ring is substituted to the C_2 position [13]. At the beginning of plant chemistry, isoflavonoids

Table 60.3 Occurrence of isoflavones in leguminous and non-leguminous plants

	Legumes	Non-legumes
Isoflavonoids	Biochanin, Daidzein, Derrisisoflavone Erysubin, Formononetin, Genistein, Glycitein, Glycyrrhiza-isoflavone Indicanine, Olibergin, Puerarin, Scanderone, Senegalensin	Non-legume plants have same isoflavonoids as legume plants, and some others are as follows: Auriculasin, Erythrin, Ficusin Garhwalin, Laburnetin Osajin, Pomiferin, Torvanol Warangalone
Plant species	*Cicer arietinum,* *Glycine max*, *Glycyrrhiza glabra,* *Phaseolus vulgaris*, *Pisum sativum,* *Psoralea corylifolia*, *Pterocarpus marsupium*, *Pueraria lobata,* *Pueraria tuberosa*, *Trifolium pratens,Vigna radiata*	*Acca sellowiana*, *Aloe vera*, *Bupleurum scorzonerifolium, Sarcolobus globosus,* *Arabidopsis thaliana*, *Erycibe expansa, Erythroxylum ulei*

were studied by phytochemists only and considered useful chemosystematic markers (chemical markers used for identification/classification of the species). Subsequently, they became known for their antifungal and insecticidal properties and were considered as phytoalexins by phytophysiologists [10]. The chemical structure of isoflavones is very similar to that of animal hormone estrogen (estradiol as female and testosterone as male hormone) (Fig. 60.2).

In plants, isoflavones occur predominantly as β−glucosides (genistin, daidzin, glycitin), or as acetyl-β-glucosides and malonyl-β−glucosides, and are, therefore, polar, water-soluble compounds. The biological and biochemical properties of isoflavonoids vary considerably with only minor modifications in structure (Table 60.4). Dietary isoflavones can be divided into four categories: (1) Aglycones (without attached glucose): daidzein, genistein, glycitein, formononetin, and biochanin A (Fig. 60.4); (2) Glucosides or glucones: daidzin, genistin, glycitin, ononin, and sissotrin; (3) Acetylglucosides or acetylglucones: 6″- acetyldaidzin, 6″-acetylgenistin, and 6″-acetylglycitin; (4) Malonylglucosides or malonylglucones: 6″- malonyldaidzin, 6″- malonylgenistin, and 6″-malonylglycitin. The low aqueous solubility of the isoflavone aglucones is due to the acidic nature of the phenolic groups and is pH-dependent. One of the most important chemical properties of isoflavones is the ability to participate in redox processes. Isoflavones act as free radical scavengers (chain-breaking antioxidants) when the phenoxilic head group encounters a free radical [14].

Table 60.4 Derivates formed due to isoflavone substitutions

Isoflavone substitution	Trivial name
7,4′-(OH)2	Daidzein
5,7,4′-(OH)3	Genistein
7,4′-(OH)2, 6-OCH3	Glycitein
5,4′-(OH)2, 7-OGlc	Genistin
4′-OH, 7-OGlc	Daidzin
4′-OH, 7-OGlc, 6-OCH3	Glycitin
7-OH, 4′-OCH3	Formononetin
5,7-(OH)2, 4′-OCH3	Biochanin A
7-OGlc, 4′-OCH3	Ononin
5-OH, 7-OGlc, 4′-OCH3	Sissotrin
7,3′-(OH)2, 4′-OCH3	Calycosin
5,4′-(OH)2, 7-OCH3	Prunetin
5,7,3′-(OH)3, 4′-(OCH3)	Pratensein
7-OH, 3′-O-CH2-O-4′	Pseudobaptigenin
5,7,3′,4′-(OH)4	Orobol
6,7-(OH)2, 4′-OCH3	Texasin
7-OH, 6,4′-(OCH3)2	Afrormosin
4′-OH, 7-OCH3	Isoformononetin

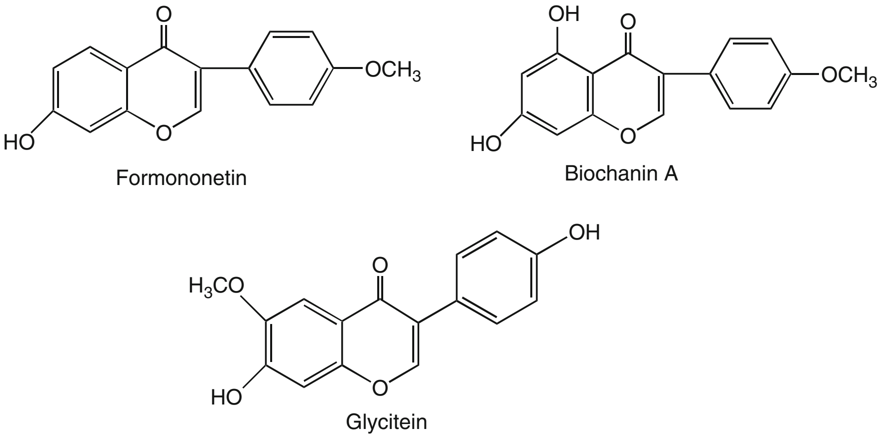

Fig. 60.4 Structure of the isoflavonoid aglycones, formononetin, biochanin A, and glycitein

4 Biosynthetic Pathway

Isoflavones are synthesized as one group of end-products (iso-flavones) in the phenylpropanoid biosynthetic pathway (Fig. 60.5). They occur in highest levels in the roots, developing seedlings, and seeds of leguminous plants, but are found in lower amounts in leaves, stems, roots, and flowers of older plants. In seeds, they are

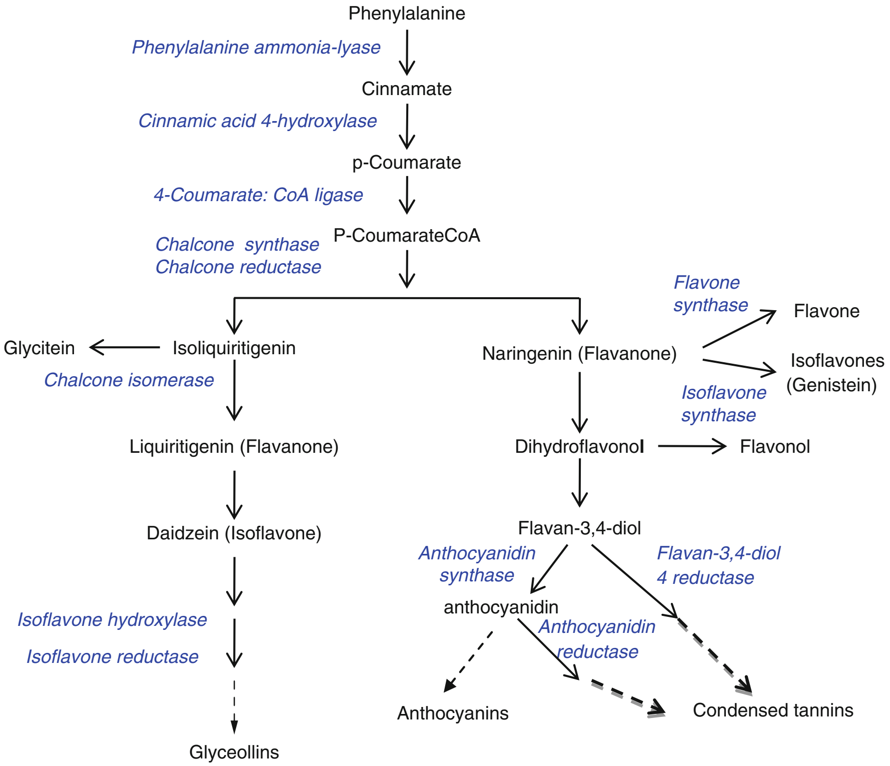
Phenylalanine
Phenylalanine ammonia-lyase
Cinnamate
Cinnamic acid 4-hydroxylase
p-Coumarate
4-Coumarate: CoA ligase
P-CoumarateCoA
Chalcone synthase
Chalcone reductase
Glycitein
Isoliquiritigenin
Chalcone isomerase
Liquiritigenin (Flavanone)
Daidzein (Isoflavone)
Isoflavone hydroxylase
Isoflavone reductase
Glyceollins
Naringenin (Flavanone)
Flavone synthase
Flavone
Isoflavones (Genistein)
Isoflavone synthase
Dihydroflavonol
Flavonol
Flavan-3,4-diol
Anthocyanidin synthase
Flavan-3,4-diol 4 reductase
anthocyanidin
Anthocyanidin reductase
Anthocyanins
Condensed tannins

Fig. 60.5 Biosynthetic pathway of major isoflavonoids

stored primarily as glucosyl conjugates (e.g., daidzin and genistin). As seeds of legumes germinate, the stored isoflavone conjugates get hydrolyzed by β-glucosidases to aglycones such as genistein and daidzein; this is accompanied by renewed synthesis of these compounds [15].

The precursors of flavonoid biosynthesis include shikimic acid, phenylalanine, cinnamic acid, and *p*-coumaric acid. Shikimic acid acts as an intermediate in the biosynthesis of aromatic acid. The basic pathways to the core isoflavonoid skeletons have been established both enzymatically and genetically [16]. The synthesis of isoflavones can be broadly divided into three main synthetic pathways: the formylation of deoxybenzoins, the oxidative rearrangement of chalcones and flavanones, and the arylation of a preformed chromanone ring. In leguminous plants, the major isoflavonoids are produced via two branches of the isoflavonoid biosynthetic pathway, and the different branches share a majority of common reactions [1]. Unlike the common flavonoid compounds, which have a 2-phenyl-benzopyrone core structure, isoflavones, such as daidzein and genistein, are 3-phenyl-benzopyrone compounds. Biochemically, the synthesis of isoflavones is an offshoot of the flavonoids biosynthesis pathway. Several attempts have aimed to increase

Table 60.5 Effects of isoflavonoid compounds on human and animal health

Compound	Effect	Model system/Species
Daidzein	Hepatoprotective, Anticancer	Rat
	Metabolizing phenotypes in relation to mammographic breast density	Women
Daidzin	Antialcohol abuse activity	Rat
Genistein	Anticancer activity, Antidiabetic	Human
	Cardiovascular diseases, Osteoporosis	Mouse
Genistin	Anticancer activity	Human
Puerarin	Protects against beta-amyloid-induced cell injury,	Rat
	Protective effect on diabetic retinopathy, Hepatoprotective	Mice
	Cardiovascular diseases, neuroprotective	Mice
	Osteoporosis,	White rabbits
	Alcohol abuse	Bone marrow cells
Biochanin A	Inhibition of stomach tumor growth	Human cell lines

isoflavone levels in soybean and introduce isoflavone biosynthesis in non-legumes [17]. Various key enzymes involved in the biosynthesis of isoflavonoid are chalcone synthase (CHS), chalcone isomerase (CHI), isoflavone synthase (IFS), 2-Hydroxyisoflavanone dehydratase (HID), *o*-methyltransferases (OMTs), UDP-glycosyltransferases (UGTs), and *o*-malonyltransferases (OMT). Chalcone reductase (CHR) is an enzyme that facilitates the biosynthesis of the 6′-deoxychalcone precursors of both 5-deoxyflavonoids and 5-deoxyisoflavonoids [18]. Genistein is biosynthetically the simplest isoflavonoid. It is a central intermediate in the formation of more complex isoflavonoids with roles in establishing or inhibiting interactions between plants and microbes. Daidzein differs from genistein by lack of a hydroxyl group at the 5-position. This hydroxyl group arises naturally from the head-to-tail condensations of malonyl CoA residues during the formation of naringenin chalcone by chalcone synthase [2].

5 Biological Activities

Dietary consumption of foods and food additives containing isoflavone phytoestrogens has been associated with several beneficial properties to human health (Table 60.5), such as prevention of coronary heart disease and osteoporosis, reduction of menopausal symptoms, and prevention of distinct cancer forms (e.g., breast, prostate, and colon cancer) [19, 20]. The potential health benefits of isoflavones for humans have been the subject of several reviews that have analyzed clinical, animal, and in vitro evidence for biological activity [5]. Several studies have reported that isoflavone consumption by postmenopausal women correlated with lower body mass index (BMI), and higher HDL levels (good cholesterol), while a number of studies have also reported absence of beneficial effects of soy on classical metabolic parameters such as bodyweight, serum lipid profiles, fat mass, blood glucose, and insulin profiles [21].

According to the USDA survey on isoflavone content, lentils do not contain significant amounts of these isoflavonoids [22]. Chickpeas contain daidzein, genistein, and formononetin (0.04, 0.06 and 0.14 mg 100 g^{-1} respectively) and approximately 1.7 mg 100 g biochanin A. Soybeans have significantly higher levels of daidzein and genistein (47 and 74 mg 100 g^{-1}, respectively) but contain less amount of formononetin and biochanin (Fig. 60.4) compared to chickpeas, 0.03 and 0.07 mg, 100 g^{-1} respectively [6]. The fact that isoflavones have been shown to exert estrogenic effects raises the possibility that this class of phytochemicals may affect glucose and lipid metabolism. In soybean, isoflavones are tightly associated with proteins. The substantial variability of phytoestrogen content found in soy products are 0.1–5 mg isoflavones/g of soy protein in mature and roasted soybeans, 0.3 mg/g soy protein in green soybeans and tempeh, and 0.1–2 mg/g soy protein in tofu and some soy milk preparations [21].

5.1 Cancer Prevention

The isoflavonoids possess many biological properties offering new strategies for cancer chemotherapy. Several papers have reviewed the potential roles of soy or its isoflavones in decreasing the risk of cancer [20]. Epidemiological data suggest that a diet rich in isoflavones provides protection against several forms of cancer, particularly those that are hormone-dependent, such as breast, prostate, and lung cancer [23]. These have shown a significant difference in cancer incidence among different ethnic groups, which is believed to be partly attributed to dietary habits. The incidences of breast and prostate cancers are much higher in the United States and European countries compared with Asian countries such as Japan and China. Genistein, the predominant isoflavones found in soy, has been shown to inhibit the carcinogenesis in animal models. There are growing body of experimental evidence that show the inhibition of human cancer cells by genistein through the modulation of genes that are related to the control of cell cycle and apoptosis. Moreover, it has been shown that genistein inhibits the activation of NF-kappa B and Akt (protein kinase also known as PKB) signaling pathways, both of which are known to maintain a homeostatic balance between cell survival and apoptosis [24]. Genistein causes inhibition of cell growth in breast [25] and prostate cancers in vivo and in vitro. Both genistein and genistin induce cell cycle arrest [26] and are able to induce significant apoptosis. Some other isoflavonoids, like puerarin was isolated from *Pueraria radix* and the study showed that puerarin also possessed anticancer properties. Methyl thiazolyl tetrazolium (MTT) assay revealed a dose-dependent reduction of HT-29 cellular growth in response to puerarin treatment. These findings indicate that puerarin may act as a chemopreventive and/or chemotherapeutic agent in colon cancer cells by reducing cell viability and inducing apoptosis [27]. Molecular mechanism of suppression of MDR1 (multidrug resistant) tumor cells by puerarin from *Pueraria lobata* via NF-κB pathway and cAMP-responsive element transcriptional activity-dependent up-regulation of AMP-activated protein kinase in breast cancer MCF-7/adr cells [28] was also reported.

5.2 Cardiovascular Diseases

Soy protein-containing foods are a rich source of isoflavone phytoestrogens, such as genistein and daidzein. There is great interest in these substances, as lower rates of chronic diseases, including coronary heart disease, have been associated with high dietary intake of soy-containing foods [29]. Genistein and daidzein were found to protect against cytotoxic effects of oxidized LDL as assessed by cellular morphologic features as well as lactate dehydrogenase released by cultured endothelial cells.

Isoflavones prevent atherosclerosis; the most cited example in this case is the inhibition of LDL oxidation, formation of which is central in atherogenesis [7]. Many investigations have demonstrated that soy protein inhibits cardiovascular diseases and reduces atherosclerosis risk in animals and humans, for example, genistein upregulates the expression of human endothelial nitric oxide synthase and lowers blood pressure in spontaneously hypertensive rats [30].

5.3 Antioxidant Activity

Soybean isoflavones may exhibit their cancer preventive function through their antioxidant properties. Genistein, the major component of soybean isoflavones, has been demonstrated to inhibit ultraviolet-B (UVB)-induced skin tumorigenesis in hairless mice. The antioxidant properties of genistein may explain the mechanisms of its anti-photocarcinogenic action either by direct quenching of reactive oxygen species or indirect anti-inflammatory effects. Genistein was found to substantially inhibit a series of oxidative events elicited by UVB irradiation, including hydrogen peroxide (H_2O_2) production, lipid peroxidation, and 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation [31]. Genistein potently scavenges both H_2O_2 and superoxide anions, which suggests genistein has potential anticarcinogenic function in photocarcinogenesis through its antioxidant property [32]. Genistein has also been shown to inhibit hydrogen peroxide production and increase the activity of antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase. Furthermore, genistein and daidzein can inhibit superoxide anion generation by xanthine/xanthine oxidase [4]. The root extract of *Pueraria lobata* (a rich source of these isoflavonoids) showed in vivo antioxidant activity in diabetic rat model [33]. Therefore, antioxidant activity of pure isoflavonoids or plant extracts has been established.

5.4 Estrogenic Activity

Because of the similarity in structure, isoflavonoids can interfere with the action of estrogen in human being. Depending on the type of estrogen receptor on the cells, isoflavones may reduce or activate the activity of estrogen. Isoflavones can compete with estrogen for the same receptor sites thereby decreasing the health risks of excess estrogen. They can also increase the estrogen activity. Estrogenic activity is dependent on the affinity of binding to the estrogen receptors, which is determined by the presence of the aromatic ring as well as hydroxyl groups at specific sites. Compared with estradiol, genistein and daidzein bind estrogen receptors with 100 and 1,000 times less affinity, respectively [4]. Isoflavonoids studies showed that developmental exposure to genistein causes deleterious effects on the reproductive system of mice. Oral exposure to genistin (25 mg/kg) increases uterine weight at 5 days of age similar to subcutaneous injection of genistein (20 mg/kg) suggesting that subcutaneous injection of genistein is a suitable model for oral exposure to genistin [34]. The root extract of *Pueraria mirifica* showed an estrogenic effect on human mammary adenocarcinoma (MCF-7) cells [35]. Root extract of *Pueraria tuberosa* also showed antifertility effect in male rate [36]. The isoflavonoid daidzein adversely affected erectile function in rats in a dose- and time-related manner that was attributable to androgen deficiency [37].

6 Biotechnological Approaches

A biotechnological approach, specifically plant tissue culture plays a vital role in search for alternatives to produce desirable medicinal compounds from plants. Plants and plant cell cultures have served as resources for flavors, aromas and fragrances, enzymes, preservatives, cosmetics, natural pigments, and bioactive compounds. These are secondary metabolites also present in unorganized callus or suspension cultures of the species. To meet the growing demand by pharmaceutical industries these compounds have been isolated from sources other than the original plants and different strategies have been adapted to improve their yield in cell cultures. These include screening and selection of high-producing cell lines, optimization of nutrient media for growth and production, organ culture, culture of immobilized cells, etc. [38, 39].

Many of plants in vitro cultures have been reported for the production of useful isoflavonoids (Table 60.6). Employment of cell and tissue culture in liquid medium (Fig. 60.6) predominantly provides uniform conditions for growth and metabolite synthesis, rapid biomass gain, better amenability for precursor feeding, elicitation, higher feasibility for bioreactor scaling up and easy extraction. Isoflavonoid production in cell cultures of *Pueraria tuberosa* as influenced by several nutrient factors, elicitors, plant growth regulators, and angiospermic parasite (*Cuscuta reflexa*) was studied in our laboratory. The optimized elicitation conditions were used in vessels of varying capacity where maximum yield of ~91 mg l^{-1} of

Table 60.6 Isoflavonoids production from in vitro cultures of different plant species

Plant species	Type of culture	Isoflavonoids produced	References
Glycine max	Cell culture	Genistein, Daidzein	[49]
Pueraria lobata	Root culture	Puerarin, Genistin, Daidzein	[50]
Psoralea corylifolia	Callus culture	Genistein, Daidzein	[51]
Pueraria tuberosa	Cell culture	Puerarin, Genistin, Daidzein	[40]
Maackia amurensis	Callus culture	Daidzein, Genistein Formononetin	[52]
Genista tinctoria	Callus culture	genistein, daidzein Formononetin	[53]
Trifolium pratense	Cell culture	Formononetin, biochanin A	[54]

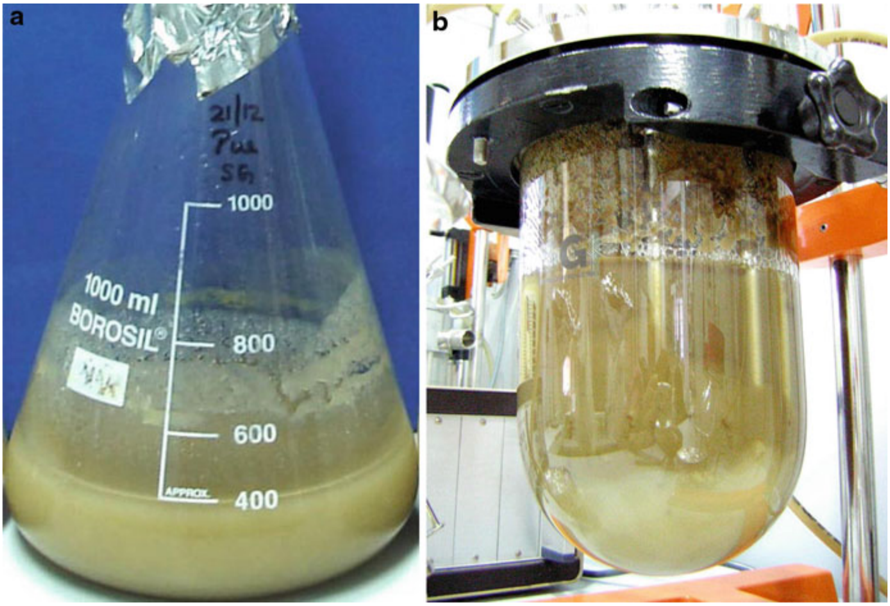

Fig. 60.6 Cell cultures of *Pueraria tuberosa* grown in shake flasks on a rotary shaker and in 2 L stirred tank bioreactor for the production of isoflavonoids

isoflavonoid was recorded in 2 l bioreactor which was about 19 % higher than the values achieved earlier [40–43]. Improved isoflavonoid production in *Pueraria candollei* hairy root cultures was achieved on day 3 of elicitation [44]. Biotechnology approaches are not limited to tissue culture but also extend up to genetic transformation and molecular biology for the production of high-level bioactive molecules. The cell cultures of *Cicer arietinum*, *Pueraria lobata*, and *P. thomsonii* have been studied for elicitor-induced manipulation of isoflavonoid production [45, 46]. The organ cultures are stable in the production of secondary metabolites than undifferentiated callus and cell cultures [47, 48]. These recent works suggest a great promise for exploitation of isoflavones production by cell cultures technology.

7 Conclusion

Pulses are traditionally consumed in Asian and east Asian countries. Health benefits of continuous consumption of soybean and pulses are now known scientifically and established. Therefore, efforts are required to improve these legumes not only for their primary nutrient contents but also for the bioactive molecules. The beneficial effects of these isoflavonoids have been established and hence these products can be either used directly or as extracts for fortification of nutraceuticals. Genetic engineering and plant cell culture technologies hold great promise for the improvement of the legume plants and production of bioactive molecules. The legume plants as well as cell cultures can be explored further for the identification of minor bioactive molecules in these materials not known so far. This will have great consequences in food and medical industries.

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Minor Flavonoids (Chalcones, Flavanones, Dihydrochalcones, and Aurones) 61

Masayuki Ninomiya and Mamoru Koketsu

Contents

1 Chalcone 1868
1.1 Introduction 1868
1.2 Occurrence of Chalcones 1869
1.3 Phytochemistry of Chalcones 1869
1.4 Biosynthesis of Chalcones 1869
1.5 Biological Activities of Chalcones 1871
2 Flavanone 1876
2.1 Introduction 1876
2.2 Occurrence of Flavanones 1877
2.3 Phytochemistry of Flavanones 1877
2.4 Biosynthesis of Flavanones 1877
2.5 Biological Activities of Flavanones 1879
3 Dihydrochalcone 1883
3.1 Introduction 1883
3.2 Occurrence of Dihydrochalcones 1883
3.3 Phytochemistry of Dihydrochalcones 1883
3.4 Biosynthesis of Dihydrochalcones 1883
3.5 Biological Activities of Dihydrochalcones 1884

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4 Aurone 1887
4.1 Introduction 1887
4.2 Occurrence of Aurones 1887
4.3 Phytochemistry of Aurones 1887
4.4 Biosynthesis of Aurones 1887
4.5 Biological Activities of Aurones 1889
5 Conclusions 1890
References 1890

Abstract

Chalcones, flavanones, dihydrochalcones, and aurones are categorized into minor flavonoids. However, these compounds take the significant roles in plant kingdom. These minor flavonoids are unique to plants and are an essential part of their success in adapting to life as sedentary organisms in diverse and inconstant surrounding. Furthermore, these compounds are subclasses of the interesting naturally occurring flavonoids in view of their structural pattern as well as biochemical and pharmacological relevance. It seems that they are important not only for plants but also for animals including human beings. This chapter deals with these minor flavonoids.

Keywords

Aurone • chalcone • dihydrochalcone • flavanone

Abbreviations

AmAS1	Aureusidin synthase
CHI	Chalcone isomerase
CHS	Chalcone synthase
CoA	Coenzyme A
COX	Cyclooxygenase
LPS	Lipopolysaccharide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF	Nuclear factor
NO	Nitric oxide
ROS	Reactive oxygen species
TNF	Tumor necrosis factor

1 Chalcone

1.1 Introduction

Chalcones, 1,3-diaryl-2-propen-1-ones, belong to the plant flavonoid family. The name “chalcone” comes from a Greek word *chalcos* (bronze). Chemically, they consist of open-chain flavonoids in which the two aromatic rings (A and B) are joined by a three-carbon α,β-unsaturated carbonyl system. Chalcones possess the conjugated double bond and a completely delocalized π-electron system on both aromatic rings. Although chalcone skeleton is the initial intermediate structure used in biosynthesis of all flavonoids, chalcones are one of the minor subclasses of flavonoids.

1.2 Occurrence of Chalcones

Chalcones were originally discovered in plants as the yellow flower pigments of *Coreopsis* and other yellow-rayed Compositae. After that, they have subsequently been found in other plant families including Solanaceae, Anacardiaceae, Caesalpiniaceae, Piperaceae, and Apiaceae. 6′-Deoxychalcones were known to be chemical constituents of leguminous plants, but it is scarcely reported that they were found in other plant species. A lot of chalcones were isolated from either Compositae (Asteraceae) or Leguminosae (Fabaceae), two families well known to accumulate these. Besides, various prenylchalcones can be found in hop plants (Cannabaceae).

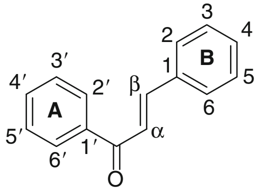
1.3 Phytochemistry of Chalcones

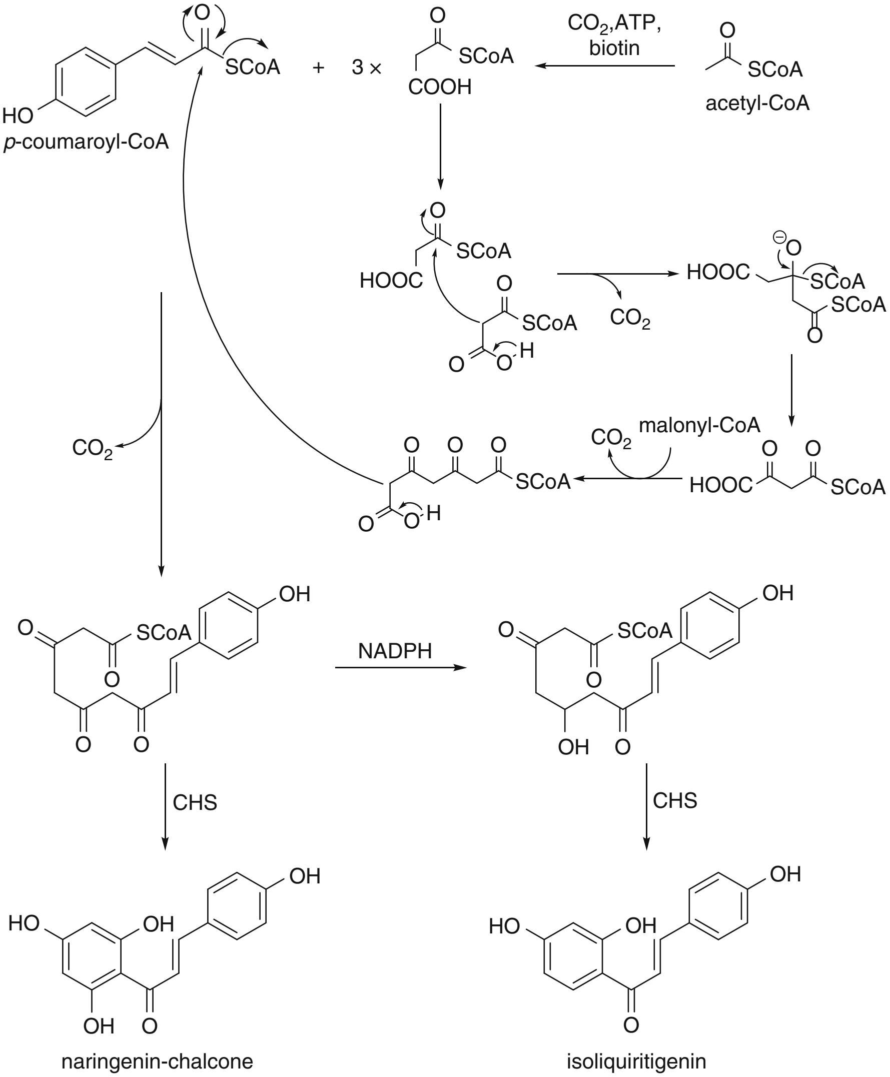
Chalcone numbering is shown in Fig. 61.1. A vast number of naturally occurring chalcones are polyhydroxylated in the aromatic rings. Usually, chalcones have hydroxyl group at C2′-, C4′-, and/or C6′-positions in A-ring because the A-ring is biosynthesized via the acetate-malonate pathway. Prenylchalcones have prenyl group(s) between the hydroxyl groups. Cyclization of the prenyl group and the adjoining hydroxyl group produces pyrano ring on the aromatic ring. Chalcones, which do not have an oxygen function at the 2′-position, are called retrochalcones. The B-ring generally has a hydroxyl group at C4-position. They are both intermediates and end products in flavonoid biosynthesis, act as defensive compounds, participate in plant-insect interactions, and contribute to the medicinal value of herbs.

1.4 Biosynthesis of Chalcones

Chalcones are biosynthesized through the combination pathway of acetate-malonate and shikimate pathways [1–3]. Malonyl-CoA is synthesized from acetyl-CoA, whereas *p*-coumaroyl-CoA originates from phenylalanine, which is produced via the shikimate pathway. Chalcone synthase (CHS) catalyzes the significant step of chalcone biosynthesis. It first condenses a phenylpropanoid-CoA (e.g., *p*-coumaroyl-CoA) with three units of malonyl-CoA and cyclizes the resulting tetraketide intermediate to afford a chalcone (e.g., naringenin-chalcone). 6′-Deoxychalcones (e.g., isoliquiritigenin) having a resorcinol-type A-ring are biosynthesized by coactions of NADPH-dependent chalcone reductase and CHS (Scheme 61.1).

Fig. 61.1 Basic skeleton of chalcones

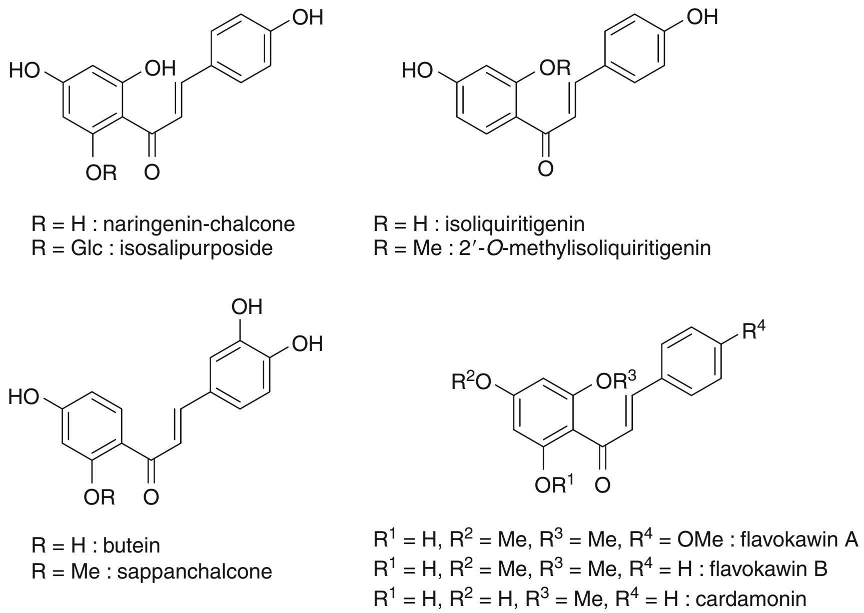

3
2
4
B
1
5
3′
4′
2′
β
6
A
α
5′
1′
6′
O


O
SCoA
HO
p-coumaroyl-CoA
+ 3 ×
O
SCoA
COOH
CO$_2$,ATP,
biotin
O
SCoA
acetyl-CoA
O
SCoA
HOOC
O
SCoA
O
O
H
CO$_2$
HOOC
O
SCoA
SCoA
O
CO$_2$
O
O
O
SCoA
O
O
H
CO$_2$
malonyl-CoA
O
O
HOOC
SCoA
OH
O
SCoA
O
O
O
NADPH
OH
O
SCoA
O
OH
O
CHS
CHS
OH
HO
OH
OH
O
naringenin-chalcone
OH
HO
OH
O
isoliquiritigenin

Scheme 61.1 Biosynthesis of chalcones

1.5 Biological Activities of Chalcones

Considerable attention has been devoted to research of chalcones [4–6], which are distributed in fruits, spices, tea, and soy-based foodstuff, because of their interesting and potential pharmacological activities. Naringenin-chalcone is found in Compositae, Lamiaceae, and Solanaceae [7]. Naringenin-chalcone is one of the predominant flavonoids found in tomatoes (*Solanum lycopersicum*) and accumulates almost exclusively in the tomato peel [8]. This compound inhibited histamine release with an IC_{50} value of 68 μg/ml and was found to be the most promising antiallergic polyphenol of this extract [9]. The effect on the production of naringenin-chalcone proinflammatory mediators in lipopolysaccharide (LPS)-stimulated macrophages has been examined. This compound inhibited the production of tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and nitric oxide (NO) by LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner [10]. Naringenin-chalcone exhibited anti-inflammatory properties by inhibiting the production of proinflammatory cytokines in the interaction between adipocytes and macrophages. In addition, the oral administration of naringenin-chalcone was shown to suppress Th2 cytokine production from CD4 T cells in the spleen and to attenuate allergic airway inflammation and airway hyperreactivity [11]. The eating naringenin-chalcone could contribute to the prevention and improvement of insulin resistance and related metabolic syndrome [12]. Isosalipurposide, the glucoside derivative of naringenin-chalcone isolated from *Nymphaea caerulea* or *Helichrysum maracandicum*, exhibited the inhibition of ROS (reactive oxygen species) generation in HL60 cells [13] and strong antiproliferative activity against cultured cells of SENCAR mouse in vitro [14]. Isoliquiritigenin is found in *Glycyrrhiza* species (Leguminosae) such as *G. uralensis*, *G. glabra*, *G. inflata*, Lardizabalaceae, and Amaryllidaceae [15–19]. Isoliquiritigenin is known as a natural aldose reductase inhibitor [20]. It was reported to possess antioxidative and super oxide scavenging activities [16], antiplatelet aggregation effect [21], estrogenic property [22], and inhibitory on xanthine oxidase activity in vitro [23]. And it demonstrates its anti-inflammatory effect by inhibiting LPS-induced iNOS and COX-2 expression via the attenuation of NF-κB in RAW 264.7 macrophages [24]. Interestingly, isoliquiritigenin was also found to inhibit cocaine-induced dopamine release by modulating $GABA_B$ receptor [25]. Besides, it was found that isoliquiritigenin had good effects on inhibition proliferation, including apoptosis and locking cell cycle progression in the G1 phase against human lung cancer A549 cells [26]. It induced cell cycle arrest and p21 expression in these cells [27], apoptosis and p53-expression in human liver carcinoma Hep G2 cells [28], and also induces apoptosis by depolarizing mitochondrial membranes in human prostate cancer DU145 cells [29]. Moreover, isoliquiritigenin induced monocytic differentiation of human leukemia HL60 cells [30]. It was reported that isoliquiritigenin has the ability to protect cells from AA (arachidonic acid) and iron-induced ROS production and mitochondrial dysfunction; the cytoprotective effect mediated via AMPK (AMP-activated protein kinase)-dependent GSK3β (glycogen synthase kinase-3β) inactivation.



R = H : naringenin-chalcone
R = Glc : isosalipurposide

R = H : isoliquiritigenin
R = Me : 2′-*O*-methylisoliquiritigenin

R = H : butein
R = Me : sappanchalcone

R1 = H, R2 = Me, R3 = Me, R4 = OMe : flavokawin A
R1 = H, R2 = Me, R3 = Me, R4 = H : flavokawin B
R1 = H, R2 = H, R3 = Me, R4 = H : cardamonin

Fig. 61.2 Structures of bioactive chalcones

Isoliquiritigenin is useful to protect mitochondria from an iron catalyzed burst of oxidative stress [31]. 2′-*O*-Methyl isoliquiritigenin isolated from the root of *Dalbergia odorifera T. Chen* had the obvious antioxidative effect and the inhibitory effect of decrease of glutathione (GSH) level of rat lens induced by UV irradiation [32] (Fig. 61.2).

Butein (2′,3,4,4′-tetrahydroxychalcone) can be isolated from stembark of cashews and *Rhus verniciflua* Stokes. Past investigations suggested that butein exhibits anticarcinogenic effects. The organic extract purified from *R. verniciflua* Stokes inhibited the growth of transformed hepatic cells but not the untransformed parent cells [33], whereas butein alone could induce G(2)/M phase arrest in Hep G2 cells [34]. Its antiproliferative or pro-apoptotic effects can be brought about through downregulating STAT3-related gene expressions [35] and inhibiting telomerase activity [36]. This compound can also resensitize the TRAIL-resistant leukemia cells undergoing apoptosis upon TRAIL treatment [37] and reduce clonogenic growth of human breast cancer UACC-812 cells [38]. In addition, butein can suppress the proliferation of many human cancers including colon carcinoma, osteosarcoma, and hepatic stellate cells in vitro [39–43]. Sappanchalcone isolated from sappan lignum (the dried heartwood of *Caesalpinia sappan*) showed rapid vasorelaxant activity on the mesenteric artery [44]. This chalcone exhibited the anti-inflammatory effect in LPS-induced human periodontal ligament HPDL cells by protecting from H_2O_2 [45]. Sappanchalcone suppressed human oral cancer cell

(HN4 and HN12) growth and induces apoptosis through the activation of p53-dependent mitochondrial, p38, ERK, JNK, and NF-κB signaling [46]. Additionally, other biological effects of this compound involve the inhibition of β-hexosaminidase release [47] and anti-influenza virus activity [48]. Flavokawin A and B were isolated from kava (*Piper methysticum*) with anti-inflammatory activity. They inhibited TNF-α-induced degradation and translocation of p50 and p65 NF-κB subunits from the cytoplasm to the nucleus [49]. Flavokawin B produced pronounced antinociception effect against both chemical and thermal models of pain in mice that exhibited both peripheral and central analgesic activity [50]. Cardamonin isolated from *Alpinia rafflesiana*, *Artemisia absinthium*, or *Syzygium samarangense* inhibited NO and prostaglandin E_2 (PGE_2) production from LPS- and IFN-γ-activated RAW 264.7 macrophages by the inhibition of p65NF-κB nuclear translocation due to prevention of I-κBα phosphorylation, which subsequently caused the accumulation of I-κBα [51, 52]. This chalcone also inhibited the generation of the stable thromboxan metabolite, thromboxan B2 (TxB_2), via both COX-1 and COX-2 pathways; generation of intracellular ROS; and secretion of TNF-α from RAW 264.7. Cardamonin demonstrated its cytotoxic activity against human colon cancer SW-480 cells [53].

Xanthohumol, desmethylxanthohumol, 4′-methylxanthohumol, and 2′-*O*-methyl-3′-prenylchalconaringenin can be found in hop plants (*Humulus lupulus*). Xanthohumol is the most abundant prenylchalcone present in hops (concentrations up to 1 %, w/w). It is accompanied by its homologue, desmethylxanthohumol, albeit in lower concentrations [54]. Xanthohumol, desmethylxanthohumol, and 4′-methylxanthohumol were reported to exhibit strong antioxidative effects in ORAC assay [55]. Xanthohumol has been suggested to have potential cancer chemopreventive activities by inhibiting human breast MCF-7, colon HT-29, ovarian cancer A-2780 [56], and B-chronic lymphocytic leukemia cell proliferation in vitro [57]. This also showed antiangiogenic properties in vitro and in vivo where it inhibited proliferation of endothelial and Kaposi's sarcoma-derived tumor cells in vitro, prevented angiogenesis in the Matrigel sponge model, and reduced Kaposi's sarcoma xenograft growth in vivo. The antiangiogenic effects of xanthohumol correlated with a block of NF-κB activation and decreased phosphorylation of Akt [58, 59]. Isobavachalcone, which is a prenylchalcone found in *Angelica keiskei*, *Dorstenia barteri*, or *Psoralea corylifolia*, has been demonstrated to exhibit cancer antipromotive and antiproliferative activity [60]. Previous studies have shown that isobavachalcone exerts inhibitory effect against skin tumor promotion in vivo mouse skin carcinogenesis [61], and the ability of this chalcone to induce apoptosis in neuroblastoma IMR-32 and NB-39 cells has been reported [62]. Isobavachalcone significantly reduced pro-caspase-3 and pro-caspase-9 and subsequently increased the level of cleaved caspase-3 and cleaved caspase-9 in both cell lines. In addition, isobavachalcone demonstrated strong antifungal activity against various fungi, *Candida albicans*, *C. glabrata*, *Microsporum audorium*, and *Trichophyton rubrum* [63]. Crotaorixin isolated from the aerial parts of *Crotalaria orixensis* and medicagenin, which is a diprenylchalcone, isolated from the roots of *Crotalaria medicagenia* exhibited

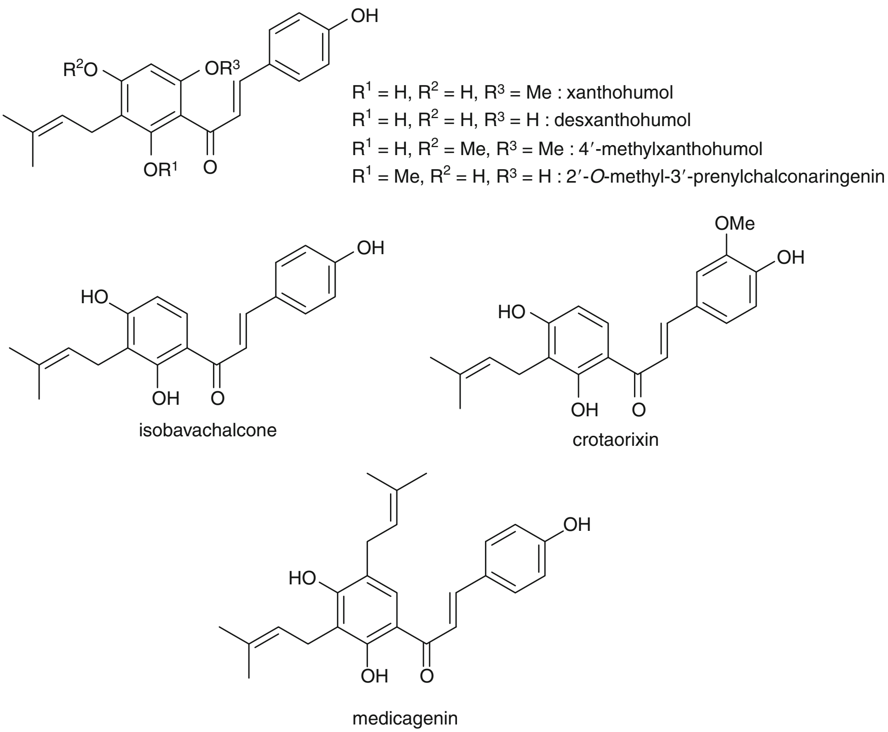

OH
R²O
OR³
OR¹
O
R¹ = H, R² = H, R³ = Me : xanthohumol
R¹ = H, R² = H, R³ = H : desxanthohumol
R¹ = H, R² = Me, R³ = Me : 4′-methylxanthohumol
R¹ = Me, R² = H, R³ = H : 2′-O-methyl-3′-prenylchalconaringenin
HO
OH
O
isobavachalcone
OMe
crotaorixin
medicagenin

Fig. 61.3 Structures of bioactive prenylchalcones

the high antimalarial activity. They showed 100 % inhibition of maturation of *Plasmodium falciparum* parasites from ring stage to schizont stage at low concentrations [64] (Fig. 61.3).

Licochalcones such as licochalcone A, B, and C isolated from licorice (the root and rhizome of *Glycyrrhiza* spp. *G. uralensis*, *G. glabra*, and *G. inflate*) are classified into the retrochalcones (chalcones which do not have an oxygen function at the 2′-position). The content of licochalcone A in licorice was found to be very high [65]. Previous studies showed that licochalcone A possessed radical scavenging [65], antileishmanial [66], and antispasmodic effects [67]. This chalcone has been used to treat various abdominal spasmodic symptoms in Japan [68]. Licochalcone A is well known to be a natural antiparasitic agent. Licochalcone A is a potent membrane-active compound that transforms normal erythrocytes into echinocytes in parallel with the inhibition of growth of *P. falciparum* cultures. The erythrocyte membrane-modifying effect was also transiently observed in vivo in mice after intravenous administration [69]. This compound exhibited the antimicrobial activity by inhibiting the growth of *Staphylococcus aureus*, *Bacillus subtilis*, and the activity of *Helicobacter pylori* [70, 71]. Furthermore, licochalcone A significantly inhibited LPS-induced NF-κB activation. This chalcone specifically inhibited the phosphorylation of p65 NF-κB at serine 276, leading to the inhibition of NF-κB transactivation [72]. Licochalcone A also has

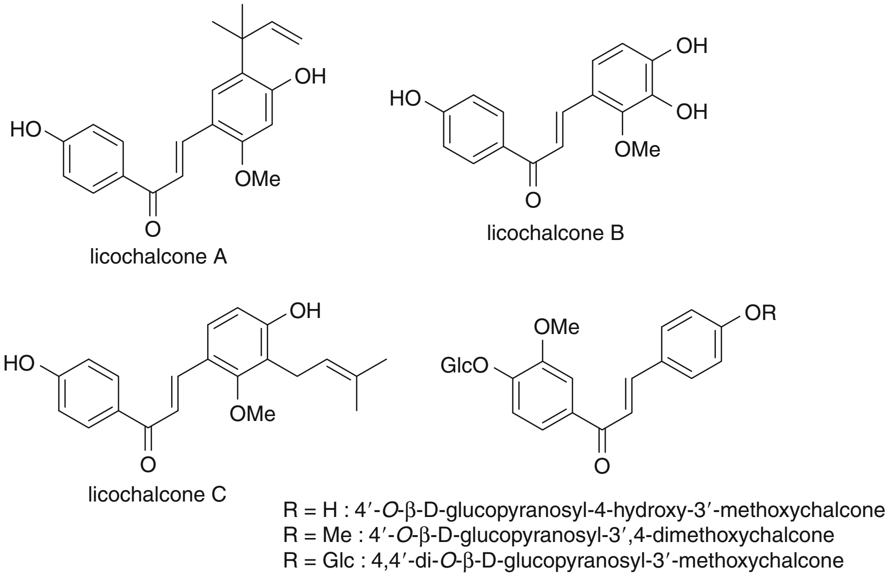

HO
OH
OMe
O
licochalcone A
HO
OH
OH
OMe
O
licochalcone B
HO
OH
OMe
O
licochalcone C
OMe
GlcO
OR
O
R = H : 4′-*O*-β-D-glucopyranosyl-4-hydroxy-3′-methoxychalcone
R = Me : 4′-*O*-β-D-glucopyranosyl-3′,4-dimethoxychalcone
R = Glc : 4,4′-di-*O*-β-D-glucopyranosyl-3′-methoxychalcone

Fig. 61.4 Structures of retrochalcones

anticancer effects, induced apoptosis in MCF-7, HL60, and human prostate cancer LNCaP cells, and arrested G2 and late-G1 in human prostate cancer PC-3 cells [73–75]. Licochalcone B showed the high antioxidative effect stronger than that of licochalcone A on the 5-lipoxygenase-dependent peroxidation in arachidonate metabolism [76]. Licochalcone C also exhibited the anti-inflammatory activity by inhibiting NF-κB signaling [77]. Recently, chalcone glycosides, which do not have an oxygen function at the 2′-position, were isolated from the aerial parts of *Brassica rapa* L. "hidabeni" [78]. These 4′-*O*-β-D-glucopyranosyl-4-hydroxy-3′-methoxychalcone and 4′-*O*-β-D-glucopyranosyl-3′,4-dimethoxychalcone markedly inhibited antigen-stimulated degranulation in rat basophilic leukemia RBL-2H3 cells. The inhibitory effects were mainly due to suppression of intracellular Ca^{2+} elevation by suppression of intracellular ROS production through NOX inactivation [79]. Moreover, 4′-*O*-β-D-glucopyranosyl-3′,4-dimethoxychalcone inhibited LPS-induced iNOS expression and NO production in rat immortalized microglia HAPI cells. The inhibitory effect is due to the prevention of phosphorylation of signal transduction and activator of translocation 1 (STAT1) [80] (Fig. 61.4).

Lonchocarpin, which is a pyranochalcone, isolated from *Lonchocarpus sericeus* showed significant antiplatelet effect. The effect was suggested to be mediated by phosphodiesterase activity by inhibition or elevation of intracellular levels of adenosine 3′:5′-cyclic monophosphate and guanosine 3′:5′-cyclic monophosphate [81]. Crotaramosmin isolated from *Crotalaria ramosissima* showed weak antimalarial activity and strong antileishmanial effect [64].

Carthamin, which is a bichalcone, occurs in the tubular flowers at a late phase of the flowering stage in safflower *Carthamus tinctorius* [82]. This compound is known to be

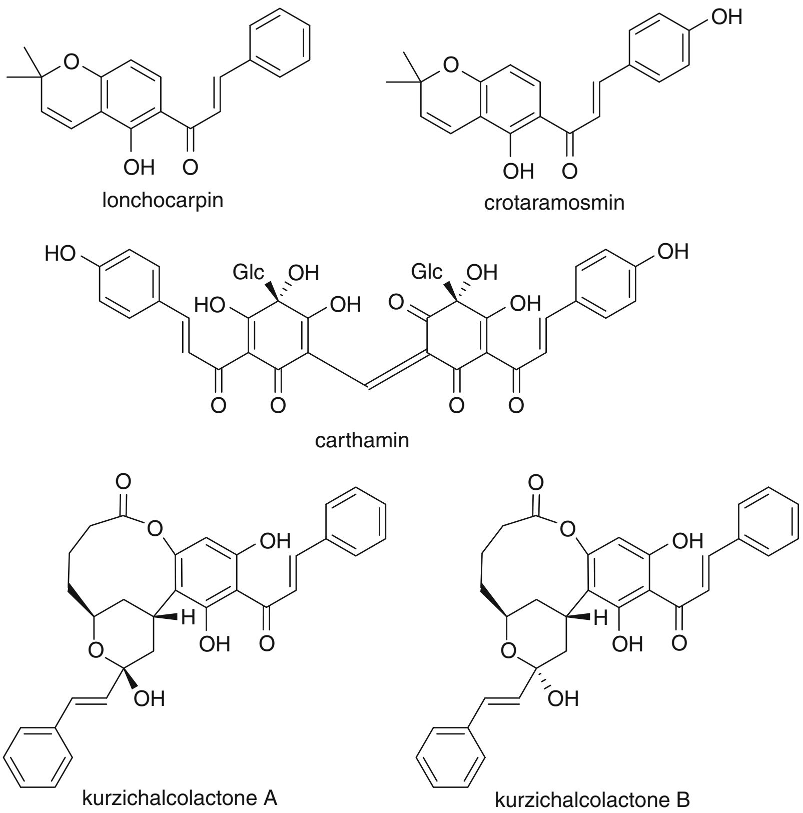


Fig. 61.5 Structures of rare chalcones

called Natural Red 26. Carthamin has been extensively used as a natural food color additive and created to cosmetics for geisha and kabuki artists for a long time in Japan. Carthamin administration could improve the blood fluidity by decreasing whole blood viscosity [83]. Kurzichalcolactones A and B, which have an unprecedented carbon side chain on the chalcone A-ring, were isolated from *Cryptocarya kurzii* with cytotoxic activity against human epidermoid carcinoma KB cells [84] (Fig. 61.5).

2 Flavanone

2.1 Introduction

Within the plant secondary metabolites of flavonoids, flavanones define one of the minor subclasses. They may be called dihydroflavones. The basic chemical structure of flavanones involves two benzene rings (A and B), which are linked by a heterocyclic ring (C). The most characteristic point of flavanone structures is that the C-ring is saturated. Flavanones have an asymmetric carbon at C2-position.

They are also interesting compounds because they are the obligate intermediates in flavonoid biosynthesis. Although flavanones are minor chemical constituents of plants, they have attracted a lot of attention in chemistry and biological sciences.

2.2 Occurrence of Flavanones

Flavanones occur in several plant families including Libiatae, Annonaceae, Acanthaceae, Compositae, Leguminosae, and also Rutaceae [85]. Flavanones having a hydroxyl group at 5-position in A-ring constitute the majority of flavonoids in Rutaceae fruits such as sweet (*Citrus sinensis*), sour oranges (*C. aurantium*), and their relatives. All orange-type citrus fruits contain the flavanone aglycones naringenin and hesperetin, but they rarely occur as free aglycones in the fruit itself. The dominant flavanone glycosides in sweet oranges (*C. sinensis*) are hesperidin and narirutin, whereas in sour oranges (*C. aurantium*), the two predominant flavanone glycosides are neohesperidin and naringin. The major difference between the flavanone glycosides of sweet and sour oranges is in their sugar moieties, which influence taste. The sugar rutinose (6-*O*-α-ʟ-rhamnosyl-β-ᴅ-glucose) causes the flavanones hesperidin and narirutin to have a neutral taste and is relatively high in sweet oranges, tangerines, and tangors. The sugar neohesperidose (2-*O*-α-ʟ-rhamnosyl-β-ᴅ-glucose) is high in tangelos and sour oranges and imparts a tangy or bitter taste to the glycosides neohesperidin and naringin [86]. The representative citrus flavanones and their glycosides are shown in Fig. 61.6. On the other hand, various flavanones, which do not have a hydroxyl group at 5-position, can be found in Leguminosae (Fabaceae) family.

2.3 Phytochemistry of Flavanones

Flavanone numbering is shown in Fig. 61.7. As a rule, flavanones have hydroxyl groups at 5- and 7-positions in A-ring and 4′-position in B-ring. And flavanones exist as glycosides in nature. Naturally occurring flavanones usually have the 2*S*-configuration, but racemization can occur during extraction. Flavanones can be easily converted to isomeric chalcones in alkaline media (or vice versa in acidic media) provided that there is a hydroxyl substituent at 2′- or 6′-position of the chalcone. In general, the physical properties of flavanones are greatly different from that of flavones. The content of flavanones may control the sweetness or bitterness of fruits. The flavanones are less soluble than the chalcones, tend to separate first in fractional crystallization, and are easily precipitated at low pH, especially if solutions are chilled or frozen.

2.4 Biosynthesis of Flavanones

The enzyme chalcone isomerase (CHI) is the second key enzyme of flavonoid biosynthesis in higher plants and catalyzes the conversion of chalcones to their

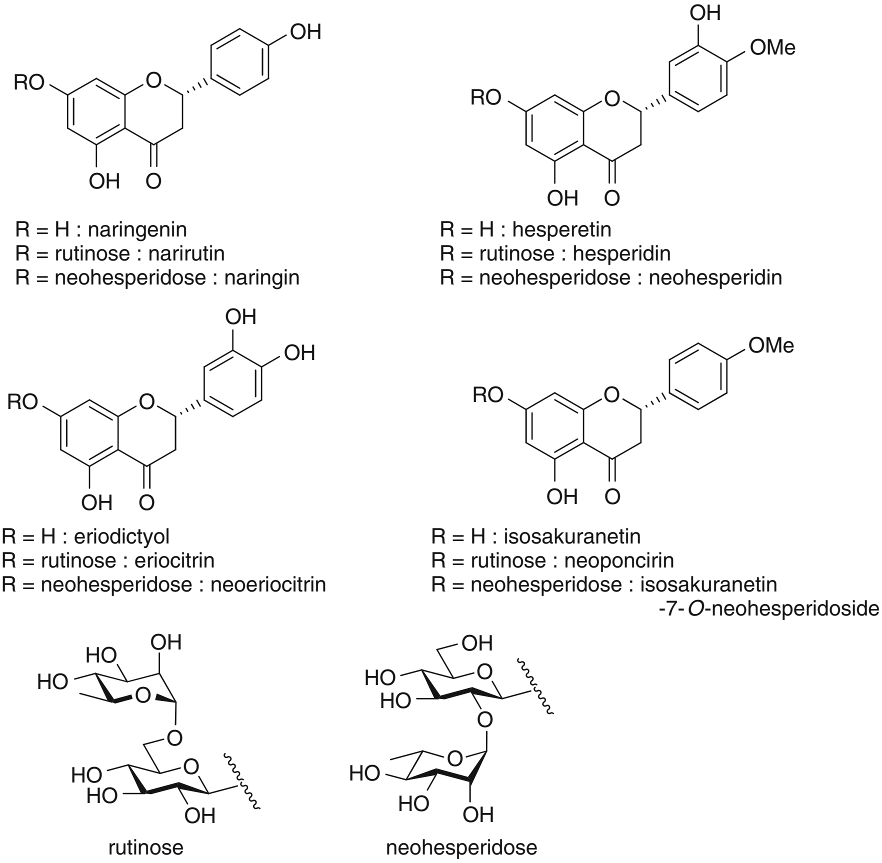

OH
RO
O
OH O
R = H : naringenin
R = rutinose : narirutin
R = neohesperidose : naringin
OH
OMe
RO
O
OH O
R = H : hesperetin
R = rutinose : hesperidin
R = neohesperidose : neohesperidin
OH
OH
RO
O
OH O
R = H : eriodictyol
R = rutinose : eriocitrin
R = neohesperidose : neoeriocitrin
OMe
RO
O
OH O
R = H : isosakuranetin
R = rutinose : neoponcirin
R = neohesperidose : isosakuranetin -7-*O*-neohesperidoside
HO
OH
HO
O
O
O
HO
HO
OH
rutinose
OH
HO
O
HO
O
O
HO
HO
OH
neohesperidose

Fig. 61.6 Structures of citrus flavanones

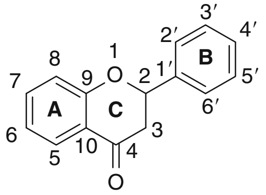
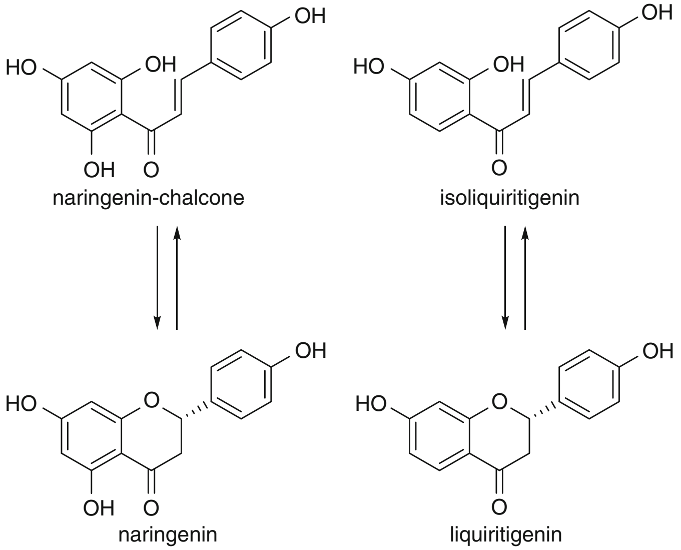

3′
2′
4′
B
1
8
1′
5′
7
9
O
2
6′
A
C
6
3
10
5
4
O

Fig. 61.7 Basic skeleton of flavanone

corresponding flavanones. Chalcones having a hydroxyl group at C2′-position, especially those further possessing a 6′-hydroxy substitution, are spontaneously converted into a racemic mixture of the respective 2 *S*- and 2*R*-flavanones. However, they are stereospecifically isomerized into 2*S*-flavanones more rapidly by CHI



Scheme 61.2 Biosynthesis of flavanones

than spontaneous conversion in plants (Scheme 61.2). Flavanones have a chiral center at C-2 position so that naturally occurring members are often optically active.

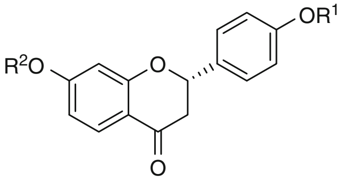
2.5 Biological Activities of Flavanones

Naringenin, which is abundant in grapefruits (*Citrus* × *Paradisi*) and other citrus fruits, has been shown to inhibit microsomal lipid peroxidation [87], nonenzymatic lipid peroxidation [88], and ascorbic acid-induced malondialdehyde (MDA) formation. Hesperetin showed a similar level of inhibition. Naringenin, however, had no effect on ferrous sulfate-induced MDA production [88]. This flavanone was found to inhibit TxB_2 production in platelets stimulated with either thrombin or AA (arachidonic acid), whereas the glycoside form naringin was inactive [89]. Naringenin also inhibited the formation of oxygenated metabolites in platelets stimulated with thrombin and inhibited AA-induced platelet aggregation. Although eriodictyol offered protection against TNF-α-induced cytotoxicity in murine fibroblast L-929 cells, naringenin was not protective [90]. Naringenin and hesperetin have beneficial effects on cardiovascular diseases involving vasodilation. They displayed a concentration-dependent inhibition of the agonist-induced contractile responses [91]. It was known that naringenin was able to traverse the blood-brain barrier [92] and exert a diverse array of neuronal effects through their ability to interact with the protein kinase C (PKC) signaling pathways [93]. Naringenin can bind to both estrogen receptors, ER-α and ER- β [94]. Importantly, naringenin competed more effectively with 17-β-estradiol for binding to ER-β than for ER-α.

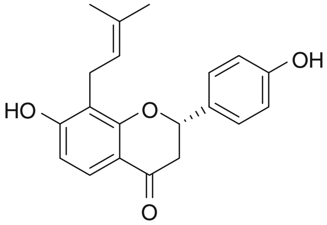
Interaction of naringenin with ER-β may be relevant for cardiovascular effects as this receptor is present in significant amount in arterial tissue [95]. Furthermore, results of anticarcinogenesis experiments indicated that naringenin, but not naringin, inhibited aflatoxin B1-induced carcinogenesis [96] and that naringenin caused cytotoxicity and apoptosis via a transient induction of caspase-3 activity in HL60 cells [97]. Additionally, it exhibited strong antiproliferative activity in various cancer cells, and its treatment dose showed no toxic effect on normal cells [98–101]. Narirutin, which is found in immature oranges, was reported to inhibit airway inflammation in the allergic mouse model [102]. The anti-inflammatory effect is likely to be associated with the reduction in the ovalbumin (OVA)-induced increases of interleukin (IL)-4 and immunoglobulin E (IgE). Naringin, which is found in the peels of citrus fruits such as grapefruit, *C. hassaku*, and others, is hydrolyzed to a major metabolite, naringenin which readily crosses the blood-brain barrier. It has been reported to possess antiviral, antihypertensive, and neuroprotective effects [103–105]. Naringin has potent antioxidative activity which has been observed in various in vitro and in vivo animal models [106, 107]. This compound also has metal chelating, free radical scavenging properties and offers some protection against mutagenesis and lipid peroxidation [108]. The antioxidative effects have been shown to be similar to GSH (glutathione). Naringin plays the important role in regulating antioxidative capacity by increasing superoxide dismutase (SOD) and catalase (CAT) activities and by upregulating the gene expression of SOD, CAT, and glutathione peroxidase (GPx) [109]. In cholesterol metabolism, naringin is known to act as an inhibitor for a hydromethylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the mevalonate pathway [110]. In addition, naringin enhanced the proliferation of cells including rat osteosarcoma UMR106, mouse osteoblastic MC3T3-E1, and mouse leukemia P388 cells [99, 111, 112]. Hesperetin is reported to be a powerful radical scavenger and a promoter of cellular antioxidant defense-related enzyme activities [113]. This compound exhibited anti-inflammatory activity by inhibiting of LPS-induced expression of the COX-2 gene in RAW 264.7 macrophages [114]. Hesperetin is a potent chemopreventive agent; its supplementation during the initiation, post-initiation, and entire period stages of colon carcinogenesis in the male rat model in vivo significantly reversed these activities [115]. Administration of hesperetin to 1,2-dimethylhydrazine (DMH)-treated rats decreased the tumor incidence and the number of aberrant crypt foci with simultaneous enhancement of tissue lipid peroxidation, glutathione S-transferase (GST), GPx, SOD, and CAT activities [116]. Hesperetin induced Notch homolog 1 (NOTCH1) expression in human gastrointestinal carcinoid (BON) cells, subsequently suppressing tumor cell proliferation and bioactive hormone production [117]. Therapeutically useful properties of hesperidin, which is found in the peels of citrus fruits (*C. aurantium* var. *daidai*, *C. natsudaidai*, and *C. unshiu*), have also been described. Hesperidin can prevent microvascular leakage by virtue of its vasoprotective action through the inhibition of the enzyme hyaluronidase which is reported to regulate the permeability of capillary walls and supporting tissues [118]. Additionally, it has been demonstrated that hesperidin can decrease blood cell and platelet aggregation, believed to be beneficial in cases of capillary permeability and fragility [119]. Besides, their effect on vascular permeability and ocular blood flow, both hesperidin and hesperetin, demonstrate strong antioxidative properties [120]. The antioxidative activity is through their ability to quench oxidative radical chain reactions and can thus help preserve neuronal health. Hesperidin also exhibited significant anti-inflammatory activity by modulating the prostaglandin synthesis and COX-2 gene expression pathways [114]. Hesperidin has been reported to possess analgesic, hypolipidemic, antihypertensive, and diuretic activity [121–123]. Another potential therapeutic application of hesperidin is its anticancer activity mediated through the suppression of cell proliferation [124, 125]. Neohesperidin also showed antiproliferative activity in Hep G2 cells [126] and the antiallergic effect on dermatitis in mice [127]. Eriodictyol, which is abundant in lemon, was found to reduce NO production from LPS-stimulated RAW 264.7 macrophages [128]. The inhibitory effect was found to be caused by blockage of NF-κB activation and phosphorylation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), and c-Jun N-terminal kinase (JNK). It has been reported that eriocitrin, which is a lemon (*C. limon*) flavanone, is effective in the prevention of oxidative damages caused by acute exercise-induced oxidative stress in the rat liver [129]. Neoeriocitrin showed free radical scavenging activity, the inhibition of superoxide formation [130], and the better effect than naringin on proliferation and osteogenic differentiation in mouse preosteoblast MC3T3-E1 cells [131]. Isosakuranetin, which is found in blood oranges (*C. sinensis*), grapefruits, and others, exhibited the neuroprotective effect. This compound increased cell viability and catalase activity (CA) and decreased membrane damage, ROS generation, intracellular calcium level ($[Ca2^+]_i$), and caspase-3 activity in H_2O_2-treated PC12 cells [132]. Interestingly, isosakuranetin is known to be an allelopathic molecule and acts by affecting K^+ uptake and K^+-dependent acid extrusion [133].

Liquiritigenin is extracted from *Glycyrrhizae radix*, a herbal that is frequently used to treat injury or swelling or for detoxification in traditional medicine. Liquiritigenin is also one of the major active compounds of menopausal formula 101 (MF101), a herbal extract used in clinical trials for the treatment of hot flushes and night sweats in postmenopausal women [134]. Liquiritigenin is shown to be a selective agonist of ER-β [135] and that targeting this receptor may be associated with anti-inflammatory effects. Liquiritigenin inhibited NO and TNF-α production induced by LPS in RAW 264.7 macrophages [136]. Studies have already proven that liquiritigenin exerts cytoprotective effects against heavy metal-induced toxicity in cultured hepatocytes [137] and has protective effects against liver injuries induced by acetaminophen and buthione sulfoximine (BSO) in rats [138]. Besides, liquiritigenin has been reported to have the choleretic effect and the ability to induce hepatic transporters and phase II enzymes [139] and inhibit amyloid β-peptide-induced neurotoxicity, not only in hippocampal neurons [140], but also in rats [141]. In addition, liquiritigenin inhibited the growth of human gastric carcinoma SGC-7901, human hepatocellular carcinoma SMMC-7721, and human colorectal cancer Lovo cells [142]. Liquiritin, an active component of *Glycyrrhiza uralensis*, might be a good candidate for treating various neurodegenerative diseases including Alzheimer's disease or Parkinson's disease [143]. This compound showed neuroprotection and neurotrophism on primary cultured hippocampal cells

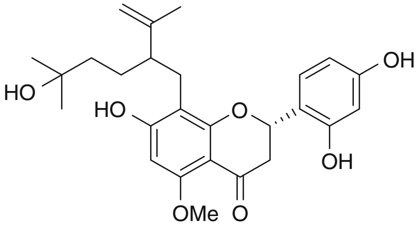
Fig. 61.8 Structures of bioactive 5-deoxyflavanones



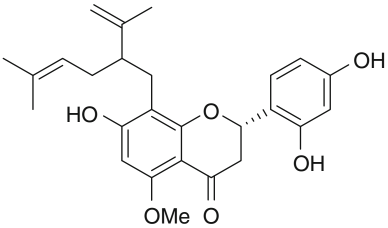
R^1 = H, R^2 = H : liquiritigenin
R^1 = Glc, R^2 = H : liquiritin
R^1 = H, R^2 = Glc : prunin



isobavachin



kurarinol



kurarinone

Fig. 61.9 Structures of prenyl and lavandulyl flavanones

[144]. Liquiritin provided obviously neuroprotective effect on middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia/reperfusion (I/R), the effect attributable to its antioxidative and antiapoptosis activities [145]. It has been reported that liquiritin produced significant antidepressant effects in the forced swimming test and tail-suspension test in mice [146]. Prunin showed the inhibitory effect on caffeine N3-demethylation, a marker activity of CYP1A2, in human liver microsomes [147]. Additionally, prunin was tested against Gram-positive and Gram-negative bacteria, yeasts, and molds. Prunin showed no inhibitory effect against the microorganisms assayed but stimulated growth of *Pseudomonas aeruginosa* and different *Bacillus* sp. [148] (Fig. 61.8).

Isobavachin, which is a prenylated flavanone, isolated from *Psoralea corylifolia* showed cytotoxicity against rat hepatoma H4IIE and rat glioma C6 cells [149], promoting effect on neurogenesis of mouse embryonic stem cells by prenylation of protein [150]. Lavandulylated flavanones such as kurarinol and kurarinone were isolated from the root of *Sophora flavescens*. Kurarinol is known to be a natural tyrosinase inhibitor; this compound markedly inhibited melanin synthesis [151, 152]. And kurarinol is a potent inhibitor of sortase A, an enzyme that plays a key role in cell wall protein anchoring and virulence in *Staphylococcus aureus* [153]. Kurarinol and kurarinone were reported to exhibit hypolipidemin effects in cholesterol-fed rats [154]. In addition, these flavanones also showed significant inhibitory activities against intracellular ROS levels as well as NF-κB activation [155] (Fig. 61.9).

3 Dihydrochalcone

3.1 Introduction

Dihydrochalcones, 1,3-diphenylpropan-1-ones, are natural phenolics related to chalcones. They consist of the C_6–C_3–C_6 skeleton structure, two aromatic rings connected by a C_3 chain. The difference with chalcones is that dihydrochalcones lack a double bond at C_2–C_3 position.

3.2 Occurrence of Dihydrochalcones

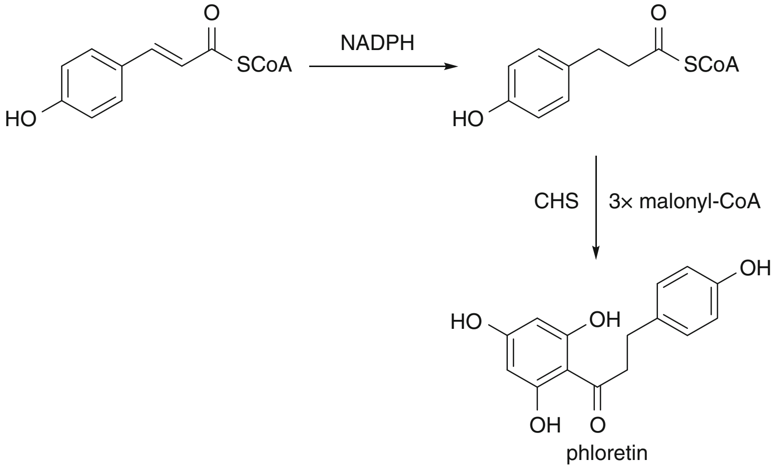
In contrast to the ubiquitously present flavonoids, occurrence of dihydrochalcones is limited. In apple trees, the major subclass of flavonoids is represented by dihydrochalcones, which are found in large amounts (up to 5% of dry weight) in leaves and immature fruits. Although they were thought for a long time to be exclusive of *Malus* sp., dihydrochalcones have been reported in several other genera like *Balanophora*, *Fragaria*, and *Symplocos*. Nowadays, dihydrochalcones seem to be restricted to approximately 30 plant families, especially Rosaceae, Rutaceae, Lauraceae, and Leguminosae.

3.3 Phytochemistry of Dihydrochalcones

Dihydrochalcone numbering is the same as the chalcone ones. The substituted pattern of hydroxyl group of dihydrochalcones resembles that of chalcones, too. Their function *in planta* remains unresolved. They have been hypothesized to act as UV filters in leaves, and a role in resistance to pathogens has been suggested.

3.4 Biosynthesis of Dihydrochalcones

Whereas *p*-coumaroyl-CoA is the precursor for the naringenin-chalcone and further flavonoid formation, *p*-dihydrocoumaroyl-CoA is required for the biosynthesis of



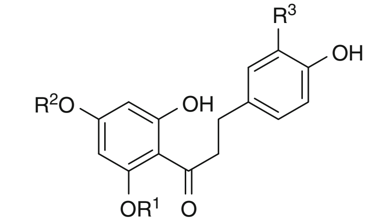
Scheme 61.3 Biosynthesis of dihydrochalcones

dihydrochalcones such as phloretin. It is assumed that *p*-dihydrocoumaroyl-CoA is formed from *p*-coumaroyl-CoA by a NADPH-dependent dehydrogenase. Dihydrochalcones are produced by the common CHS with equal chalcone biosynthesis [156, 157] (Scheme 61.3). However, in past radiolabeled biosynthetic experiments, *p*-dihydrocoumaric acid was not detected as the intermediate.

3.5 Biological Activities of Dihydrochalcones

Phloretin and its glycoside phloridzin are abundantly present in apples (*Malus* × *domestica*), especially in the peel, and strawberries. Phloretin has been reported to display antioxidative properties [158] and to prevent cytokine-induced expression of endothelial adhesion molecules and to reduce activation of human platelet activation [159]. In addition, phloretin may be beneficial for reducing insulin resistance through its potency to regulate adipocyte differentiation and function [160, 161]. Phloretin is a penetration enhancer in the delivery of lidocaine, which is a common local anesthetic and antiarrhythmic drug, through skin [162, 163]. Phloretin has been reported to inhibit the growth of human acute lymphoblastic leukemia MOLT4 cells in vitro [164] and Fisher bladder carcinoma and rat mammary adenocarcinoma cells in vivo [165]. And phloretin induced apoptosis B16 melanoma 4A5 cells by inhibition of glucose transmembrane transport [166]. Phloridzin, which is mainly distributed in plants of *Malus*, is known to be an antidiabetic agent. This compound inhibited intestinal glucose uptake via the sodium D-glucose cotransporter and similarly inhibited renal glucose reabsorption

Fig. 61.10 Structures of bioactive dihydrochalcones

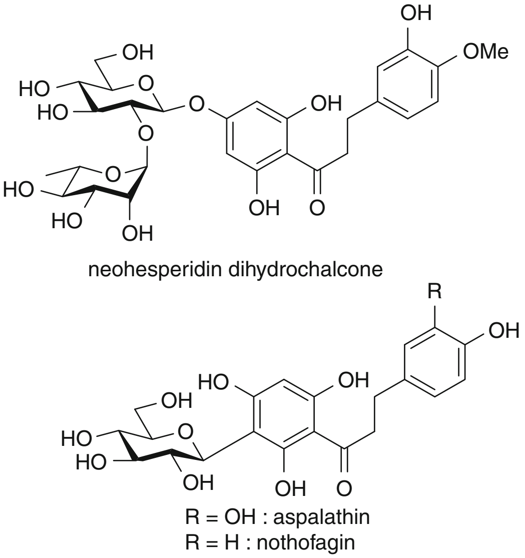


$R^1 = H, R^2 = H, R^3 = H$: phloretin
$R^1 = Glc, R^2 = H, R^3 = H$: phloridzin
$R^1 = H, R^2 = Glc, R^3 = OH$: sieboldin
$R^1 = H, R^2 = Glc, R^3 = H$: trilobatin

[167, 168]. Correction of hyperglycemia with phloridzin has been shown to normalize the effects of insulin on glucose metabolism in the liver and other peripheral tissues such as muscle and adipose tissue in diabetic rat models [169]. In addition, phloridzin improved hyperglycemia but not hepatic insulin resistance in a transgenic mouse model of type 2 diabetes [170]. Other biological functions of phloridzin involve estrogenic and antiaging activity and the inhibitory effect against the three human concentrative nucleoside transporters hCNT1, hCNT2, and hCNT3 [171–173]. Sieboldin and trilobatin, which can be found in apple leaves, were reported to contribute to the antioxidative activity and blocking effects of bacterial spread of apples [174]. Trilobatin inhibited against α-glucosidase and α-amylase linked to type 2 diabetes [175] (Fig. 61.10).

Neohesperidin dihydrochalcone, which is a non-nutritive sweetening agent of oranges, inhibited DPPH radical, lipid peroxidation, inflammation-related ROS, and xanthine oxidase activity [176–178]. Aspalathin, a dihydrochalcone *C*-glycoside, is the most abundant flavonoid in rooibos (*Aspalathus linearis*), which is well known as a herbal tea in many countries. Unfermented rooibos plant material contains between 4% and 12% aspalathin. Aspalathin also has beneficial effects on glucose homeostasis in type 2 diabetes through stimulating glucose uptake in muscle tissues and insulin secretion from pancreatic β-cells [179]. Aspalathin appeared to have in vitro antioxidative and antimutagenic effects [180–182]. Nothofagin, which is found in *Aspalathus linearis*, has been reported to exhibit the antioxidative and antimutagenic effects as same as aspalathin [182, 183] (Fig. 61.11).

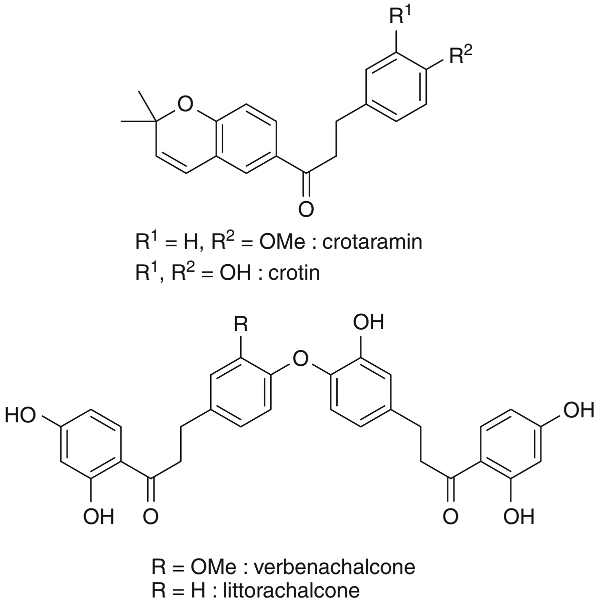
Crotaramin and crotin, which are pyranodihydrochalcones isolated from *Crotalaria ramosissima*, exhibited weak inhibition of maturation of *Plasmodium falciparum* (NF-54) parasites [64]. Dimeric dihydrochalcones verbenachalcone and littorachalcone were isolated from the aerial parts of *Verbena littoralis*. These compounds were reported to act as enhancers of nerve growth factor (NGF)-mediated neurite outgrowth and axonal branching in rat pheochromocytoma PC12D cells [184, 185] (Fig. 61.12).


OH
OMe
OH
HO
O
HO
O
OH
O
HO
O
HO
OH
OH
O
R
OH
HO
OH
OH
HO
O
HO
OH
OH
O

neohesperidin dihydrochalcone

R = OH : aspalathin
R = H : nothofagin

Fig. 61.11 Structures of bioactive dihydrochalcone glycosides

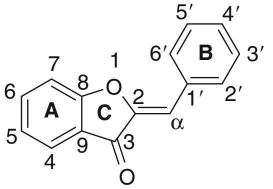

R^1
R^2
O
O
R
OH
O
HO
OH
OH
O
O
OH

R^1 = H, R^2 = OMe : crotaramin
R^1, R^2 = OH : crotin

R = OMe : verbenachalcone
R = H : littorachalcone

Fig. 61.12 Structures of rare dihydrochalcones

Fig. 61.13 Basic skeleton of aurone



4 Aurone

4.1 Introduction

Aurones, 2-benzylidene-coumaran-3-ones, belong to the subclass of plant flavonoids that provides the bright yellow color of some important ornamental flowers. The name "aurone" comes from a Latin word *aurum* (= gold) because of the golden yellow color of the pigments. They consist the three-ring C_6—C_3—C_6 system, and the heterocyclic C-ring is the five-membered ring [186]. Aurones are structurally the isomeric of flavones.

4.2 Occurrence of Aurones

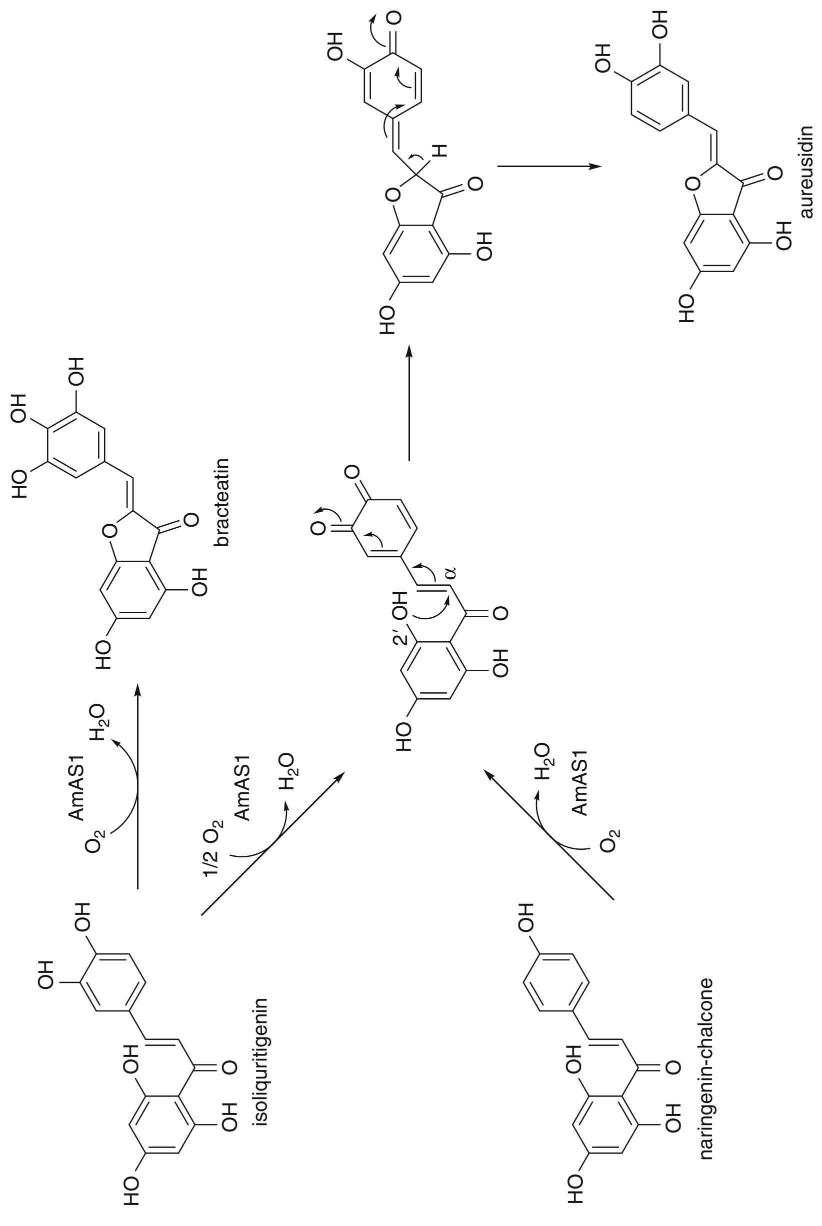
Aurones are found in a number of flowers of some Scrophulariaceae (e.g., snapdragon [*Antirrhinum majus*]) and Compositae (e.g., *Coreopsis*, *Cosmos*, and *Dahlia*). The yellow snapdragon flower is probably one of the best sources of aurones in the vacuoles of the epidermal cells of the flowers. In some other plant species, however, aurones are also found in the bark, wood, leaves, seedlings, and nectar. In 2001, the occurrence of aurone (4′-chloroaurone) in marine organisms has been reported [187]. However, its structure was revised to 3-(4′-chloroisocoumarin) later [188].

4.3 Phytochemistry of Aurones

Aurone numbering is shown in Fig. 61.13. Naturally occurring aurones are polyhydroxylated in the aromatic rings. On the whole, aurones have hydroxyl groups at 4- and 6-positions in A-ring and 4′-position in B-ring. Aurones have been described as phytoalexins, used by the plant as defense agents against various infections.

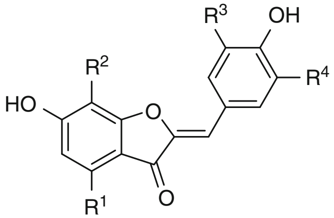
4.4 Biosynthesis of Aurones

Biosynthesis of aurones from chalcones involves dual chemical transformation of chalcones such as hydroxylation of the B-ring moiety and oxidative cyclization (2′,α-dehydrogenation) to give the aurone structure [2]. Several lines of evidence


isoliquiritigenin
naringenin-chalcone
bracteatin
aureusidin
AmAS1
O_2
H_2O
1/2 O_2
AmAS1
H_2O
H_2O
AmAS1
O_2
2′ OH
α
H
HO
OH
O

Scheme 61.4 Biosynthesis of aurones

Fig. 61.14 Structures of bioactive aurones



R^1 = OH, R^2 = H, R^3 = OH, R^4 = H : aureusidin
R^1 = OH, R^2 = H, R^3 = OH, R^4 = OH : bracteatin
R^1 = H, R^2 = OH, R^3 = OH, R^4 = H : maritimetin
R^1 = OH, R^2 = H, R^3 = H, R^4 = H : 4,4′,6-trihydroxyaurone
R^1 = H, R^2 = H, R^3 = H, R^4 = H : hispidol
R^1 = H, R^2 = H, R^3 = OH, R^4 = H : sulfuretin

suggest that a single enzyme, which is called aureusidin synthase (AmAS1), catalyzes both these transformations. Aureusidin can be produced from either naringenin-chalcone or isoliquiritigenin, whereas bracteatin arises solely from isoliquiritigenin. The mechanistic investigations showed that AmAS1 acts as an oxygenase. It was established that AmAS1 is responsible for the transformation of a variety of chalcones to aurones [189]. According to the screening of a panel of hydroxylated and glycosylated chalcones, it is found that only chalcones, which are hydroxylated at C2′ and C4′ positions, are transformed to aurones (Scheme 61.4). Furthermore, the AmAS1 is definitely specific to chalcones because flavanones are inert for the enzyme action.

4.5 Biological Activities of Aurones

Aureusidin which is found in snapdragon, maritimetin which is an anthochlor pigment of *Coreopsis tinctoria* and *Baeria chrysostoma*, and bracteatin which is isolated from *Helichrysum bracteatum* have been studied with regards to their radical scavenging potential using density functional theory (DFT) [190–192]. 4,4′,6-Trihydroxyaurone isolated from *Pterocarpus santalinus* and *Smilax bracteata* and hispidol which can be found in *Trichilia hispida* were able to induce significant tyrosinase inhibition. In particular, 4,4′,6-trihydroxyaurone was highly active when compared to kojic acid [193]. Sulfuretin isolated from the heartwood of *Rhus verniciflua* is an active antirheumatoid arthritis agent. This compound showed significant inhibitory effects on hind paw edema and trypsin inhibitor activity induced by Freund’s complete adjuvant reagent (FCS reagent) and on vascular permeability caused by acetic acid [194]. In addition, sulfuretin exhibited the anti-inflammatory effect by the suppression of NF-κB transcription activity via the inhibitory regulation of IκB kinase β-phosphorylation in LPS-induced RAW 264.7 macrophages [195]. Also, sulfuretin demonstrated the antidiabetogenic effect by the suppression of NF-κB activation [196] (Fig. 61.14).

5 Conclusions

The minor flavonoids are important subclasses of plant polyphenols. Especially, chalcones and flavanones are the obligate intermediates in flavonoid biosynthesis. These naturally occurring compounds also play significant roles as pigments, phytoalexins, and signaling molecules in pathogenesis and symbiosis. Additionally, considerable attention has been devoted to these compounds because of their potential pharmaceutical applications in recent years. For this reason, they are an object of continuously growing interest among the scientists. The attention is mainly drawn to the common skeleton and possibilities for its modifications guided by mechanistic and structure-activity relationship studies.

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Stilbenoids 62

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Contents

1 Introduction 1902
2 Phytochemical Aspects 1902
2.1 Stilbenes 1903
2.2 Oligostilbenes 1906
2.3 Bibenzyls 1914
2.4 Bisbibenzyls 1917
2.5 Phenanthrenes 1923
3 Biosynthesis 1925
3.1 Biosynthesis of Stilbenes 1925
3.2 Biosynthesis of Oligostilbenes 1925
3.3 Biosynthesis of Bibenzyls and Bisbibenzyls 1928
3.4 Biosynthesis of Phenanthrenes 1931
4 Biological Activities 1932
4.1 Antitumor Activity 1932
4.2 Antioxidant Activity 1934
4.3 Antiplatelet Activity 1936
4.4 Antidiabetic Activity 1937
4.5 Antimicrobial Activity 1937
4.6 Anti-inflammatory Activity 1938
4.7 Neuroprotective Activity 1940
4.8 Hepatoprotective Activity 1940
4.9 Cardioprotective Activity 1941
4.10 Phytotoxicity 1941
5 Conclusion 1941
References 1942

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Abstract

Stilbenoids are a class of plant phenolics containing C6–C2–C6 unit in their structures and classified into five groups, covering stilbenes, oligostilbenes, bibenzyls, bisbibenzyls, and phenanthrenes. They have been a hot research topic for their intricate structures and diverse biological activities. Resveratrol and combretastatin A-4 are the star compounds due to their potent cardioprotective, chemopreventive, and antitumor properties and have the potential to be developed as new drugs. The stilbenoids do not enjoy a wide distribution and are only found in special genus. Although the constituent unit is simple, the structures of stilbenoids highlight the chemical diversity by different substitutes and various oligomeric styles. In a biogenesis viewpoint, they are formed by a branch of the flavonoid biosynthetic pathway. This chapter provides a summary of the occurrence, phytochemisty, biosynthesis, and biological aspects of the stilbenoids.

Keywords

Bibenzyls • bioactivities • biosynthesis • bisbibenzyls • occurrence • oligostilbenes • phenanthrenes • phytochemistry • stilbenes • stilbenoids

1 Introduction

The term “stilbenoids” was proposed by Gorham in 1980 [1, 2], which refers to a class of plant phenolics with 1,2-diphenylethylene or 1,2-diphenylethane nucleus in their structures. Stilbenoids are regarded as plant phytoalexins and have been a hot research topic for their intricate structures and diverse biological activities. The phytochemical research concerning the stilbenoids developed quickly in recent years. More than 1,000 compounds belonging to this group have been discovered, compared with just over 100 listed in 1980 and about 300 in 1995 [2]. Recent advances in analytical and spectroscopic techniques, especially the NMR methods, speed up the discovery and elucidation of the intricate structures of stilbenoids. The intricate structures and stereochemistry of oligostilbenes and bisbibenzyls were established based on the modern techniques. Furthermore, these compounds demonstrated diverse biological activities, including antitumor, antimicrobial, antioxidant effects, antiplatelet aggregation, phytotoxicity, etc. These bioactive compounds and their derivatives are of great interest for drug research and development as a result of their potential in therapeutic or preventive applications, exemplified by resveratrol and combretastatin A-4. In this chapter, we will give an overview of structural features, occurrence, phytochemical aspects, biosynthesis, and biological activities of the stilbenoids.

2 Phytochemical Aspects

According to their structural characteristics, stilbenoids are mainly divided into five categories, stilbenes, oligostilbenes, bibenzyls, bisbibenzyls, and phenanthrenes.

In the section of phytochemical aspects, the structural characteristic, distribution, typical representatives, and their structures of each group are introduced.

2.1 Stilbenes

Stilbenes possess a skeleton with two aromatic rings joined by a methylene bridge. The simple stilbene nucleus is generally substituted by different groups of hydroxyl, methyl, methoxy, prenyl, geranyl, etc., and combined with sugars to form glycosides. The double bonds in naturally occurring stilbenes are usually *E*-configuration, but stilbenes with *Z*-configuration are also observed. The compounds of this group highlight the chemical structural diversity through the modification of above styles on the nucleus. About 125 new stilbenes have been discovered between the year of 1995 and 2008 [3]. They mainly occur in the families of Aceraceae, Anchinoidae, Asteraceae, Bombycidae, Burseraceae, Combretaceae, Cyperaceae, Dipterocarpaceae, Euphorbiaceae, Gnetaceae, Hepaticae, Iridaceae, Leguminosae, Lejeuneaceae, Liliaceae, Meliaceae, Moraceae, Ophioglossaceae, Orchidaceae, Polygonaceae, Rosaceae, Stemonaceae, Vitaceae, and Zingiberaceae.

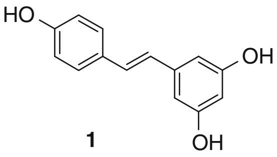
Resveratrol **1** is the most famous representative of this group and occurs in *Polygonum cuspidatum* root and *Vitis* species. It is a phytotoxin produced by several plants in response to infection or other stresses and attracted attention for its cardioprotective effect in red wine. In addition, it is the most important unit for the construction of oligomeric stilbenes.

Combretastatins, a series of bioactive stilbenes (combretastatin A series), bibenzyls (B series), phenanthrenes (C series), and macrocyclic lactone (D series), were obtained from the African willow tree *Combretum caffrum* (Combretaceae). Among them, the A series of combretastatins belonging to stilbenes, including combretastatins A-1 to A-6 **2–7**, are found to be tubulin polymerization inhibitors.

Typical stilbenes substituted with hydroxyls, methyl, methoxy, menthane groups, and their origin were listed as follows. *Trans*-4-[2-(3,5-dimethoxyphenyl) ethenyl]-1,2-benzenediol **8** was isolated from *Sphaerophysa salsula* (Leguminosae) [4]. Thunalbene **9** was obtained from *Thunia alba* (Orchidaceae), and the structure was designated as 3,3′-dihydroxy-5-methoxystilbene [5]. The *Phragmipedium* species produced three new stilbenes including 2,3′-dihydroxy-5′-methoxystilbene **10**, 2,3-dihydroxy-3′,5′-dimethoxystilbene **11**, and 2,3′-dihydroxy-5,5′-dimethoxystilbene **12** [6]. Phoyunbenes A-D **13–16** were found in *Pholidota yunnanensis* (Orchidaceae) [7]. 5,4′-Dihydroxy-3-methoxystilbene **17**, 3,5-dihydroxy-4′-methoxystilbene **18**, and (*E*)-3,3′-dimethoxy-4,4′-dihydroxystilbene **19** were isolated from *Rumex bucephalophorus* and *Leuzea carthamoides* [8, 9], respectively. Two *Z*-type stilbenes named (*Z*)-3-methoxy-5-hydroxystilbene **20** and (*Z*)-3,5-dihydroxystilbene **21**, together with a menthane-substituted stilbene (*E*)-1-(1-terpinen-4-olyl)-3-methoxystilbene **22**, were obtained from aerial parts of *Alpinia katsumadai* (Zingiberaceae) [10, 11].

There are two stilbene representatives isolated from special origins. Bryophytes are characterized by the production of bisbibenzyls, and no stilbene has been obtained before the isolation of 3,4-dihydroxy-3′-methoxystilbene **23** from *Marchesina bongardiana* (Lejeuneaceae) [12]. *Kirkpatrickia variolosa*, a kind of Antarctic red sponge of Anchinoidae family, yielded a triacetate derivative 3,4,5-triacetoxystilbene **24** which was the only marine natural stilbene [13].

Two stilbene glycosides, named (*E*)-3,4′-dimethoxyl-5-rutinosyl stilbene **25** and 3,5-dimethoxy-4′-*O*-(β-rhamnopyranosyl-(1→6)-β-glucopyranoside)stilbene **26**, were isolated from *Guibourtia tessmanii* (Leguminosae) [14, 15]. *Acer mono* (Aceraceae), a Korean folk medicine for hemostasis, produced two new stilbene glycosides 5-*O*-methyl-(*E*)-resveratrol 3-*O*-β-D-glucopyranoside **27** and 5-*O*-methyl-(*E*)-resveratrol 3-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside **28** [16].



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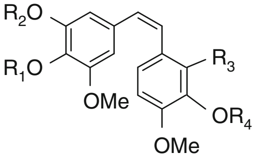
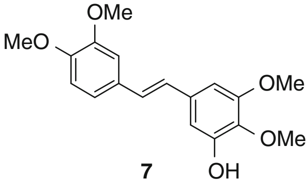


2 $R_1=R_2=Me, R_3=OH, R_4=H$
3 $R_1,R_2= –CH_2–, R_3=R_4=H$
4 $R_1=Me, R_2=R_3=R_4=H$
5 $R_1=R_2=Me, R_3=R_4=H$
6 $R_1=R_4=Me, R_2=R_3=H$



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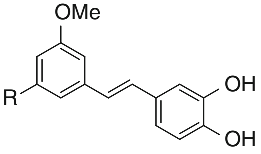
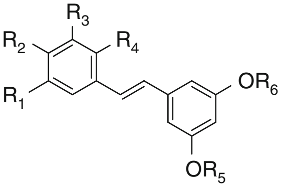
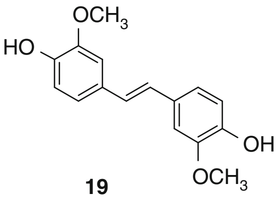


8 R=OMe
23 R=H



9 $R_1=R_2=R_4=R_5=H, R_3=OH, R_6=Me$
10 $R_1=R_2=R_3=R_5=H, R_4=OH, R_6=Me$
11 $R_1=R_2=H, R_3=R_4=OH, R_5=Me, R_6=Me$
12 $R_1=OMe, R_2=R_3=H, R_4=OH, R_5=H, R_6=Me$
13 $R_1=R_2=R_4=OMe, R_3=OH, R_5=H, R_6=Me$
14 $R_1=R_5=H, R_2=OH, R_3=OMe, R_4=OMe, R_6=Me$
15 $R_1=R_2=R_5=H, R_3=OH, R_4=OMe, R_6=Me$
16 $R_1=R_2=R_5=H, R_3=OMe, R_4=OMe, R_6=Me$
17 $R_1=R_3=R_4=H, R_2=OH, R_5=H, R_6=Me$
18 $R_1=R_3=R_4=H, R_2=OMe, R_5=R_6=H$



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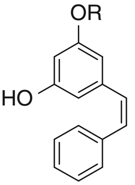
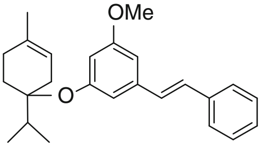


20 R=Me
21 R=H



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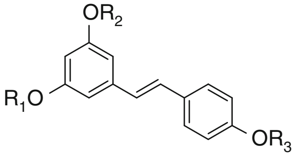
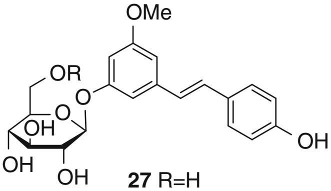
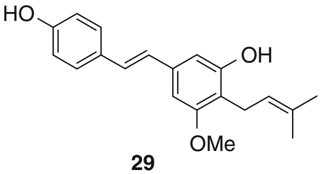


24 R_1=R_2=R_3=Ac
25 R_1=rutinosyl,R_2=R_3=Me
26 R_1=R_2=Me,R_3=rutinosyl



27 R=H
28 R=apiose

Prenyl substitution is a conventional derivatization style in stilbenes, exemplified by artoindonesianin N **29** from *Artocarpus gomezianus* [17]. The prenyl groups in stilbenes cyclize to form new derivatives. 4-Hydroxy-5′-methoxy-6″,6″-dimethylpyran[2″,3″:3′,4′]stilbene **30**, 3,5′-dimethoxy-4-hydroxy-6″,6″-dimethylpyran[2″,3″:3′,4′]stilbene **31**, and 3,4,5-trimethoxy-6″,6″-dimethylpyran [2″,3″:3′,4′]stilbene **32**, with dimethylchromene ring in their structures, have been obtained from *Lonchocarpus utilis* (Leguminosae) [18]. Furthermore, schweinfurthins A-C **33–35** from the leaves of *Macaranga schweinfurthii* (Euphorbiaceae) are typical samples of the prenylated stilbenes [19].



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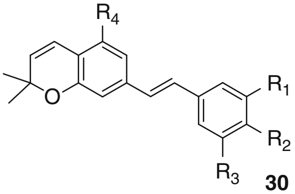
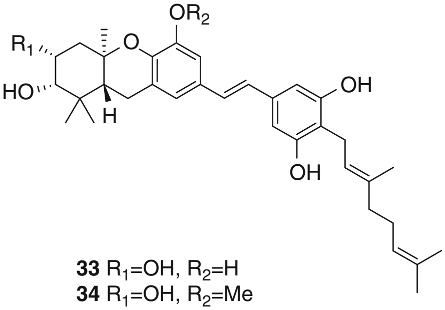
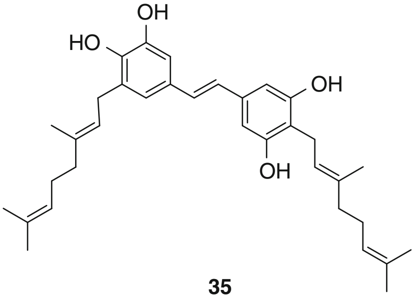


30 R_1=R_3=H,R_2=R_4=OMe
31 R_1=R_4=OMe,R_3=H,R_2=OH
32 R_1=R_2=R_3=OMe,R_4=H

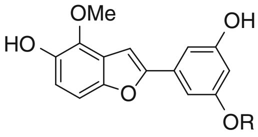


33 R_1=OH, R_2=H
34 R_1=OH, R_2=Me

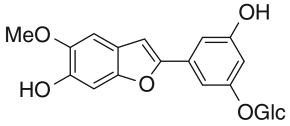


35

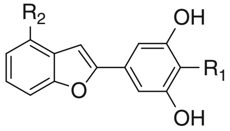
Arylbenzofuran derivatives are a group of special stilbenes formed by C_7–O–C_7 linkage, for instance, gnetofurans B **36** and C **37** from *Gnetum klossii* [20]. In addition, schoenoside **38**, a phenylbenzofuran glucoside discovered from *Schoenocaulon officinale* (Liliaceae), as well as stemofurans A-C **39–41** from *Stemona collinsae* belongs to this group [21, 22].



36 R=Me
37 R=H



38



39 R_1=R_2=H
40 R_1=H,R_2=OH
41 R_1=Me,R_2=H

2.2 Oligostilbenes

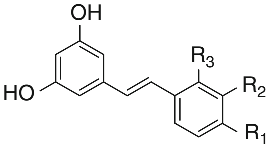
The structures of oligostilbenes are produced by coupling between homogeneous or heterogeneous monomeric stilbenes, leading to the construction of dimer, trimer, and even the octamer. They do not enjoy a wide distribution in plant kingdom and have been found in the family of Agavaceae, Apiaceae, Arecaceae, Celastraceae, Cyperaceae, Dipterocarpaceae, Gnetaceae, Haemodoraceae, Iridaceae, Leguminosae, Moraceae, Musaceae, Orchidaceae, Pinaceae, Polygonaceae, Ranunculaceae, Vitaceae, and Welwitschiaceae. Thereinto, Vitaceae, Leguminosae, Gnetaceae, and Dipterocarpaceae are particular rich resource of this group.

Oligostilbenes are constructed by C–C or C–O–C linkage of various stilbene units with diverse coupling patterns and producing structures with diverse skeletons, complex configurations, and different degrees of oligomerization. The most common monomeric stilbene units which comprised the oligostilbenes are resveratrol, isorhapontigenin, piceatannol, oxyresveratrol, etc. (Fig. 62.1). Therefore, oligostilbenes are classified into six groups which are resveratrol oligomers, isorhapontigenin oligomers, piceatannol oligomers, oxyresveratrol oligomers, resveratrol and oxyresveratrol oligomers, and finally miscellaneous oligomers [3].

2.2.1 Resveratrol Oligomers

The group of resveratrol oligomers comprises the largest number of oligostilbenes and is characterized by the polymerization of two to eight resveratrols. About 180 constituents of this group covering dimer to octamers have been reported, which is produced by diverse polymeric styles.

Vitisinol A **42** isolated from *Vitis thunbergii* is a dimer linked by four C–C or C–O–C bonds [23]. Two dimeric stilbene glycosides **43** and **44** were reported from *Polygonum cuspidatum* (Polygonaceae) [24]. Thereinto, **44** is a symmetrical molecule and possesses a novel four-membered ring which is very rare in natural products. Isoampelopsin F **45** linked by three C–C bonds and heimiol A **46** with seven-member ring were isolated from *Parthenocissus tricuspidata* and *Neobalanocarpus heimii* [25]. A novel resveratrol dimer with a five-membered lactone ring, namely, shorealactone **47**, was obtained from *Shorea hemsleyana* [26]. Schneide reported the isolation of anigopreissin A **48** from the *Anigozanthos*


OH
HO
R3
R2
R1

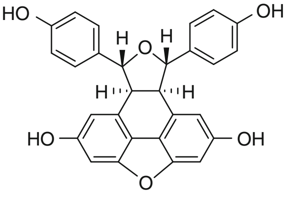
R_1=OH,R_2=R_3=H	resveratrol
R_1=R_2=OH,R_3=H	piceatannol
R_1=R_3=OH,R_2=H	oxyresveratrol
R_1=OH,R_2=OCH_3,R_3=H	isorhapontigenin

Fig. 62.1 Units comprising oligomeric stilbenes

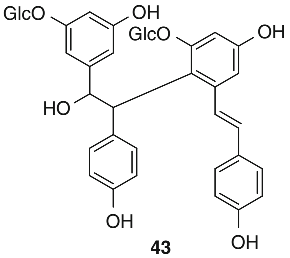
preissii (Haemodoraceae) and *Musa cavendish* (Musaceae), which is the first dimer containing unsaturated benzofuan moiety [27]. Moreover, an aldehyde-substituted derivative, (−)-viniferal **49**, was isolated from *Vitis vinifera* [28].

Two trimers containing rare tribenzobicyclo[3.3.2]decatriene system were isolated from *Vatica rassak* and *V. pauciflora*, named vaticanol G **50** and vaticaside D **51** [29, 30]. Caragaphenol A **52**, with a nine-membered ring in the molecules, was found in *Caragana stenophylla* [31]. Three isomers which have bicyclo[5.3.0]decane ring system were obtained including amurensin G **53** from *Vitis amurensis* [32] and suffruticosols A **54** and B **55** from *Paeonia suffruticosa* (Ranunculaceae) [33].

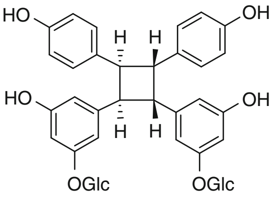
Two new resveratrol pentamers, named amurensins E **56** and F **57**, have been isolated from *Vitis amurensis* [34]. The isolation and structural elucidation of a hexamer vaticanol D **58** and a heptamer vaticanol J **59** from *Vatica rassak* have been reported [29]. An octamer vateriaphenol A **60** from *Vateria indica* was reported by Ito and coworkers, and it is the largest molecules of stilbenoids [35].


HO
OH
H
O
H
H
H
HO
OH
O

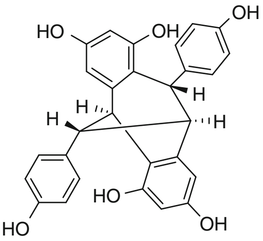
42


GlcO
OH
GlcO
OH
HO
OH
OH

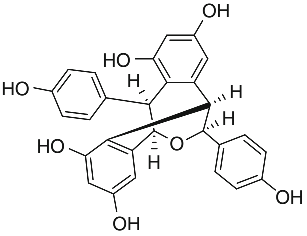
43


HO
OH
H
H
HO
OH
H
H
OGlc
OGlc

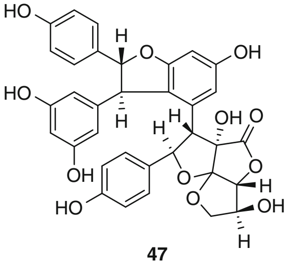
44


HO
OH
OH
H
H
H
H
HO
OH
HO

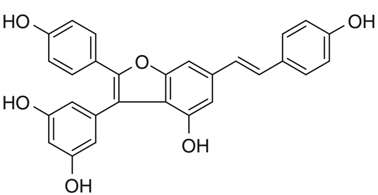
45


OH
HO
HO
H
H
H
HO
O
H
OH
OH

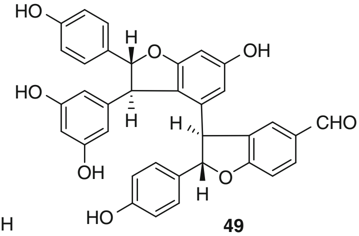
46


HO
H
O
OH
HO
H
H
H
OH
O
O
OH
O
H
HO
O
OH
H

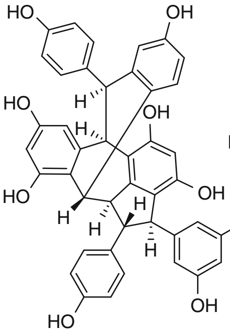
47



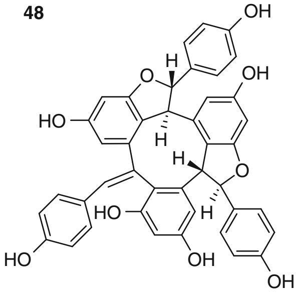
48



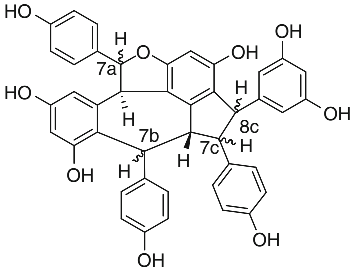
49



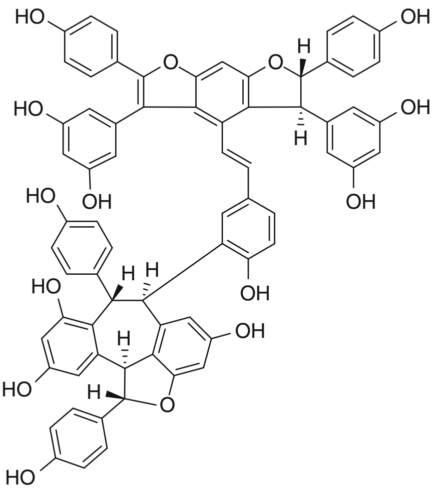
50 R=H
51 R=Glc



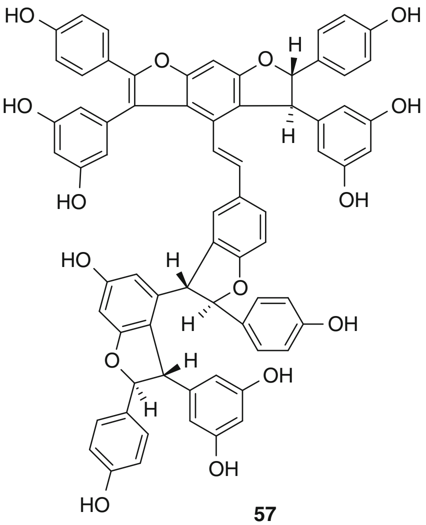
52



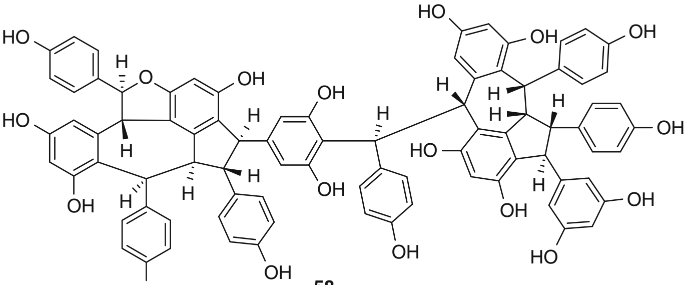
53 H-7a=H-8c=H-7b=β,H-7c=α
54 H-7a=H-8c=α,H-7b=H-7c=β
55 H-7a=H-7b=H-8c=α,H-7c=β



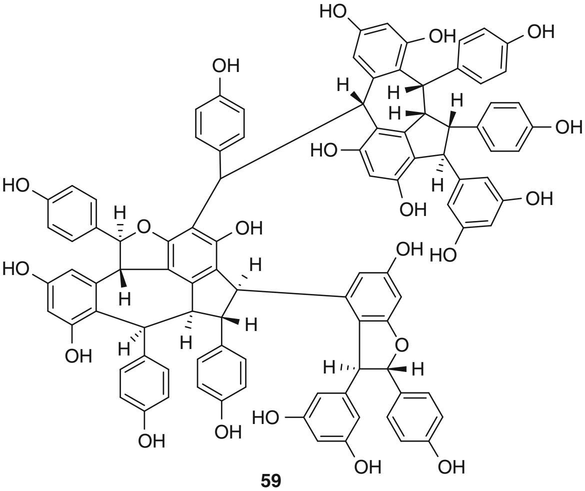
56



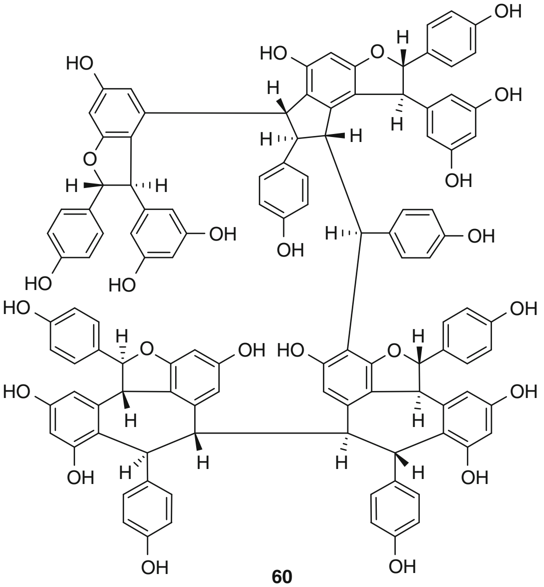
57


HO
OH
H
O
HO
OH
H
H
H
H
H
H
OH
OH
OH
OH
H
HO
OH
OH
H
H
H
H
HO
OH
OH
OH
HO

58


HO
OH
OH
H
H
H
H
OH
HO
OH
H
O
OH
HO
H
H
H
OH
HO
OH
H
H
O
H
H
OH
OH
HO
OH
OH

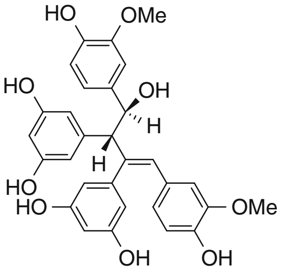
59


HO
HO
O
H
OH
H
OH
O
H
H
H
H
H
OH
OH
H
HO
HO
OH
OH
HO
H
O
OH
HO
O
H
OH
HO
H
H
OH
H
H
OH
OH
H
H
OH
OH
OH

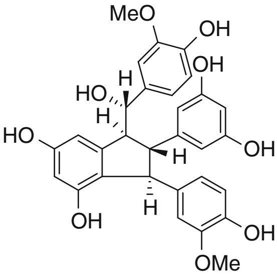
60

2.2.2 Isorhapontigenin Oligomers

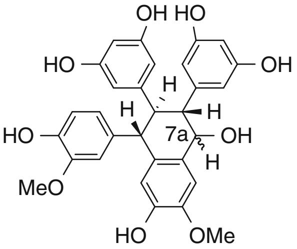
Oligostilbenes of this group mainly occur in the genus of *Gnetum*. The representatives comprise gnetuhainins P **61** and I **62** from *G. hainanense* [36, 37], dimeric stilbene epimers gnetifolins M **63** and N **64** from *G. montanum* [38], gnemonol M **65** from *G. gnemon* [39], gneafricanin F **66** found in *G. africanum* [40], as well as bisisorhaphontigenin B **67** obtained from *G. africanum* [41]. With the exception of *Gnetum* species, *Salacia lehmbachii* produced three isorhapontigenin dimers, named lehmbachols A-C **68–70** [42]. The only two isorhapontigenin trimers, gnetuhainins N **71** and O **72**, which are stereoisomers have been found in *Gnetum hainanense* [43]. A tetramer named gnetuhainin R **73** was obtained from the same species (*G. hainanense*) [44].


HO
OMe
HO
OH
H
H
HO
HO
OMe
OH
OH

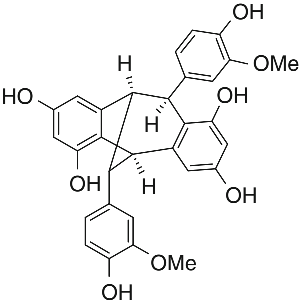
61


MeO
OH
H
HO
H
OH
HO
H
OH
OH
H
OH
OMe

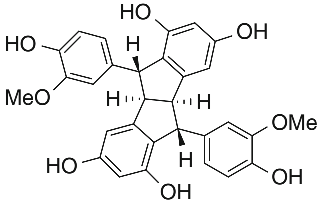
62


OH HO
HO
OH
H
H
H
HO
7a
OH
H
MeO
HO
OMe

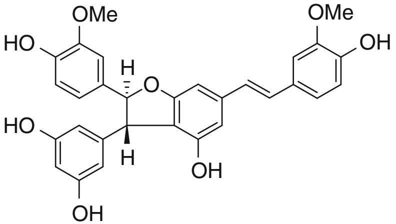
63 H-7a=β
64 H-7a=α


OH
OMe
H
HO
OH
H
OH
H
OH
OMe
OH

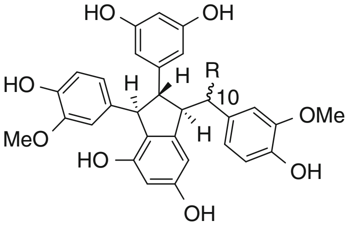
65


HO
OH
HO
H
MeO
H
H
OMe
HO
H
OH
OH

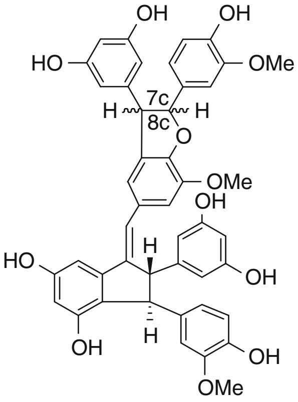
66


OMe
OMe
HO
OH
H
O
HO
H
OH
OH

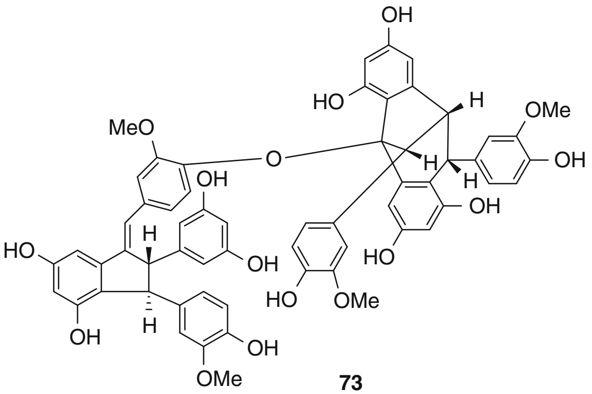
67


HO
OH
HO
H
H
R
10
OMe
MeO
H
HO
OH
OH

68 R=OMe
69 R=OMe diastereoisomer of **322**
70 R=OCH$_2$Me


OH
OH
HO
OMe
7c
H
H
8c
O
OMe
OH
H
HO
OH
H
OH
OH
OMe

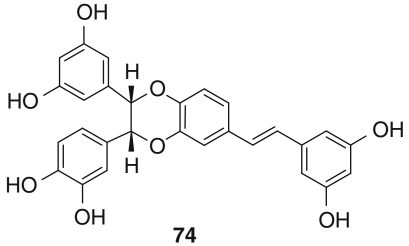
71 H-7c=α,H-8c=β
72 H-7c=β,H-8c=α


OH
HO
H
OMe
MeO
O
OH
H
H
OH
H
OH
HO
OH
HO
HO
OMe
OH
H
OH
OMe

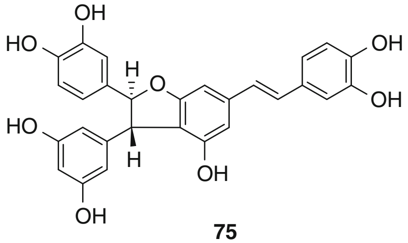
73

2.2.3 Piceatannol Oligomers

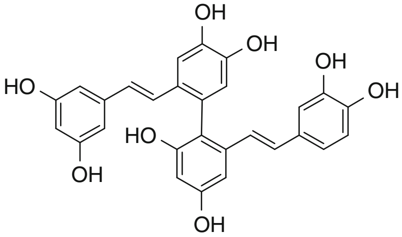
Only piceatannol dimers have been discovered from the plant species. Longusol C **74** and gneafricanin C **75** were isolated from *Cyperus longus* and *Gnetum africanum* [40, 45]. Tibeticanol **76** was obtained from *Caragana tibetica* [46]. Two piceatannol dimer glycosides named piceasides A–B **77–78** were isolated from Norway spruce *Picea abies* as a mixture in a ration of 1:1 [47].


OH
HO
H
O
O
H
OH
HO
OH
OH

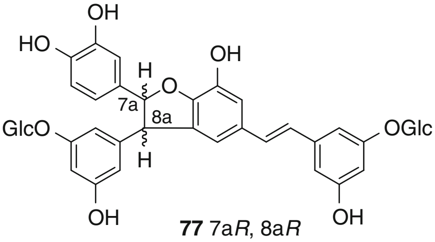
74


OH
HO
H
O
OH
OH
HO
H
OH
OH

75


OH
OH
OH
HO
OH
HO
OH
OH

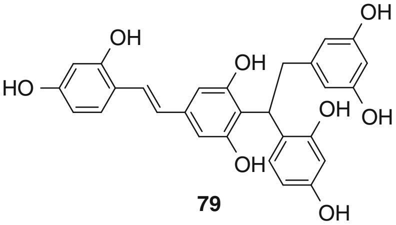
76


OH
HO
H
O
OH
7a
8a
GlcO
OGlc
H
OH
OH

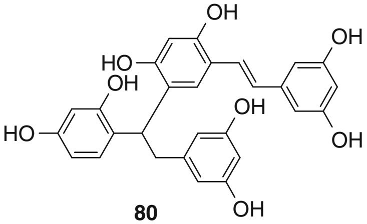
77 7a*R*, 8a*R*
78 7a*S*, 8a*S*

2.2.4 Oxyresveratrol Oligomers

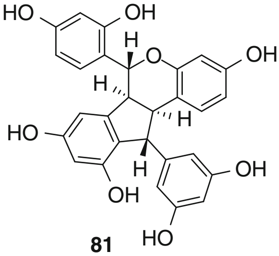
Andalasin A **79** and artogomezianol **80** were isolated from *Artocarpus gomezianus* (Moraceae) [48]. Structures **79** and **80** are possible intermediates in the biogenesis of oxyresveratrol dimers. Parvifolol C **81** and gnetumontanin A **82** were discovered in two *Gnetum* species *G. parvifolium* and *G. montanum* [49, 50].



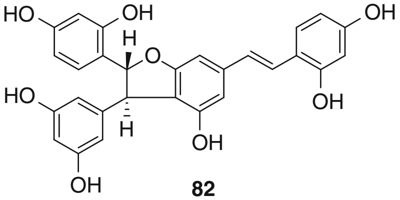
79



80



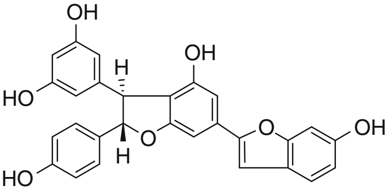
81



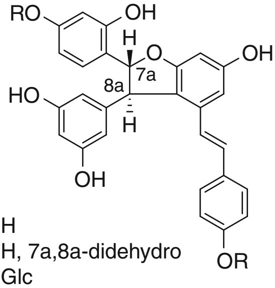
82

2.2.5 Resveratrol and Oxyresveratrol Oligomers

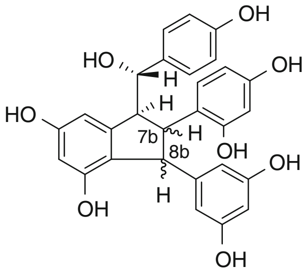
The resveratrol and oxyresveratrol oligomers are only obtained from the genus *Gnetum* (Gnetaceae) and polymerize by oxidative coupling between resveratrol and oxyresveratrol. About 24 compounds of this group have been elucidated. A dimer containing a benzofuran and a dihydrobenzofuran moiety, named gnemonol G **83**, was isolated from *G. gnemon* [51]. Four stilbene dimers, gnetuhainins A **84**, B **85**, D **86** and E **87**, were obtained from *G. hainanense* [52]. Gnemonoside J **88**, a diglucoside of **84**, was isolated from *G. africanum* [53]. Three stereoisomers including parvifolols A **89** and B **90** and gnetuhainin S **91** have been founded from *G. parvifolium* and *G. hainanense* [44, 49, 54]. Gnemonol A **92** and its stereoisomers and gnemonol I **93** composed of two resveratrol units and one oxyresveratrol unit were discovered in *G. gnemon* [51, 55]. Gnemonol J **94** from *G. gnemon* possesses the same skeleton of **92** and **93**; however, it is coupled by one resveratrol unit and two oxyresveratrol units [51]. Gnemonol C **95** from *G. gnemonoides* is a stilbene tetramer constructed by three resveratrol and one oxyresveratrol units [55].



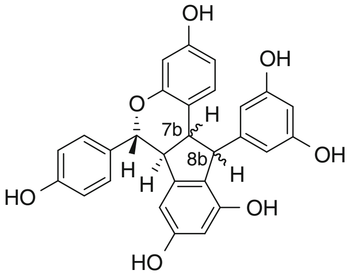
83



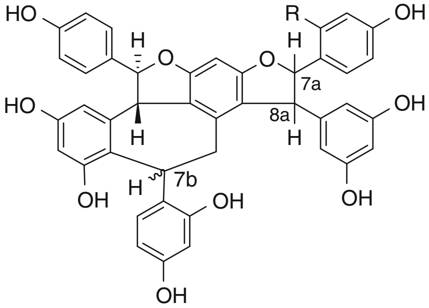
84 R=H
85 R=H, 7a,8a-didehydro
88 R=Glc


OH
HO
H
H
OH
HO
7b
H
8b
OH
OH
H
OH
OH

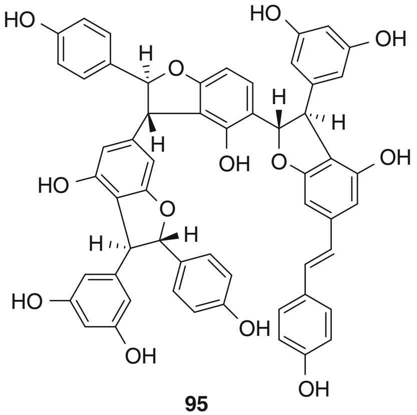
86 H-7b=β,H-8b=α
87 H-7b=α,H-8b=β


OH
OH
O
7b
H
8b
H
OH
H
H
HO
OH
HO

89 H-8b=α,H-7b=β
90 H-8b=β,H-7b=β
91 H-8b=β,H-7b=α


HO
R
OH
H
O
O
H
7a
HO
8a
OH
H
H
H
7b
OH
OH
OH
OH

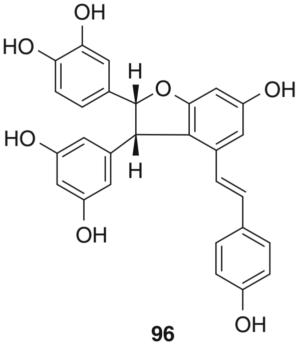
92 R=H,H-7b=α,H-7a/8a=*trans*
93 R=H,H-7b=β,H-7a/8a=*trans*
94 R=OH,H-7b=α,H-7a/8a=*trans*


HO
HO
OH
H
O
H
H
H
OH
O
OH
HO
O
H
H
HO
OH
OH
OH

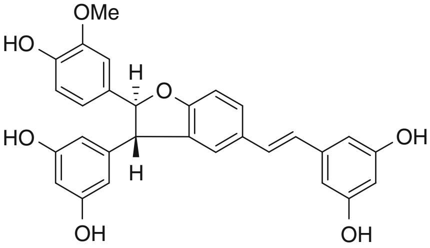
95

2.2.6 Miscellaneous Oligomers

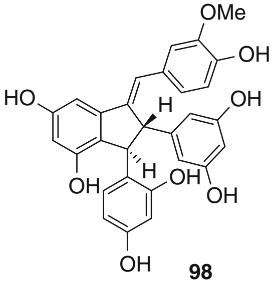
Oligostilbenes polymerize from different stilbene units with the exception of resveratrol and oxyresveratrol oligomers, and containing miscellaneous structural skeleton will be classified into this group. Longusol B **96** from *Cyperus longus* [45] is a stilbene dimer composed of resveratrol and piceatannol units. Gnetuhainin Q **97**, an isorhapontigenin and resveratrol dimer, was found in *Gnetum hainanense* [36]. The first isorhapontigenin and oxyresveratrol dimer named gnetuhainin J **98** was isolated from *G. hainanense* [37]. An isorhapontigenin and piceatannol dimer, gneafricanin B **99** was discovered in *Gnetum africanum* [41]. Two isorhapontigenin and 2-hydroxyisorhapontigenin dimers were obtained from *G hainanense* and named gnetuhainin G **100**. An isorhapontigenin and gnetol dimer gnetuhainin K **101** was isolated from *G*. *hainanense* [36].


OH
HO
H
O
OH
HO
H
OH
OH

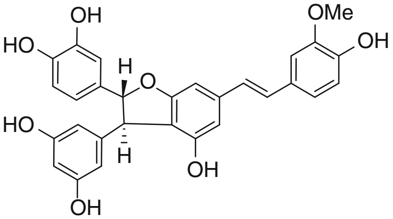
96


OMe
HO
H
O
HO
H
OH
OH
OH
OH

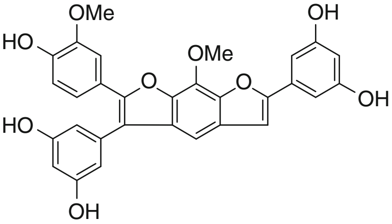
97


OMe
OH
HO
H
OH
OH
H
OH
OH
OH

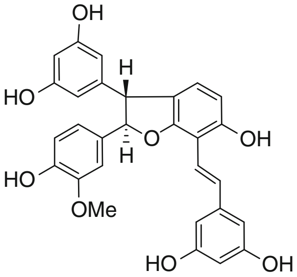
98


OH
HO
H
O
OMe
OH
HO
H
OH
OH

99


OMe
HO
OMe
O
O
OH
OH
HO
OH

100


OH
H
HO
O
OH
H
HO
OMe
HO
OH

101

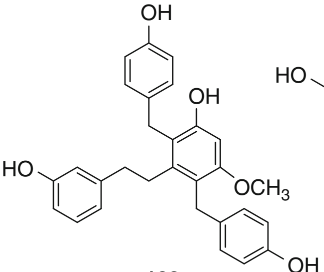
2.3 Bibenzyls

The bibenzyls are characterized by the presence of one 1,2-diphenylethane structure in their molecules. Similar with the stilbene structures, there are hydroxyls, methyl, methoxy, prenyl, geranyl, etc., located in the structures of bibenzyls. Bibenzyls have been mainly isolated from bryophytes. In addition, a few compounds of this group were reported from the genera of *Stemona*, *Dendrobium*, and *Polygonum*.

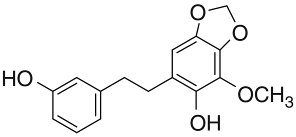
According to the suggestion given by Gorm et al. [56], bibenzyl compounds are classified into four groups. However, a few groups of bibenzyls are new addition to the growing list of naturally occurring bibenzyls, such as tyrolobibenzyls. In addition, bibenzyls containing isoprene units, regardless of branched or heterocyclic ring-forming isoprene units, are regarded as the same group. Therefore, the bibenzyls are reclassified into five groups according to their substitute patterns on both the benzene nucleus and ethylidene bridge.

2.3.1 Group A: Simple Bibenzyls

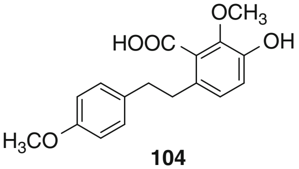
Bibenzyls having halogenated, hydroxylated, methyled, methoxylated, carboxylate, benzoyl, and/or methylenedioxy substitutes constitute the group of simple bibenzyls. The representatives are 2′,6′-bis(*p*-hydroxybenzyl)-3,3′-dihydroxy-5-methoxybibenzyl **102** from *Bletilla formosana* [57], bulbophyllum **103** from *Bulbophyllum protractum* [58], 2-carboxy-4-hydroxy-3,4′-dimethoxybibenzyl **104** from *Plagiochila* species [59], 2-chloro-3-hydroxybibenzyl **105** from *Riccardia marginata* [60], 1-(3′,5′-dihydroxyphenyl)-2-(4′-hydroxyphenyl)-ethane-1,2-diol **106** from *Polygonum cuspidatum* [61], methyl 4-hydroxy-4′-*O*-methyllunularate **107** from *Plagiochila spinulosa* [62], tragopogonic acid **108** from *Tragopogon porrifolius* [63], and 2,4,6-trichloro-3-hydroxybibenzyl **109** from *Riccardia marginata* [60].



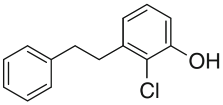
102



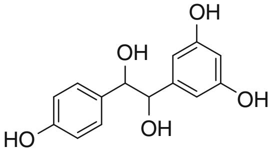
103



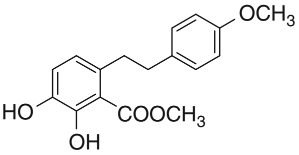
104



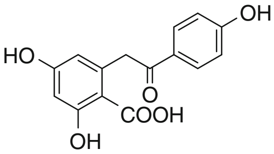
105



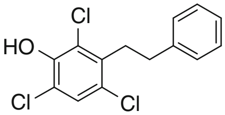
106



107



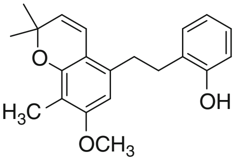
108



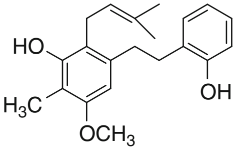
109

2.3.2 Group B: Isoprene Unit–Substituted Bibenzyls

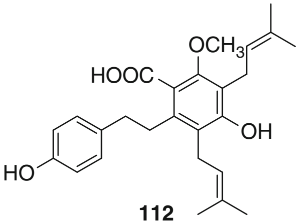
Bibenzyls of this group are characterized by the presence of prenyl, geranyl, and/or farnesyl substitutes. Isoprene units in the structures may be branched and/or form five- to seven-member rings. The typical compounds of this group are bauhinols A **110** and B **111** from *Bauhinia saccocalyx* [64], 2-carboxy-3-methoxy-4,6-di-(3-methyl-2-butenyl)-5,4′-dihydroxy-bibenzyl **112** from *Lethocolea glossophylla* [65], 2-isopropenyl-6-hydro-4-(2-phenylethyl) dihydrobenzoluran **113** from *Radula perrottetii* [66], and 3,5,4′-trihydroxy-4-(3,7,11-trimethyl-2,6,10-dodecatrienyl)bibenzyl **114** from *Radula* species [67].



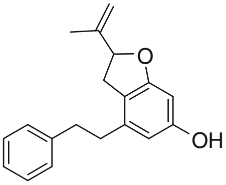
110



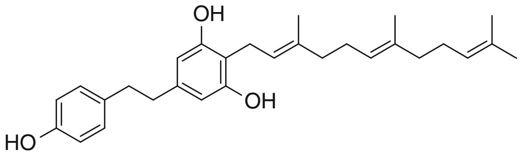
111



112



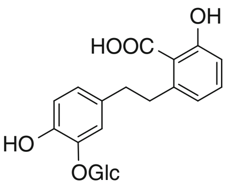
113



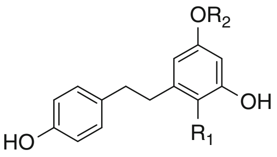
114

2.3.3 Group C: Glycosylated Bibenzyls

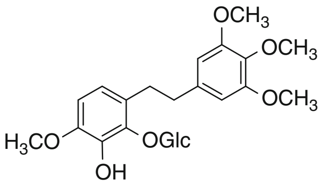
Bibenzyls conjugated with glycosyl substitutes on the aromatic rings or benzylic methylenes (excluding tyrolobibenzyls) belong to this group. The representatives of this group include 2′-carboxy-4,3′-dihydroxybibenzyl-3-*O*-β-D-glucopyranoside **115** from *Ricciocarpus natans* [68], 2-carboxyl-3,4′-dihydroxy-5-β-D-xylopyranosyloxybibenzyl **116** and 5,4′-dihydroxy-3-α-L-rhamnopyranosyl-(1→3)-β-D-xylopyranosyloxybibenzyl **117** from *Tragopogon porrifolius* [63], and combretastatin B-1,2′-β-D-glucoside **118** from *Combretum erythrophyllum* [69].



115



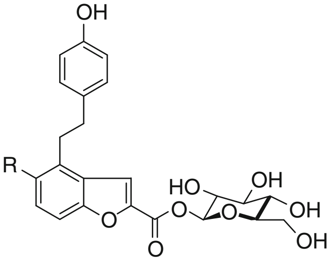
116 R_1=COOH, R_2=β-D-xylopyranosyl
117 R_1=H, R_2=α-L-rhamnopyranosyl-(1→3)-β-D-xylopyranosyl



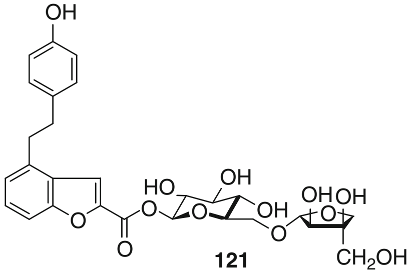
118

2.3.4 Group D: Tyrolobibenzyls

Tyrolobibenzyls are a new class of naturally occurring bibenzyl derivatives possessing a unique phenylethyl-benzofuran skeleton. Tyrolobibenzyls A **119**, B **120**, D **121**, and F **122** from *Scorzonera humilis* L. (Asteraceae) are the typical constituents of this group [70–72].


OH
R
HO
OH
OH
OH
O
O
O
O

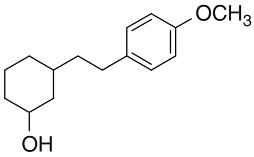
119 R=H
120 R=OH
122 R=OGlc


OH
HO
OH
OH
OH OH
O
O
O
O
O
O
CH_2OH

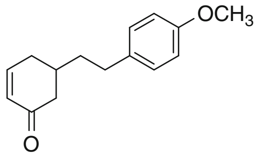
121

2.3.5 Group E: Other Bibenzyls

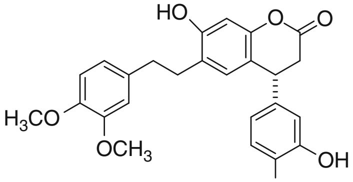
The hydrogenated bibenzyls **123–124** from *Plagiochila longispina* [73], dihydrocoumarin-type bibenzyl **125** from *Vittaria anguste-elongata* [74], cannabinoid-type bibenzyl **126** from *Radula marginata* [75], and dihydrophenanthrene hybrids such as shancilin **127** from *Pleione bulbocodioides*, and terpenoid hybrid spinuloplagin A from *Plagiochila spinulosa* **128** are classified into this group.



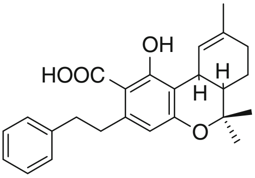
123



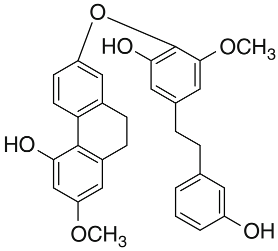
124


HO
O
O
H_3CO
OCH_3
OH
OH

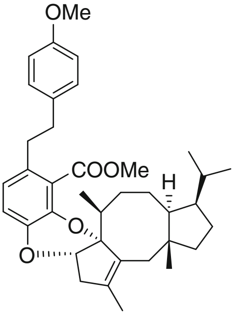
125



126


O
HO
OCH_3
HO
OCH_3
OH

127

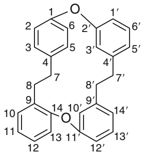
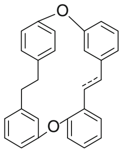
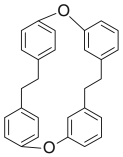
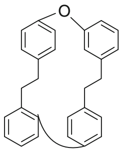
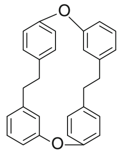
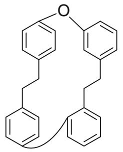
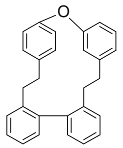

OMe
COOMe
H
O
O

128

2.4 Bisbibenzyls

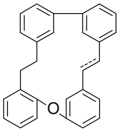
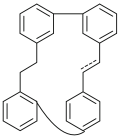
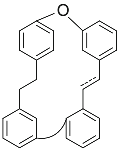
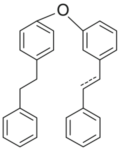
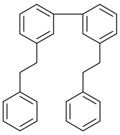
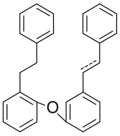
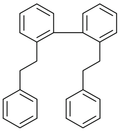
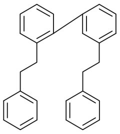
Bisbibenzyls are dimeric bibenzyls and chemically constructed by two lunularin moieties with diarylether and/or biphenyl linkages, and producing cyclic and acyclic four aromatic rings system. They are usually distributed in liverworts and

Table 62.1 Cyclic and acyclic bis(bibenzyl) types and their distribution

Subclass	Type	Structure	Linkage pattern	Family member	Distribution
Cyclic	Marchantin	 1 O 1′ 2 6 2′ 6′ 3 5 3′ 5′ 4 4′ 7 7′ 8 8′ 9 9′ 14 10′ 14′ 10 O 11 13 11′ 13′ 12 12′	C_1-O-$C_{2'}$ and C_{14}-O-$C_{11'}$ linkage	Marchantins A-P, Marchantinquinone	Aneuraceae Grimaldiaceae Jungermanniaceae Marchantiaceae Monocleaceae
	Isomarchantin		C_1-O-$C_{2'}$ and C_{13}-O-$C_{10'}$ linkage	Isomarchantins B and C Ptychantols A-C	Jungermanniaceae Lejeuneaceae Marchantiaceae
	Neomarchantin		C_1-O-$C_{2'}$ and C_{12}-O-$C_{11'}$ linkage	Neomarchantins A and B; Pakyonol	Grimaldiaceae Marchantiaceae Monocleaceae Schistochilaceae
	Riccardin I		C_1-O-$C_{2'}$ and C_{14}-$C_{12'}$ linkage	Riccardins A, C-H	Grimaldiaceae Marchantiaceae Monocleaceae Aneuraceae Jungermanniaceae
	Riccardin II		C_1-O-$C_{2'}$ and C_{13}-O-$C_{12'}$ linkage	Riccardin B	Aneuraceae Marchantiaceae
	Isoriccardin		C_1-O-$C_{2'}$ and C_{12}-$C_{10'}$ linkage	Isoriccardin C Isoriccardinquinones A and B	Marchantiaceae
	Plagiochin		C_1-O-$C_{2'}$ and C_{14}-$C_{10'}$ linkage	Plagiochins A-E	Plagiochilaceae

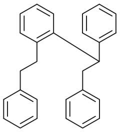
(*continued*)

Table 62.1 (continued)

Subclass	Type	Structure	Linkage pattern	Family member	Distribution
	Isoplagiochin I		C_6-$C_{2'}$ and C_{14}-O-$C_{11'}$ linkage	Isoplagiochins A-B, E-G.	Plagiochilaceae
	Isoplagiochin II		C_6-$C_{2'}$ and C_{14}-$C_{12'}$ linkage	Isoplagiochins C and D; Bazzanins A-J, L-S	Blasiacea Herbertaceae Plagiochilaceae
	Planusin		C_6-O-$C_{2'}$ and C_{13}-$C_{10'}$ linkage	Planusin A	Plagiochilaceae
Acyclic	Perrottetin		C_1-O-$C_{2'}$ linkage	Perrottetins E-H	Jungermanniaceae Pelliaceae Radulaceace
	Isoperrottetin		C_6-$C_{2'}$ linkage	Isoperrottetin A	Moraceae Radulaceace
	Paleatin		C_{14}-O-$C_{11'}$ linkage	Paleatins A-B	Marchantiaceae
	Plagilin		C_5-$C_{3'}$ linkage	Plagilin, Vitamin E	Plagiochilaceae
	Isoplagilin		C_5-$C_{2'}$ linkage	Isoplagilin	Plagiochilaceae

(*continued*)

Table 62.1 (continued)

Subclass	Type	Structure	Linkage pattern	Family member	Distribution
	Plagiolin		C_5-$C_{7'}$ linkage	Plagiolin	Plagiochilaceae

rarely found in other plant species. The first bisbibenzyl not obtained from liverworts is perrottetin H **149**, which was isolated from a pteridophyte *Hymenophyllum barbatum* [2]. It is also believed that the distribution of bisbibenzyls in both pteridophytes and liverwort is an important marker of determining the evolutionary ladder of terrestrial spore-forming plants.

Bisbibenzyls are classified into ten types of cyclic bisbibenzyls and six types of acyclic bisbibenzyls on the basis of basic bisbibenzyls skeletons (see Table 62.1). Over 100 cyclic and acyclic bisbibenzyls have been obtained from plant kingdom to date.

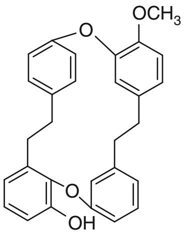
2.4.1 Macrocyclic Bisbibenzyls

According to the constitution of the macrocyclic ring, these bisbibenzyls can be divided into three main groups, those with two diarylether bonds (marchantins, isomarchantins, neomarchantins, and riccardins II), those with two biphenyl bonds (isoplagiochin II), or those with one diarylether bond and one biphenyl bond (riccardin I, isoriccardin C, plagiochins, isoplagiochin I, and planusin A) (see Table 62.1). The range of macrocyclic structures is extended ultimately and derived from additional functions (e.g., carbonyl, hydroxyl, and methoxyl) on both the benzene nucleus and ethylidene bridge and the ways in which aromatic rings are linked.

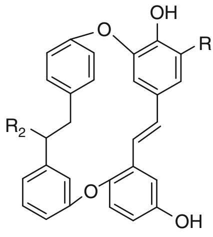
Asakawa and his coworkers reported the isolation of a marchantin-type macrocyclic bisbibenzyl marchantin P **129** from the liverwort *Marchantia chenopoda* collected in Venezuela [76]. Three isomarchantin-type macrocyclic bisbibenzyls were isolated from the liverwort *Ptychantus striatus*, belonging to the Lejeuneaceae, and designated them as ptychantols A-C **130–132** [77]. Neomarchantins A and B **133–134** were obtained from *Schistochila glaucescens* [78].

Two riccardin I-type compounds, riccardins F **135** and H **136**, were isolated from *Blasia pusilla* [79] and *Marchantia polymorpha* [80], respectively. Riccardin B **137** from *Preissia quadrata* belongs to the riccardin II-type bisbibenzyls. Isoriccardin C **138**, a compound of isoriccardin group, was obtained from *Plagiochila sciophila* [81].

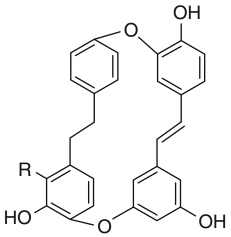
Plagiochins A-B **139** and **140** were plagiochin-type constituents from *Plagiochila fruticosa* [81]. Isoplagiochins E-F **141–142**, belonging to the isoplagiochin I-type bisbibenzyls, have been isolated from several *Plagiochila* species. Isoplagiochins C **143** and D **144** with two biphenyl linkages, which are different from isolagiochin I-type bisbibenzyls, represent isolagiochin II-type of macrocyclic bisbibenzyls. Planusin A **145** with a *cis*-stilbene moiety was discovered from cultured cells of the liverwort *Heteroscyphus planus* and classified into planusin-type bisbibenzyls [82].



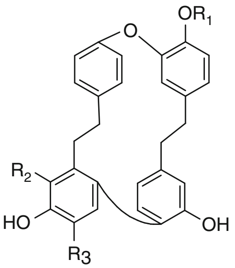
129


OH
O
R$_1$
R$_2$
O
OH

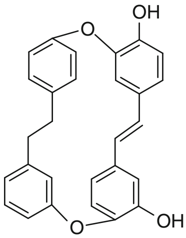
130 R$_1$=R$_2$=H
131 R$_1$=OH, R$_2$=H
132 R$_1$=H, R$_2$=OH



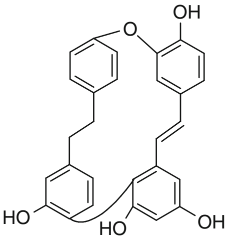
133 R=H
134 R=OH


OR$_1$
O
R$_2$
HO
OH
R$_3$

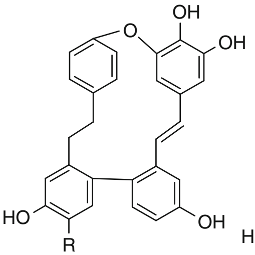
135 R$_1$=CH$_3$, R$_2$=R$_3$=H
136 R$_1$=H, R$_2$=R$_3$=OH



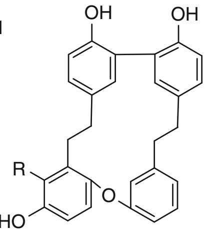
137



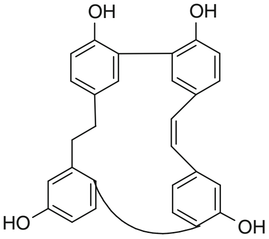
138


OH
O
OH
HO
R
OH

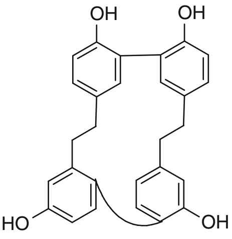
139 R=OH
140 R=H



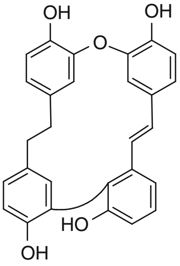
141 R=H
142 R=OH



143



144



145

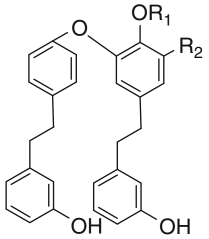
2.4.2 Acyclic Bisbibenzyls

Compared with cyclic bisbibenzyls, acyclic bisbibenzyls receive less attention due to their small number and poor structural diversity. Some novel acyclic bisbibenzyls, however, have been obtained in the past few years. These bisbibenzyls can be divided into two main groups, those with one diarylether bond (perrottetins and paleatins) or those with one biphenyl bond (isoperrottetin A, plagilins, isoplagilin, plagiolin, artogomezianol, and andalasin). The linkages occurred between two aromatic rings or between one benzene nucleus and ethylidene bridge (see Table 62.1).

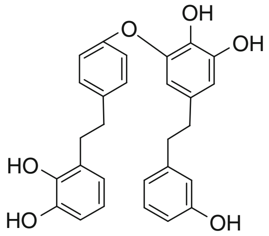
Perrottetins and isoperrottetins represent the most frequently encountered skeletal types of acyclic bisbibenzyls. They are of much interest for the investigation of biogenesis of macrocyclic bisbibenzyls, for example, the derivatives of riccardin, plagiochin, ptychantol, and isoplagiochin types. Examples of the perrottetin-type compounds are perrottetins E-H **146–149**. They have been found in different liverwort species and a fern *Hymenophyllum barbatum* [83]. Isoperrottetin A **150** and its chlorinated derivative **151** belong to isoperrottetin-type compounds [66, 84], which contain one biphenyl bond between aromatic rings instead of one diarylether bond for perrottetins.

Plagilin **152**, isoplagilin **153**, and plagiolin **154** were obtained from a neotropical *Plagiochila* species [85]. Another plagilin-type bisbibenzyl, vittarin-E **155**, has been isolated from the whole plant of *Vittaria anguste-elongata* [74]. This is one more evidence for the occurrence of acyclic bisbibenzyls in the pteridophytes.

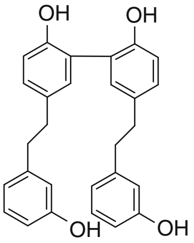
Paleatins A **156** and B **157** were isolated from the methanol extract of *Marchantia paleacea* var. *diptera* [86]. These phenolic compounds are of interest because they are the linear analogues of the macrocyclic bisbibenzyl ethers and possible biogenetic precursors of the plagiochins and riccardins.


OR1
O
R2
OH
OH

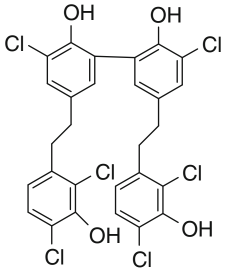
146 $R_1=R_2=H$
147 $R_1=H$, $R_2=OH$
148 $R_1=CH_3$, $R_2=OH$


OH
O
OH
HO
HO
OH

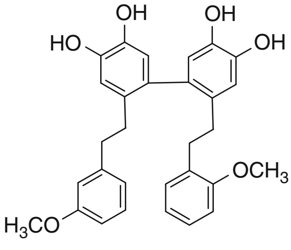
149



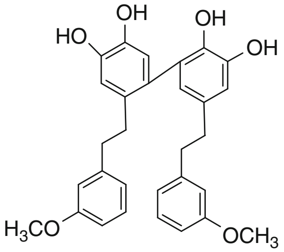
150


OH
OH
Cl
Cl
Cl
Cl
OH
OH
Cl
Cl

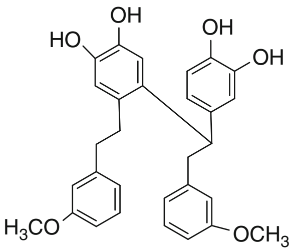
151


OH
OH
HO
OH
OCH3
H3CO

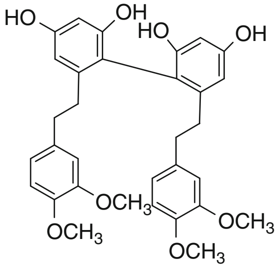
152


OH
OH
HO
OH
H3CO
OCH3

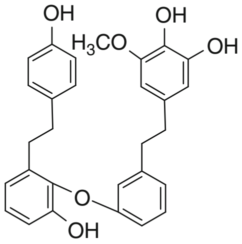
153


OH
HO
OH
OH
H3CO
OCH3

154


HO
OH
HO
OH
OCH3
OCH3
OCH3
OCH3

155


OH
OH
H3CO
OH
O
OH

156 R=OCH3
157 R=H

2.5 Phenanthrenes

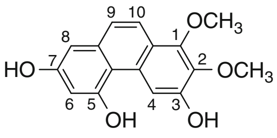
The phenanthrenes are a rather uncommon group of aromatic constituents formed by oxidative coupling of the aromatic rings of stilbene precursors and existed in the form of monomers, dimers, and even trimers [87]. A large number of phenanthrenes have been isolated from higher plants (mainly in the Orchidaceae family) and covering 49 species. The genera *Dendrobium*, *Bulbophyllum*, *Eria*, *Maxillaria*, *Bletilla*, *Coelogyna*, *Cymbidium*, *Ephemerantha*, and *Epidendrum* were particularly rich resources of phenanthrenes. In addition, a few phenanthrenes have been discovered in the family of Dioscoreaceae, Combretaceae and Betulaceae, and the Hepaticae class. The greatest number of phenanthrenes has been obtained from the *Juncus* species.

2.5.1 Monomeric Phenanthrenes

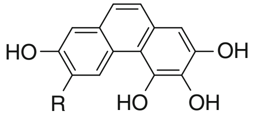
Most of the phenanthrenes are present in the form of monomers, containing about 210 compounds. Hydroxyl and methyloxyl are the most common substitutes located in the phenanthrene skeletons and occupy about 50% of all monomers. For instance, coeloginanthrin **158** from *Coelogyne cristata* [88], 4-methoxyphenanthrene-2,3,7-triol **159** and 4-methoxyphenanthrene-2,3,6,7-tetrol **160** from *Bulbophyllum vaginatum* [89], as well as 2-methoxy-3,4,7-trihydroxy-phenanthrene **161** from *B. inconsipicum* [90] are hydroxyl- and/or methyloxyl-substituted ones.

With the exception of hydroxy and methyloxy groups, methyl-, hydroxymethyl-, carboxy-, formyl-, prenyl-, and vinyl-substituted compounds are observed, with stemanthrenes A-D **162–165**, **166**, **167**, dehydroeffusal **168**, and gancaonin U **169** as the representatives [91–94]. Furthermore, glycosides were isolated from the plants of *Juncus effusus*, *Epimedium koreanum*, *Dendrobium chrysanthum*, and *Bulbophyllum striata*, for example, effuside I **170**, epimedoicarisoside A **171**, and denchryside A **172** [95–97].

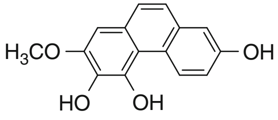
Phenanthraquinones are special phenanthrenes with quinone group in the structures. Ephemeranthoquionone **173** from *Dendrobium plicatile*, cymbinodin A **174** from *C. aloifolium*, and moniliformin **175** from *D. moniliforme* were typical constituents of this group [98–100].



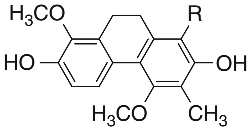
158



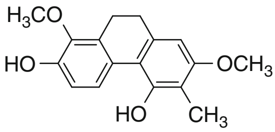
159 R=H
160 R=OH



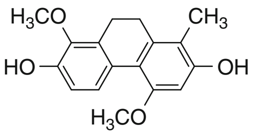
161



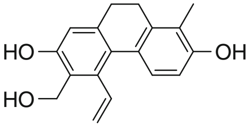
162 R=H
164 R=CH3



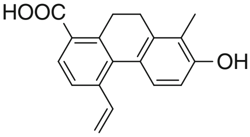
163



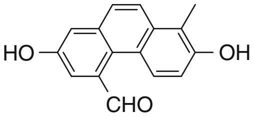
165



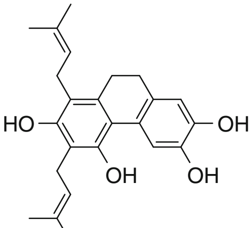
166



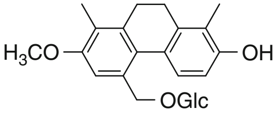
167



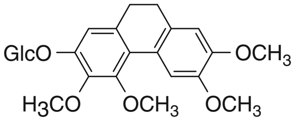
168



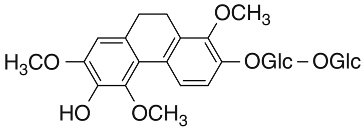
169


H3CO
OH
OGlc

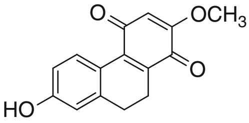
170


GlcO
OCH3
H3CO
OCH3
OCH3

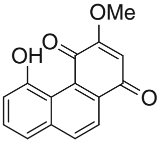
171


OCH3
H3CO
OGlc – OGlc
HO
OCH3

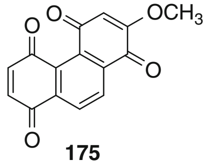
172



173



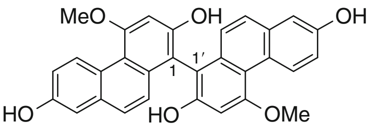
174



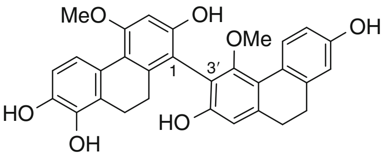
175

2.5.2 Dimeric Phenanthrenes

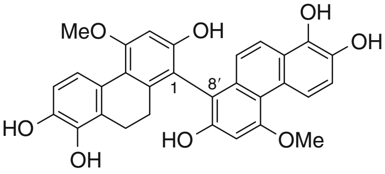
The dimeric phenanthrenes are commonly constructed by 1-1′ linkage of monomers, and the dimers with 1-3′, 1-8′, and 3-3′ link patterns also existed. Nearly 40 dimeric phenanthrenes have been found in the plants [87]. The representatives are cirrhopetalanthrin **176** from *Cremastra maculosum* [101], blestrianol A **177** from *Bletilla striata* [102], blestriarene B **178** from *Bletilla formosana* [57], and spiranthesol **179** from *Spiranthes sinensis* [103].


MeO
OH
OH
1
1′
HO
HO
OMe

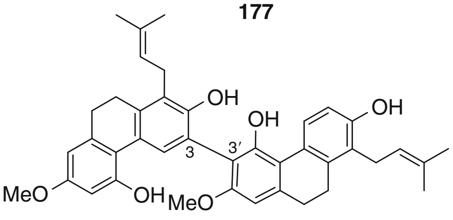
176


MeO
OH
OMe
OH
1
3′
HO
HO
OH

177


OH
MeO
OH
OH
1
8′
HO
HO
OMe
OH

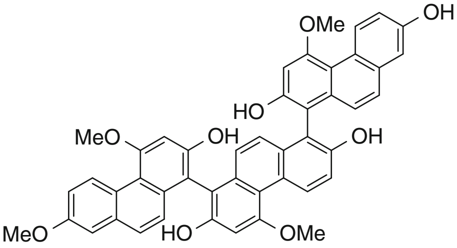
178


OH
OH
OH
3
3′
MeO
OH
MeO

179

2.5.3 Triphenanthrene

Hitherto, only one triphenanthrene **180** has been reported from the tubers of an orchidaceous plant *Cremastra appendiculata* [104].



180

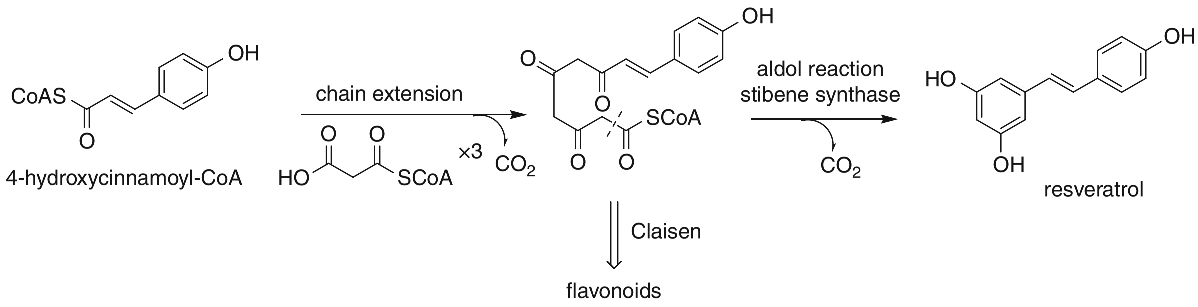
3 Biosynthesis

3.1 Biosynthesis of Stilbenes

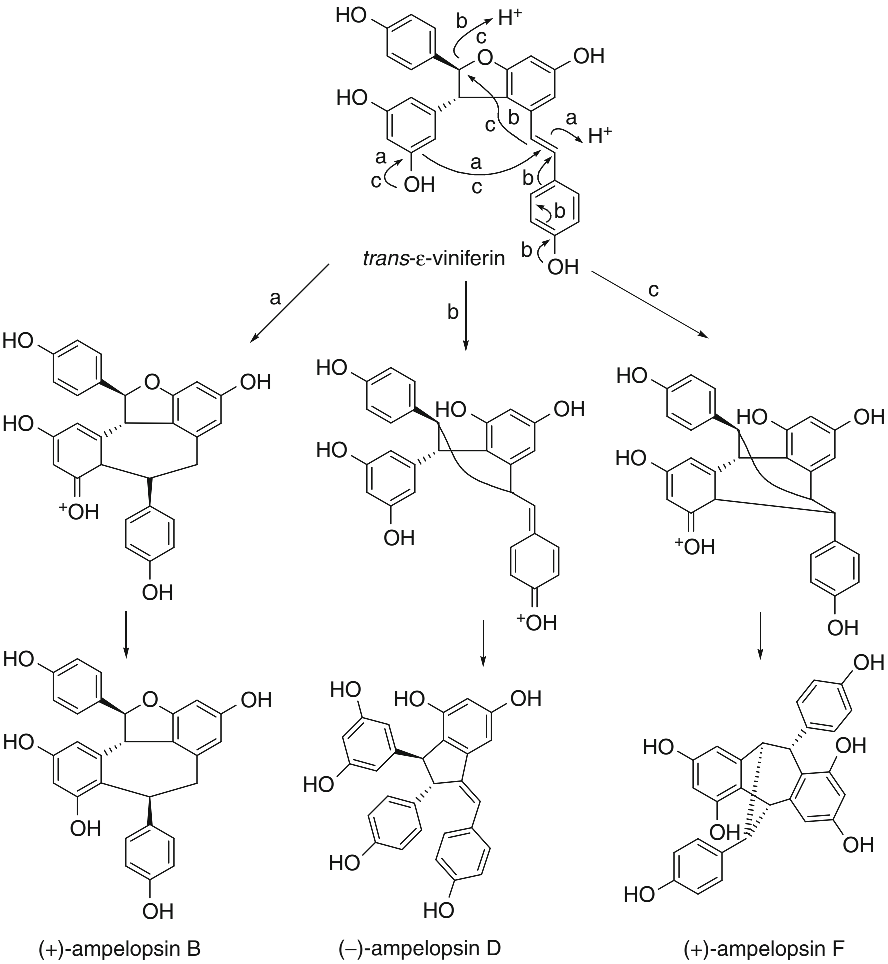
The biosynthesis of simple stilbenes has been found out, and it shared a similar biosynthetic pathway with the flavonoids. Taking resveratrol for example, it starts from a cinnamoyl-CoA unit and extended the chain with three malonyl-CoA molecules (Scheme 62.1) [105]. Then, the resveratrol structure is produced by aldol reaction with the presence of stilbene synthase. Nevertheless, the flavonoids are formed depending on chalcone synthase and Claisen reaction.

3.2 Biosynthesis of Oligostilbenes

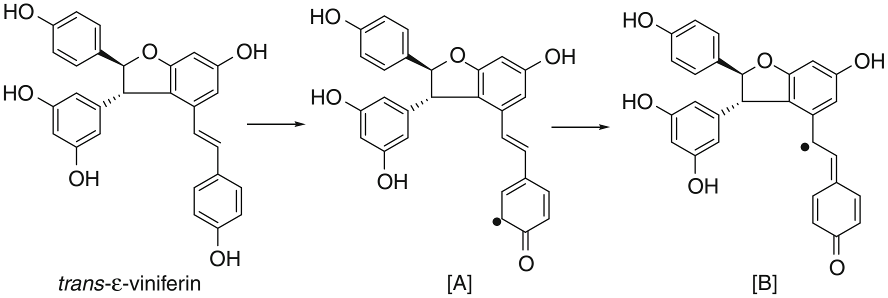
Sotheeswaran has mentioned that the oligostilbenes with dihydrobenzofuran moiety are biosynthesized through an important intermediate *trans*-ε-viniferin [106]. Combined with the reported work of oligostilbenes, the biosynthesis of oligostilbenes is summarized. For instance, the dimers named (+)-ampelopsin B, (−)-ampelopsin D, and (+)-ampelopsin F are produced by isomerization and/or rearrangement of *trans*-ε-viniferin (Scheme 62.2). The differences in their structures are caused by the different protonation position at the initial stage of reaction. Furthermore, *trans*-ε-viniferin is able to transform to the isomers (Scheme 62.3) and then forming the tetramers, (+)-vitisin A, (−)-vitisin B, (+)-hopeaphenol, and (+)-viniferol A, etc., by oxidative coupling (Scheme 62.4) [2, 107].


CoAS
O
OH
4-hydroxycinnamoyl-CoA
chain extension
HO
O
O
SCoA
×3
CO_2
O
O
O
O
SCoA
OH
Claisen
flavonoids
aldol reaction
stibene synthase
CO_2
HO
OH
OH
resveratrol

Scheme 62.1


HO
OH
HO
OH
OH
H+
a
b
c
trans-ε-viniferin
+OH
(+)-ampelopsin B
(–)-ampelopsin D
(+)-ampelopsin F

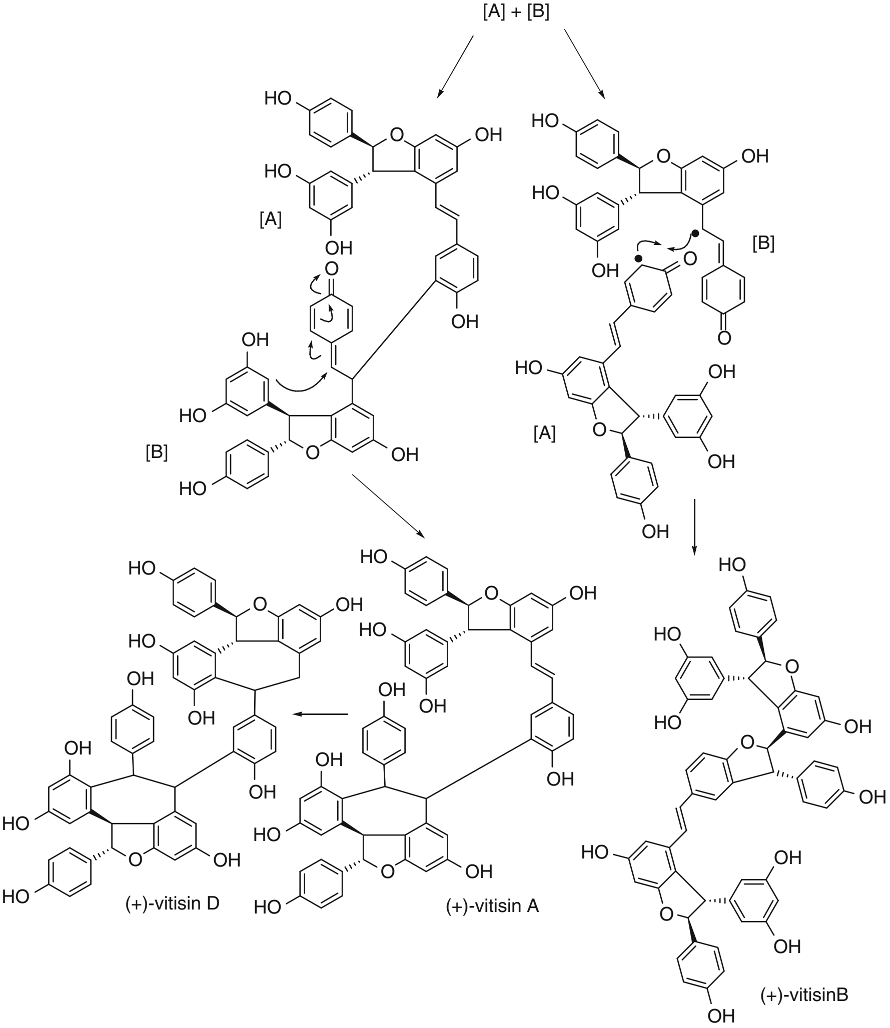
Scheme 62.2


HO
OH
O
trans-ε-viniferin
[A]
[B]

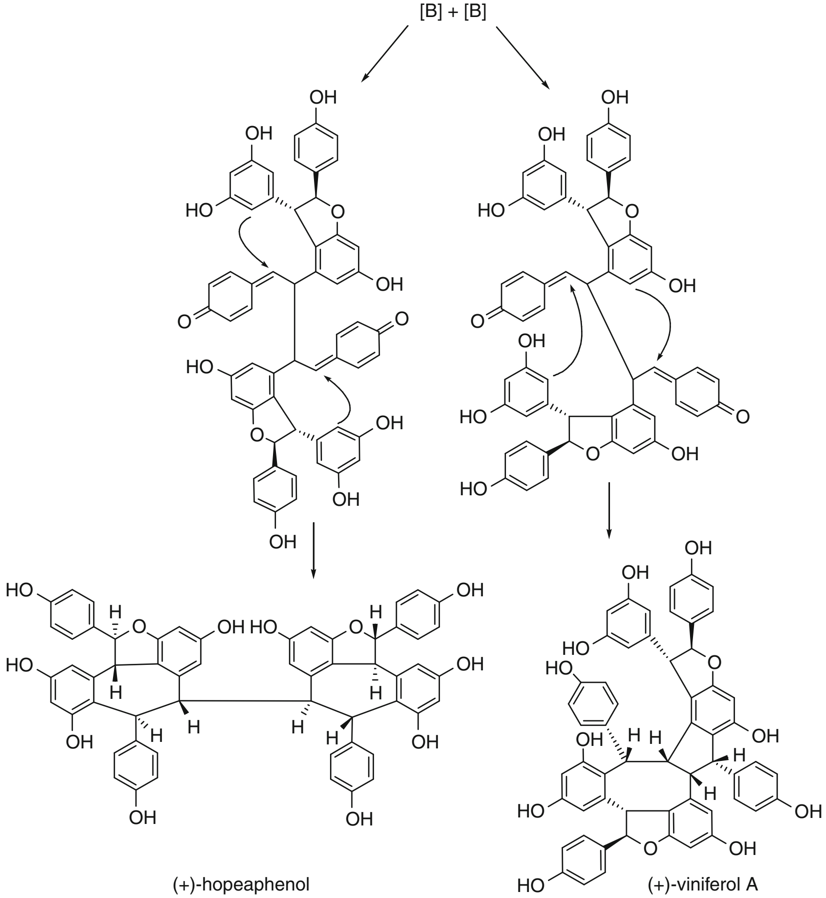
Scheme 62.3

3.3 Biosynthesis of Bibenzyls and Bisbibenzyls

The biosynthesis of marchantins A and C has been certified by a C-labeled precursor feeding experiment. It shows that rings A and C of the marchantin molecules are derived from the benzene ring of L-phenylalanine. The bibenzyl


[A] + [B]
[A]
[B]
[B]
[A]
(+)-vitisin D
(+)-vitisin A
(+)-vitisinB

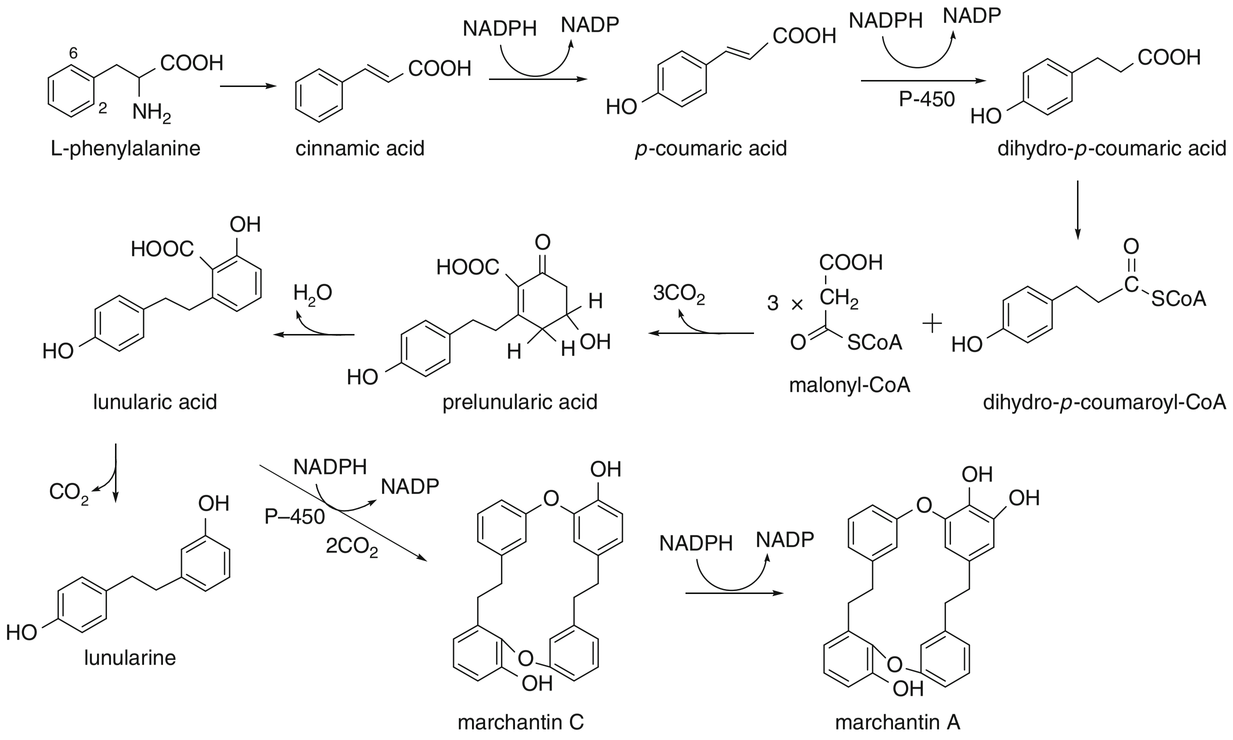
Scheme 62.4 (continued)



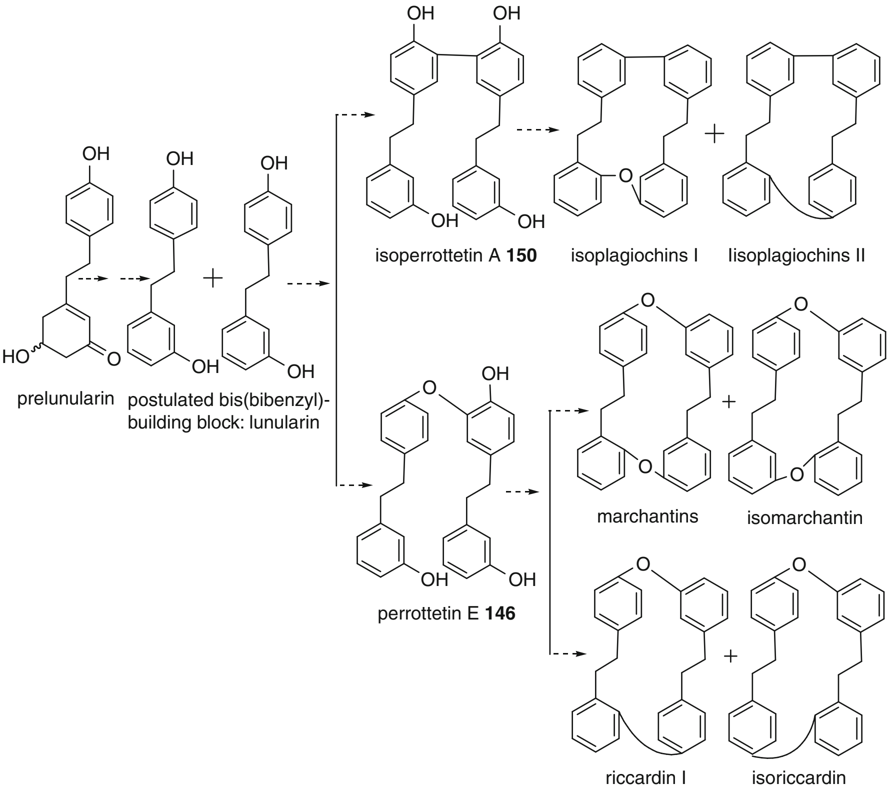
Scheme 62.4

lunularic acid is biosynthesized through dihydro-*p*-coumaric acid coupling with three malonyl-CoA units and then coupled in typical ways to form bisbibenzyls marchantins A and C (Scheme 62.5) [108].

Evidences have confirmed that bisbibenzyls can be produced by the coupling of two phenolic systems by means of free-radical reactions. These reactions can be mediated by oxidase enzymes. C–C bonds involving positions *ortho* or *para* to the original phenols, or ether linkages, may be formed in coupling of two of these bibenzyl structures [105]. A previous hypothesis for the biogenesis of


6
COOH
2
NH$_2$
L-phenylalanine
COOH
cinnamic acid
NADPH
NADP
COOH
HO
p-coumaric acid
NADPH
NADP
P-450
COOH
HO
dihydro-*p*-coumaric acid
O
C
SCoA
HO
dihydro-*p*-coumaroyl-CoA
COOH
3 ×
CH$_2$
O
SCoA
malonyl-CoA
3CO$_2$
O
HOOC
H
H
H
OH
HO
prelunularic acid
H$_2$O
OH
HOOC
HO
lunularic acid
CO$_2$
OH
HO
lunularine
NADPH
NADP
P–450
2CO$_2$
OH
O
O
OH
marchantin C
NADPH
NADP
OH
OH
O
O
OH
marchantin A

Scheme 62.5

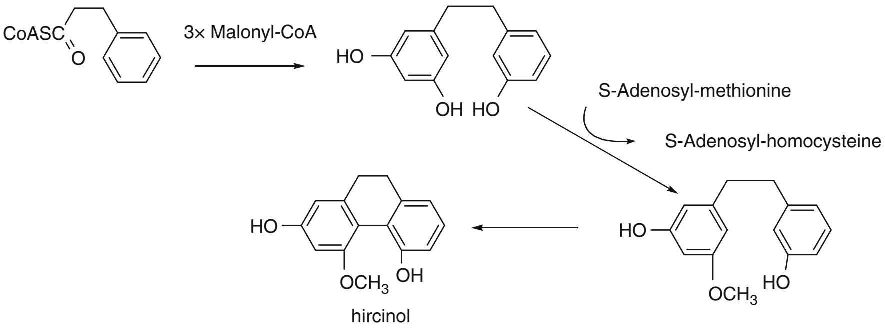

OH
OH
OH
HO
O
OH
OH
prelunularin
postulated bis(bibenzyl)-
building block: lunularin
OH
OH
OH
OH
isoperrottetin A **150**
O
isoplagiochins I
Iisoplagiochins II
O
OH
O
OH
OH
perrottetin E **146**
O
O
O
O
marchantins
isomarchantin
O
O
riccardin I
isoriccardin

Scheme 62.6

marchantins, riccardins, and plagiochins considers cyclization of open-ring precursors, such as perrottetin E **146**, through intramolecular phenol oxidation, accompanied by C–O or C–C linkage of the terminal *m*-hydroxyphenyl units [109]. Likewise, isoplagiochins A-D might be biosynthesized from isoperrottetin A **150** (Scheme 62.6) [110].

3.4 Biosynthesis of Phenanthrenes

Bibenzyls are regarded as the bicyclic intermediates of 9,10-dihydrophenanthrenes, and the biosynthetic pathway of 9,10-dihydrophenanthrenes was proposed as showed in Scheme 62.7. Oxidative coupling of the bibenzyl intermediate leads to the formation of phenanthrene [2].



Scheme 62.7

4 Biological Activities

4.1 Antitumor Activity

4.1.1 Stilbenes and Oligostilbenes

Resveratrol, as a representative compound of stilbene, possesses diverse pharmacological activities. The antitumor property has taken the spotlight for its cancer preventive effect on skin cancer in a mice model. Subsequently, plenty of in vivo experiments targeting different tumor model were carried out to evaluate its therapeutic effects on tumors [111]. The results definitely show that resveratrol is able to inhibit or possess chemopreventive functions on different tumors, including breast cancer, liver cancer, gastric cancer, colorectal cancer, prostate cancer, leukemia, lung cancer, neuroblastoma, etc. In these experiments, the incidences, tumor volume, and metastasis are improved. Clinical trials of investigating resveratrol's effects on colon cancer and melanoma (skin cancer) are intending to launch.

Combretastatins attract a lot of interests for their potent antitumor properties by inhibiting tubulin polymerization and disrupting the formation of tumoral vasculature. Combretastatin A-4 **5** was proved to be the most potent candidate of combretastatins with GI_{50} of 3.20 nM in an antitumor screening project against the NCI-60 human tumor cell lines, followed by combretastatins A-1 **2** and A-2 **3** [112]. Further studies focused on the antitumor mechanism of combretastatins, and the results suggested that compounds **2**, **3**, and **5** possessed potent antimitotic effect through binding to the tubulin at colchicine site [113]. Combretastatin A-4, its prodrug combretastatin A-4 phosphate (CA-4-P), and other analogues are currently being investigated in the clinical trials. CA-4-P, being developed as vascular targeting agents, in combination with carboplatin has entered into phase III clinical trial for the treatment of anaplastic thyroid cancer.

The seeds of *Iris halophila* (Iridaceae) produced halophilol A, which possesses moderate cytotoxicity against KB cells and human mammary epithelial cells

(HMECs) with IC_{50} values of 17.28 and 22.47 μM [114], respectively. The prenyl stilbenes **30–32** with dimethylchromene ring exhibit cytotoxic activity against Hepa-1clc7 cells with IC_{50} values of 8.5, 13.0, and 7.0 μM [18], respectively. Tested in the NCI-60 cell line human cancer screen, schweinfurthins A **33** and B **34** show significant cytotoxic activity with mean panel GI_{50} of 0.36 and 0.81 μM. Schweinfurthins E-H were isolated from *M. alnifolia* [115] and display potent antiproliferative effect against A2780 human ovarian cancer cell line with IC_{50} values ranging from 0.26 to 5.0 μM, respectively. Lakoochins A and B also possess cytotoxic activity against breast cancer cell line (6.1 and 3.1 μg/mL) and nasopharyngeal carcinoma cell line (20 and 6.1 μg/mL) [116].

4.1.2 Oligostilbenes

Three trimers nepalensinols A, C, and D and three tetramers nepalensinols B, G, and F were obtained from *Kobresia nepalensis* (Cyperaceae) [117, 118]. The inhibitory effect of the above six oligomers against the decatenation activity of topoisomerase II on kinetoplast DNA is evaluated with IC_{50} values ranging from 0.02 to 10.8 μg mL^{-1}. Among them, nepalensinol B exhibits the strongest activity with an IC_{50} of 0.02 μg mL^{-1}, much better than the clinical antitumor drugs daunorubicin (IC_{50} 4.8 μg mL^{-1}) and etoposide (IC_{50} 70.0 μg mL^{-1}). Vaticanol C is a resveratrol tetramer with dibenzobicyclo[3.2.1]octadiene moiety, is widely distributed in Dipterocarpaceae species, and shows potent growth suppressive activity with IC_{50} values of 5.9 μM against HL60 cells. Upunaphenol A is a hexamer obtained from *Upuna borneensis* and was found to suppress cell growth in HL60 cells through induction of apoptosis with IC_{50} at 9.2 μM [119].

4.1.3 Bibenzyls and Bisbibenzyls

Erianin (also named dihydrocombretastatin A-4), a dihydro derivative of combretastatin A-4 which has been initiated phase II clinical trial as antitumor agent, possessed potent cytotoxicity toward diverse cancer cell lines. It was evaluated against A-549 lung carcinoma, MCF-7 breast carcinoma, HT-29 colon adenocarcinoma, SKMEL-5 melanoma, and MLM melanoma cell lines with ED_{50} ranging from 0.002 to 0.33 μM, respectively [120]. It also showed potent inhibitory activity on the proliferation of HL-60 cells (IC_{50} 38 nM) and was able to alter expression of *bcl*-2 and *bax* genes in HL-60 cells [121].

Bauhinols A **110** and B **111** exhibit significant cytotoxicity against NCI-H187 (small-cell lung cancer) and BC (breast cancer) cell lines with IC_{50} values ranging from 1.1 to 9.7 μg/mL. In addition, bauhinol A **110** is active toward KB cells (IC_{50} 4.5 μg/mL) [64]. 3,5-Dihydroxy-4-methylbibenzyl shows cytotoxic activity, which is able to inhibit the growth rate of P-388 leukemia and hepatoma cell lines by 99.7% and 83.6% at 10 μg/mL, respectively [122].

The bisbibenzyls neomarchantins A **133** and B **134**, marchantin C, and Glaucescens Bis Bibenzyl A and B possess moderate cytotoxicity against P-388 leukemia cells with IC_{50} ranging from 8 to 18 μg/mL [123]. Riccardin C and pusilatins B-C display moderate cytotoxicity against KB cells with ED_{50} of 7.1 to 16.4 μg/mL [79].

Table 62.2 In vitro cytotoxic activity of **181** and **182** with human cancer cell lines (ED_{50} μg/mL)

	Human cancer cell lines											
	A-431	BC1	Col2	HT	KB	KB-V (+VLB)	KB-V (−VLB)	Mel2	LNCaP	Lu1	U373	ZR-75-1
181	11.3	6.9	13.1	5.5	15.0	0.8	3.0	10.0	19.0	6.1	>20	11.2
182	9.4	13.4	>20	3.1	6.4	3.6	5.9	9.2	10.3	19.9	3.2	10.5

A-431, human epidermoid carcinoma; BC1, human breast cancer; Co12, human colon cancer; HT, human fibrosarcoma; KB, human oral epidermoid; KB-V(+VLB), drug-resistant KB + vinblastine (1 lag/mL); KB-V(−VLB), drug-resistant KB (no vinblastine); LNCaP, human prostate cancer; Lul, human lung cancer; Mel2, human melanoma; U373, human glioma; ZR-75-1, hormone-dependent human breast cancer

4.1.4 Phenanthrenes

The cytotoxic activities both in vitro and in vivo of lusianthridin and denbinobin isolated from *Dendrobium nobile* are evaluated. Both of them exhibit potent antitumor effects against A549 human lung carcinoma, SK-OV-3 human ovary adenocarcinoma, and HL-60 human promyelocytic leukemia with EC_{50} values ranging from 0.11 to 9.8 μg/mL [124].

Dimeric phenanthrenes denthyrsinol, denthyrsinone, and monomer denthirsinin demonstrate potent cytotoxicity against cervix adenocarcinoma HeLa, K-562, and MCF-7 cells with IC_{50} values from 1.6 to 9.9 μM [125]. A series of phenanthrenes, including 7-hydroxy-2,3,4,8-tetramethoxyphenanthrene, 3-hydroxy-2,4,-dimethoxy-7,8-methylenedioxyphenanthrene, 2-hydroxy-3,5,7-trimethoxyphenanthrene, 2-hydroxy-3,5,7-trimethoxy-9,10-dihydrophenanthrene, and confusarin are evaluated on their antitumor properties against HeLa cell line with IC_{50} values of 0.97–14.21 μM. [126, 127].

3,6-Dihydroxy-l,7-dimethyl-9-methoxyphenanthrene **181** and 3,6-dihydroxy-L-hydroxymethyl-9-methoxy-7-methylphenanthrene **182** are found to demonstrate significant cytotoxic responses against several tumor cell lines (Table 62.2). Compound **181** is more active against drug-resistant KB cells, while **182** is active against HT (fibrosarcoma) and U373 (glioma) cell lines [128].

4.2 Antioxidant Activity

4.2.1 Stilbenes and Oligostilbenes

Lespedezavirgatol was obtained from *Lespedeza virgata* and shows potent antioxidant property. Its oxygen radical absorbance capacity (ORAC) value for Trolox equivalents is 762.96 at 1.5 μM, much better than 164.56 of vitamin C. Inhibitory effects of lespedezavirgatol against lipid peroxidation toward malondialdehyde levels in rat kidney homogenate and plasma are also evaluated with IC_{50} values of 0.16 and 0.18 mM, better than the control vitamin C, with IC_{50} values of 5.54 and 3.05 mM. The above results suggest that lespedezavirgatol is a potent candidate for antioxidants [129]. Tibeticanol **76** was obtained

Table 62.3 Effect of compounds from *V. thunbergii* on ABTS$^+$ scavenging

Compounds	Free-radical scavenging activity (EC_{50} μM)
Vitisinol B	3.6 ± 0.1
Vitisinol C	4.5 ± 0.1
Vitisinol D	4.1 ± 0.1
(+)-ε-Viniferin	2.8 ± 0.1
(−)-Viniferal	4.4 ± 0.1
Ampelopsin C	5.4 ± 1.2
Miyabenol A	6.6 ± 1.2
(+)-Vitisin A	13.8 ± 2.7
(+)-Vitisin C	4.8 ± 0.1
Trolox (positive control)	28.4 ± 5.2

from *Caragana tibetica* and exhibits strong superoxide anion scavenging activity with an IC_{50} of 1.33 μM [46].

A series of oligostilbenes isolated from *Vitis thunbergii* are evaluated on their antioxidant properties based on the radical scavenging effect of the stable ABTS$^{\bullet+}$ free radical. The results are shown in Table 62.3. All of the tested compounds are more active than the positive control Trolox and display free-radical scavenging activity with EC_{50} values from 2.8 to 13.8 μM. Among them, (+)-ε-viniferin shows the most potent radical scavenging potency with EC_{50} of 2.8 μM [23].

4.2.2 Bibenzyls and Bisbibenzyls

Marchantin H is able to inhibit nonenzymatic iron-induced lipid peroxidation in rat brain homogenates and NADPH-dependent microsomal lipid peroxidation with an IC_{50} of 0.51 and 0.32 μM [130]. It also possesses inhibitory effects of copper-catalyzed oxidation of human low-density lipoprotein. Marchantinquinone exhibits inhibitory effects of Fe^{2+}-induced lipid peroxidation in rat brain homogenates (IC_{50} 15.3 μM) and displays radical scavenging activity [131]. Schwartner et al. reported the antioxidative potential of three macrocyclic bisbibenzyls [marchantins A, B, and D], one acyclic bisbibenzyl (paleatin B) and a prenylated bibenzyl (perrottetin D) by pulse-radiolytic and EPR-spectroscopic techniques. The results confirm that these compounds are effective antioxidants [132]. Isoamoenylin and 5,4′-dihydroxy-3-α-L-rhamnopyranosyl-(1→3)-β-D- xylopyranosyloxybibenzyl showed radical scavenging activity, comparable with the control vitamin C and ascorbic acid [63, 133]. Five prenylated dihydrostilbenes, α,α-dihydro-3,5,3,4-tetrahydroxy-4,5-diisopentenylstilbene, α,α-dihydro-3,5,3,4-tetrahydroxy-5-isopentenylstilbene **183**, α,α-dihydro-3,5,4-trihydroxy-4,5-diisopentenylstilbene, α,α-dihydro-3,5,4-trihydroxy-5-isopentenyl stilbene, and α,α-dihydro-3,5,3-trihydroxy-4-methoxy-5-isopentenylstilbene **184**, from *Glycyrrhiza glabra* were tested for antioxidant effects by measuring the absolute inhibition rate constant (k_{inh}) of the oxidation process. Compounds **183** and **184** display potent antioxidant properties with k_{inh} values of 1.1×10^4 and $0.9 \times 10^4\ M^{-1}s^{-1}$ [134].

Table 62.4 Effect of compounds isolated from *V. thunbergii* on the platelet aggregation induced by AA and U46619

	IC_{50} (μM)	
Compounds	AA	U46619
Vitisinol B	>100	7.8 ± 2.2
Vitisinol C	13.4 ± 2.2	10.5 ± 3.4
Vitisinol D	15.0 ± 4.8	5.7 ± 1.4
(−)-Viniferal	7.0 ± 2.9	3.1 ± 2.5
Ampelopsin C	8.1 ± 1.1	5.9 ± 0.9
Miyabenol A	9.0 ± 1.6	7.5 ± 2.0
(+)-Vitisin A	10.3 ± 1.2	13.3 ± 2.1
(+)-Vitisin C	5.7 ± 1.3	3.9 ± 0.7
Aspirin (positive control)	32.7 ± 6.4	Not detected

4.3 Antiplatelet Activity

Resveratrol prevents platelet aggregation and thrombus formation in vitro. In a hypercholesterolaemic diet-induced rabbit model, administration of resveratrol inhibited the platelet aggregation. This effect was also verified by reducing the atherosclerotic area and the size of the thrombus generated by laser-induced damage to the endothelium in mice.

Vitis thunbergii (Vitaceae), a folk medicine in Taiwan, produced several resveratrol oligomers, including vitisinol C with a tropilene structure in molecule, vitisinols B and D, and some known compounds (−)-viniferal, (+)-ε-viniferin, and (+)-vitisin C, etc. The isolated compounds were evaluated for antiplatelet aggregation activities induced by arachidonic acid (AA) and 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin F2α (U46619). Most of the tested oligomers demonstrate potent antiplatelet aggregation property with $IC_{50} < 10$ μM, more positive than aspirin (EC_{50} 32.7 μM). In the above bioassay, (−)-viniferal and (+)-vitisin C are most effective against aggregation induced by AA and U46619, with IC_{50} values of 5.7 and 3.1 μM, respectively [23]. The results are summarized in Table 62.4.

Marchantinquinone displays potent inhibitory activity on the aggregation of washed rabbit platelets induced by thrombin, arachidonic acid (AA), collagen, U46619, and platelet-activating factor (PAF) [135, 136]. It inhibits thromboxane B_2 (TxB_2) formation induced by thrombin, PAF, and collagen. In addition, marchantinquinone is able to inhibit the rising intracellular Ca^{2+} concentration stimulated by five inducers mentioned above [136]. Gigantol exhibits antiplatelet aggregation activity on SD-rat platelet aggregation [137]. 3-Methylgigantol possesses significant inhibitory effects against aggregation induced by AA, collagen, and PAF [138]. Among them, 3-methylgigantol is most potent effective against AA-induced aggregation (IC_{50} 30 μM). Moscatilin is able to inhibit AA- and collagen-induced platelet aggregations [139]. Perrottetin E **146** shows inhibitory activity of thrombin (IC_{50} 18 μM) [140].

The phenanthrenes, erianthridin, and denbinobin display potent antiplatelet activity on washed rabbit platelets against aggregation induced by either thrombin, arachidonic acid (AA), collagen, or PAF at a dose of 100 μg/mL. Erianthridin is proved to be the most potent compound with an IC_{50} of 9 μM against AA-induced aggregation [138].

4.4 Antidiabetic Activity

5,4′-Dihydroxystilbene-3-*O*-α-arabinopyranoside, named rumexoid, was found in *Rumex bucephalophorus* (Polygonaceae). This compound and resveratrol display potent α-glucosidase inhibitory activity even better than the commercial antidiabetic agent acarbose [141]. 2′-*O*-Demethylbidwillol B and addisofurans A-B were prenyl-substituted arylbenzofurans isolated from *Erythrina addisoniae*. Those compounds are inhibitors of type II diabetes target protein tyrosine phosphatase 1B with IC_{50} values of 13.6–15.7 μM. The linear prenyl chain was responsible for its inhibitory activity, and the cyclization of prenyl group decreased this effect [142]. In a bioassay-guided fractionation against α-glucosidase, 13-hydroxykompasinol A and scirpusin C were obtained from the seeds of *Syagrus romanzoffiana* and possess potent inhibitory activity against α-glucosidase type IV from *Bacillus stearothermophilus* with the IC_{50} value of 6.5 and 4.9 μM [143].

4.5 Antimicrobial Activity

4.5.1 Stilbenes and Oligostilbenes

Machaeriol B, a compound with hexahydro-6*H*-benzo[c]chromene system, was obtained from *Machaerium multiflorum* (Leguminosae), and it demonstrates potent antimalarial activity (IC_{50} 0.12 μg/mL) against *Plasmodium falciparum* [144]. Preracemosols A and B exhibit moderate antimalarial activity with EC_{50} of 18.0 and 3.0 mg/mL, respectively [145]. *Trans*-4-isopentenyl-3,5,2′,4′-tetrahydroxystilbene was discovered in *Artocarpus integer* (Moraceae) and possesses antimalarial activity against *Plasmodium falciparum* ($EC_{50}=1.7$ μg mL^{-1}) [146]. This is the first report of antimalarial activity of stilbenes.

A series of arylbenzofuran-type stilbenes, guided by bioautographic assay for antifungal activity against *Cladosporium herbarum*, have been isolated from the root of *Stemona collinsae* (Stemonaceae) and tested in microwells against another four microfungi *Alternaria citri*, *Fusarium avenaceum*, *Pyricularia grisea*, and *Botrytis cinerea* [22]. Among them, stemofuran B shows the highest antifungal potency against above four parasitic fungi with EC_{50} values of 1.4 μg/mL. Stemofuran E exhibited antifungal property against *C. herbarum* with EC_{50} of 0.09 μg/mL.

Stilbene derivatives were obtained from *Calligonum leucocladum*, and the structures were determined as (*E*)-resveratrol 3-(6-galloyl)-*O*-β-d-glucopyranoside.

Although ineffective when tested alone, it is able to restore oxacillin's effectiveness against oxacillin/methicillin-resistant *Staphylococcus aureus* when used in combination. The galloyl group may play a role in this synergistic activity [147].

Hopeanolin was obtained from the stem bark of *Hopea exalata* and exhibits potent antifungal properties against six types of pathogenic fungi *Alternaria attenata*, *Alternaria solani*, *Colletotrichum lagenarium*, *Fusarium oxysporum f.* sp. *vasinfectum*, *Pyricularia oryzae*, and *Valsa mali* with MIC values ranging from 0.10 to 22.5 μg mL^{-1} [148].

4.5.2 Bibenzyls and Bisbibenzyls

Bryophytes normally grow in humid habitats; however, they are seldom damaged by fungi. It indicates that bryophytes are able to elaborate constitutive and inducible antifungal natural products against adverse effects. A large amount of bibenzyls and bisbibenzyls with antifungal activity have been found in plants, primarily in bryophytes (Table 62.5), which provided important sources for research and development of antifungal agents. The antifungal effects of bibenzyls and bisbibenzyls were summarized in Table 62.5.

The bibenzyls 4-hydroxy-3′-methoxybibenzyl, 2,4,6-trichloro-3-hydroxybibenzyl, 2, 4-dichloro-3-hydroxylbibenzyl, 2-chloro-3-hydroxybibenzyl, together with bisbibenzyls neomarchantins A **133** and B **134**, and marchantin C show antimicrobial activity against the gram-positive bacterium *Bacillus subtilis* [60, 123, 150]. In addition, 4-hydroxy-3′-methoxybibenzyl is active toward *Escherichia coli* [150].

4.6 Anti-inflammatory Activity

Anti-inflammatory properties of resveratrol have been confirmed, which is an effective inhibitor of cyclooxygenase (COX) in vitro. It is also found that resveratrol significantly reduces acute and chronic chemically induced edema, lipopolysaccharide-induced airway inflammation, and osteoarthritis. Resveratrol could also present as an alternative, instead of aspirin, for treatments of chronic inflammation because of the latter's side effect on the stomach. Resveratrol derivatives displayed similar anti-inflammatory effects. For instance, resveratrol (*E*)-dehydrodimer 11-*O*-β-D-glucopyranoside and resveratrol (*E*)-dehydrodimer from *Vitis vinifera* show significant inhibitory activity against cyclooxygenase-1 (COX-1) with IC_{50} of 5.2 and 4.3 μM and against cyclooxygenase-2 (COX-2) with IC_{50} of 7.5 and 3.7 μM. From the above results, these two compounds seem to be the worthy candidates for further research to find application in anti-inflammatory treatment [151].

(+)-Vitisifuran A and heyneanol A were found in *Vitis* genus and display potent inhibition on biosynthesis of LTB_4 with inhibitory rate of 72% and 76% at a concentration of 10 μM [152]. A tetramer named gnetuhainin R **73** was obtained from the same species (*G. hainanense*) and shows potent histamine receptor antagonism (IC_{50} 0.1 μM) [44]. Aiphanol exhibited significant inhibitory activities against COX-1 (IC_{50} 1.9 μM) and COX-2 (9.9 μM) [153].

Table 62.5 Antifungal bibenzyls and bisbibenzyls

Compound	Fungi	Dose	References	Compound	Fungi	Dose	References
Bauhinol B	*Candida albicans*	28.9^{a}	[64]	2,4-Dichloro-3-hydroxy bibenzyl	*Candida albicans*	2^{f}	[60]
Bazzanin B	*Botrytis cinerea*	18.9^{a}	[149]		*Cladosporium resinae*	2^{f}	
	Cladosporium cucumerinum	17.5^{a}			*Trichophyton mentagrophytes*	12^{f}	
	Pyricularia oryzae	3.9^{a}		Dihydropinosylvin	*Botrytis cinerea*	69^{b}	[91]
	Septoria tritici	23.5^{a}			*Cladosporium herbarum*	32^{b}	
Bazzanin S	*Cladosporium cucumerinum*	30.8^{a}	[149]		*Fusarium avenaceum*	56^{b}	
	Phythophthora infestans	29.2^{a}			*Pyricularia grisea*	44^{b}	
	Pyricularia oryzae	2.6^{a}		Isoplagiochin D	*Botrytis cinerea*	7.6^{a}	[149]
	Septoria tritici	4.5^{a}			*Cladosporium cucumerinum*	13.0^{a}	
2-Chloro-3-hydroxybibenzyl	*Candida albicans*	2^{f}	[60]		*Pyricularia oryzae*	4.0^{a}	
	Trichophyton mentagrophytes	3^{f}			*Septoria tritici*	15.9^{a}	
Riccardin H **136**	*Candida albicans*	0.4^{c}	[80]				

[a] IC_{50} (μg/mL)
[b] EC_{50} (μg/mL)
[c] MID (μg)
[d] LC_{90} (ppm)
[e] MIC (μg/mL)
[f] Width of the zone of inhibition (mm) at 30 μg/disk

Stemofurans B, D, G, and J and stilbostemin G were reported possessing anti-inflammatory effects with IC_{50} values ranging from 3.7 to 26.3 μM by inhibiting leukotriene formation [154]. The inhibition of lipopolysaccharide-induced nitric oxide synthase (NOS) by 19 bisbibenzyls in RAW 264.7 macrophages has been reported, and marchantin A is most effective with IC_{50} values of 1.44 μM. The structure-activity relationship (SAR) is discussed, and the phenolic hydroxyl groups and diarylether bonds play important roles in its inhibitory effect [155]. Pusilatins B-C exhibit selective DNA polymerase-β inhibitory activity with IC_{50} of 13.0 and 5.16 μM [79]. Bauhinol B **111** and 3,5-dihydroxy-2-(3-methyl-2-butenyl)bibenzyl are potent inhibitors of COX-1 and COX-2 with IC_{50} ranging from 1.3 to 9.0 μg/mL [134].

Phenanthrenes obtained from the *Stemona* species were evaluated on their leukotriene biosynthesis inhibition property using human neutrophile granulocytes in vivo. Stemanthrenes A **162** and D **165** display inhibitory activity in a dose-dependent manner, with IC_{50} values of 8.5 and 4.8 μM. Stemanthrenes B **163** and C **164** possess 100% inhibition against leukotriene biosynthesis at 25 μM. The phenanthrenes might be responsible for the anti-inflammatory and antiasthmatic principles of the *Stemona* species [154]. Denbinobin, a phenanthraquinone from *Dendrobium moniliforme*, shows in vitro anti-inflammatory activity. It inhibits the formation of tumor necrosis factor and prostaglandin E2 induced by lipopolysaccharide in RAW 264.7 and N9 cells at a dose of 1 μM [100].

4.7 Neuroprotective Activity

In vivo pharmacological studies have indicated that resveratrol has a neuroprotective effect, including reduced lipid, peroxidation and neurological cell destruction, attenuation of induced lesion areas, induced tolerance to brain injury, reduced frequency of seizures, impairment of motor coordination, and enhancement of learning [111].

Stilbostemin B 3′-β-D-glucopyranoside, stilbostemin H 3′-β-D-glucopyranoside, and stilbostemin I 2″-β-D-glucopyranoside possess significant neuroprotective activity against 6-hydroxydopamine-induced neurotoxicity in human neuroblastoma SH-SY5Y cells [156]. Hopeahainol A from *Hopea hainanensis* contains an unprecedented carbon skeleton and shows potent acetylcholinesterase inhibitory effect with an IC_{50} value of 4.33 μM, comparable even to that of huperzine A (IC_{50} 1.6 μM) [157]. The tetramer neohopeaphenol A from the same species also displays significant inhibitory action against AChE with an IC_{50} value of 7.66 μM [158].

4.8 Hepatoprotective Activity

Acer mono (Aceraceae), a Korean folk medicine for hemostasis, produces two potent hepatoprotective stilbene glycosides, 5-*O*-methyl-(*E*)-resveratrol 3-*O*-β-D-glucopyranoside and 5-*O*-methyl-(*E*)-resveratrol 3-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside [16]. Those two compounds significantly prevent the depletion of glutathione (GSH) in H_2O_2-injured primary cultured rat hepatocytes and potently restore the level of GSH depleted by buthionine sulfoximine or diethylmaleate in the presence or absence of H_2O_2. In addition, they preserve the effects of antioxidant enzymes such as superoxide dismutase, glutathione reductase, and glutathione peroxidase reduced by H_2O_2 insults. Therefore, it is concluded that both compounds exerted significant hepatoprotective effects against H_2O_2-induced hepatotoxicity by maintaining the antioxidant defense system [159].

4.9 Cardioprotective Activity

It is well known that drinking wine and grape juices will reduce cardiovascular, cerebrovascular, and peripheral vascular risks due to the presence of resveratrol. As a natural antioxidant, resveratrol is able to prevent LDL oxidation, scavenge intracellular reactive oxygen species, lower the oxidative stress, and induce NO synthesis. Resveratrol modulates various aspects of cardiovascular diseases and is effective against atherosclerosis, hypertension, ischemia reperfusion injury and heart failure, and many other cardiac dysfunctions [111].

4.10 Phytotoxicity

The phenanthrenes, ephemeranthol-A and fimbriol A from *Epidendrum rigidum*, together with erianthridin from *M. densa* demonstrated phytotoxicity against *Amaranthus hypochondriacus* with IC_{50} values of 0.12, 5.9, and 58.2 μM [160]. The phenanthrenes from the *Juncus* genus show growth inhibitory effects against the green alga *Selenastrum capricornutum* with IC_{50} values ranging from 11.1 to 19.9 μM [161].

Bibenzyls are elaborated to confer the producing plants' selective advantage against the competition from the other plants and microbial attack. Gigantol, batatasin III, 2,3-dimethoxy-9,10-dihydrophenathrene-4,7-diol, and 3,4,9-trimethoxyphenanthrene-2,5-diol from the orchid *Epidendrum rigidum* inhibit radicle growth of *Amaranthus hypochondriacus* with IC_{50} of 0.65, 0.1, 0.12, and 5.9 μM [160].

5 Conclusion

Stilbenoids represent a group of important natural products in the plant kingdom. The developments of modern analytical methods accelerate the discovery of these compounds, and to this day more than 1,000 stilbenoids have been isolated. For the stilbenes, bibenzyls, and phenanthrenes, these three groups shared the feature of their nucleus with hydroxyls, methyl, methoxy, prenyl, geranyl, glycosyl, etc., substituents. The oligostilbenes and bisbibenzyls are formed by polymerization of stilbene and bibenzyl units, and the diverse polymerized patterns produced their diverse structures. They display diverse biological activities and have the potential to be developed as new drugs, especially in the field of antitumor, anti-inflammation, and cardioprotective drug research. The representative compounds resveratrol and combretastatin A-4 phosphate are currently being evaluated as drugs for the treatment of Alzheimer's disease and tumors in clinical trials and have shown satisfactory therapeutic effects. Furthermore, resveratrol displays developing prospects as a cardioprotective drug.

There are problems that need to be noted. Concerning the oligostilbenes, they commonly possess large molecules, intricate structures, and complex stereochemistry, and these characteristics cause troubles in structure identification and chemical total synthesis, therefore, limiting the probability to be developed to new drug. The bisbibenzyls are mainly distributed in the bryophytes, which are very small terrestrial spore-forming green plants, and different for collections. Therefore, getting enough plant materials for phytochemical investigation and new drug research is really a Gordian knot and resorts to the chemical synthesis for resolving the resource problems.

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Phenolic Acids

63

Marta Goleniowski, Mercedes Bonfill, Rosa Cusido, and
Javier Palazón

Contents

1 Introduction ... 1952
1.1 Hydroxybenzoic Acids ... 1953
1.2 Hydroxycinnamic Acids ... 1954
1.3 Biosynthesis of Hydroxycinnamic Acids ... 1955
1.4 Biosynthesis of Hydroxybenzoic Acids ... 1956
2 Functions in Plants ... 1958
2.1 Biotechnological Production of Phenolic Acids ... 1959
2.2 Phenolic Acids in Foods ... 1959
2.3 Phenolic Acids in Human Health ... 1960
2.4 Presence and Therapeutic Properties of the Main Phenolic Acids ... 1961
2.5 Extraction and Determination of Phenolic Acids ... 1966
3 Conclusion ... 1967
References ... 1968

Abstract

Phenolic acids are plant metabolites widely spread throughout the plant kingdom. Recent interest in phenolic acids stems from the potential protection they offer against oxidative damage diseases (e.g., coronary heart disease, stroke, and cancers) when consumed in fruits and vegetables. This chapter discusses the function of plant phenolic acids associated with diverse roles, including nutrient uptake, protein synthesis, photosynthesis, and allelopathy. It also provides an update of the

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health-promoting benefits of their important biological and pharmacological properties, especially anti-inflammatory, antioxidant, and antimutagenic and anticarcinogenic activities. The main methodologies currently being used for the extraction and quantification of these important phenolic compounds are also discussed.

Keywords
Biosynthesis • food • health • phenolic acids • plant cell culture

Abbreviations

4CL4	Coumarate:coenzyme A ligase
CA	Caffeic acid
CA4H	Cinnamic acid 4-hydroxylase
CO	p-coumaric acid BA-Benzoic acid
DAD	Diode array detection
E	Ellagic
FA	Ferulic
G	Gentisic
GA	Gallic acid
GC-MS	Gas chromatography–mass spectrometry
HCA	Hydrocinnamic acid
HPLC	High-performance liquid chromatography
JA	Jasmonic acid
LC-MS	Liquid chromatography–mass spectrometry
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PAL	Phenylalanine ammonia lyase
PDAD	Photodiode array detection
RA	Rosmarinic acid
RP	Reversed phase
SA	Salicylic acid
SIA	Sinapic acid
TLC	Thin-layer chromatography
UV	Ultraviolet

1 Introduction

Secondary metabolites are products of plant interaction with the environment and have a restricted occurrence. Many have economical importance, for example, as drugs, antioxidants, flavors, fragrances, dyes, insecticides, and pheromones [1]. Secondary metabolites can be classified according to their biosynthetic building blocks or their carbon skeleton. Approximately 8,000 naturally occurring compounds belong to the category of “phenolics,” all of which share a common structural feature: an aromatic ring bearing at least one hydroxyl substituent, that is, a phenol [2]. The hydroxyl group(s) can be free or engaged in another function as ethers, esters, or glycosides [3].

A straightforward classification attempts to divide the broad category of phenolics into simple phenols and polyphenols, based exclusively on the number of phenol subunits present, but many plant phenolic compounds are polymerized into larger molecules. Thus, the term “plant phenolics” encompasses simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins [4].

Widely distributed in plants, phenolic compounds are synthesized either as soluble or cell wall–bound compounds during plant growth and especially in response to conditions such as infection, wounding, UV radiation, etc. [5].

Phenolics have generated considerable interest due to their important biological and pharmacological properties, especially their anti-inflammatory, antioxidant, and antimutagenic and anticarcinogenic activities [6–8].

Phenolic acids are a subclass of the larger phenolics category (Fig. 63.1), occurring in food plants as esters or glycosides conjugated with other natural compounds such as flavonoids, alcohols, hydroxyfatty acids, sterols, and glucosides. Although much knowledge is still needed on the role of phenolic acids in plants, they have been associated with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy [9].

Phenolic acids (phenolcarboxylic acids) are phenols that include substances containing a phenolic ring and at least one organic carboxylic acid function. Depending on the carbon units of the lateral chain attached to the phenolic ring, the phenolic acids can be divided into C6-C3, C6-C2, and C6-C1 compounds, the most important being C6-C3 (derived from the hydroxycinnamic acid) and C6-C1 (compounds with a hydroxybenzoic structure) (Fig. 63.1). Although the basic skeleton remains the same, phenolic acids differ in the number and position of the hydroxyl groups on the aromatic ring.

The majority of phenolic acids are linked through ester, ether, or acetal bonds either to structural components of the plant (cellulose, proteins, lignin) or to larger polyphenols (flavonoids), or smaller organic molecules (e.g., glucose, quinic, maleic, or tartaric acids) or other natural products (e.g., terpenes) [10–14]. Many phenolic acids like cinnamic and benzoic acid derivatives exist in all plant and plant-derived foods (e.g., fruits, vegetables, and grains) [15]. However, only a minor fraction exists in the free acid form. Due to their ubiquitous presence in plant-based foods, humans consume phenolic acids on a daily basis. The estimated range of consumption is 25 mg to 1 g a day depending on diet (fruit, vegetables, grains, teas, coffees, spices, etc.).

Phenolic acids have been the subject of a great number of chemical, biological, agricultural, and medical studies [1].

1.1 Hydroxybenzoic Acids

Hydroxybenzoic acids have a general structure of C6-C1 derived directly from (BA), for example, p-hydroxybenzoic acid (p-HBA); salicylic acid (SA);

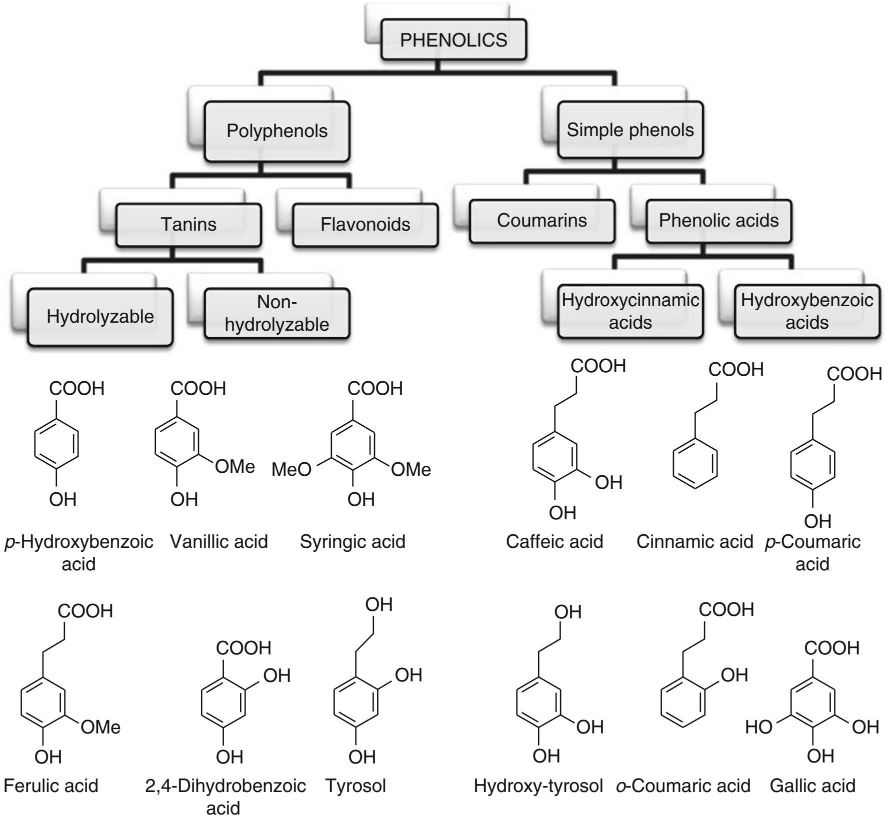

PHENOLICS
Polyphenols
Simple phenols
Tanins
Flavonoids
Coumarins
Phenolic acids
Hydrolyzable
Non-hydrolyzable
Hydroxycinnamic acids
Hydroxybenzoic acids
p-Hydroxybenzoic acid
Vanillic acid
Syringic acid
Caffeic acid
Cinnamic acid
p-Coumaric acid
Ferulic acid
2,4-Dihydrobenzoic acid
Tyrosol
Hydroxy-tyrosol
o-Coumaric acid
Gallic acid

Fig. 63.1 Schematic representation of the different groups of the phenolic compounds in plants

2,3-dihydroxybenzoic acid (2,3-DHBA); 2,5-dihydroxybenzoic acid (2,5-DHBA); 3,4-dihydroxybenzoic acid (3,4-DHBA); 3,5-dihydroxybenzoic acid (3,5-DHBA); gallic acid (GA); and vanillic acid (Fig. 63.2).

Variations in the structures of individual hydroxybenzoic acids lie in the hydroxylations and methylations of the aromatic ring [16]. Four acids commonly occur: p-hydroxybenzoic, vanillic, syringic, and protocatechuic acid. They may be present in soluble form conjugated with sugars or organic acids as well as bound to cell wall fractions, for example, lignin [8].

1.2 Hydroxycinnamic Acids

The hydroxycinnamic acids (HCAs) include PA, CA, FA, SIA, and their esterified/etherified conjugates such as chlorogenic acids. Chemical structures of these compounds are depicted in Figs. 63.1 and 63.2. The most commonly found naturally occurring HCAs is chlorogenic acid (5-*O*-caffeoylquinic acid), which is caffeic acid

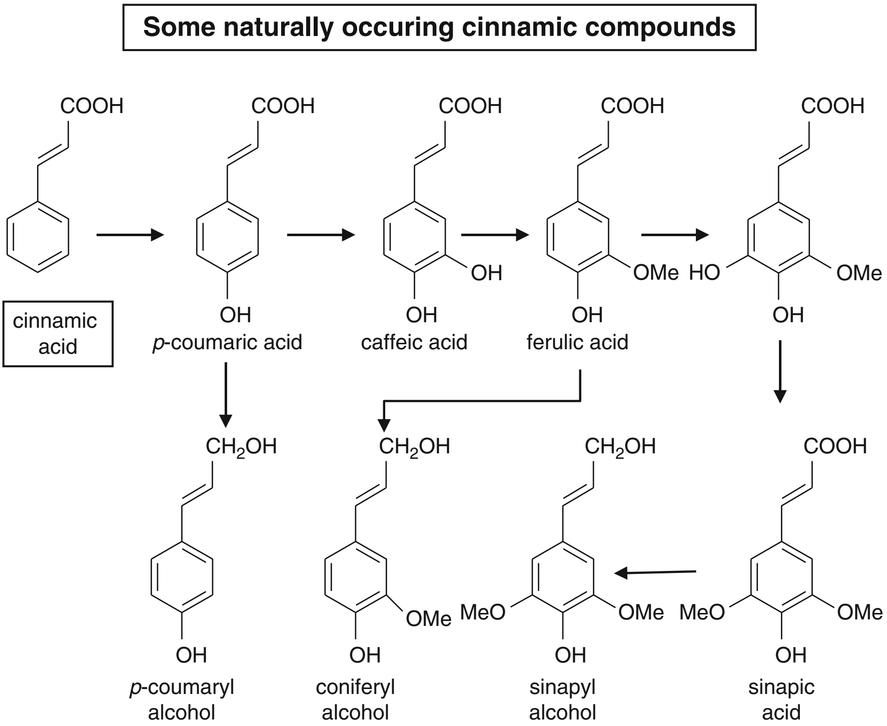

Some naturally occuring cinnamic compounds
COOH
cinnamic acid
OH
p-coumaric acid
caffeic acid
OMe
HO
ferulic acid
CH2OH
p-coumaryl alcohol
coniferyl alcohol
MeO
sinapyl alcohol
sinapic acid

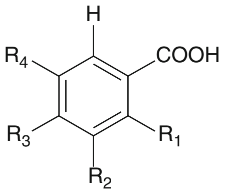
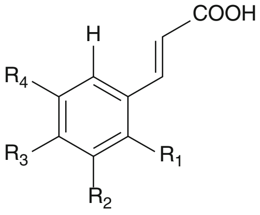
Fig. 63.2 Some naturally occurring cinnamic acid in plants

esterified with quinic acid. Hydroxycinnamates are found in food as monomers, dimers, and bound forms esterified with hydroxy acids, mono/disaccharides, and polymers. They also occur as amides (with amino acids and amines), particularly in coffee and cocoa [4]. The conjugated forms are esters of hydroxy acids such as quinic, shikimic, and tartaric acid, as well as their sugar derivatives [15] (Table 63.1).

1.3 Biosynthesis of Hydroxycinnamic Acids

The formation of hydroxycinnamic acids (caffeic, ferulic, 5-hydroxyferulic, and sinapic acids) from p-coumaric acid requires two types of reactions: hydroxylation and methylation. The phenylalanine/hydroxycinnamate pathway is defined as general phenylpropanoid metabolism. It includes reactions leading from L phenylalanine to the hydroxycinnamates and their activated forms [15]. The enzymes catalyzing the individual steps in general phenylpropanoid metabolism are (PAL), (CA4H), and (C4L). The introduction of a second hydroxyl group into p-coumaric acid to give caffeic acid is catalyzed by monophenol mono-oxygenases, a well-known group of plant enzymes [8, 16] (Fig. 63.3).

Table 63.1 Phenolic acids: hydroxybenzoic and hydroxycinnamic acids

Hydroxybenzoic acids					
	Name	R1	R2	R3	R4
	Benzoic acid	H	H	H	H
	p-Hydroxybenzoic acid	H	H	OH	H
	Vanillic acid	H	OCH_3	OH	H
	Gallic acid	H	OH	OH	OH
	Protocatechuic acid	H	OH	OH	H
	Syringic acid	H	OCH_3	OH	OCH_3
	Gentisic acid	OH	H	H	OH
	Veratric acid	H	OCH_3	OCH_3	H
	Salicylic acid	OH	H	H	H
Hydroxycinnamic acids					
	Cinnamic acid	H	H	H	H
	o-Coumaric acid	OH	H	H	H
	m-Coumaric acid	H	OH	H	H
	p-Coumaric acid	H	H	OH	H
	Ferulic acid	H	OCH_3	OH	H
	Sinapic acid	H	OCH_3	OH	OCH_3
	Caffeic acid	H	OH	OH	H

Methylation of caffeic acid leads to the formation of ferulic acid, which, together with p-coumaric acid, are the precursors of lignins. The methylation is catalyzed by an *O*-methyltransferase. Caffeic acid is the substrate for the rare 5-hydroxyferulic acid, which yields sinapic acid as a result of *O*-methylation. The formation of hydroxycinnamic acid derivatives requires the formation of hydroxycinnamate-CoAs (e.g., p-coumaroyl-CoA) catalyzed by hydroxycinnamoyl-CoA ligases or by the action of *O*-glycosyl transferases.

Moreover, hydroxycinnamate-CoAs can conjugate with organic acids. In the biosynthesis of sugar derivatives of hydroxycinnamic acids, the transfer of glucose from uridine diphosphoglucose to hydroxycinnamic acid is catalyzed by glucosyl transferase [8, 16].

1.4 Biosynthesis of Hydroxybenzoic Acids

Benzoic acids are structurally simple natural products, so it is quite surprising that many different pathways may be employed for their biosynthesis. Pathways may differ according to the organism, and sometimes more than one pathway may exist in a single

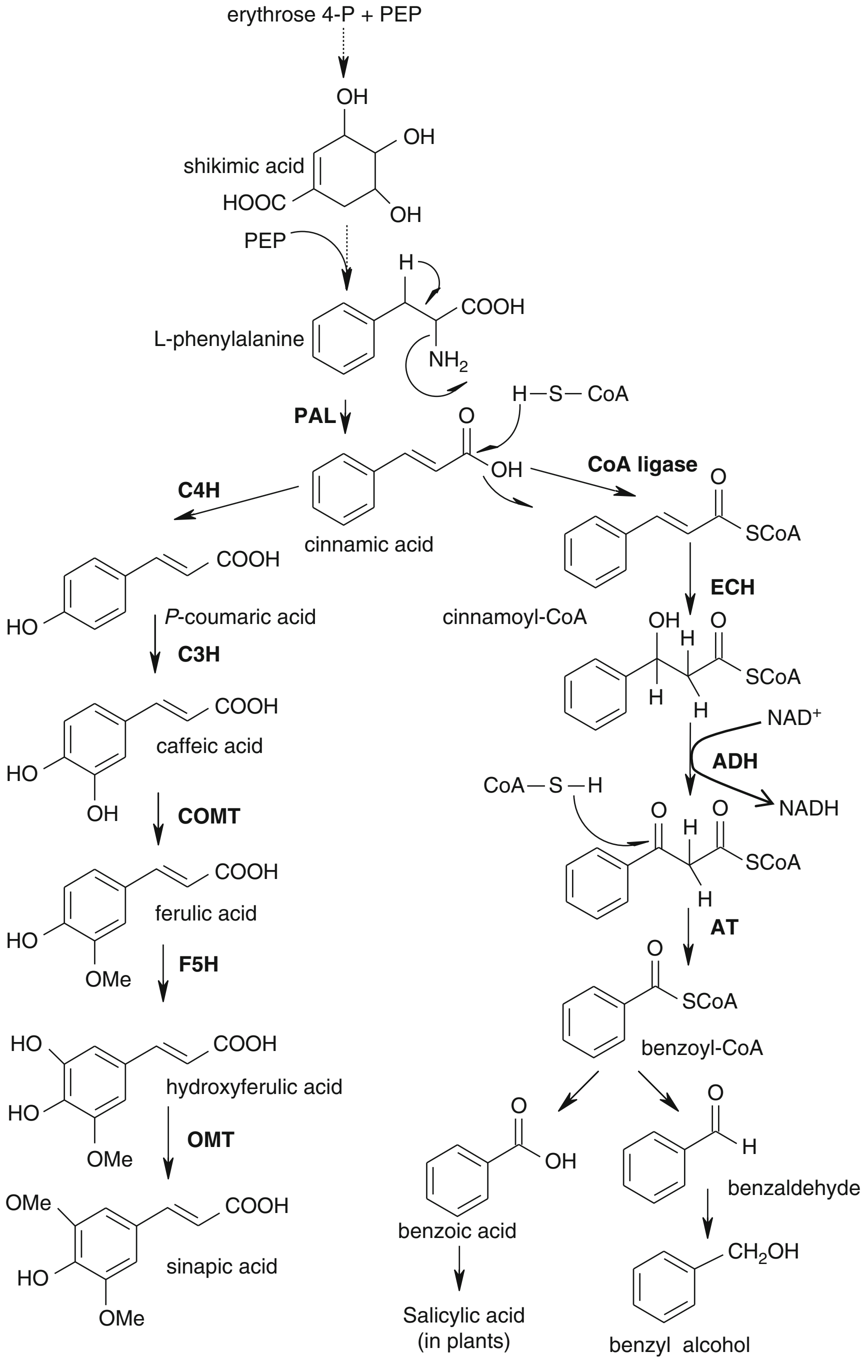

erythrose 4-P + PEP
shikimic acid
OH
OH
HOOC
OH
PEP
H
COOH
L-phenylalanine
NH_2
H—S—CoA
PAL
O
OH
CoA ligase
C4H
O
SCoA
cinnamic acid
COOH
HO
P-coumaric acid
ECH
cinnamoyl-CoA
OH
H
O
C3H
SCoA
H
H
COOH
caffeic acid
NAD+
ADH
HO
CoA—S—H
OH
COMT
NADH
O
H
O
SCoA
COOH
ferulic acid
H
HO
AT
F5H
O
OMe
SCoA
HO
COOH
benzoyl-CoA
hydroxyferulic acid
HO
O
O
OMe
OMT
H
OH
OMe
COOH
benzaldehyde
benzoic acid
sinapic acid
CH_2OH
HO
OMe
Salicylic acid
(in plants)
benzyl alcohol

Fig. 63.3 Pathway of the shikimic acid in the biosynthesis of phenolic acids

organism. Some of the simple hydroxybenzoic acids (C6-C1 compounds), such as GA and 4-hydroxybenzoic acid, can be formed directly from intermediates early in the shikimate pathway, especially from dehydroshikimic acid; this reaction is the main route to gallic acid [3]. However, they can also be produced by the degradation of hydroxycinnamic acids, in a similar manner to the b-oxidation of fatty acids; the main intermediates are cinnamoyl-CoA esters (Fig. 63.3). Hydroxybenzoates are also produced occasionally by the degradation of flavonoids. Moreover, hydroxylations and methylations of hydroxybenzoic acids are known to occur in an analogous way to the phenylalanine/hydroxycinnamate pathway. Knowledge of the mechanisms and above all the enzymes involved in the biosynthesis of hydroxybenzoic acids and their derivatives is rather limited, especially regarding fruits, although gallic acid and its derivatives play an important role in the formation of hydrolyzable tannins [8, 16].

2 Functions in Plants

Although a great deal is still unknown regarding the roles of phenolic acids in plants, they have been connected with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy [17].

A variety of evidence suggests that phenolic compounds influence the pools and fluxes of inorganic and organic soil nutrients. Phenolic compounds can directly affect the composition and activity of decomposer communities, thus influencing the rates of decomposition and nutrient cycling. To determine how individual phenolic acids in a mixture might affect phosphorus (P) uptake, cucumber seedlings grown in solution culture were treated with ferulic, vanillic, p-coumaric, or equimolar mixtures of these phenolic acids. The dose required for 50% inhibition of P uptake was approximately two to three times higher for vanillic acid (6.73 mM) than for ferulic (2.27 mM) and p-coumaric acids (3.00 mM) when the dose was based on the initial treatment concentrations [18], even though the concentrations of individual compounds are well below their inhibitory levels. Different types of soluble phenolics, such as ferulic acid and gallic acid, have been found to inhibit either seed germination or root growth. The allelopathic effects of these phenolic acids seem not to be species specific, but they act like a broad-spectrum preemergence herbicide that affects seedling establishment while not affecting established turfgrass. The allelopathic effects of phenolic acids may be at least partly responsible for preventing the establishment of new plants in the sward [19].

Either biotic or abiotic stress, or a combination of both, increases the production of phenolic compounds. Different kinds of stress may affect different parts of the Systemic Acquired Resistance (SAR) pathways and may determine whether (SA), (JA), ethylene or more than one signaling compound is employed in different plant species [20]. Simple phenolic acids are signaling molecules involved in plant-microbe interactions. Methyl salicylate is synthesized in the site of primary infection and protects the plant from the spread of infection and future attack [21].

2.1 Biotechnological Production of Phenolic Acids

Tissue and cell cultures are a potential model to elucidate the biosynthetic pathways of many secondary compounds and understand their regulation, as well as to improve the production of secondary metabolites of chemical-pharmaceutical interest [1, 2]. Compared with the traditional approach of whole plant cultivation in the natural environment or on agricultural farms, plant tissue culture in shake-flasks or bioreactors has the advantages of offering better controlled and more sustainable culture systems without the limitations of natural factors, such as geographical location and seasonal variation [21–25]. It has been regarded as a promising way for the mass production of valuable secondary metabolites, such as food additives, pharmaceuticals and nutraceuticals, and antimicrobials and pesticides, particularly from rare and slow-growing plant species [26–30].

2.2 Phenolic Acids in Foods

Phenolic acids account for almost one third of the dietary phenols and are associated with organoleptic, nutritional, and antioxidant properties of foods [4, 31]. Phenolic acids are abundant in a balanced diet that includes adequate amounts of fruits, vegetables, and whole grains. Sources include mangoes, berries, apples, citrus fruits, plums, cherries, kiwis, onions, tea, coffee, red wine, and wheat, rice, corn, and oat flours. Since they are widespread in plant-based foods, humans consume phenolic acids on a daily basis. They are easily absorbed due to their simplicity. The estimated range of consumption is 25 mg to 1 g a day, depending on diet [15, 32]. Among fruits, the highest contents are in rowanberry (28 mg/100 g). Coffee (97 mg/100 g) as well as green and black teas (30–36 mg/100 g) are the best sources among beverages. Additionally, the food industry has investigated the content and profile of phenolic acids, their effect on fruit maturation and prevention of enzymatic browning, and their roles as food preservatives [15].

Plant antioxidant activity is due to phenolic acid content, especially caffeic and p-coumaric. The greater antioxidant activity of caffeic acid than p-coumaric acid stems from the 3,4-position of dihydroxylation on the phenolic ring [33].

Phenolics behave as antioxidants due to the reactivity of the phenol moiety (hydroxyl substituent) on the aromatic ring [34]. Caffeic acid is expected to have higher antioxidant activity because of additional conjugation in the propanoic side chain, which might facilitate the electron delocalization, by resonance, between the aromatic ring and proanoic group [35].

Although the exact nature of the antioxidant or protective effects in vivo is not well established, some preliminary investigations have been carried out. Caffeic, ferulic, chlorogenic, and sinapic acid have shown antioxidant or protective effects in vivo by several mechanisms. The predominant mode is believed to be radical scavenging via hydrogen atom donation or established antioxidant, radical quenching mechanisms via electron donation, and singlet oxygen quenching [36, 37].

2.3 Phenolic Acids in Human Health

Phenolic acids have been reported to have important biological and pharmacological properties and may have benefits for human health. These compounds are important components of the human diet, due to their potential antioxidant activity, their capacity to diminish oxidative stress-induced tissue damage resulting from chronic diseases, and their potentially important anticancer activities [38, 39].

The literature provides information that correlates a diet rich in phenolic acids with the maintenance of health and disease prevention. The high antioxidant contents in fruits and vegetables inhibit oxidative damage diseases such as coronary heart disease, stroke, and cancers [40–42]. Certain foods are even classified as functional foods, owing to their established protective effects on human health.

Caffeic acid, one of the most prominent naturally occurring cinnamic acids, is known to selectively block the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma, and allergic reactions [35].

Phenolic acids have been reported to exert an antibacterial effect. Ferulic, isovanillic, p-hydroxycinnamic, p-hydroxybenzoic, syringic, caffeic, gentisic, protocatechuic, p-coumaric, vanillic, and p-hydroxybenzoic acids, isolated from different plant sources, have exhibited potent antibacterial activities. The mechanism of action of phenolic compounds is described as being nonspecific and resulting in alterations of the cytoplasmic membrane [32]. Caffeic acid and some of its esters might possess antitumor activity against colon carcinogenesis [43]. This compound also inhibits aflatoxin production of *Aspergillus flavus* without inhibiting the fungal growth and shows bactericidal activity toward *Pseudomonas aeruginosa* and *Staphylococcus aureus* [32].

Gallic acid can reduce allergic symptoms by acting as an antihistamine. Recent investigations by Maggi-Capeyron et al. [100] have linked a series of phenolic acids with the inhibition of AP-1 transcriptional activity. AP-1 is an activator protein implicated in the processes that control inflammation, cell differentiation, and proliferation, so gallic acid is a potent anti-inflammatory agent.

The human immunodeficiency virus (a virus that is often abbreviated to HIV and causes acquired immunodeficiency syndrome (AIDS) which ultimately destroys the immune system) is extremely difficult to treat because it hosts itself inside the body's cells and constantly mutates into new strains. Caffeic acid derivatives (e.g., dicaffeoylquinic and dicaffeoyltartaric acids) have been shown to be potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase. This enzyme catalyzes the integration of viral DNA into the host chromatin. Therefore, these hydroxycinnamate derivatives are currently being investigated for their potential antiviral therapy [44]. Cancer develops when the deoxyribonucleic acid (DNA) in cells becomes damaged, resulting in rapid, uncontrollable cell growth, ultimately leading to the formation of a tumor. It is known that some phenolic acids exert a direct antiproliferative action [44]. Furthermore, their direct interaction with the aryl hydrocarbon receptor, nitric oxide synthase inhibition, and their pro-apoptotic effect provide some insights into the phenolic acid biological mode of action [45, 46].

Consumption of products rich in phenolic acids, such as wine and the "Mediterranean diet," correlates with a reduced risk of cardiovascular disease. Since phenolic acids may exhibit antioxidant properties, a role in protecting low-density lipoprotein (LDL) from oxidative modification has been proposed [47, 99].

2.4 Presence and Therapeutic Properties of the Main Phenolic Acids

The phenolic acids found in natural sources are present in variable quantities and those with therapeutic properties have a higher added value in the market. An overview of the general therapeutic and chemical properties of phenolic acids has already been given, and the most important compounds are now described in more detail, including their present applications.

2.4.1 Ferulic Acid

FA (Fig. 63.1) is a ubiquitous plant constituent that arises from the metabolism of phenylalanine and tyrosine. It occurs primarily in seeds and leaves both in its free form and covalently linked to lignin and other biopolymers. Due to its phenolic nucleus and an extended side chain conjugation, it readily forms a resonance stabilized phenoxy radical, which accounts for its potent antioxidant potential.

FA is mainly conjugated with mono- and oligosaccharides, polyamines, lipids, and polysaccharides and seldom occurs in a free state in plants. It is a phenolic acid of low toxicity and can be absorbed and easily metabolized in the human body. FA has been reported to have many physiological functions, including antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, and anticancer activities [48, 49]. It also protects against coronary disease, lowers cholesterol, and increases sperm viability. In addition to this, provisional evidence suggests that FA may have further health benefits in the human body, although further studies are required for confirmation. These potential health benefits include possibly preventing bone degeneration, cancer, hot flushing symptoms in menopausal women, protecting the skin from ultraviolet (UV) damage, reducing blood levels of LDL cholesterol (a type of cholesterol which can cause blockages in the arteries and increase risk of heart disease), and treating diabetes by reducing blood glucose levels [50].

Due to its properties and low toxicity, FA is now widely used in the food and cosmetic industries. It serves as the raw material for the production of vanillin and preservatives, as a cross-linking agent for the preparation of food gels and edible films, and as an ingredient in sports foods and skin protection agents.

2.4.2 Salicylic Acid

SA (Fig. 63.3), also called 2-hydroxybenzoic acid, is used chiefly in the preparation of aspirin and other pharmaceutical products. Salicylic acid occurs in such diverse plants as *Salix* spp., *Sauromatum guttatum*, *Grindelia* spp., and *Mespilus germanica* and is also produced by the bacterium *Pseudomonas cepacia* [51].

SA has keratolytic, anti-inflammatory, antipyretic, analgesic, antiseptic, and antifungal properties for several skin conditions such as dandruff and seborrhoeic dermatitis, ichthyosis, psoriasis, acne, etc. [52]. It functions as a hormonal mediator of plant resistance responses to environmental stress and pathogen attacks. The free acid occurs naturally in small amounts in many plants.

Plants have developed an array of structural, chemical, and protein-based defenses designed to detect invading organisms and stop them before they are able to cause extensive damage. Disease can be caused by living (biotic) agents, including fungi and bacteria, or by environmental (abiotic) factors such as nutrient deficiency, drought, lack of oxygen, excessive temperature, ultraviolet radiation, or pollution. In order to protect themselves from damage, plants have developed a wide variety of constitutive and inducible defenses. This phenomenon is called systemic acquired resistance (SAR) and involves the mobilization of plant resources in case of further attack. Plants respond with a salicylic-dependent signaling cascade that leads to the systemic expression of a broad spectrum and long-lasting disease resistance that is efficient against fungi, bacteria, and viruses [53].

SA in plants is thought to be derived from the phenylalanine pathway by the cinnamic acid chain oxidative pathway [54].

2.4.3 Cinnamic Acids

The antioxidant activity of CA (Fig. 63.1) and four homologous derivatives has been shown to differ according to the kind of aromatic substitution (p-hydroxy, p-hydroxymethoxy, p-hydroxydimethoxy, dihydroxy). The antioxidant activity was studied using a competition kinetic test that measured the relative capacity to quench peroxyl radicals and the in vitro oxidative modification of human low-density lipoprotein. In both models, cinnamic acids were more efficient than their benzoic counterparts [55].

2.4.4 3-Hydroxybenzoic Acid

3-Hydroxybenzoic acid is found in common plants such as *Citrus paradisi*, *Olea europaea*, and *Mespilus germanica*. p-Hydroxybenzoic acid (4-hydroxybenzoic acid) has been isolated from many sources, including *Daucus carota*, *Elaeis guineensis*, *Vitis vinifera*, and numerous other species including East African *Fagara macrophylla*, *Xanthophyllum rubescens*, *Paratecoma peroba*, *Tabebuia impetiginosa*, *Pterocarpus santalinus*, *Catalpa bignonioides*, *Vitex negundo*, *Areca catechu*, *Roystonea regia*, and *Mespilus germanica* [56]. It shows antifungal, antimutagenic, antisickling, and estrogenic activities [57, 58].

2.4.5 Gentisic Acid

G (2,5-dihydroxybenzoic acid) is an analgesic, anti-inflammatory, antirheumatic, antiarthritic, and cytostatic agent, and inhibits low-density lipoprotein oxidation in human plasma [59].

G is thought to be responsible for the anti-carcinogenic activity of China-rose hibiscus (*Hibiscus rosa-sinensis*) extract, and a recent study has shown that gentisic acid is a Fibroblast Growth Factor (FGF) inhibitor [60, 61].

2.4.6 Gallic Acid

GA (3,4,5-trihydroxybenzoic acid) (Fig. 63.1) is a widespread phytochemical that occurs in *Allanblackia floribunda*, *Garcinia densivenia*, *Bridelia micrantha*, *Caesalpinia sappan*, *Dillenia indica, Diospyros cinnabarina*, *Paratecoma peroba*, *Psidium guajava*, *Syzygium cordatum*, *Rhus typhina*, *Tamarix nilotica*, *Vitis vinifera*, *Hamamelis virginiana*, and *Toona sinensis* [62].

GA exhibits a ROS-mediated anticancer activity in human prostate cancer cells [63] and possesses antimelanogenic properties. A phenolic fraction from evening primrose (*Oenothera biennis*) containing 55% of GA showed antitumor activity [64]. It has been proposed as a candidate for treatment of brain tumors as it suppresses cell viability, proliferation, invasion, and angiogenesis in human glioma cells. GA induces the death of HeLa cervical cancer cells via apoptosis and/or necrosis [17].

GA has shown promising results as an anti-HSV-2 (Herpes simplex virus) agent [65]. It is also used in the pharmaceutical industry as an astringent and styptic agent.

2.4.7 Vanillic Acid

Vanillic acid (4-hydroxy-3-methoxybenzoic acid) (Fig. 63.1) occurs in many plants such as *Fagara spp*., *Alnus japonica*, *Elaeagnus pungens*, *Erica australis*, *Gossypium mexicanum*, *Melia azedarac*h, *Panax ginseng*, *Paratecoma koraiensis*, *Pterocarpus santalinus*, *Rosa canina*, *Picrorhiza kurroa*, *Trachelospermum asiaticum*, *Amburana cearensis*, and *Lentinula edodes*. Besides antisickling and anthelmintic activities, vanillic acid could suppress hepatic fibrosis in chronic liver injury [66]. It is also found to be an inhibitor of snake venom 5′-nucleotidase [67].

2.4.8 Sinapic Acid

SIA (4-Hydroxy-3,5-Dimethoxy-Cinnamic Acid) (Fig. 63.2) is a phenylpropanoid compound found in various herbal materials and high-bran cereals. It has been reported that sinapic acid has antioxidant efficacy as a metal chelator due to the orientation of functional groups. Administration of sinapic acid exhibited significant reversal of arsenic-induced toxicity in hepatic tissue [68].

Diabetes is a major public health problem and the development of new therapies that are able to improve glycemia management and even to cure diabetes is of great interest. In a study designed to evaluate the effect of sinapic acid on physiological and biochemical parameters in streptozotocin-induced diabetic rats, oral administration of sinapic acid for a period of 35 days restored all these biochemical parameters to near normal [69].

2.4.9 Digallic Acid

Digallic acid ([3,4-dihydroxy-5-(3,4,5-trihydroxybenzoyl)- oxy]benzoic acid) is isolated from *Acacia farnesiana*, *Acacia arabica*, *Metasequoia glyptostroboides*, *Castanopsis spp*., *Quercus aliena*, *Mangifera indica*, *Rhus chinensis*, *Adenia cissampeloides*, *Terminalia chebula*, and *Pistacia lentiscus*. It is an HIV reverse transcriptase inhibitor and has cytotoxic/antiapoptotic activity and also shows antigenotoxic and antioxidant activities [70].

2.4.10 Diferulic Acids

They have been found in the cell walls of most plants, but are present at higher levels in the grasses and also sugar beet. The 8-*O*-4′-DiFA tends to predominate in grasses, but also predominate in barley bran. In sugar beet, the predominant diferulic acids are 8-*O*-4′-DiFA and 8,5′-DiFA, respectively [71]. Ferulates are thought to play an important role in modifying the mechanical properties of cell walls as well as in limiting polysaccharide degradation by exogenous enzymes by acting as a cross-link between polysaccharides and between polysaccharides and lignin [72]. Dimerization of ferulates is a mechanism for cross-linking cell wall polysaccharides. Ferulates have been extracted attached to a few sugar molecules at both ends, but so far no definitive proof of them linking separate polysaccharide chains has been found. In suspension-cultured maize cells, dimerization of ferulic acid and their esterification to polysaccharides occurs mostly in the protoplasm, but may occur in the cell walls when peroxide levels increase due to pathogenesis. In suspension-cultured wheat cells, only the 8,5′-diferulic acid is formed intraprotoplasmically with the other dimers being formed in the cell wall [73, 74].

2.4.11 Rosmarinic Acid

RA (α-*O*-caffeoyl-3,4-dihydroxyphenyllactic acid, RA) is a phenolic acid:(R)-alpha-[3-(3,4-Dihydroxyphenyl)-1-oxo-2E-propenyl]oxydihydroxybenzenepropanoic acid is found in many medicinal species belonging to *Lamiaceae* and *Boraginaceae*, including basil, sage, rosemary, mint, and *Perilla frutescens* [75].

RA has been shown to exert antioxidant effects in vitro against superoxide radicals and hydroxyl radicals [76].

RA exhibits various pharmacological activities including antiviral, antithrombotic, and antiplatelet suppression of cytokine-induced proliferation of murine-cultured mesangial cells, prevention of oxidation of low-density lipoprotein, inhibition of murine cell proliferative activity, and of cyclooxygenase, and anti-allergic action [77, 78, 101]. RA is used to treat peptic ulcers, arthritis, cataract, cancer, rheumatoid arthritis, and bronchial asthma. RA and its derivatives possess promising biological activities, such as improvement of cognitive performance, prevention of the development of Alzheimer's disease, cardioprotective effects, reduction of the severity of kidney diseases and cancer chemoprevention.

RA has been used topically as an anti-inflammatory drug. In order to alleviate inflammation, it seems to be able to suppress 5-lipoxygenase and 5-HETE synthesis (a pro-inflammatory compound in the omega-6 metabolic chain) [79]. During this process of inflammation several pathological changes occur: cyclooxygenase-2 (COX-2), a key enzyme in the inflammatory response, is thought to affect carcinogenesis indirectly via its modulatory effect on the inflammatory response and the immune system. RA is capable of reducing or eliminating leukotriene production and modulating the complement system without adverse reactions.

In vivo, RA has been reported to inhibit cobra venom factor (CVF)-induced paw edema, immune complex-mediated passive cutaneous anaphylaxis, and complement-dependent stimulation of prostacyclin synthesis.

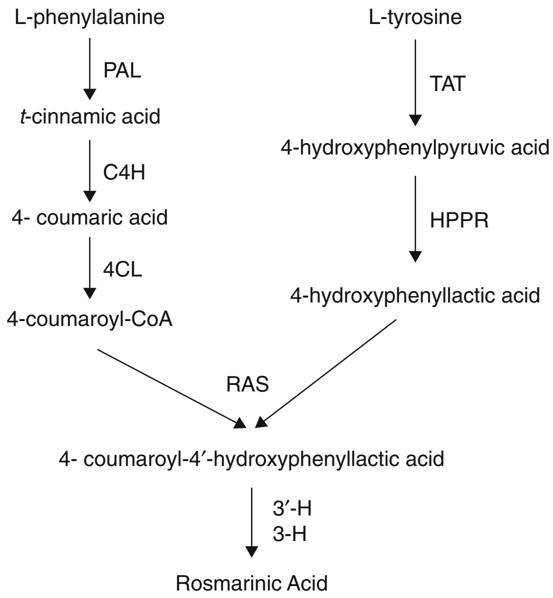

L-phenylalanine
PAL
t-cinnamic acid
C4H
4- coumaric acid
4CL
4-coumaroyl-CoA
L-tyrosine
TAT
4-hydroxyphenylpyruvic acid
HPPR
4-hydroxyphenyllactic acid
RAS
4- coumaroyl-4′-hydroxyphenyllactic acid
3′-H
3-H
Rosmarinic Acid

Fig. 63.4 Biosynthesis of rosmarinic acid

Sanbongi et al. (2004) have shown that the oral administration of RA has an effective intervention for allergic asthma. Oral supplementation with this compound has been shown to suppress allergic reactions in mice and, more recently, in humans. RA relieves allergy symptoms by kill allergy-activated T cells and neutrophils during allergic prevention by activation of immune responder cells and by inducing apoptosis.

RA furnished notable antibacterial activity against *Bacillus subtilis*, *Micrococcus luteus*, and *Escherichia coli* [80].

The biosynthesis production of RA have been extensively studied using cell and organ culture systems because. RA biosynthesis represents an interesting model system in which two parallel biosynthetic pathways must be regulated in a coordinated manner, caffeic acid moiety and the 3,4-dihydroxyphenyllactic acid moiety are derived from phenylalanine and tyrosine, respectively (Fig. 63.4). RA synthase catalyzed the condensation of 4-hydroxyphenyllactic acid, derived from tyrosine, with 4-coumaroyl-CoA formed through the phenylpropanoid pathway, to yield α-*O*-4-coumaroyl-4′-hydroxyphenyllactic acid (CHPL). CHPL is then converted to RA through two consecutive hydroxylation reactions as shown in Fig. 63.4.

Biotechnology offers an opportunity to sustainably exploit plant cells, tissues, organs, and the entire plant organism, using in vitro cultures and genetic manipulation, for the production of plant secondary metabolites, including rosmarinic acid, that are beneficial for human health.

Rosmarinic acid (RA) is one of the first secondary metabolites produced in plant cell cultures in extremely high yields, up to 19 % of the cell dry weight. More complex derivatives of RA, such as rabdosiin and lithospermic acid B, were also later obtained in cell cultures at high yields (Table 63.2).

Table 63.2 Phenolic acid in plant cell cultures

Plant species	Compounds	References
Agastache rugosa	Rosmarinic acid	[81]
Artemisia	Hydroxycinnamic	[85]
Beta vulgaris	Chlorogenic acid	[86]
Beta vulgaris	Caffeic acid	[86]
Calendula officinalis	Hydroxycinnamic	[85]
Capsicum annuum	Ferulic acid	[87]
Catharanthus roseus	2,3-Dihydroxybenzoic acid	[20]
Catharanthus roseus	trans-Cinnamic acid	[88]
Catharanthus roseus	2,3-dihydroxybenzoic-5-O-glicoside	[89]
Catharanthus roseus	Salicylic acid	[90]
Catharanthus roseus	Salicylic acid	[89]
Catharanthus roseus	Salicylic acid glucoside	[90]
Catharanthus roseus	Benzoic acid	[90]
Catharanthus roseus	Vanillic acid	[91]
Catharanthus roseus	Glucovanillic acid	[90]
Cocos nucifera	p-Hydroxybenzoic acid	[92]
Coleus forskohlii	Rosmarinic acid	[29]
Cynara scolymus	Hydroxycinnamic	[85]
Echinacea angustifolia	Hydroxycinnamic	[85]
Larrea divaricata	Ferulic acid	[24]
Larrea divaricata	p-Coumaric acid	[91]
Larrea divaricata	Ferulic acid	[23]
Lithospermum erythrorhizon	p-Coumaric acid	[93]
Mentha piperita	Hydrocinnamic acid	[94]
Mentha piperita	Dehydrocaffeic acid derivates	[95]
Nicotiana tabacum	Hydroxycinnamic	[85]
Nicotiana tabacum	Benzoic acid	[95, 98]
Ocimum basilicum	Rosmarinic acid	[27]
Oryza sativa	Feruloyl	[95]
Oryza sativa	Diferuloyl	[95]
Salvia miltiorrhiza	Caffeic acid	[96]
Salvia miltiorrhiza	Rosmarinic acid	[97]
Salvia miltiorrhiza	Rosmarinic acid	[28]
Salvia officinalis	Rosmarinic acid	[30]
Silybum marianum	Hydroxycinnamic	[85]

2.5 Extraction and Determination of Phenolic Acids

The most common solvents used for the extraction of phenolic acids from plant matrices are ethyl acetate, diethyl ether, methanol or aqueous methanol [24]. Enzymatic hydrolysis with b-glucosidase or hydrocinnamoyl-quinate esterase has been applied for the analysis of phenolic acids. However, acid and alkaline hydrolysis are more commonly used for the determination of phenolic acids in plants [81].

2.5.1 Chromatographic Techniques

HPLC, particularly RP-HPLC, is the method of choice in the chromatographic analysis of phenolic acids. The solvent systems used in the analytical HPLC usually include binary gradient elutions using solvents of aqueous acetic, formic, or phosphoric acids with methanol or acetonitrile as an organic modifier. The pH and ionic strength of the mobile phase are known to influence the retention of phenolics in the column depending on protonation, dissociation, or a partial dissociation [82]. A change in pH that increases the ionization of a sample could reduce the retention in a reversed-phase separation. Thus, small amounts of acetic (2–5 %), formic, phosphoric, or trifluoroacetic acid (0.1 %) are included in the solvent system to suppress ionization of phenolic and carboxylic groups and hence to improve resolution and reproducibility of chromatographic runs.

2.5.2 Detection and Identification of Phenolic Acids

Phenolic compounds absorb in the UV region and the most commonly used detector for HPLC is a variable-wavelength UV or UV-vis detector [23].

Most benzoic acid derivatives display their maxima at 246–262 nm, except for gallic acid and syringic acid, which have absorption maxima at 271 and 275 nm, respectively. Hydroxycinnamic acids absorb in two UV regions, one maximum being in the range of 225–235 nm and the other in the range of 290–330 nm. At 320 nm, cinnamic acid derivatives can be detected without any interference from benzoic acid derivatives, which have a higher absorptivity at 254 nm. However, detection at 280 nm is the best alternative for the determination of both classes of phenolic compounds. The extensive use of photodiode array detection (PDAD) in the analysis of phenolic acids can be attributed to the ability to collect online spectra without using stopped-flow techniques [23, 83, 84]. This has led to a considerable improvement in the HPLC analysis for identification purposes and has demonstrated the usefulness of qualitative information in phenolic analysis that is based on the absorption spectrum [32].

HPLC-MS is a fast and reliable method for structural analyses of nonvolatile phenolic compounds, since better techniques (interfacing systems) have been developed for the removal of the liquid mobile phase before ionization.

3 Conclusion

Phenolic compounds constitute a very large and complex group of secondary products found in plants. Among these phenolic compounds the phenolic acids are a large family of natural compounds widely distributed in plants, with very important and useful properties both for the producer plants and for the human health. In this review, the nature of the various phenolic acids present in food sources and the influence on physiological process in the plants and in human health are reviewed. The phenolic acids have a very high antioxidant effect and different studies in human health are discussed. According to modern theory of free radical biology and medicine, reactive oxygen species are involved in several disorders. The harmful action of the free radicals can, however, be blocked by antioxidant substances which scavenge the free radicals and detoxify the organism. Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers and neurodegenerative diseases. Therefore, plant-derived antioxidants are now receiving special attention. A large number of phenolic compounds, including most part of the phenolic acids present in vegetable foods, such as fruits and nuts, have been reported to possess these good antioxidant properties.

The biosynthetic pathways of phenolic acids in plants are quite well known; special emphasis was given to the hydrocinnamic, benzoic acids and rosmarinic acid biosynthetic pathways. The biotechnological production of secondary compounds of interest for the chemical-pharmaceutical industry is one of the main aims of the plant biotechnology. For these reasons, the in vitro culture of phenolic acid producer plants, mainly those endangered, is one aspect of the biotechnology that can offer an important response to the high world demand of these compounds. In this chapter, the major studies about the production of phenolic acids in plant cell cultures are included.

Several reviews have been published on the analysis of phenolic acids in plants and the optimization of the extraction methods, as well as to their chromatographic separation and identification techniques. The processing methods on extraction and purification presented in this review are the most employed in phenolic acids analysis.

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Hydrolyzable Tannins: Gallotannins and Ellagitannins

64

Michaël Jourdes, Laurent Pouységu, Denis Deffieux, Pierre-Louis Teissedre, and Stéphane Quideau

Contents

1 Classification of Hydrolyzable Tannins 1976
1.1 Gallotannins and Ellagitannins 1976
1.2 The *C*-Glycosidic Ellagitannins: A Special Subclass of Ellagitannins 1985
2 Biosynthesis 1988
3 Involvement of *C*-Glucosidic Ellagitannins in Wine Chemistry 1993
3.1 Occurrence of Flavano-ellagitannins in Red Wine 1993
3.2 Reactions of *C*-Glucosidic Ellagitannins with Non-phenolic Wine Nucleophiles 1996
3.3 Impact of the *C*-glucosidic Ellagitannins on Red Wine Color Modulation 1997
3.4 Effect of Oxidation on the Flavano-ellagitannin Profile 1999
4 Biological and Pharmacological Activities 2000
4.1 Hydrolysable Tannins in Folk Medicine 2000
4.2 Antiviral Activities 2002
4.3 Antimicrobial Activities 2002
4.4 Antitumoral Activities 2003
References 2003

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Abstract

The structural diversity displayed by these gallic acid metabolites is comparable to that of other classes of plant secondary metabolites such as alkaloids and terpenoids. The first aim of this chapter is to provide a condensate the state-of-the art on ellagitannin natural products and their significance as plant secondary metabolites, as well as interesting leads in the continuous search of new pharmaceutical drugs with original structural features. The following sections of this chapter will address research performed on *C*-glucosidic ellagitannins subclass over the last decade and will, in particular focus on their implication in wine chemistry as well as their putative role as active principles in traditional herbal remedies to their potential applications in more conventional medicinal approaches.

Keywords

Anthocyano-ellagitannins • *C*-glycosidic ellagitannins • Complex tannins • Ellagitannins • Gallotannins • Wine chemistry

1 Classification of Hydrolyzable Tannins

1.1 Gallotannins and Ellagitannins

The chemical structures of the hydrolyzable tannins are basically composed of a central sugar core, typically a glucose unit, to which gallic acid (**1**) and gallic acid-derived moieties are esterified. β-Glucogallin (**2**), the simplest glucosyl gallate and first precursor of this class of phenolic natural products was isolated in 1903 from Chinese rhubarb (*Rheum officinale*) [1]. The variety of hydrolyzable tannin structures thus resulting from glucose and gallic acid is so large that subdividing it into distinct categories in a logically ordered manner was far from being a trivial task [2]. Hydrolyzable tannins are first grossly divided into two subclasses: (1) the gallotannins, which are characterized by the presence of depsidically-linked galloyl units, and (2) the ellagitannins, which are characterized by the presence of biarylic dehydrodigalloyl so-called hexahydroxydiphenoyl (HHDP) units [3]. Haslam and co-workers subdivided the ellagitannins into two differents groups and proposed a three-group classification: (A) (B) (C) [4, 5]. Group A corresponding to the gallotannins featuring a core of β-penta-*O*-galloyl-ᴅ-glucopyranose (**3**) to which several other galloyl ester groups are linked in depside fashion (Fig. 64.1) [6]. The presence of two C–C-coupled galloyl ester groups at the 2,3- and/or 4,6-positions of a $^{4}C_{1}$-glucopyranose core was used to define ellagitannins, such as tellimagrandins I (**5**) and II (**6**), as belonging to group B of the hydrolyzable tannins [7]. This 6,6′-dicarbonyl-2,2′,3,3′,4,4′-hexahydroxybiphenyl bisester group, commonly referred to as the hexahydroxydiphenoyl (HHDP) unit, is the structural characteristic that defines hydrolyzable tannins as ellagitannins. Hydrolytic release of HHDP units from ellagitannins gives rise to their facile and unavoidable conversion into

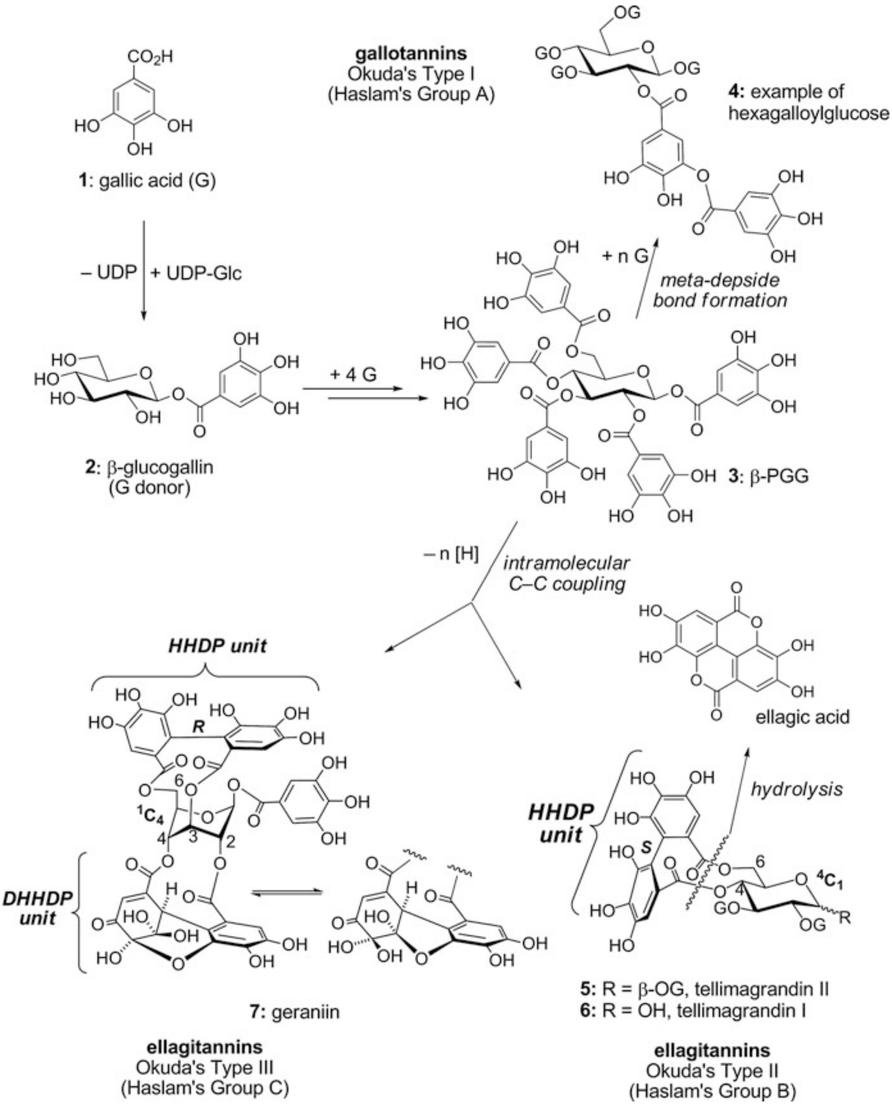

1: gallic acid (G)
– UDP
+ UDP-Glc
2: β-glucogallin
(G donor)
+ 4 G
3: β-PGG
gallotannins
Okuda's Type I
(Haslam's Group A)
4: example of hexagalloylglucose
+ n G
meta-depside bond formation
– n [H]
intramolecular C–C coupling
ellagic acid
hydrolysis
HHDP unit
DHHDP unit
7: geraniin
5: R = β-OG, tellimagrandin II
6: R = OH, tellimagrandin I
ellagitannins
Okuda's Type III
(Haslam's Group C)
ellagitannins
Okuda's Type II
(Haslam's Group B)

Fig. 64.1 General biosynthetic pathway of hydrolyzable tannins (i.e., gallotannin and ellagitannins) as well as Okuda's and Haslam's classification

the bislactone ellagic acid for which these natural products are named. Haslam's group C gathers ellagitannins in which HHDP units are connected to the 1,6-, 2,4- and/or 3,6-positions of the D-glucopyranose ring in its least thermodynamically favored $^1C_4$-conformation, as exemplified by the structure of geraniin (**7**) [8]. However, this classification was soon found too simple to accommodate many of the newly identified structures. More recently, Okuda proposed four main types of

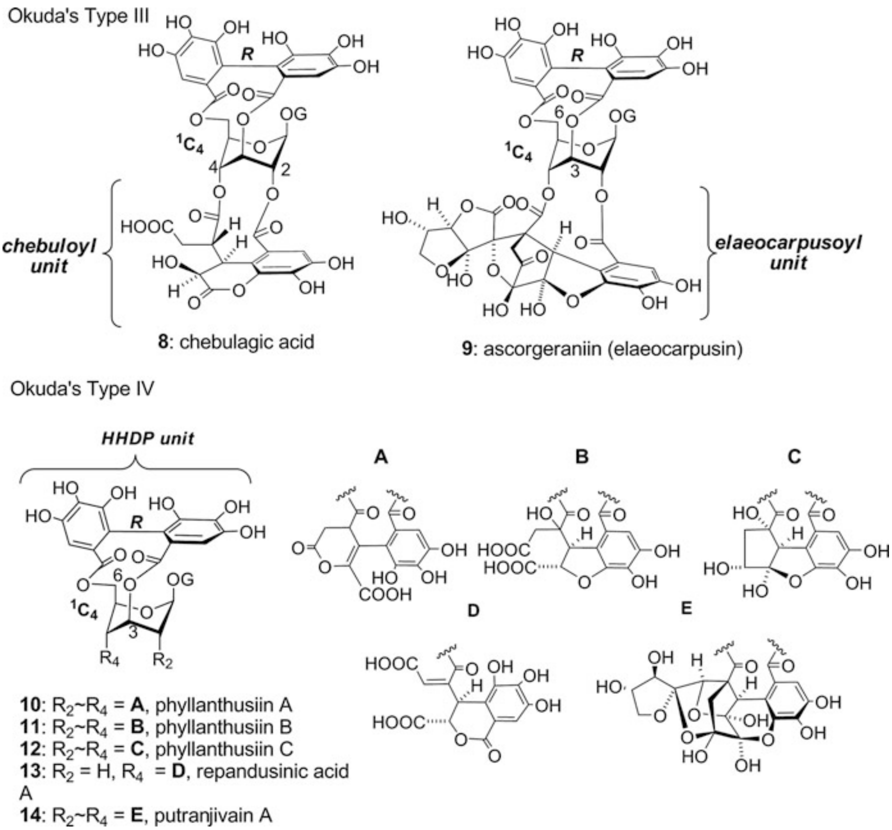
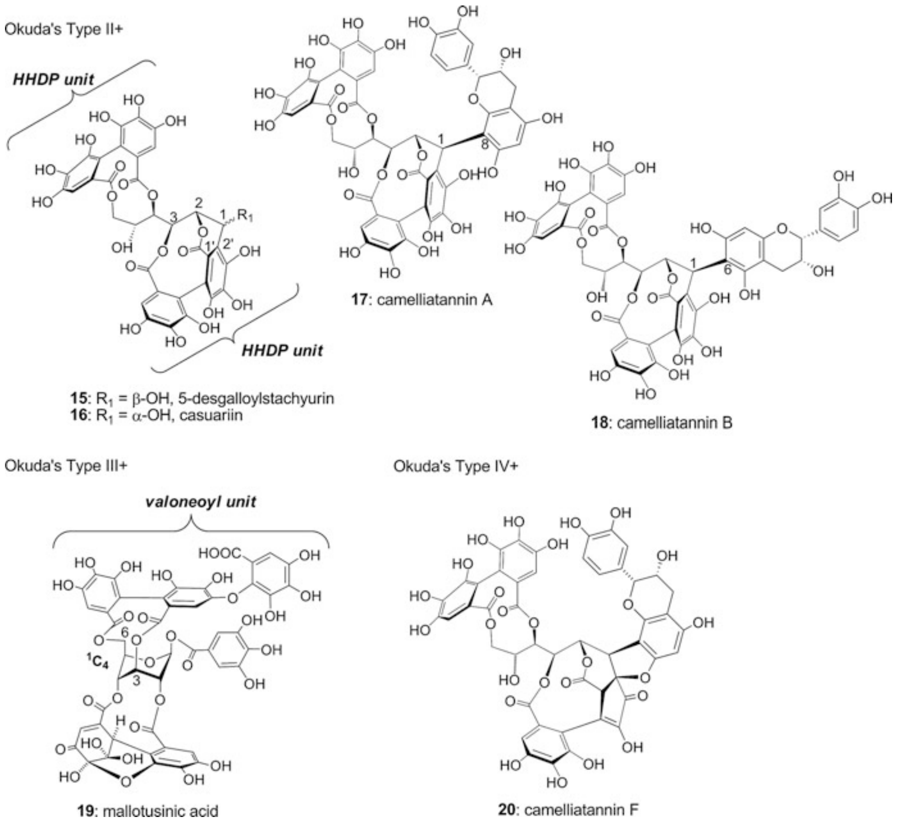

Okuda's Type III
chebuloyl unit
8: chebulagic acid
elaeocarpusoyl unit
9: ascorgeraniin (elaeocarpusin)
Okuda's Type IV
HHDP unit
A
B
C
D
E
10: R2~R4 = A, phyllanthusiin A
11: R2~R4 = B, phyllanthusiin B
12: R2~R4 = C, phyllanthusiin C
13: R2 = H, R4 = D, repandusinic acid A
14: R2~R4 = E, putranjivain A

Fig. 64.2 Examples of Okuda's type III and type IV ellagitannins

hydrolyzable tannins on the basis of the oxidation level of their galloyl ester groups [2, 9, 10]. This revised classification, which is modeled on a plausible progressive biogenetic elaboration of hydrolyzable tannins, first hypothesized by Schmidt and Mayer [11], defines gallotannins as type-I hydrolyzable tannins. Type II gathers the HHDP-bearing ellagitannins such as the monomeric tellimagrandins II (**5**) and I (**6**) [12, 13], and type III those featuring the dehydrohexahydroxydiphenoyl (DHHDP) unit (i.e., dehydroellagitannins), as exemplified by geraniin (**7**, Fig. 64.1). Ellagitannins in which the DHHDP unit has suffered additional transformations, such as chebulagic acid (**8**) and ascorgeraniin (**9**), belong to the type-IV group (Fig. 64.2). The chebuloyl and elaeocarpusoyl ester groups are only two examples out of a large series of ester units derived from the parent DHHDP unit [14]. Several other DHHDP-derived units have been identified such as *inter alia* in phyllanthusiins A-C (**10**–**12**), repandusinic acid (**13**) [15, 16], and putranjivain A (**14**) [17], whose 2,4-DHHDP-derived unit results from decarboxylation of the elaeocarpusoyl unit of ascorgeranin (**9**) [14]. All of these ellagitannins also belong to Okuda's type-IV hydrolyzable tannin group (Fig. 64.3).



15: R_1 = β-OH, 5-desgalloylstachyurin
16: R_1 = α-OH, casuariin

17: camelliatannin A

18: camelliatannin B

19: mallotusinic acid

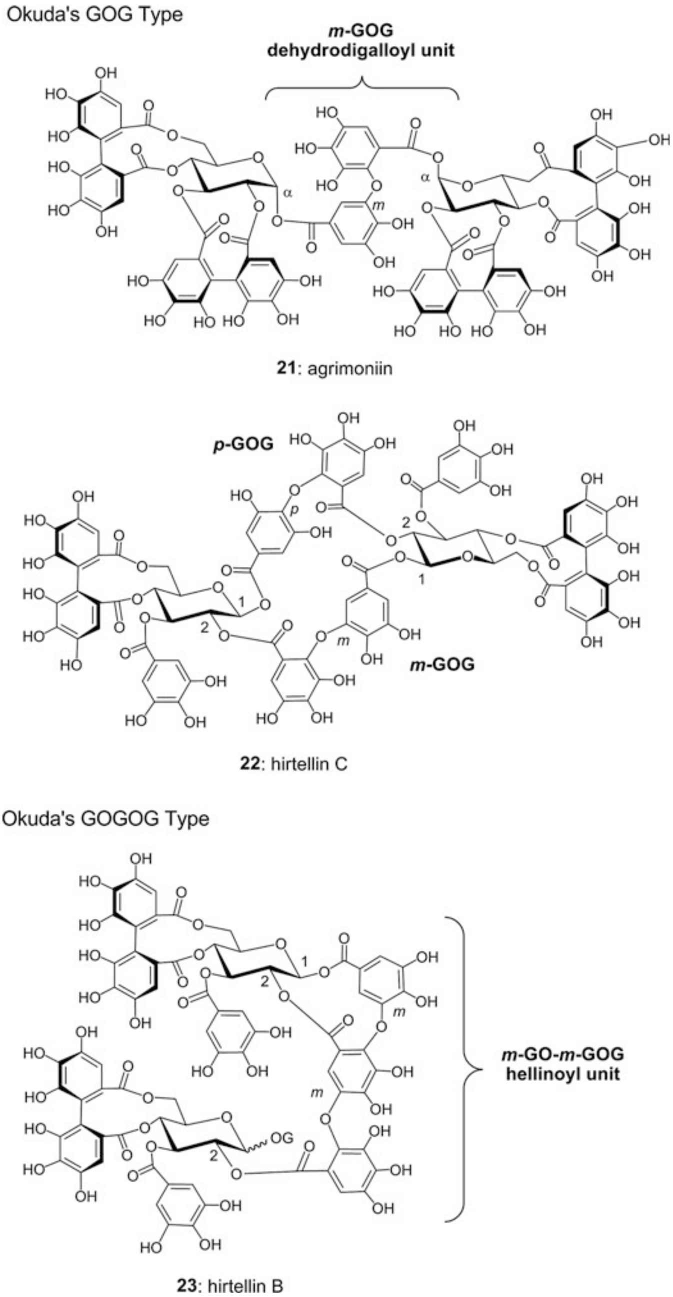
20: camelliatannin F

Fig. 64.3 Examples of Okuda's type II+ and type III+ ellagitannins

However, this classification still left out many monomeric ellagitannins, the structures of many of which resulting from chemical transformations other than those strictly mediated by oxidative processes. In this vein, some important structural modifications have to do with the opening of the D-glucopyranose core, the formation of *C*-arylglucosidic bonds and condensation reactions taking place at the glucose C-1 locus. Since these ellagitannins still mostly display the characteristic structural features of compounds belonging to primary types II–IV, Okuda refined his classification system and accordingly added four subtypes [9]. Type II+ principally gathers HHDP-bearing *C*-glucosidic ellagitannins, such as 5-*O*-desgalloylstachyurin (**15**) and casuariin (**16**), and their flavanoid hybrids (sometimes referred to as complex tannins), such as camelliatannins A/B (**17/18**), which result from condensation reactions with the flavan-3-ols catechin or epicatechin at the C-1 center of their open-chain glucose core (Fig. 64.3). Dehydroellagitannins that feature moieties resulting from a diaryl ether linkage with another phenolic or polyphenolic unit belong to the type-III+ group. An example of this type is mallotusinic acid (**19**, Fig. 64.3), having a valoneoyl group (*vide infra*) linked to the 3,6-positions of a 2,4-DHHDP-bearing $^{1}C_4$-glucopyranose core [18, 19]. Transformed dehydroellagitannins that feature moieties resulting from a C–C linkage with another phenolic or polyphenolic unit belong to the type-IV+ group. The *C*-glucosidic epicatechin-containing complex tannin camelliatannin F (**20**, Fig. 64.3) is an example of a structure of this type that is derived from the oxidative metabolism of a structure of type II+ (i.e., camelliatannin A (**17**, Fig. 64.3).

This classification work does not stop there and becomes further complicated by the fact that ellagitannins of essentially types II and II+ oligomerize via various modes based on oxidative coupling reactions between free and C–C-coupled galloyl groups (i.e., HHDP units) on different glucopyranosic ellagitannins, as well as on condensation reactions at the C-1 center of *C*-glucosidic ellagitannins. Once again, Okuda brought order into this seemingly chaotic ensemble of oligomeric structures. After a first proposal based on the structures of the constituting monomers [20], they instead classified oligomers into five types according to the nature of the interunit linkage between monomers [21]: (i) GOG (and GOGOG), (ii) DOG, (iii) GOD, (iv) $D(OG)_2$ and (v) *C*-glucosidic type, for which G = galloyl, O = oxygen and D = HHDP. In GOG- and GOGOG-type oligomers, the interunit linkages are composed of two (or three, not shown) G units linked together via a diaryl ether bond such as in agrimonin (**21**, Fig. 64.4) [12], having a *meta*-GOG type linking unit (i.e., one of the oxygen atoms *meta*-positioned to the carboxyl group-bearing carbon of one G unit is C-linked to one of the unsubstituted *ortho*-positions of the other G unit). This type of unit is also referred to as the dehydrodigalloyl (DHDG) unit. Although less frequently encountered, isodehydrodigalloyl units (i.e., *para*-GOG type) are observed in some oligomers isolated from plant species of the family Tamaricaceae such as *Reaumuria hirtella*, which produces a dimer named hirtellin C (**22**, Fig. 64.4) that results from a double oxidative coupling of two molecules of tellimagrandin II (**5**) [22]. This double coupling mutually occurs between the *O*-1-galloyl group of one monomer and the *O*-2-galloyl group of the other monomer and *vice versa*, but one C–O coupling gives rise to the *m*-GOG type unit, whereas the other one led to the more sterically encumbered *p*-GOG type unit [22]. Interestingly, the same plant species in Tamaricaceae are also capable of combining the same monomeric ellagitannin tellimagrandin II (**5**) in different ways via oxidative C–O coupling of galloyl groups. The structure of hirtellin B (**23**) illustrates one of these alternative oligomerization processes [23]. The two *O*-2-galloyl groups are linked together by a *m*-GOG type unit, the oxygen-donating *O*-2-galloyl group being similarly linked to the *O*-1-galloyl group of the same monomeric unit. The resulting *m*-GO-*m*-GOG type unit is also referred to as the hellinoyl group (Fig. 64.4).

The DOG-type units are also subdivided into their *meta* and *para* variants. In these units, a HHDP unit is O–C-linked to a G unit. The *m*-DOG type unit that is also referred to as the valoneoyl unit is found in numerous oligomeric ellagitannins [21], as well as in some monomeric ellagitannins such as the aforementioned type-III+ mallotusinic acid (**19**). The dimer oenothein B (**24**) [24], isolated in significant quantities from *Oenothera* and *Epilobium* species in Onagraceae and from *Lythrum*



Okuda's GOG Type

m-GOG
dehydrodigalloyl unit
α
m

21: agrimoniin

p-GOG
p
2
1
m
m-GOG

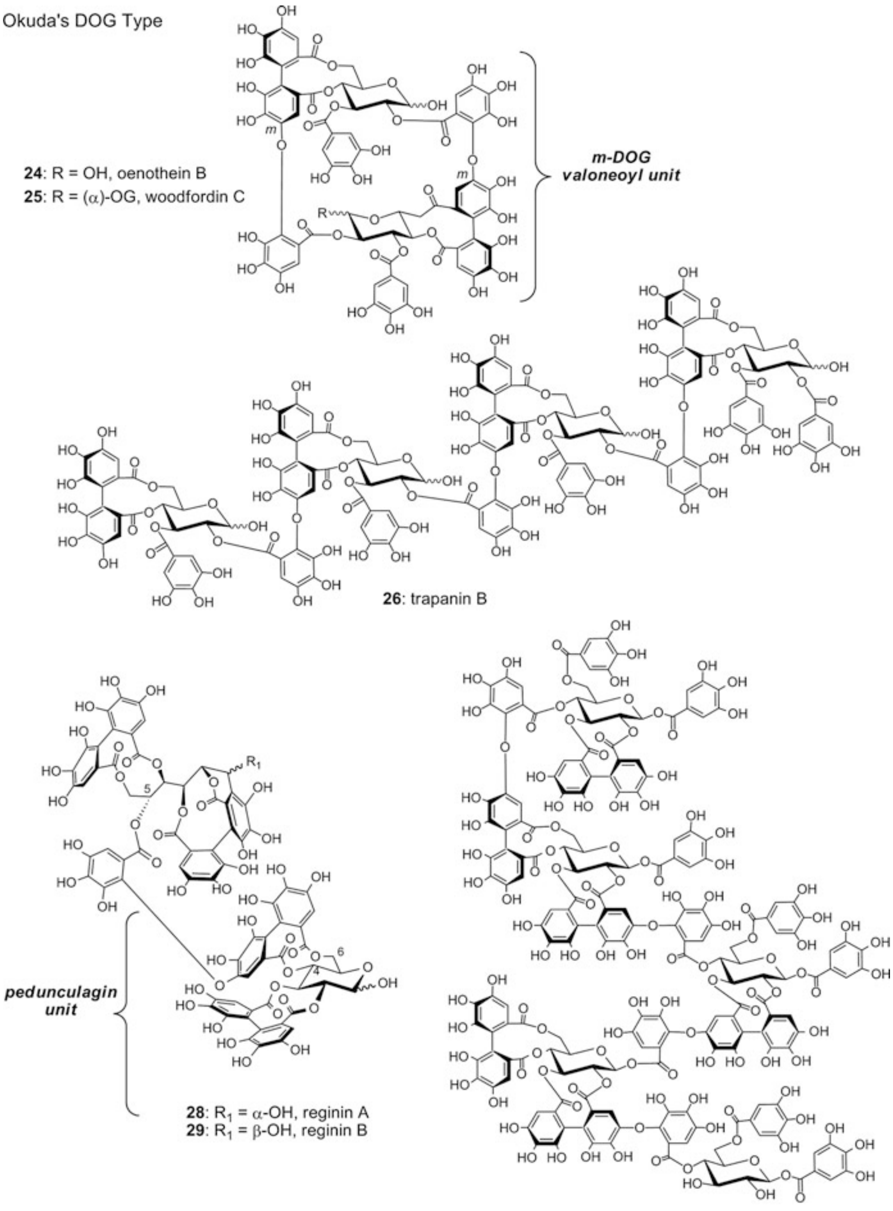
22: hirtellin C

Okuda's GOGOG Type

1
2
m
m
OG
m-GO-*m*-GOG
hellinoyl unit

23: hirtellin B

Fig. 64.4 Examples of Okuda's GOG and GOGOG type ellagitannins


Okuda's DOG Type
m
m
m-DOG valoneoyl unit
pedunculagin unit
R
R_1
5
6
4

24: R = OH, oenothein B
25: R = (α)-OG, woodfordin C

26: trapanin B

28: R_1 = α-OH, reginin A
29: R_1 = β-OH, reginin B

27: melastoflorin A

Fig. 64.5 Examples of Okuda's DOG type ellagitannins

anceps in Lythraceae, and its α-monogalloylated variant woodfordin C (**25**) [25, 26], isolated together with **24** from *Woodfordia fruticosa* in Lythraceae, constitute structurally fascinating examples of macrocyclic ellagitannin structures featuring two valoneoyl groups as macroring-forming interunit linkages (Fig. 64.5).

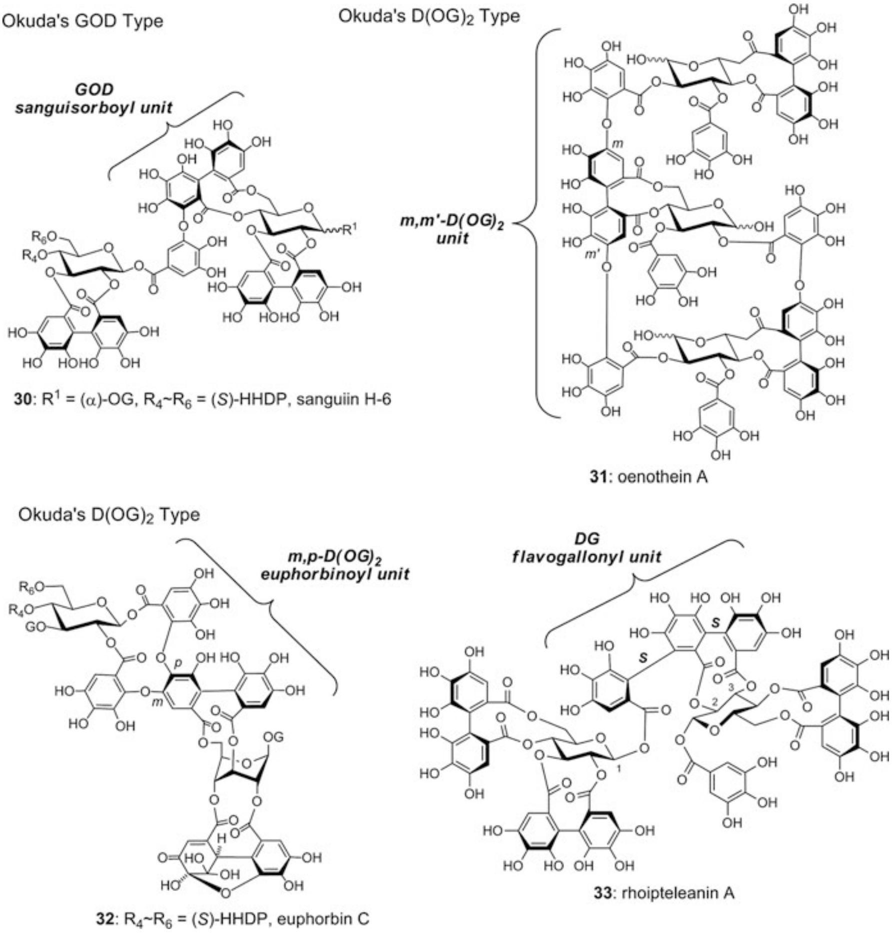
The *m*-DOG-type valoneoyl unit is probably the most often encountered interunit linkage in ellagitannin oligomerization via oxidative coupling processes. The construction of the highest oligomers fully characterized to date such as the tetramer trapanin B (**26**) [27], isolated from *Trapa japonica* (Trapaceae) and the pentamers melastoflorins A (**27**)-D [28], isolated from *Monochaetum multiflorum* (Melastomataceae), relies on multiple occurrence of this unit type (Fig. 64.5). The valoneoyl group also occurs in some biogenetically intriguing dimers composed of a glucopyranosic monomer *m*-DOG-linked to an open-chain *C*-glucosidic monomer. Reginins A (**28**) and B (**29**, Fig. 64.5) are examples of this type of dimers in which the 4,6-HHDP of a pedunculagin (**58**) monomer is linked to the 5-*O*-galloyl group of either stachyurin (**37**) or casuarinin (**38**) [29].

In the GOD type unit, which appears to exist only in its *meta* version, a HHDP unit of one monomer is C–O-linked to a G unit of another monomer. This type of interunit linkage is also referred to as the sanguisorboyl unit and is found in a small number of oligomers, here exemplified by the dimer sanguiin H-6 (**30**, Fig. 64.6), isolated from *Sanguisorba officinalis* (Rosaceae) [30].

The D(OG)$_2$ type unit is composed of a HHDP unit that has engaged two of its hydroxyl groups in oxidative O–C coupling with two G units. Two isomeric versions exist in which the diaryl ether bonds involve either the two oxygen atoms *meta* to the HHDP carboxyl group-bearing carbons and *para* to the biaryl axis (i.e., *m*,*m*′-D(OG)$_2$), or one of these two oxygen atoms and its adjacent oxygen atom *para* to the carboxyl group-bearing carbon of the same HHDP galloyl-derived moiety (i.e., *m*,*p*-D(OG)$_2$, also referred to as the euphorbinoyl unit). These two units are also found in a small number of oligomers, here exemplified by the trimer oenothein A (**31**), which is commonly isolated from Onagraceae and Lythraceae plant species [31], and euphorbin C (**32**, Fig. 64.6), a type of dimer that is characteristic of Euphorbiaceae plant species and composed of the type-II monomer tellimagrandin II (**1**) and the type-III dehydroellagitannin geraniin (**7**) [26].

To these four types of interunit linkages in oligomeric ellagitannins derived from phenolic C–O oxidative coupling processes should be added a fifth one derived from intermolecular (and atroposelective) C–C coupling between HHDP (D) and G groups. This "DG" type unit is known as the flavogallonyl unit and is found in a handful of dimeric structures known as the rhoipteleanins A (**33**)-F isolated from *Rhoiptelea chiliantha* (the sole species of the Rhoipteleaceae in the Juglandales order of the *Hamamelidae* subclass) (Fig. 64.6) [32, 33].

The fifth and last type of oligomeric ellagitannins proposed by Okuda and associates corresponds to oligomers of open-chain *C*-glucosidic ellagitannins, which, by virtue of the special reactivity expressed at their benzylic C-1 locus lead to three major subtypes of structures in this category [21]. Thus, the CD-type oligomers result from a C–C connection between the C-1 center of one *C*-glucosidic (C) unit and the 4,6-HHDP (D) of another such as in the dimer roburin D (**46**) [34], and so on up to the pentamer castaneanin D (**34**) [35], the highest known member of this class of oligomers commonly isolated from *Quercus* and *Castanea* species (Fagaceae) (Fig. 64.7). Casuglaunin A (**35**) [36], isolated from *Casuarina glauca* (Casuarinaceae), is an example of a CG type dimer in which the C-1 center of one


Okuda's GOD Type
GOD
sanguisorboyl unit
Okuda's D(OG)$_2$ Type
m,m'-D(OG)$_2$
unit
m
m'
Okuda's D(OG)$_2$ Type
m,p-D(OG)$_2$
euphorbinoyl unit
p
m
DG
flavogallonyl unit
S
S
1
2
3

30: R^1 = (α)-OG, R_4~R_6 = (*S*)-HHDP, sanguiin H-6

31: oenothein A

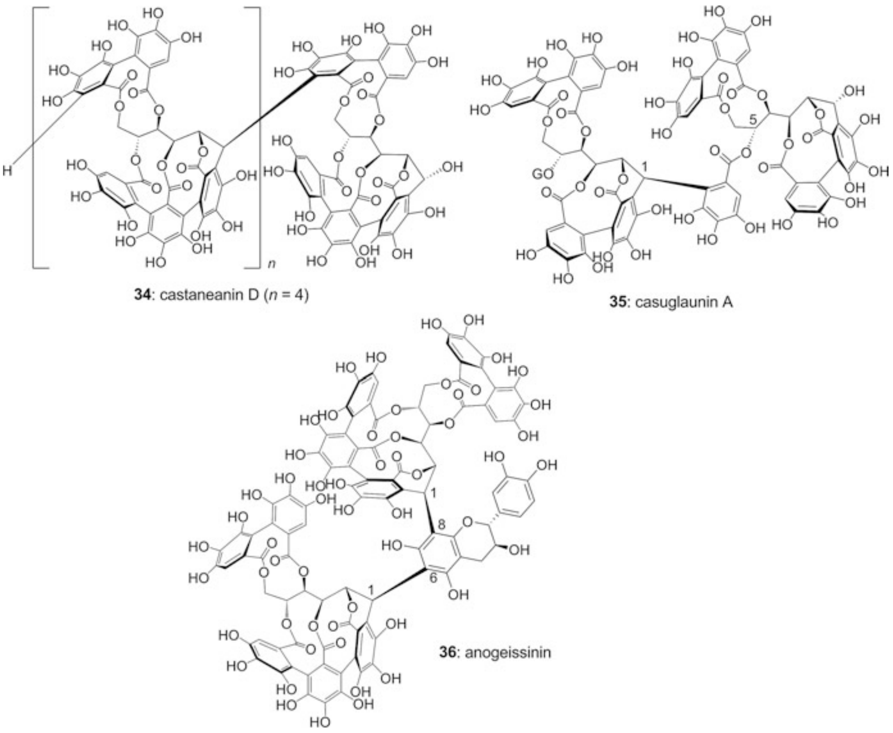
32: R_4~R_6 = (*S*)-HHDP, euphorbin C

33: rhoipteleanin A

Fig. 64.6 Examples of Okuda's GOD and D(OG)$_2$ type ellagitannins

C-glucosidic (C) unit is linked to a galloyl (G) group of another; in this case, the 5-*O*-galloyl group of casuarinin (**38**) (Fig. 64.7). Finally, the CFC type dimers correspond to complex tannin structures in which a flavanoid unit serves as a linker between two *C*-glucosidic units such as in anogeissinin (**36**), isolated from *Anogeissus acuminata* (Combretaceae) [37].

This rather extensive but outstandingly comprehensive classification should enable natural products chemists to rapidly determine the type of structure displayed by any ellagitannin compounds. Furthermore, phytochemists and botanists should find use for this classification as a guiding tool to delineate plant systematics. Indeed, Okuda and associates made some interesting and valuable observations that led them to correlate the apparent progressive metabolism of the simplest monomeric ellagitannins into more complex species and oligomers, mainly through oxidative processes, to plant taxonomy and hierarchic ranking in Cronquist's system of plant



34: castaneanin D ($n = 4$)

35: casuglaunin A

36: anogeissinin

Fig. 64.7 Examples of open-chain *C*-glucosidic ellagitannins and flavano-ellagitannins (complex tannins)

evolution [9, 28, 38]. Thus, for example, only types II and II+ ellagitannins are produced in the *Magnoliidae*, the earliest subclass in the *Dicotyledoneae*, whereas oxidized ellagitannins of types III, III+ and IV are additionally and frequently found in the *Rosidae*, a highly developed subclass. The occurrence of certain types of ellagitannins and their oligomers can be restricted to some plant families within an order of a given subclass, and sometimes even to a few genera of a particular family [9, 21]. Some ellagitannins can hence be used as chemotaxonomic markers because of their specific occurrence, and this becomes even more relevant that their structures get more and more complex. Thus, late-stage biosynthesized oligomeric structures can be more specifically correlated than their constituting monomers with plant systematics, as evidenced *inter alia* for GOG- and DOG-type oligomers in the Rosaceae and in the Melastomataceae [10, 28].

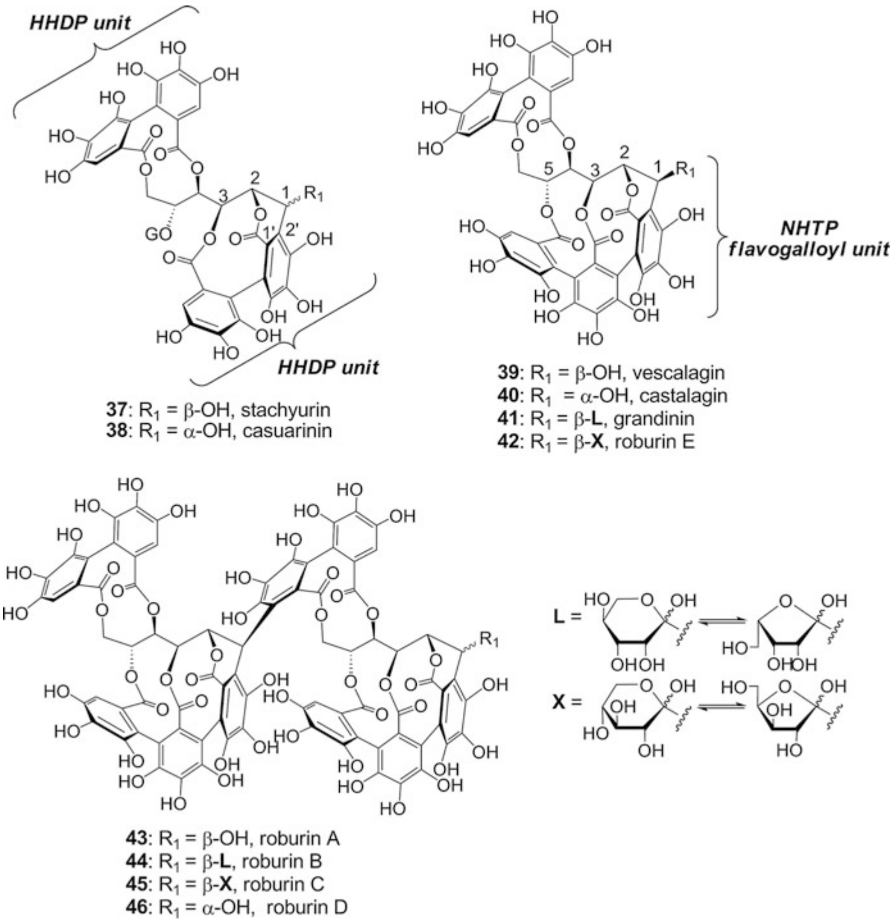
1.2 The *C*-Glycosidic Ellagitannins: A Special Subclass of Ellagitannins

Numerous ellagitannins of the *C*-glucosidic type, including monomers, oligomers and complex tannins (*vide infra*), have been isolated over the years from various plant species belonging to the Hamamelidaceae, Fagaceae, Betulaceae, Casuarinaceae, Juglandaceae and Rhoipteleaceae families in the *Hamamelidae* subclass, to the Rosaceae, Combretaceae, Lythraceae, Melastomataceae, Myrtaceae, Punicaceae, Trapaceae and Elaeagnaceae families in the *Rosidae* subclass, and to the Theaceae and Stachyuraceae families in the *Dilleniidae* subclass [9, 21, 38]. The *C*-glucosidic ellagitannins are a subclass of hydrolyzable ellagitannins that present the structural particularity of having a highly characteristic C–C linkage between the carbon C-1 of the open chain of glucose core and the carbon C-2 of the 2-*O*-galloyl unit. Their C-1-linked galloyl-derived unit is either part of a HHDP ester group bridging the 2- and 3-positions of the glucose core, as exemplified in the structures of stachyurin (**37**) and casuarinin (**38**), or part of a terarylic nonahydroxyterphenoyl (NHTP) variant (also known as the flavogalloyl group) that is attached *via* three ester bonds to the 2-, 3- and 5-positions of the glucose core, as exemplified in the structures of vescalagin (**39**) and castalagin (**40**) (Fig. 64.8). In contrast with the plant distribution of other *C*-glucosidic ellagitannins, the NHTP-bearing *C*-glucosidic ellagitannins appear to be limited to the family Fagaceae, Combretaceae, Lythraceae, Melastomataceae, Myrtaceae [10].

Vescalagin (**39**) and its C-1 epimer castalagin (**40**) are the first members of this sub-class that have been isolated from *Castanea* (chestnut) and *Quercus* (oak) species in 1971 [39–42]. Their content in the heartwood of oak species, such as *Q. petraea* and *Q. robur* together with the grandinin (**41**), roburins A (**43**), B (**44**), C (**45**), D (**46**), and E (**42**) [34, 43, 44] can vary from *ca.* 3 to 24 mg/g of dry wood, depending on the species, age and sampling height of the sampled tree [45]; chestnut heartwood can even contain up to 63 mg/g of dry wood [46] (Fig. 64.8). Since the characterization of **39** and **40**, a large variety of *C*-glucosidic ellagitannin oligomers has been isolated. To date, the largest known *C*-glucosidic ellagitannin oligomer is the vescalagin-based pentamer castanaenin D (**34**), which was isolated from heartwood of the Japanese chestnut tree (*Castanea crenata*) [35] (Fig. 64.9).

Like in the previously described ellagitannin groups, the *C*-glucosidic ellagitannin subclass also displays a large diversity of structures, as illustrated for example by the structures of the punicacorteins C (**47**) and D (**48**) isolated from the bark of *Punica granatum* (Punicaceae), which have the particularity of featuring a tetraphenyl ellagic acid-derived bislactone bisester group, known as the gallagyl unit, linked to the 4- and 6-positions of the glucose core [8] (Fig. 64.9). Another example of this structural diversity is paeonianin E (**49**) isolated from *Paeonia lactiflora* (Paeoniaceae), which is an unusual *C*-glucosidic ellagitannin featuring a gallic acid methyl ester group C–C-linked to the C-1 position of the open-chain glucose core. Also isolated from the same plant species, the paeonianins A-D are composed of a 5-*O*-desgalloylstachyurin (**15**) unit C_1–C-linked, respectively, to the 2- (**50**), 3-, 4- or 6-galloyl group of a β-PGG unit [48]. Many additional *C*-glucosidic ellagitannins, including monomers and oligomers, have been identified over the years from species belonging to the selection of plant families mentioned above [2, 10, 49].

The *C*-glucosidic ellagitannin subclass also includes complex tannins known as flavano-ellagitannins, which are hydrolysable tannin hybrids with a *C*-glucosidic ellagitannin moiety such as stachyurin (**37**) or vescalagin (**39**) and a flavan-3-ol



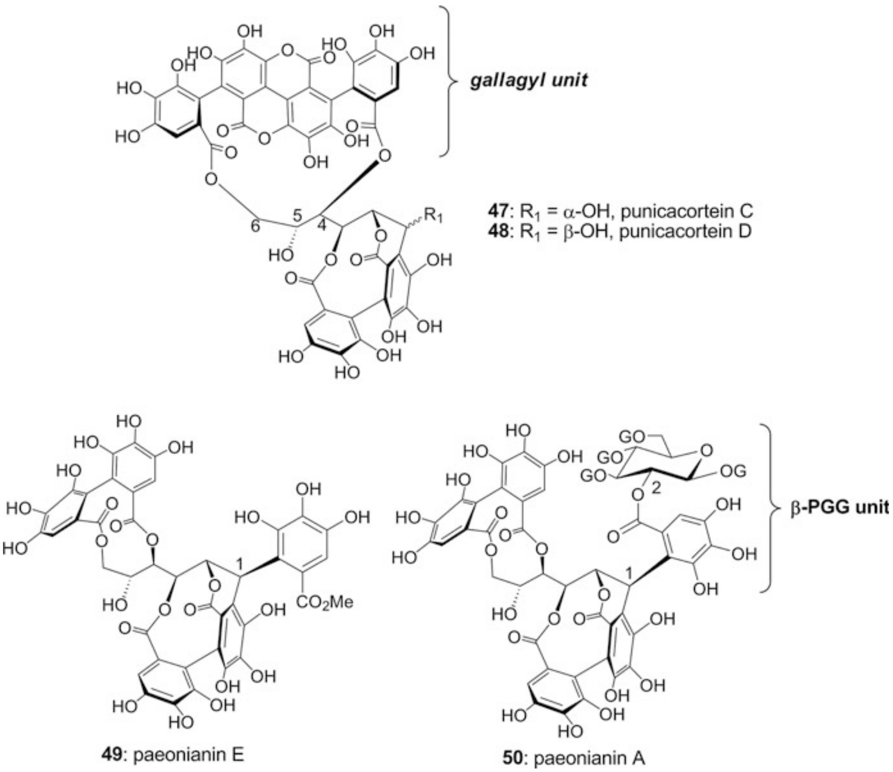
37: R_1 = β-OH, stachyurin
38: R_1 = α-OH, casuarinin

39: R_1 = β-OH, vescalagin
40: R_1 = α-OH, castalagin
41: R_1 = β-**L**, grandinin
42: R_1 = β-**X**, roburin E

43: R_1 = β-OH, roburin A
44: R_1 = β-**L**, roburin B
45: R_1 = β-**X**, roburin C
46: R_1 = α-OH, roburin D

Fig. 64.8 Main NHTP-bearing *C*-glucosidic ellagitannins isolated from *Castanea* (chestnut) and *Quercus* (oak) species

moiety such as catechin or epicatechin. In these complex tannins, both moieties are connected via a C–C linkage between the C-1 locus of the *C*-glucosidic ellagitannin moiety and the C-8 or C-6 centers of the A-ring of the flavan-3-ol unit. Variations of the nature of each moiety lead to complex tannins that display a large diversity of structures such as the catechin-based stenophyllanins A (**51**) and B (**52**) [50] and acutissimins A (**53**) and B (**54**) [51] or the epicatechin-based camelliatannins A (**17**), B (**18**), and F (**20**) (Fig. 64.10). Dehydrogenerative oxidation of these complex tannins leads to additional derivatives such as mongolicains A (**55**) and B (**56**) [52], which contain a hydroxyphenylcyclopentenone (HPCP) motif. Like for the simpler *C*-glucosidic ellagitannins, complex tannins are only found in plant species belonging to a very few number of families such as the Fagaceae, Combretaceae, Myrtaceae, Theaceae and Melastomataceae [53].



47: R_1 = α-OH, punicacortein C
48: R_1 = β-OH, punicacortein D

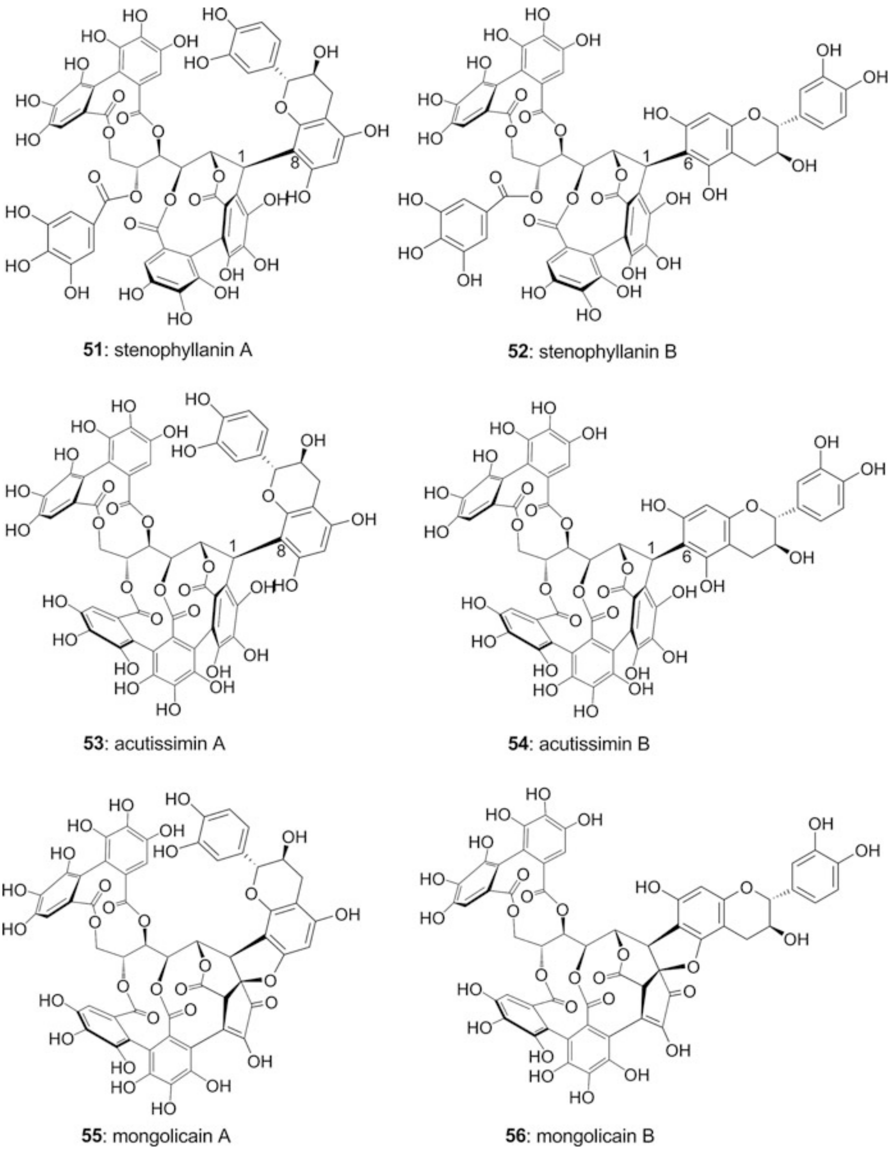
49: paeonianin E

50: paeonianin A

Fig. 64.9 Other example of *C*-glucosidic ellagitannins derivatives

2 Biosynthesis

β-Glucogallin (**2**) is the simplest glucosyl gallate known and it notably serves as a galloyl unit donor in the biosynthesis of the fully galloylated β-D-glucopyranose (**3**, β-PGG), which is itself considered as the branching point leading to either gallotannins or ellagitannins [54–57] (Fig. 64.1). Gallotannins are the result of further galloylations of β-PGG and are characterized by the presence of one or more depsidically-linked galloyl moieties, as exemplified with the hexagalloylglucose 2-*O*-digalloyl-1,3,4,6-tetra-*O*-galloyl-β-D-glucopyranose (**4**). Alternatively, β-PGG (**3**) can evolve through intra- and intermolecular oxidative phenolic coupling processes leading to the formation of covalent bonds such as C–C biaryl and C–O diaryl ether bonds between galloyl groups. The intramolecular coupling leads to the formation of biarylic hexahydroxydiphenoyl (HHDP) unit, which is the characteristic structural motif of ellagitannins. Acidic hydrolysis of such tannins releases the bislactonic ellagic acid (Fig. 64.1). Okuda's type-II ellagitannins are characterized by the presence of this HHDP unit [2, 10]. The atropoisomerism of these axially chiral biaryl motifs, such as the (*S*)-HHDP unit of the tellimagrandins or the



51: stenophyllanin A

52: stenophyllanin B

53: acutissimin A

54: acutissimin B

55: mongolicain A

56: mongolicain B

Fig. 64.10 Example of (+)-catechin-containing flavano-ellagitannins (complex tannins)

(*R*)-HHDP unit of geraniin (**7**) is determined by the positioning of the galloyl groups on their glucopyranosic precursors in either its $^4C_1$- or its $^1C_4$-conformation. Furthermore, the HHDP motif can go through an additional oxidative transformation leading to the so-called dehydrohexahydroxydiphenoyl (DHHDP) unit that is the structural characteristic of Okuda's type-III ellagitannins or dehydroellagitannins, such as geraniin (**7**) [58–61]. The DHHDP unit can undergo further transformations notably leading to the formation of the chebuloyl or elaeocarpusoyl groups, which characterized Okuda's type-IV ellagitannins (Fig. 64.2) [2, 10].

A β-pentagalloylglucopyranose oxidizing enzyme has recently been shown to mediate the formation of the 4,6-HHDP-containing tellimagrandin II (**5**) [62]. A different laccase-type phenol oxidase has also been shown to promote the conversion of tellimagrandin II (**5**) into the diaryl ether-linked dimer cornussiin E [63]. This is all we know today about enzymatic implications in the ellagitannins biosynthetic pathway. One has to admit that the task is enormous, due to the large structural diversity expressed by ellagitannins simply derived for the most part from glucose and gallic acid (**1**). More dedicated investigations are in demand to identify the enzymes involved in the biosynthetic pathway leading to glucopyranosic ellagitannins, as well as their *C*-glucosidic anabolites. As stated above, the occurrence of ellagitannins harboring *C*-glucosidic bonds appears to be limited to species of specific plant families.

A proposal for the biosynthetic pathway leading to *C*-glucosidic ellagitannins would start from β-PGG (**3**), the presumed pergalloylated glucopyranosic parent of all ellagitannins [56, 62, 63]. Two successive and diastereoselective oxidative galloyl coupling reactions would then result in the formation of two (*S*)-HHDP units at the 4,6- and 2,3-positions of the glucopyranose core. A 1-*O*-desgalloylation would then afford pedunculagin (**58**) (Fig. 64.11) [64]. The first key step leading from such a glucopyranosic ellagitannin to the *C*-glucosidic ellagitannins is the opening of the glucose core from its anomeric hemiacetal position. The thus-released hydroxyl group at the *O*-5 position of the resulting aldehydic open-chain glucose would be galloylated to form liquidambin (**59**), which has notably been isolated from the leaves of *Liquidambar formosana* [65]. The ellagitannin content of the leaves of this Hamamelidaceae plant has been thoroughly investigated, since members of both the glucopyranosic ellagitannin and the open-chain *C*-glucosidic ellagitannin classes co-exist [13, 66]. Interestingly, the ratio between these two classes of ellagitannins change over the leaves growing season. In early spring, the leaves of *Liquidambar formosana* contain high amounts of tellimagrandin II (**5**), whereas in summer, the amount of tellimagrandin II (**5**) becomes negligible and two other ellagitannins, pedunculagin (**58**) and the *C*-glucosidic casuarinin (**57**) become predominant throughout summer until autumn. This seasonal variation of the ellagitannin content in *Liquidambar formosana* was proposed to reflect the following biogenetic filiation (Fig. 64.11): from tellimagrandin II (**5**) to casuarinin (**38**) via pedunculagin (**58**) and liquidambin (**59**) [66]. Liquidambin (**59**) thus constitutes the molecular keystone between the glucopyranosic ellagitannins and the open-chain *C*-glucosidic ellagitannins. The second key step leading to the *C*-glucosidic ellagitannins is the intramolecular aldol-type nucleophilic addition of the 2,3-HHDP unit to the aldehyde function of **59**, which establishes the characteristic *C*-glucosidic bond, first featured in stachyurin (**37**) and its C-1 epimer casuarinin (**38**) [12, 13, 67–69]. An additional oxidative coupling between the glucose C–C-linked 2,3-HHDP unit and the free 5-*O*-galloyl group of **37** and **38** would then leads to the characteristic nonahydroxyterphenoyl (NHTP) unit of, respectively vescalagin (**39**) and castalagin (**40**) [43].

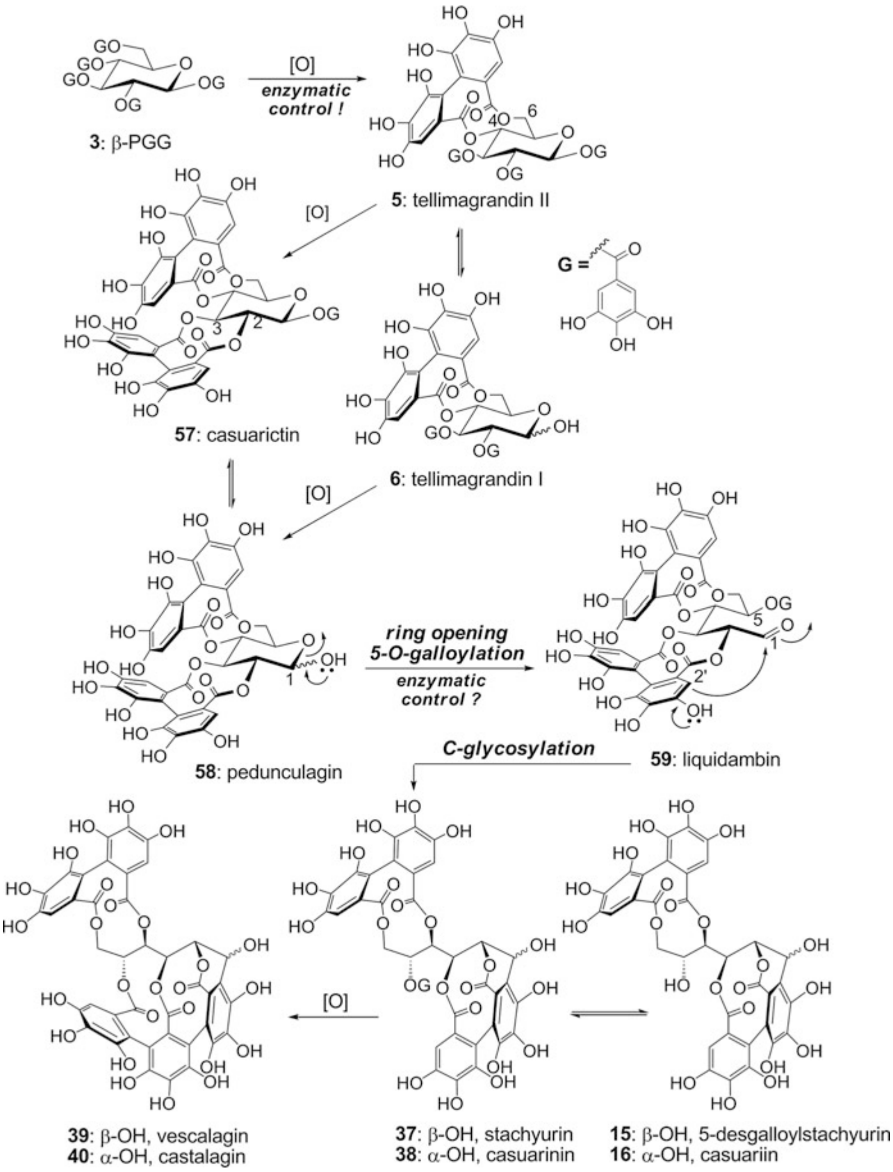

3: β-PGG
[O]
enzymatic control !
5: tellimagrandin II
G =
[O]
57: casuarictin
6: tellimagrandin I
[O]
58: pedunculagin
ring opening
5-O-galloylation
enzymatic control ?
59: liquidambin
C-glycosylation
[O]
39: β-OH, vescalagin
40: α-OH, castalagin
37: β-OH, stachyurin
38: α-OH, casuarinin
15: β-OH, 5-desgalloylstachyurin
16: α-OH, casuariin

Fig. 64.11 Putative biosynthetic pathway leading to the formation of the *C*-glycosidic ellagitannins

Once monomeric *C*-glucosidic ellagitannins such as the epimeric couples vescalagin (**39**)/castalagin (**40**) and stachyurin (**37**)/casuarinin (**38**) are formed, they can serve as precursors of numerous other *C*-glucosidic ellagitannins, as well as several so-called complex tannins (Fig. 64.12), such as the NHTP-bearing

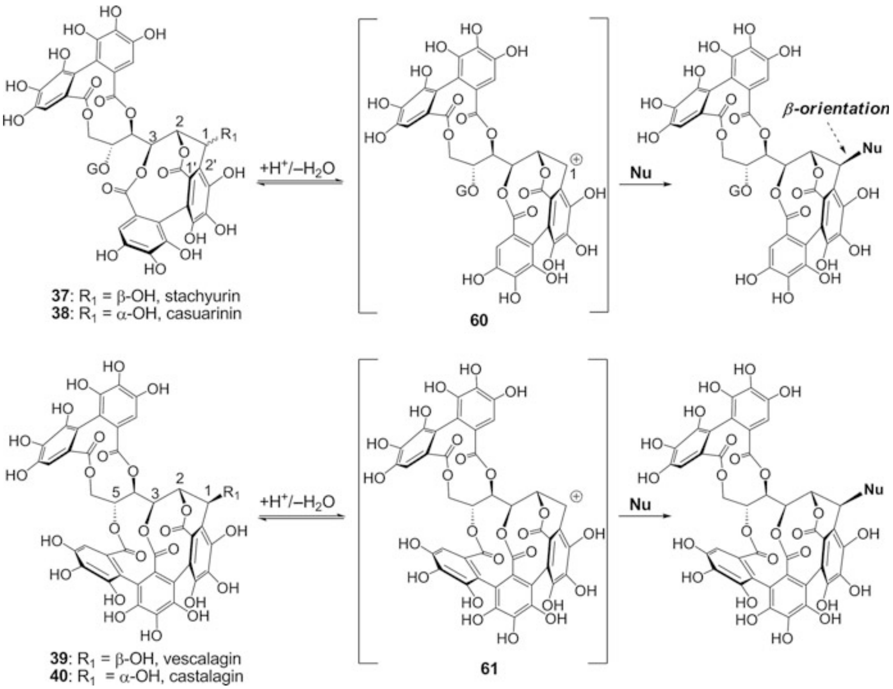

+H$^+$/–H$_2$O
Nu
β-orientation
60
61
37: R$_1$ = β-OH, stachyurin
38: R$_1$ = α-OH, casuarinin
39: R$_1$ = β-OH, vescalagin
40: R$_1$ = α-OH, castalagin

Fig. 64.12 Putative pathway from monomeric *C*-glycosidic ellagitannins to oligomeric and glycosilated *C*-glycosidic ellagitannins as well as to flavano-ellagitannins. Acid-catalyzed condensation reactions of *C*-glycosidic ellagitannins via benzylic-type carbocation intermediates

C-glucosidic grandinin (**41**), roburins A (**43**), B (**44**), C (**45**), D (**46**), and E (**42**) [34, 43] and the flavano-ellagitannins acutissimins A (**53**) and B (**54**). The acid-catalyzed deshydroxylation of the benzylic alcohol at the C-1 position of the glucose core of monomers **37** or **39** results in the formation of benzylic cation intermediates **60** or **61**. Various nucleophilic species, including **37–40**, as well as lyxose, xylose, flavan-3-ols and many more, can trap these cationic intermediates, hence building up the structural diversity expressed by the *C*-glucosidic ellagitannins. This S_N1-type nucleophilic substitution process proceeds under a strict stereochemical control that results in a β-orientation of the newly formed bond at the C-1 position. This stereochemical preference is observed in all known vescalagin (**39**)/castalagin (**40**) and stachyurin (**37**)/casuarinin (**38**) derived *C*-glucosidic ellagitannins, including all of the flavano-ellagitannins. The stereoelectronic nature of the benzylic cation intermediate **61** has been characterized through molecular modeling and offered a sound rationale for this remarkable diastereoselective S_N1-type nucleophilic substitution reaction (*vide infra*) [2, 43, 70–72].

3 Involvement of *C*-Glucosidic Ellagitannins in Wine Chemistry

The presence of *C*-glucosidic ellagitannins in beverages such as wines and spirits is the result of their presence in high amounts in fagaceous wood species such as *Quercus petraea*, *robur* and *alba*, for the heartwood of these oak species is used to manufacture barrels in which those beverages are stored or aged. The main *C*-glucosidic ellagitannins extracted by wines during their aging in oak barrels are the two monomers vescalagin (**39**) and castalagin (**40**), as well as the glycosilated monomers **41** and **42** and dimers **43–46** [73]. Once in the wine solution, the *C*-glucosidic ellagitannins are slowly but continuously transformed through condensation, hydrolysis, and oxidation reactions. Taken together the fact that the hydro-alcoholic wine solution aged in barrels extracts *C*-glucosidic ellagitannins from the oak wood, the fact that wine (especially red wine) contains a large amount of flavan-3-ols such as catechin and epicatechin with concentrations of about 115–190 mg/L and 80 mg/L, respectively [74, 75], and the fact that wine is a slightly acidic solution (pH ~ 3–4 in the case of red wine), the presence of the flavano-ellagitannins acutissimins **53** and **54** in red wine has been verified and confirmed.

3.1 Occurrence of Flavano-ellagitannins in Red Wine

Flavano-ellagitannin hybrids such as acutissimins A (**53**) and B (**54**) present a characteristic structure with a *C*-glucosidic ellagitannin moiety derived from vescalagin (**39**) or castalagin (**40**) connected, respectively, to the carbon C-8 and C-6 of the flavan-3-ol catechin (**62**) moiety. Acutissimins A (**53**) and B (**54**) were first isolated from the bark of *Quercus acutissima* [51], and were later found to express interesting biological activities, notably as inhibitors of human DNA topoisomerase II [43, 70, 71].

The hemisynthesis of **53** and **54** has been achieved in a high yield of about 87 % by simply reacting vescalagin (**39**) with catechin (**62**) in an acidic medium (1.5 vol% of TFA in THF) at 60 °C over a period of 7 h [70, 71]. Interestingly, the mixture of **53** and **54** thus obtained by hemisynthesis exhibit a ratio similar to that observed from the isolation of these two regioisomers from *Quercus acutissima*, i.e., 75:25 and 81:19, respectively [51]. The formation of acutissimin A (**53**) as the main regioisomer in both cases results from the more accessible and higher nucleophilic character of the carbon C-8 of the catechin (**62**) [70, 71]. The hemisynthesis of the not yet isolated epiacutissimin A (**64**) and B (**65**) has also been achieved by reacting vescalagin (**39**) with epicatechin (**63**) using the same procedure. Both epiacutissimin regioisomers were obtained in similar yield (~78 %) as for the reaction leading to the acutissimins, but with a regioisomeric ratio slightly different (67:33) [70, 71].

The mechanistic description of those hemisyntheses corresponds, as stated above, to a classical acid-catalyzed S_N1-type nucleophilic substitution that starts

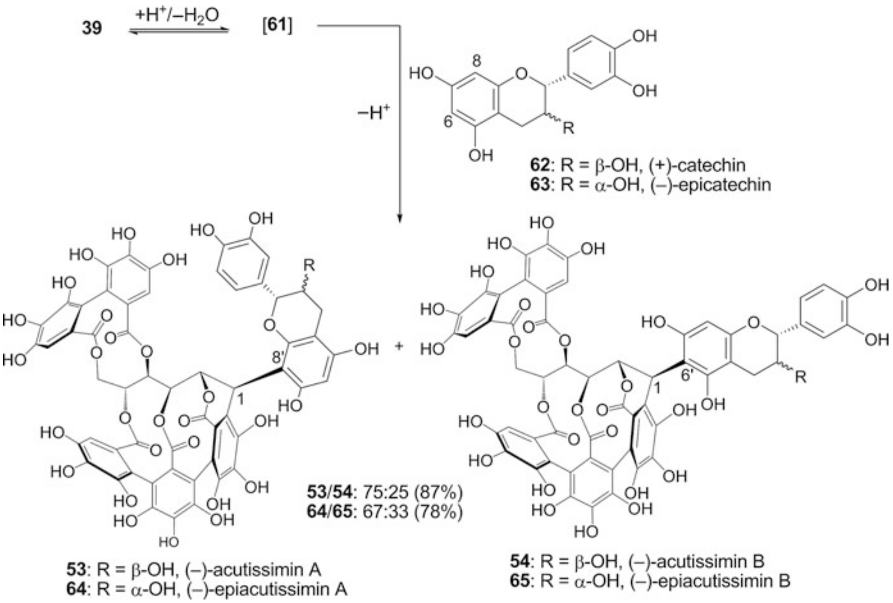

39
+H⁺/–H₂O
[61]
–H⁺
62: R = β-OH, (+)-catechin
63: R = α-OH, (–)-epicatechin
53/54: 75:25 (87%)
64/65: 67:33 (78%)
53: R = β-OH, (–)-acutissimin A
64: R = α-OH, (–)-epiacutissimin A
54: R = β-OH, (–)-acutissimin B
65: R = α-OH, (–)-epiacutissimin B

Fig. 64.13 Hemisynthesis of acutissimins **53**, **54** and epiacutissimins **64**, **65** from vescalagin (**39**) and either (+)-catechin (**62**) or (−)-epicatechin (**63**) respectively, in acidic media

with the protonation of the OH-1 group of vescalagin and the departure of H_2O from this locus to first afford the benzylic cation **61** (Fig. 64.13) [1]. This stable cationic intermediate **61** is then predominantly attacked by the carbon C-8 of the flavan-3-ol units and, to a lesser extent, by the carbon C-6, which is more encumbered and less nucleophilic than its C-8 counterpart [76, 77]. These C–C bond-forming nucleophilic substitution reactions proceed with full diastereofacial differentiation, resulting in retention of configuration at C-1 relatively to that of the starting vescalagin (i.e., β-orientation of the flavan-3-ol units linked at C-1).

Interestingly, attempts to generate the acutissimins **53** and **54** by using castalagin (**40**) instead of vescalagin (**39**) under the same mild conditions as those described above was totally unsuccessful. The inefficiency of **40** to form flavano-ellagitannins in a mildly acidic medium has been rationalized by molecular modeling calculations [2, 70–72]. This marked difference in reactivity between the two epimers castalagin (**40**) and vescalagin (**39**) is quite remarkable when considering their structural similarity, the only difference being the orientation of their OH-1 group. The deshydroxylation of the benzylic alcohol group of castalagin (**40**) appears to be very difficult and requires very drastic conditions, whereas that of vescalagin (**39**) easily occurs under mildly acidic conditions and leads to an expeditive formation of the benzylic cation **61**. Those opposite features have been rationalized by separate computational examination of each epimers [70–72].

The hemisynthesis of the flavano-ellagitannins **53**, **54**, **64** and **65**, which was initially performed in organic media, was also achieved in a wine model solution,

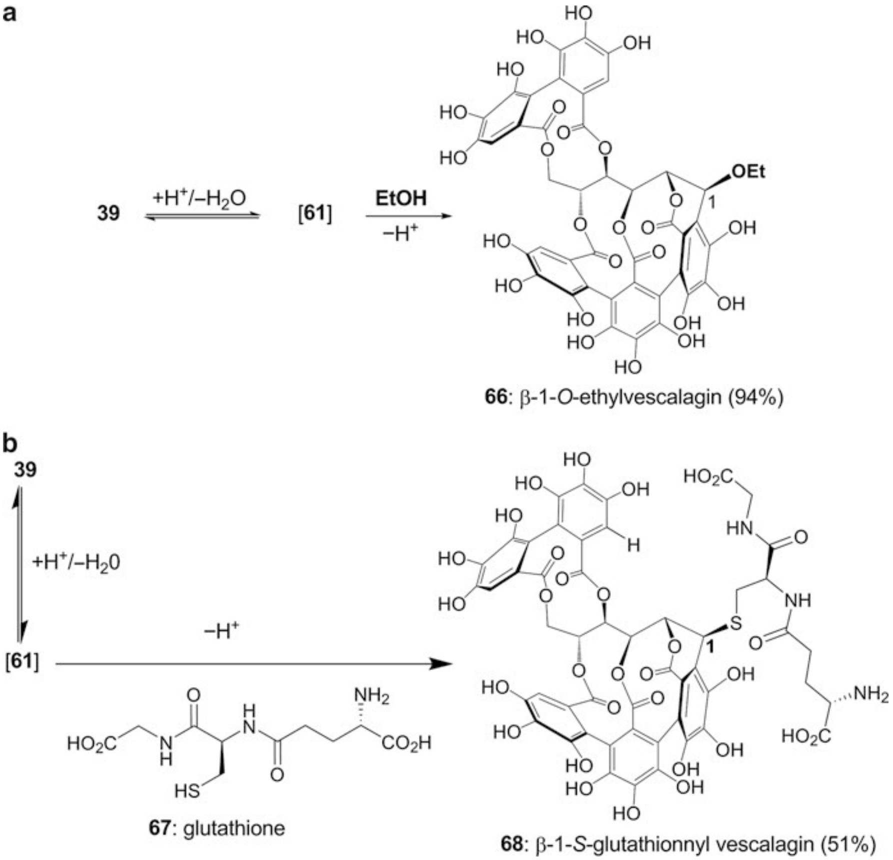

a
39
+H⁺/–H₂O
[61]
EtOH
–H⁺
OEt
1
66: β-1-O-ethylvescalagin (94%)
b
39
+H⁺/–H₂0
[61]
–H⁺
67: glutathione
S
1
68: β-1-S-glutathionnyl vescalagin (51%)

Fig. 64.14 (**a**) Hemisynthesis of β-1-*O*-ethylvescalagin (**66**) from vescalagin (**39**) and ethanol in acidic media. (**b**) Hemisynthesis of β-1-*S*-glutathionyl vescalagin (**68**) from vescalagin (**39**) and glutathione (**67**) in acidic media

consisting of a 12 vol% hydro-alcoholic (ethanol) solution with 5 g/L of tartaric acid at pH 3.2, in order to confirm their possible formation in wine. After several days at room temperature, the reaction mixture between **39** and **62** or **63** resulted in the formation of the corresponding flavano-ellagitannins, together with that of the β-1-*O*-ethylvescalagin (**66**) (Fig. 64.14a). The formation of **66** in such a wine model solution is the result of the nucleophilic attack of ethanol on the intermediate benzylic cation **61**. With this evidence that flavano-ellagitannins **53**, **54**, **64** and **65** can be formed in wine, an HPLC/ESI/MS-based identification and quantification procedure has been used to confirm their presence, as well as that of β-1-*O*-ethylvescalagin (**66**), in a sample of red wine aged during 18 months in oak barrels. Their concentrations were determined to range from 0.30 to 0.40 mg/L [78].

After this first detection and quantification in red wine, the kinetic evolution of the concentrations of the native oak-derived *C*-glucosidic ellagitannins and their wine hybrids was comparatively monitored during red wine aging in either oak barrels or in stainless steel tanks in which oak chips were added. Although the use of oak chips during red wine aging can lead to red wines with a woody aromatic profile (vanilla, smoky, coconut) close to that of a red wine traditionally aged in oak barrels, the global *C*-glucosidic ellagitannin concentration in those red wines appeared to be four times lower than that in the wine aged in oak barrels. Moreover, under the wine aging conditions used, the concentration in *C*-glucosidic ellagitannins increased regularly to reach its maximum after 3–4 months and then slowly decreased during the rest of the aging in barrels [73]. For the red wine aged in stainless steel tanks with oak chips, the *C*-glucosidic ellagitannin maximum concentration was obtained after only 1.5–2 months. These differences in reaching maximum concentration levels could have an impact on the wine gustative properties such as astringency, bitterness, amplitude and roundness of the wine. The rates of formation of the flavano-ellagitannins **53**, **54**, **64** and **65** and of the β-1-*O*-ethylvescalagin **66**, as well as the evolution of their concentrations was thus monitored for the first time in a red wine aged in either oak barrels or in stainless steel tanks with oak chips [73]. Interestingly, the total amount of these vescalagin derivatives **53**, **54**, **64–66** appeared to be lower for the red wine aged in stainless steel tanks with oak chips than for the red wine aged in oak barrels. However, despite these overall concentration differences, the relative composition between these five vescalagin derivatives **53**, **54**, **64–66** was similar under both red wine aging conditions.

Even if these quantitative analyses indicated relatively low concentrations of these vescalagin derivatives **53**, **54**, **64–66** (i.e., around 2 mg/L in red wine aged in new oak barrels), their occurrence constitutes compelling evidences that oak-derived *C*-glucosidic ellagitannins do react, via condensation at their C-1 position, with various nucleophilic species in wine. Moreover, red wine is a slow but continuously evolving mildly acidic and aerobic medium in which the newly formed flavano-ellagitannins **53**, **54**, **64** and **65** certainly go through further phenolic oxidation processes and can, for example, generate products such as mongolicain A (**55**), an oxidation product derived from acutissimin A (**53**). However, as long as grape-derived flavan-3-ols and oak-derived vescalagin (**39**) are present in wine, **53**, **54**, **64** and **65** will be continuously formed. Furthermore, one must not forget that red wine contains an even much broader panel of nucleophilic species, such as other flavonoids (anthocyanins, proanthocyanidins) and, more generally, different kinds of nucleophilic phenols, alcohols, amines, carboxylic acids, enolizable carbonyl compounds, and thiols that may all also compete in the trapping event of the benzylic cation intermediate **61** [2, 71].

3.2 Reactions of *C*-Glucosidic Ellagitannins with Non-phenolic Wine Nucleophiles

The formation of any adduct between vescalagin (**39**) and the cysteine-containing tripeptide glutathione (**67**) has also been investigated. This study originates from empiric observations made by oenologists showing that the level of glutathione (**67**) considerably diminishes during the aging of white wines in new oak barrels [79]. Glutathione (**67**) plays an important antioxidant role in preserving some organoleptic properties of white wines, because of its protective action against oxidative degradation of volatile thiols, such as 4-mercapto-4-methylpentan-2-one and 3-mercaptohexanol, which contribute to the fruity aroma of white wines. It has been previously observed that the thiol function of the cysteine residue featured in **67** can engage in nucleophilic addition reactions with electrophilic *ortho*-quinones derived from the oxidation of caftaric acid in white must [80, 81]. However, before our investigations highlighted below, no covalent interaction between oak *C*-glucosidic ellagitannins and glutathione (**67**), which is present in white must at concentrations ranging from 3 to 24 mg/L [77, 78], had been proposed and evidenced as a chemical event conceivably affecting the amount of glutathione (**67**) in wines.

The hemisynthesis of β-1-*S*-glutathionyl vescalagin (**68**) has been achieved using conditions similar to those used for the hemisynthesis of the flavano-ellagitannins **53**, **54**, **64** and **65** [71] (Fig. 64.14b). The hemisynthesis of **68** has also been confirmed in a wine model solution, in which the sulfoxide variant of **68** and β-1-*O*-ethylvescalagin (**66**) were also formed. The contribution of *C*-glucosidic ellagitannins on the decrease of the concentration of glutathione (**67**) in wine was then definitely evidenced by the detection of β-1-*S*-glutathionyl vescalagin (**68**) in a white wine aged during 12 months in oak barrels [82].

3.3 Impact of the *C*-glucosidic Ellagitannins on Red Wine Color Modulation

Together with astringency and bitterness, color is another main organoleptic criterium for red wine quality. The maceration and alcoholic fermentation step of the wine-making process allow the extraction of anthocyanins from red grape skins and result in the intense color of young red wines. However, these native grape pigments progressively disappear during wine maturation, especially during the aging in oak barrels and are replaced by more stable wine-specific pigments through various chemical reactions [83, 84]. A considerable amount of investigations have been conducted in order to understand, characterize and eventually control the physicochemical mechanisms causing the red wine color modulation. Most of these studies reveal that several chemical reactions between the anthocyanins and nucleophilic or electrophilic components in wine, such as flavan-3-ols, ethanal, and pyruvic acid, occur and generate new pigments displaying different coloring properties (i.e., either bluer or more orange tints) [85–89]. Moreover, physical co-pigmentation phenomena that result from stacking of the colored anthocyanin flavylium cations with other wine phenolic species [84, 90, 91], as well as complexation with metallic cations [92, 93], have also been shown to contribute to this color modulation. Surprisingly, covalent interactions between grape anthocyanins and oak *C*-glucosidic ellagitannins have not been considered as a possible means

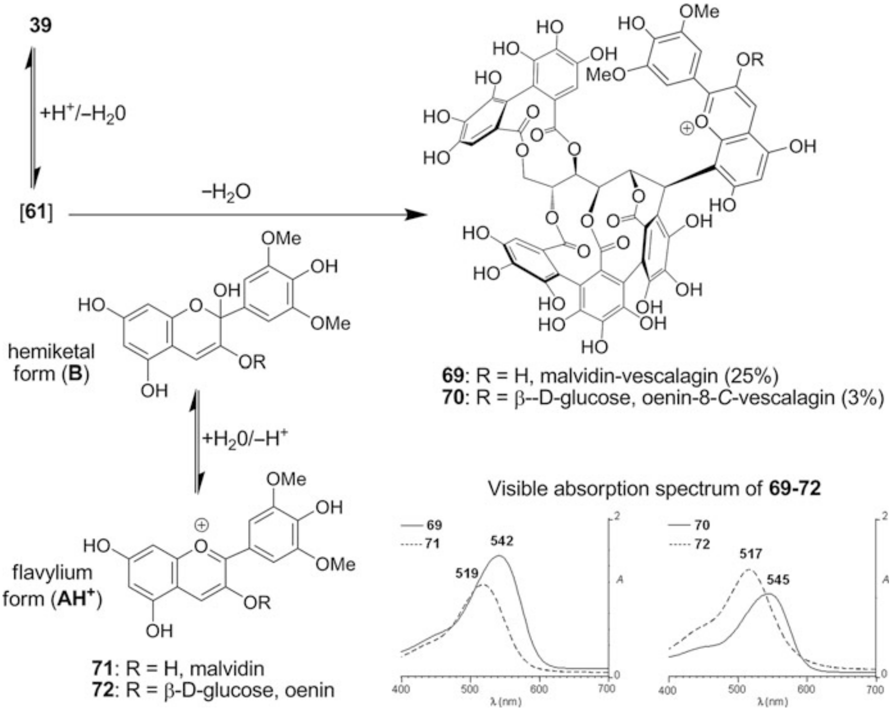

39
+H+/–H20
[61]
–H2O
hemiketal form (B)
flavylium form (AH+)
+H20/–H+
71: R = H, malvidin
72: R = β-D-glucose, oenin
69: R = H, malvidin-vescalagin (25%)
70: R = β--D-glucose, oenin-8-C-vescalagin (3%)
Visible absorption spectrum of 69-72
69
71
542
519
70
72
517
545
A
λ (nm)
400
500
600
700
0
2

Fig. 64.15 Hemisynthesis of the two first anthocyano-ellagitannin **69** and **70** from vescalagin (**39**) and either malvidin (**71**) or oenin (**72**) respectively, in acidic media as well as their visible spectra in aqueous 1 M HCl solutions (pH 1)

underpinning the color modulation of red wine aged in oak barrels. The remarkably efficient hemisynthesis of the flavano-ellagitannins **53**, **54**, **64** and **65** from the oak *C*-glucosidic ellagitannin vescalagin (**39**) in an acidic organic solution and in a wine model solution supports the assumption that similar condensation reactions should occur between **39** and a grape-derived anthocyanin pigment.

The first hemisynthesis of two first anthocyano-ellagitannins, **69** and **70**, was thus performed under conditions similar to those used for the formation of the flavano-ellagitannins **53**, **54**, **64** and **65** [71, 94] (Fig. 64.15). Both anthocyano-ellagitannins **69** and **70** also result from an acid-catalyzed nucleophilic substitution at the C-1 center of vescalagin (**39**) by either oenin (**72**) or its aglycone malvidin (**71**), respectively. The oenin (**72**) was selected as a model substrate for the anthocyanins present in red wine obtained from *Vitis vinifera* grape, since **72** is the main anthocyanin with concentrations ranging from 24 to 240 mg/L [74, 95].

From an oenological perspective, the fascinating aspect of the formation of these anthocyano-ellagitannins is their color compared to that of their respective anthocyan(id)ins precursors, as observed during their hemisynthesis, the initial bright red color progressively becoming deep purple. The visible absorption spectra of both anthocyano-ellagitannins **69** and **70** have been recorded in water at pH 1 and

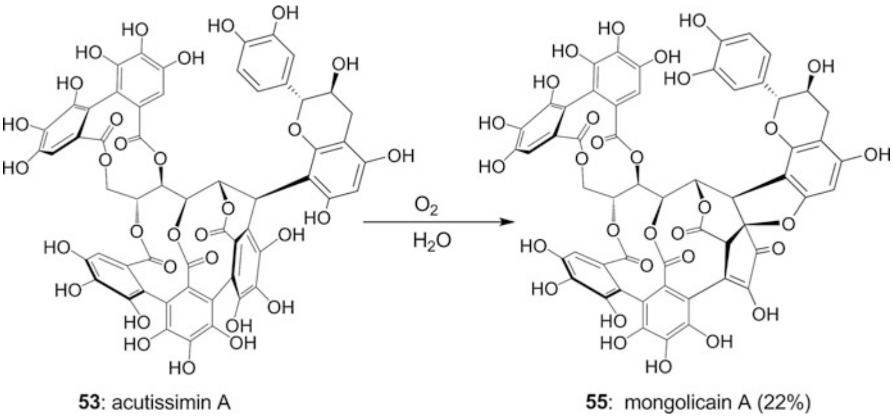


Fig. 64.16 Oxidative hemisynthesis of the mongolicain A (**55**) form the acutissimin A (**53**) in aqueous solution

revealed an important bathochromic shift of about 20 nm (Fig. 64.15). This bathochromic shift was initially recorded from aqueous solutions at pH 1 to ensure that the anthocyanidins moieties of **69** and **70** were entirely in their cationic flavylium forms, but the same shift was also observed in solutions at wine pH (i.e., 3.2). Such a bathochromic shift is generally observed when anthocyan(id)ins are covalently linked by their C-8 position to colorless phenolic compounds [96, 98]. This color modulation involving a chemical reaction with a *C*-glucosidic ellagitannin such as vescalagin (**39**), as well as the pH-dependent equilibrium of the resulting anthocyano-ellagitannins **69** and **70**, have been investigated in details and rationalized [94].

3.4 Effect of Oxidation on the Flavano-ellagitannin Profile

Red wine is a slow but continuously evolving mildly acidic and aerobic media in which the main polyphenolic constituents such as tannins and anthocyanins go through various oxidative transformations that generate a large variety of oxidized derivatives. Once dissolved in wine, oxygen is thus progressively and rather rapidly consumed by various substrates, such as phenolic acids (e.g., gallic acid, caftaric acid), anthocyanins, flavanols and proanthocyanidic oligomers [92, 99–101]. However, very few studies have addressed what happens to oak ellagitannins in this context at the molecular level, and the information available from the literature is rather contradictory [102, 103].

The formation of oxidation products derived from the acutissimin A (**53**), as well as their occurrence in red wine, have been investigated [82] (Fig. 64.16). The formation of mongolicain A (**55**) as the main product of the oxidation of acutissimin A (**53**) (22 % isolated yield) has been achieved in a de-ionized aqueous solution, as well as in a wine model solution, under atmospheric air pressure. The presence of

55 in a red wine aged in oak barrels, together with that of its precursor acutissimin A (**53**), were confirmed by mass spectrometry [82]. The mongolicain A (**55**) presents a connection between the catechin (**62**) moiety and the vescalagin (**39**) unit similar to that of its precursor **53**, as expected [52]. The oxidative formation of mongolicain A (**55**) in aqueous media implies a participation of both the vescalagin (**39**) and the catechin (**62**) moieties of acutissimin **53** to forge the characteristic *o,m*-hydroxyphenylcyclopentenon (HPCP) motif of mongolicain A (**55**). This characteristic motif is the result of a multi-step oxidative process that starts with the deshydrogenation of the NHTP-galloyl ring-I of the vescalagin moiety, leading to the formation of an α-hydroxy-*ortho*-quinone intermediate, which is followed by an intramolecular nucleophilic attack of the OH-7 of the catechin moiety of **53**. This nucleophilic addition is then followed by prototropic rearrangement, decarboxylation, and a second oxidative deshydrogenation finally leading to the formation of the HPCP motif of **55** [2, 72].

4 Biological and Pharmacological Activities

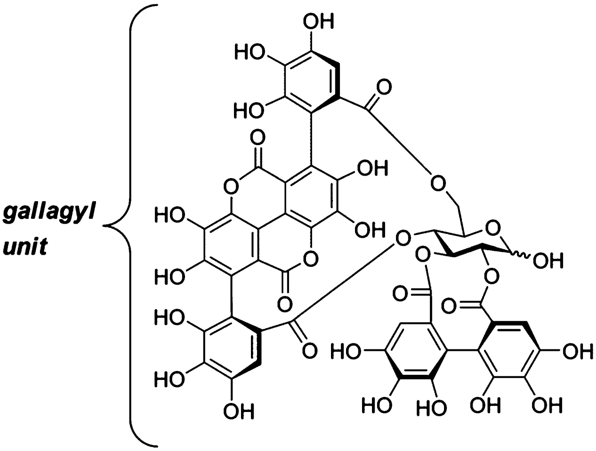
Hydrolysable tannins share with other polyphenols two fundamental physicochemical properties that are often cited as the origin of their health beneficial actions in traditional herbal remedies. The first one is their capability to scavenge reactive oxygen species. This antioxidant activity is commonly thought to enable *inter alia* the prevention of age-related health deteriorations such as carcinogenesis, neurodegenerative and cardiovascular diseases, including atherosclerosis. Their second property is their ability to participate in complexation processes with macromolecules, like proteins and polysaccharides. These associations of polyphenols with such biomolecules have been proposed to underlie beneficial health effects of herbal remedies against internal inflammations, hemorrhages, stomach disorders and other digestive problems [104, 105].

Besides these general activities based on chemical reactivity and structural features that are inherent in any polyphenolic substance (i.e., antioxidation and complexation) [1], ellagitannins are also capable of expressing remarkable biological activities such as potent antibacterial and antiviral activities, inhibition of mutagenicity of carcinogens and tumor promotion, as well as remarkable host-mediated antitumor effects [28, 106–108].

4.1 Hydrolysable Tannins in Folk Medicine

Most of the plants species used in folk medicine, especially in Asia, contain ellagitannins and gallotannins [108]. For example, one of the most popular medical plants in Japan (i.e., *Geranium thumbergii*) contains in its aerial parts an important amount of geraniin (**7**), which is used wildly as an antidiarrheic agent, as well as as a preventing agent against constipation. Both of these antagonist effects results from the action of geraniin (**7**), which expresses a protective action on the intestinal

Fig. 64.17 Punicalagin, a glucopyranosic monomeric ellagitannins found in *Punica granatum*



73: punicalagin

mucous membrane. The potent antioxidant and related activities of geraniin (**7**) may also participate in these effects [10]. Similarly, the leaves of *Mallotus japonica* that also yield geraniin (**7**), and mallotusinic acid (**19**), are also used for their stomach protective effects [27, 109, 110], like the fruits of *Trapa japonica*, which contain trapanin B (**26**) [27].

The *Agrimonia pilosa* herb, which contains agrimoniin (**21**), the first isolated dimeric ellagitannin, is used as an anti-cancer agent in Chinese medicine [10]. It is also used as an antidiarrheic and as a hemostatic agent in Japan. Agrimoniin (**21**) has also been subjected to detailed investigations related to its host-mediated antitumor activities [111, 112].

The root and fruit peels of *Punica granatum* (Punicaceae) have also been used in central and western Asia for their medicinal purposes (i.e., as a gargling liquid for throat diseases) [10]. Pomegranate fruits are consumed fresh and in processed form as juice, wines, flavors, and extracts. Commercial pomegranate juice has one of the highest antioxidant activities compared to other fruit juices, red wine, and green tea [113]. This can be attributed to its high content of polyphenols and in particular to its ellagitannins, the major ones being punicalagin (**73**, Fig. 64.17) [113, 114].

Plant extracts from *Quercus* and *Castanea* species containing acutissimins A (**53**) and B (**54**) are used to treat diseases such as gastritis and gastric ulcer, diarrhea, various inflammations (e.g., oral, genital and anal mucosa, skin) [115], and are also used as tonic and antitussive medicines [10, 51].

This short list of plants used in Asian folk medicines and furnishing ellagitannin-rich decoction and extracts are thus used to treat a large variety of diseases. To date, most of the investigations on ellagitannin-containing herbal medicines focus on the search of compounds capable of exhibiting antiviral or antitumor activity. The remarkable stability of most ellagitannins, despite the presence of several phenolic groups on their chemical structure, allows evaluation of their biological and pharmacological activities.

4.2 Antiviral Activities

Some monomeric and dimeric ellagitannins, as well as some gallotannins, also act as potent inhibitors of herpes simplex HSV-1 (i.e., labial herpes) and HSV-2 (i.e., genital herpes) infection [116]. In this study it has been observed that, among all of the studied compounds, tannins having galloyl groups or hexahydroxydiphenoyl groups (i.e., ellagitannins) express the most potent anti-HSV activity. Their 50 % effective doses (0.03–0.1 μg/mL) were by two to three orders of magnitude lower than their 50 % cytotoxic doses (>10 μg/mL). More recently, the monomeric *C*-glucosidic ellagitannins casuarinin (**38**) was also found to express antiviral activity against herpes simplex virus of type 2 (HSV-2) in plaque reduction assay. Interestingly, this antiherpetic activity of casuarinin (**38**) remained active even when it was added 12 h after infection, thus indicating that it affects some late event(s) of HSV-2 infection [117]. The NHTP-containing *C*-glucosidic ellagitannins extracted from oak wood such as vescalagin (**39**), castalagin (**40**), grandinin (**41**), roburin C (**45**), and roburin D (**46**) were also evaluated against four HSV-1 (labial herpes) and HSV-2 (genital herpes) strains, two of which were resistant to acyclovir (ACV), the reference drug against HSV-mediated diseases [43]. All five compounds effectively inhibited the replication of ACV-resistant mutants in cultured cells with activities at least 10^4 to 10^5 times higher than that of ACV.

Several ellagitannin monomers and dimers, as well as complex tannins show inhibition of HIV-induced cytopathic effects and HIV-specific antigen expression [118]. Interestingly, condensed tannins and simpler polyphenols including flavan-3-ol (e.g., epicatechin) also tested at the same time were found inactive [118]. Similarly, punicacortein C (**47**), a gallagyl-bearing ellagitannin, has been identified as a potent inhibitor of the human immunodeficiency virus (HIV) replication in infected H9 lymphocyte with little cytotoxicity [119].

4.3 Antimicrobial Activities

Gallotannins and ellagitannins also exhibit strong and very interesting antimicrobial activities, especially against some drug-resistant bacteria in the presence of some other antimicrobial agent. For example, *Helicobacter pyroli*, which is related to chronic gastritis, gastric ulcer, duodenal ulcer and also stomach cancer, was potently inhibited by tellimagrandin I (**6**), geraniin (**7**) and casuarictin (**57**). Interestingly, the ellagitannins tested were not toxic to *E. coli*, which is a normal inhabitant of the human intestinal microflora. Therefore, ellagitannins are potential agents that could be used to suppress *H. pylori* without affecting gastric epithelial cells and nonpathogenic intestinal bacteria. Moreover, corilagin and other ellagitannins also exhibit activities against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* [10, 49, 120].

4.4 Antitumoral Activities

Over the last decades, numerous studies related to inhibitory activities on tumor incidence and propagation have been performed ranging from the evaluation of complex plant or fruit extracts to the specific cytotoxicities activities of purified or hemisynthesized ellagitannins. Studies on in vitro activities on cancer cell lines of ellagitannin-rich berries such as strawberries [121–125], raspberries [122, 125, 126], cloudberries [125], and pomegranate [114, 127, 128] demonstrated inhibition of cell proliferation, induced apoptosis and cell cycle arrest in human colon, liver, lung breast or cervical cancer. Even if most of these studies were performed on crude extracts, it was suggested that the antiproliferative activity was mostly associated with the ellagitannin content of these plant extracts.

Several gallotannins, ellagitannins, and complex tannins have been evaluated for their cytotoxicities against a series of six different human tumor cell lines [129]. In this study, *C*-glucosidic ellagitannins, such as vescalagin (**39**), castalagin (**40**), grandinin (**41**) and acutissimin A (**53**), exhibited moderate but selective cytotoxicity against PRMI-7951 melanoma cells with IC_{50} values ranging from 0.1 to 0.8 μg/mL [130]. Moreover, antitumor promoting activity was also observed for monomeric and dimeric *C*-glucosidic complex ellagitannin such as stenophyllanin A (**51**) [131].

Similarly, several *C*-glucosidic ellagitannins, including complex tannins, were identified as potent in vitro inhibitors of human DNA topoisomerase II (top2), a current target of chemotherapeutic anticancer strategies [132]. Castalagin (**35**), vescalagin (**39**), casuarinin (**38**), punicacortein C (**40**), acutissimin A (**53**) and mongolicain A (**55**) were found to be from 100 to 250 times more potent than etoposide (VP-16), a clinically used drug against top2. More recently, the activity of some NHTP-bearing ellagitannins as potent anti-top2 has been evaluated. In addition to vescalagin (**39**), castalagin (**40**) and acutissimin A (**53**) previously investigated [132], three novel compounds, the epiacutissimins A/B (**64/65**), and ethylvescalagin (**66**) were tested using a standard kDNA decatenation assay. All of these *C*-glucosidic ellagitannins inhibited top2-mediated decatenation of kDNA at concentrations as low as 1 μM and showed a much higher activity than VP-16. Moreover, at 10 μM concentrations, an almost complete inhibition of top2-mediated decatenation was observed for the acutissimin A/B (**53/54**) and epiacutissimin A/B (**64/65**) (i.e., 85–97.5 % of inhibition) [2, 70, 71].

References

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Part VII

Phenolics: Methods of Analysis

Preparative Extraction and Separation of Phenolic Compounds

65

Winny Routray and Valérie Orsat

Contents

1 Introduction 2014
2 Phenolic Compounds 2015
3 Methods of Extraction 2017
3.1 Solvent Extraction 2017
3.2 Accelerated Solvent Extraction 2021
3.3 Supercritical Fluid Extraction 2022
3.4 Ultrasonic Extraction 2027
3.5 Microwave-Assisted Extraction (MAE) 2028
4 Methods of Separation 2032
4.1 Liquid-Liquid Phase Separation 2033
4.2 Chromatographic Separation/Purification Methods 2034
4.3 Solid-Phase Extraction (SPE) 2035
5 Conclusion 2037
References 2037

Abstract

Phenols are an important group of phytochemicals with significant health beneficial effects. Extraction of phenols from the biological sources is a growing field of interest and is an integrated part of analytical methods. Some of the common methods of extraction of phenolic compounds are solvent extraction, accelerated solvent extraction, supercritical fluid extraction, ultrasonic extraction, and microwave extraction. Separation is the next important step of analytical methods, which is done to separate the required phenolic components from the unwanted part of the extract. In case of phenols, methods such as

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liquid-liquid separation and chromatographic separation are applied; however, solid-phase extraction has been found to be one of the most popular methods of separation of phenolic compounds. All the above-mentioned different extraction and separation methods, along with some other analytical methods applied for phenols, have been discussed in detail in this chapter.

Keywords

Extraction • polyphenol • purification • separation • solvent

Abbreviations

ASE	Accelerated solvent extraction
EM	Electromagnetic
GC	Gas chromatography
HPLC	High-performance liquid chromatography
LDL	Low-density lipoprotein
MAE	Microwave-assisted extraction
SFE	Supercritical fluid extraction
SPE	Solid-phase extraction
*t*BHQ	tert-Butylhydroquinone
TLC	Thin-layer chromatography
UE	Ultrasonic extraction

1 Introduction

Phenols are an important group of naturally occurring compounds which have many particular and interesting properties. They have a potential to be a major contributor in the nutraceutical sector. According to the Bureau of Nutritional Sciences, of the Food Directorate of Health Canada [1], “A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease,” and phenolic compounds fulfill the criteria mentioned in the definition. Phenolic compounds are mainly popular as potential antioxidants, and their antioxidant effects have been found to generally increase with the amount of phenolic content of a food or sample [2–4]. They have been reported to have anticarcinogenic [5], antidiabetic, antibacterial, positive ocular, and gastric effects [6]. They have been also reported to have protective effects against cardiovascular disorders [5], hepatic damage, and neurodegenerative disorders [6] and help in the prevention of cytotoxic effects of oxidized low-density lipoprotein (LDL) and consequently atherosclerosis [7]. These health beneficial effects not only encourage the consumption of fresh fruits but also promote the utilization of food industry wastes as the source of phenolic compounds extract, which can be further utilized in enrichment of other food products and production of nutraceutical additive concentrates or powders.

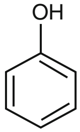


Fig. 65.1 Basic structure of phenol

Bioavailability, that is, the availability of the bioactive compounds at the cellular level to an organism when consumed, is one of the major factors which govern the effectiveness of different bioactive compounds. Bioavailability is affected by various factors which have been discussed in different reviews [8, 9]. It has been observed that sometimes either the pure form of a polyphenol is more helpful or combinations of some particular polyphenols are more effective. Hence, extraction of the bioactive compounds with efficient processes, proper separation with least losses, and encapsulation with appropriate methods are required. For all the studies related to bioactive compounds such as polyphenols, including characterization, quantification, or biomedical in vivo or in vitro studies, extraction is the fundamental method which can be divided into the various steps, mainly preparation, extraction, separation, and purification [10]. This chapter concentrates on the phenols and properties of phenol which affect the extraction efficiency. Other factors affecting the extraction efficiency have also been discussed along with the different methods of extraction. Different methods of separation applied for the polyphenols have also been discussed.

2 Phenolic Compounds

All compounds which have one or more hydroxyl groups attached directly to an aromatic ring are known as phenolic compounds. The entire group is based on the structure of phenol (Fig. 65.1) [11]. These are the widely distributed and important aromatic secondary metabolites present in plants. Fruits have been reported to have higher polyphenolics than vegetables [12]. Parts of the plants other than fruits have also been observed to be rich in polyphenols [13–16]. The industrial wastes obtained after the processing of fruits and vegetables have also been found to be a good source of polyphenols [17, 18].

There are several ways in which phenols have been categorized. Harborne and Simmonds categorized polyphenols based on the number of carbon atoms, which includes simple phenols (C_6); phenolic acids and related compounds (C_6—C_1); acetophenones and phenyl acetic acids (C_6—C_2); cinnamic acids, cinamyl aldehydes, and alcohols (C_6—C_3); coumarins, isocoumarins, and chromones (C_6—C_3); flavonoids (C_{15}); biflavonyls (C_{30}); stilbenes (C_6—C_2—C_6); benzophenones and xanthones (C_6—C_2—C_6); quinones (C_6, C_{10}, C_{14}); betacyanins (C_{18}); and lignans, lignins, tannins, and phlobaphenes (which are dimmers, oligomers, or polymers) [19]. Polyphenols have also been categorized by some researchers based on their distribution such as “common and less common” [11, 20] and “widely distributed, less widely distributed and polymers” [11, 21]. The detailed description regarding the classification of polyphenols and their properties have been discussed in other books such as “Plant Phenolics” edited by J.B. Harborne [22] and “Phenolic Compound Biochemistry” by Vermerris and Nicholson [11] and also in chapters of Part I of this book.

The structural differences between the different groups of polyphenols decide the chemical and physical properties of polyphenols. Some of these properties also affect the extraction efficiency of the phenols. Because of the presence of aromatic ring which is a common feature of polyphenols, the hydrogen of phenolic hydroxyl is labile, making phenols weak acids [11]. The level of acidity of phenols affects their properties related to extraction. As stated by Vermerris and Nicholson, pK_a is important and plays an important role in “the way certain polyphenolics are extracted” [11]. They have mentioned that, in case of a mixture of phenols, which include strong acids and weak acids, the addition of sodium carbonate (Na_2CO_3) or sodium bicarbonate ($NaHCO_3$) to the mixture allows separation of these phenols. “The weak base picks up the H^+ from the strong acids or the more acidic phenols” [11], which results in formation of phenolate salts (derived from the more acidic phenols after losing the H^+) of the phenols, soluble in water. “The less acidic phenols are not neutralized or do not lose a H^+ and tend to remain as free phenols and are not as soluble in water. These compounds can then be extracted with organic solvents” [11]. Hence, some of the common solvents used in the extraction of polyphenols include ethanol, methanol, and in some cases butanol as well.

Hydrogen bond formation can be considered as another important factor affecting the various aspects of extraction and separation of phenolic compounds. The intramolecular hydrogen bonding has been reported to reduce the reactivity of phenolic hydroxyl group, thus reducing the solubility in alcohol (which may affect the extraction efficiency, as methanol and ethanol are two of the most used solvents). It has also been mentioned by some authors that intermolecular hydrogen bonding makes it “difficult to purify phenolic compounds from mixtures, because of the interactions between different molecules including the solvent” [11].

Structural differences between different polyphenols decide their chemical properties and thus affect their extractability. Antioxidant effect, which is one of the most commonly studied effects of polyphenols, has been found to generally increase with the amount of polyphenols in the extracts [23–25]. However, the factors such as solvents and the reactivity and solubility of different polyphenols in different solvents also affect the extraction efficiency and antioxidant activity of the different polyphenols. Hence, the extraction processes and the solvents to be chosen are very much dependent on the type of polyphenols to be extracted, and the processes are chosen to obtain the highest amount of polyphenols possible. Study of the mechanisms of different extraction methods, their basic principles, and factors affecting the extraction processes can be helpful in deciding the best method for the extraction of different polyphenols from different sources.

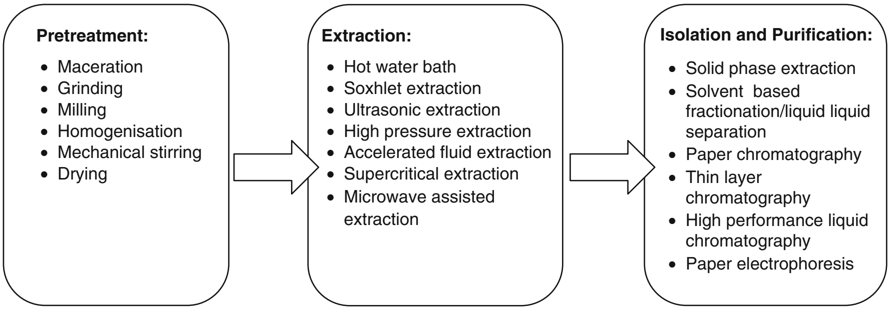

Pretreatment:
Maceration
Grinding
Milling
Homogenisation
Mechanical stirring
Drying
Extraction:
Hot water bath
Soxhlet extraction
Ultrasonic extraction
High pressure extraction
Accelerated fluid extraction
Supercritical extraction
Microwave assisted extraction
Isolation and Purification:
Solid phase extraction
Solvent based fractionation/liquid liquid separation
Paper chromatography
Thin layer chromatography
High performance liquid chromatography
Paper electrophoresis

Fig. 65.2 Different processing steps in the extraction of phenolics

3 Methods of Extraction

Extraction methods are selected mainly depending on the nature of the compounds, the importance of the facilities, and the end use of the manufactured substance. Several pretreatment steps have been also applied in different studies including drying, maceration, homogenization, grinding, and/or milling [10]. These processes increase the interaction between the solvent and the analyte (the polyphenols) during the step of extraction. Drying generally reduces the mass of the raw material required to be used for extraction. Pretreatment methods such as maceration and grinding lead to breakdown of cellular structure to increase the surface area of the biomaterial exposed for extraction and increase the amount of polyphenols extracted. The most common method of extraction is solvent extraction, and many of the above-mentioned treatments are combined with solvent extraction to optimize the final yield. The different pretreatment/preparation, extraction, and separation methods have been summarized in Fig. 65.2.

There are several other advanced methods of extraction for polyphenols which have been found to be more efficient methods than standard solvent extraction. These advanced methods of extraction are also based on solvent extraction, that is, they use the most suitable solvents with advanced processes to drive the extraction. With concerns regarding energy conservation, lower solvent consumption, and prevention of pollution, methods such as microwave extraction and ultrasonic extraction have been recently highly encouraged. Other efficient methods include accelerated solvent extraction (ASE), supercritical extraction, and pressurized fluid extraction. All these methods along with the operating factors affecting the extraction of polyphenols are discussed in the following subsections.

3.1 Solvent Extraction

Solvent extraction is the most common method of extraction. Solvent-based extraction method such as Soxhlet extraction and hot-water-bath extraction has been used

Table 65.1 Solvents used for phenolic compound extraction

I. Most common solvents used for polyphenolic extraction	
Methanol	
Ethanol	
Ethyl acetate	
Aqueous solutions of methanol and ethanol	
II. Other solvents used for some specific phenolic groups	
Phenolic groups	*Solvents*
Lipophilic flavonoids	Chloroform [22]
	Dichloromethane [22]
	Acetone [22]
Phenolic acids and their glycosides	Alcohol and water mixtures rather than pure alcohols [22]
Anthocyanins	Methanol and ethanol with different acids [30]
	Acetone with chloroform [10]
	Acetonitrile [10]
Tannins	0.5% vanillin solution in methanol containing 4% HCl (v/v) [31]
Stilbenes	Polar to mildly polar solvents (acetone to ethyl acetate) [22]
Flavones and flavonol glycosides	Ether, hexane, or dichloromethane [22]

Note: The numbers in the square brackets “[]” represent the respective references

quite often to extract different polyphenolic compounds. Soxhlet extraction has been used for extraction of polyphenolic compounds from solid samples and is a quite inexpensive method which needs little specialized training [26, 27]. It has been modified according to the modern requirements and has been combined with many other modern technologies to increase the efficiency of extraction. In the case of hot-water-bath extraction, the water bath is the medium of maintenance of the temperature required for extraction of the phytochemical from the matrix into the surrounding solvent in which it is dispersed. There are several factors affecting the efficiency of solvent-based extraction methods, which include nature and type of solvent, time of extraction, and temperature maintained during the extraction. Other methods such as vortexing followed by centrifugation [28] and mechanical stirring can also be used along with the solvent extraction to further increase the extraction efficiency by increasing the contact surface area, which is a controlling factor as well [29]. Another important factor affecting the extraction is the source of the compound, that is, the type of tissue from which it is extracted. For example, extraction of biflavonoids from plant tissues such as powdered heartwood is done using a long extraction process by Soxhlet reflux or using hot solvent for up to 40 h [22]. However, in other cases, the time taken can be shorter or longer than this.

Most common solvents for extraction of polyphenols from plant sources include methanol, ethanol, ethyl acetate, and their aqueous solvents. However, the choice of solvent depends on the type of polyphenol to be extracted (Table 65.1). Because of the high boiling point and economic availability, aqueous methanol has been reported as a popular choice [12]. Ethanol and aqueous ethanol solutions are chosen as solvents in most of the cases where the extracted phytochemical is to be used as a nutraceutical or as a clinical standard. However, methanol and ethanol are not appropriate solvents for the phenolics bound to insoluble carbohydrates and proteins [22]. Choice of extraction method depends on the type of phenolic compound to be extracted, and some solvents have been used more frequently for certain phenolic compounds. Lipophilic flavonoids are solvent extracted using chloroform, dichloromethane, and acetone [22]. In order to avoid contamination with lipid material or to avoid partial esterification, phenolic acids and their glycosides are preferably extracted with alcohol and water mixtures rather than pure alcohols. Also when the plant sample is devoid of lipophilic phenols, it is suggested to be pretreated with petroleum or hexane to remove chlorophyll, carotenoids, and sterols. “Test extractions with labelled compounds should always be performed in order to check the validity of the proposed procedure and, if required, correction factors should be employed” [22]. In case of anthocyanins, combinations of solvents such as methanol and ethanol with different acids [30], acetone with chloroform, and acetonitrile are used for extraction [10]. Hydrochloric acid is comparatively the most common acid used for anthocyanin extraction; however, to prevent the hydrolysis and change in the native form of the anthocyanins, weaker organic acids such as formic, citric, or tartaric acid, or small amount of highly volatile acids such as trifluoroacetic acid, or lower concentrations of hydrochloric acid are preferred [12]. For other phenolic compounds such as tannin, several other variations of solvents have been used for successful extraction, which include 0.5% vanillin solution in methanol containing 4% HCl (v/v) [31]; for stilbenes, polar to mildly polar solvents are used for extraction ranging from acetone to ethyl acetate. Flavones and flavonol glycosides need comparatively less polar solvents for extraction which can be ether, hexane, or dichloromethane, and soaking the biomaterial in the solvent for a short period of time has been found sufficient for the extraction of these compounds. Biflavonoids have been found to be soluble in all solvents except aliphatic hydrocarbons such as hexane [22].

Other than the type and nature of solvents, temperature is another important factor and is controlled depending on the nature of biomaterial from which the compound is to be extracted and also depending on the stability of the compound itself. For instance in a study, it was observed that temperatures higher than 100 °C lead to a decrease in the recoveries of catechin and epicatechin whereas caffeic acid was not affected [32]. High temperature increases solubility of compounds in general because of the increased intermolecular interactions within the solvent and the molecules of biomaterial, giving rise to relatively higher molecular motion with decreased viscosity of the solvent with increased temperature. Temperature may also aid in the breaking of analyte-matrix bond, thus increasing the extraction efficiency further. Also cell rupture occurs because of the increased pressure induced by high temperature inside the cellular structure leading to opening of cell matrix and increased interaction of the solvent with compounds present in the cell matrix [29]. However, it has been observed that the polyphenolic content increase in the extract is limited to a certain temperature during extraction, and beyond that, the concentration of several polyphenolic compounds decreases because of oxidation and degradation at higher temperature [32]. It has been reported that polyphenolic compounds with higher number of hydroxyl-type substituents are more prone to destruction at higher temperatures [33].

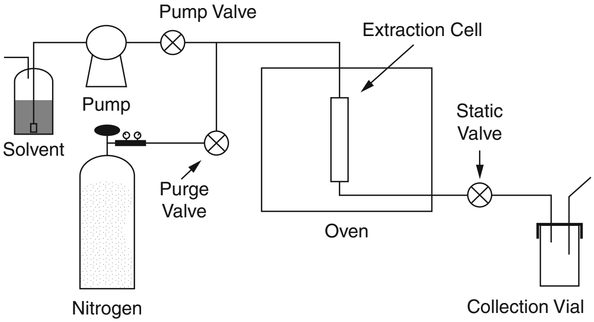
However, the destructive effect of temperature can be controlled by controlling the time of application of high temperature and pressure. With an increase in time up to an optimum time, the yield of extraction has been found to increase. However, after that optimum time, the extraction yield decreases if the extraction process still continues. There are certain cases of solvent extraction where high hydrostatic pressure is also applied, in high-pressure extraction. These are generally batch processes and are fast extraction processes, where low temperature can be maintained for extraction [34, 35]. Hence, the optimum time of extraction differs with the pressure level, the temperature of extraction, and the additional processes if applied, such as mechanical stirring, ultrasound, microwave, or vortexing. Another important factor is the mass of the sample used for extraction which will affect the volume of solvent and time of extraction. Solvent volume is an important factor. In extraction methods oriented toward the quantification of polyphenolic compounds, several iterations of the extraction process are applied to fully extract the compounds from the biological matrix. The iterations are conducted with fresh solvents to prevent solvent saturation with the compounds and maintain the extraction kinetics [29]. All the extraction parameters are interdependent; hence, all bioproduct extraction parameters have to be optimized for every extraction method.

The solvent extraction can be enhanced by utilizing carbohydrate-hydrolyzing enzymes, for instance pectinase, cellulase, hemicellulase, and many other enzymes, which can be used to disintegrate the plant cell-wall matrix leading to more efficient release of phenolic compounds [36]. Enzymatic-assisted extraction has been applied for superior extraction of polyphenolic compounds from different biomaterials, such as black currant juice press residues [37, 38], pigeon pea leaves [39], and grape pomace [40].

Light and oxygen are some of the normally present environmental factors during standard solvent extraction, and exposure of the polyphenolic extract to light and oxygen can lead to polyphenol oxidation [11]. "Formation of cross-linked structures as a result of exposure to light and oxygen" is known as autoxidation [11]. As mentioned by Vermerris and Nicholson, "under the influence of light, oxygen can abstract a proton," thus leading to the formation of radical [11]. The radical generated through oxidation can subsequently react with other radicals to form dimer. "Since the radical electron is delocalized, several structures can be formed depending on the precise location of the radical electrons at the time of the reaction" [11]. This is an important point to be considered carefully in case of extraction, separation, and extract storage methods. Oxidation can lead to formation of some unwanted complex compounds in the extract; hence, technological modifications in the solvent extraction have to be applied to prevent these degradation processes as well.

Considering all the factors (factors supporting and opposing the extraction) mentioned above, many new advanced processes of solvent extraction have been devised, which utilizes many modern technologies with specific advantages.

Fig. 65.3 Schematic of accelerated solvent extraction (Source: Richter et al. 1996 [45])



3.2 Accelerated Solvent Extraction

Accelerated solvent extraction (ASE^{TM}, which is a Dionex trademark) is a fast and efficient extraction method mainly operated with the control of two major factors affecting solvent extraction: temperature and pressure. Generally, high temperature and high pressure are applied during the process, and extraction is conducted for a very short period of time. However, there are certain exceptions where lower temperature levels are also applied [41], as for thermolabile polyphenolic compounds, lower temperature levels might be preferable [42]. This method of extraction is also known as pressurized liquid extraction, pressurized fluid extraction, or pressurized solvent extraction. The matrices of the samples of extraction are either solid or semisolid. Extraction systems for both single extraction and simultaneous multiple extractions are available, and the Dionex ASE^{TM} has been reported to be used in most of the reported studies [41, 43, 44]. According to the schematic of ASE described by Richter et al. (Fig. 65.3) [45], which is one of the earliest report on ASE, the system consists of a pump (for pumping solvent), extraction chamber, gas cylinder, collection vial, tubing, and three high-pressure valves (purge valve, static valve, and pump valve). As described by Camel [46], the extraction takes place in several steps. The extraction cell is filled with the sample and the liquid solvent, then they are pressurized to the level required, and the mixture is heated (the temperature is achieved in a short time because of the high pressure). Static extraction is carried out for a certain period of time, and in certain cases, a multiple number of extraction cycles are repeated [41]. The pressure is released after the static extraction phase, and the extract gets collected in the collection vials. The extraction cell is rinsed with fresh solvent to avoid losses of extracts. To ensure total extraction and avoid memory effects, the cell is purged with inert gas. The typical volume collected depends on the cell size [46]. To avoid oxidation of the analytes during the operation, the solvents used are degassed beforehand [41]. In certain cases to prevent clogging of the extraction cell with the biological matrix during extraction, layers of diatomaceous earth are added, and cellulose filters are provided at the bottom of the cell to avoid discharge of suspended particles into the collection vial [41, 47].

High pressure and high temperature accelerate the extraction process by ensuring increased contact of the polyphenolic compounds with the solvents [32]. Increased diffusion rate is observed because of the increased temperature leading to increased extraction kinetics with reduced viscosities. The pressure helps in rapid filling of the extraction cell and forcing the liquid solvent into the matrix; however, high-pressure application mainly helps in maintaining the solvent in liquid state even at very high temperatures [42, 46]. Even though high pressure and temperature are applied in this case, the solvents are still below their critical conditions. This process allows extraction of polyphenolics under an inert atmosphere and protection from light, which are some of the major advantages of the process as polyphenolic compounds are both prone to oxidation as well as light deterioration or degradation. The increased rate of extraction also helps in reducing the chance of certain negative reaction which takes place in the extract with increased time of exposure leading to the inactivity of certain compounds due to oxidation or isomerization [32].

Generally organic solvents are used in ASE in their pure form or as mixtures of several organic solvents with water. Several cases of ASE with the specific solvents (emphasizing some of the recent studies and some of the most cited studies), the corresponding temperature and pressure levels, and the different matrices have been summarized in Table 65.2. Water can also be used as an extraction solvent utilizing ASE apparatus for extraction of polyphenolic compounds [48]. This process using pressurized hot water or subcritical water (as mentioned before, the solvents are still under their critical conditions) with ASE apparatus is generally known as pressurized hot-water extraction or subcritical-water extraction [42, 49].

3.3 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is obtained at particular temperature and pressure combinations, which are higher than their critical values for a specific solvent where the solvent is said to be in its supercritical state. “The critical point of a pure substance is defined as the highest temperature and pressure at which the substance can exist in a vapour liquid equilibrium” [36]. Supercritical fluids possess certain properties similar to both gases and liquid. The density and dissolving power of these fluids depend on their density which can be adjusted by adjusting the pressure and temperature. At this high temperature and pressure, viscosity of the supercritical fluid is lower than that of the liquids. These solvents also have higher diffusion coefficient leading to higher mass transfer and efficient extraction [42, 46]. A supercritical fluid can be easily recovered as it is highly volatile. CO_2 is the most commonly used solvent (CO_2 is less expensive, nonflammable) in SFE, and it is a nontoxic solvent extraction method which generally does not leave any harmful residue in the extract (environmentally friendly process) [36]. The process uses high-pressure conditions and has the flexibility of changing temperature and pressure conditions to retain the supercritical condition of the solvent fluid. Boiling point increases with increase of pressure, and some solvents have very low boiling

Table 65.2 Examples of application of ASE for the extraction of polyphenolic compounds from biological matrices

Biological matrix	Solvent	Parameters (temperature/pressure)	Phenolic compound	References
Peel and pulp of Basque cider apple	Methanol	40 °C/6.895 MPa	Many polyphenolic compounds	Alonso-Salces et al. 2001 [41]
Different types of samples (dried and fresh) of apricot	Mixture of methanol, water (70:30 v/v) including 0.1 g of tert-butylhydroquinone (*t*BHQ) (best combination)	60 °C/10.342 MPa	17 polyphenols	Erdoğan and Erdemoğlu 2011 [44]
Different propolis samples (from honey-producing locations of Anatolia)	Ethanol:water: HCl; (70:25:5, v/v/v) containing 0.1% *t*BHQ	40 °C/10.342 MPa	Gallocatechin (GCT), catechin [50], epicatechin gallate (ECTG), caffeic acid (CA), chlorogenic acid (ChA)	Erdogan et al. 2011 [51]
[a]Spinach	Water AND 70% aqueous ethanol	<130 °C AND <150 °C/13.8 MPa	Flavonoids	Howard and Pandjaitan 2008 [52]
Dried ground sunbelt red grape pomace	10–70% Ethanol	40–120 °C/6.8 MPa	Anthocyanins	Monrad et al. 2010 [48]
Almond skin powder	30–90% aqueous methanol in 5% acetic acid	100 °C/10.342 MPa	Flavonoids and phenolic acids	Bolling et al. 2009 [53]
Pomegranate peels	Deionized water	40 °C/10.345 MPa	Different phenolic compounds	Çam and Hışıl 2010 [54]
Oak wood chips	Dichloromethane	150 °C/20 MPa	Lignin dimer derivatives	Vichi et al. 2007 [55]
Epimedium sagittatum	70% aqueous ethanol	120 °C/10.342 MPa	15 Flavonoids	Chen et al. 2007 [56]
Green parts of *Trifolium* L.	Methanol, 75% aqueous methanol, acetone, and acetone-water (75:25, v/v)	75–125 °C/10 MPa	Isoflavones	Grażyna 2009 [57]

[a] Two separate temperature conditions with two different solvents were found suitable; hence, the solvents and temperatures are written in sequence separated by AND

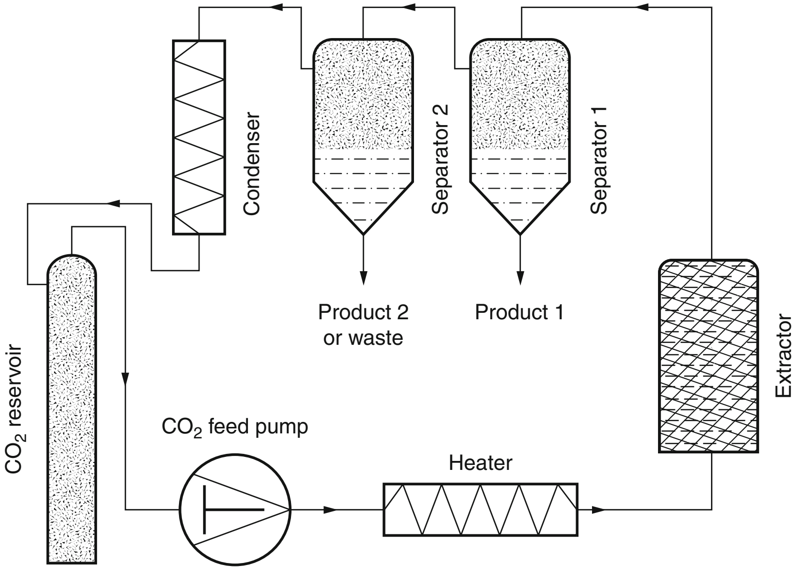


Fig. 65.4 Schematic diagram of a process-scale supercritical fluid extraction system (Source: Wang and Weller 2006 [42])

point temperatures at atmospheric pressure; the hazards caused due to boiling of the liquid can be avoided at elevated pressures. Low temperature level can be used for extraction which is helpful in case of thermolabile compounds, and for thermostable compounds with high boiling points, very high temperature can also be applied for extraction [36, 42, 46]. The dissolved phenolic compounds can be separated from the solvents by decreasing the density of supercritical solvents achieved through decreasing pressure. Hence, SFE can reduce or eliminate the requirement of concentration, which saves a lot of time and also minimizes the deteriorating effect on polyphenols, by reducing exposure of the compounds to high temperature and oxidation [42]. One of the earliest accounts of SFE is the extraction of resorcin derivatives from *Ginkgo biloba* fruits and leaves dated back to 1993 [58]. In the 1990s, most of the application of SFE was in the environmental sector and soil analysis. Since then SFE has been increasingly used in the food, plants, and waste utilization sector to optimize operating factors to obtain the highest yield of extractable polyphenolic content.

A SFE system basically consists of a high-pressure pump to deliver the fluid solvent at high pressure, an extraction cell provided with temperature controllers, and valves at both ends to maintain the high temperature and high pressure. Process scale extraction systems (Fig. 65.4) are provided with separators to separate the extracts and waste present in the solvent (unwanted compounds present in solvent other than extracts) from the solvent fluid which is generally CO_2 and condensers to condense CO_2 and reuse it in subsequent extraction processes. In case of SFE, sometimes the commercially manufactured extraction systems are used [59], and in certain cases, SFE extractor units are assembled in their respective laboratory units [60].

In the preparation of SFE samples, ground, dry materials are preferred for efficient extraction. During SFE, after loading the biological matrix in the extraction cell, it is pressurized with solvent fluid (generally supercritical CO_2) from the pump (modifiers, which are generally organic co-solvents, are added to the biological matrix that increases solubility of polar phenolic compounds, and then CO_2 is pressurized) [42, 46]. Both static extraction (while the mixture is in the extraction cell) and dynamic extraction take place for a relatively short period of time, after which the pressure and temperature levels of the solvent fluid with the extract and impurities are adjusted which leads to selective separation of extract from the solvent and then CO_2 is condensed in the condenser to be reused [42, 59]. Other solvents can be used, for example, in lignin extraction, supercritical fluids such as supercritical alcohols [61] and supercritical acetone have also been used. SFE has been applied successfully for lignin as it has been observed that the high-pressure application does not alter the wood structure [22]. Phenolic acids and their esters have been reported to be soluble in supercritical CO_2 [62]. Hydroxycinnamic acid and coumaric acid derivatives have been found to be partially soluble in supercritical CO_2 without co-solvent [36]. However, in the case of polar polyphenols, since CO_2 is nonpolar, modifiers are used for extraction. Organic co-solvents such as methanol, ethanol, and acetone, which are used as modifiers, increase the solubility of polar phenolic compounds (due to hydrogen bonding and dipole interaction [63]); however, the critical temperature is increased when a polar co-solvent is added, which implies that the process could be disadvantageous for thermolabile compounds [36, 42]. The modifiers can be added to the biological matrix in the extraction chamber through an additional pump [46]. Methanol appears to be the most used co-solvent; however, for nutraceutical purposes, ethanol is more acceptable. A mixture of modifiers can also be applied [42].

Along with factors such as the type of compound to be extracted, the nature of solvents, and the temperature, pressure, and time of extraction, the efficiency of SFE also depends on the nature of modifier applied. The dependence on so many factors increases the selectivity of SFE which is another beneficial characteristic property of SFE other than higher extraction kinetics. Selective extraction and fractionations are also possible because “the solubility of a chemical in a supercritical fluid can be manipulated by changing pressure and/or temperature of the fluid” [42]. However, depending on the quantity and nature of the modifier, its addition can also reduce the extraction selectivity, while more nontarget analytes can get co-extracted [46]. Yet there are many reports of successful extraction of polyphenols using a solvent with modifiers in case of SFE [10]. Some of the successful reports of different polyphenolic compounds extraction have been summarized in Table 65.3. Use of aqueous solution of methanol has been reported to provide higher yield than the case where pure solvents were added as modifiers individually [64]. Water increases the polarity further leading to efficient extraction of polar polyphenols [10]. The extract composition has been found to be varying with time, since less polar and low molecular weight compounds are more readily extracted while the extraction mechanism is generally controlled by internal diffusion [42]. Overall, different combinations of factors lead to the extraction of different compounds varying in terms of their quantities as well (Table 65.3).

Table 65.3 Examples of application of SFE for extraction of polyphenolic compounds from biological matrices

Biomaterial matrix	Solvent/ modifier	Temperature/ pressure	Phenolic compounds extracted	References
Arbutus unedo fruits	CO_2/19.7% ethanol[a]	48 °C/60 bar[a]	Total phenolic	Akay et al. 2011 [65]
Leaves of *Jatropha curcas* Linn.	CO_2/70% methanol	50 °C/20 MPa[a]	Gallic acid	Manpong et al. 2009 [60]
Vitis vinifera seeds	CO_2/20% or 15% ethanol	50 or 30 °C/ 300 bar, 30 °C/ 250 bar	Gallic acid, epigallocatechin, epigallocatechin gallate/catechin, epicatechin/ epicatechin gallate[b]	Yilmaz et al. 2011 [66]
Physalis peruviana L.	CO_2/4 and 5% ethanol	60 °C/400 bar	Flavonoids and phenol	Wu et al. 2006 [59]
Solid waste generated by industrial berry juice	CO_2/ethanol	60 °C/ 80–300 bar	Total phenolic content	Laroze et al. 2010 [67]
Guava seeds	CO_2/ethanol	40, 50, 60 °C/ 10, 20, 30 MPa	Total phenolic content	Castro-Vargas et al. 2010 [68]
Pine barks	CO_2/ethanol	60 °C/200 bar	Flavonoid content	Yesil-Celiktas et al. 2009 [69]
Roasted wheat germs	CO_2	58 °C/336 bar[a]	Total phenolic content	Gelmez et al. 2009 [62]
Phyllanthus niruri Linn.	CO_2/water; 30, 50, and 70% ethanol	60, 100 °C/100, 200 bar	Gallic acid, ellagic acid	Markom et al. 2007 [70]
Theobroma cacao hulls	CO_2	50 °C/100, 150, 200 bar	Phenolic compounds	Arlorio et al. 2005 [71]
Pistachio (*Pistachia vera*) hull	CO_2/methanol	35, 45, 55 °C/ 100, 200, 350 bar	Total phenolics	Goli et al. 2005 [72]
Wine industry by-product extract	CO_2/methanol[c]	45 °C/250, 150 or 100 bar	Polyphenols	Louli et al. 2004 [73]
Mellissa officinalis	CO_2/methanol	60 °C/40 MPa	Phenolic compounds	Ziaková and Brandšteterová 2002 [74]
Grape seeds	CO_2/methanol	313 K/20 MPa and above	Low polymerized proanthocyanidins, catechin, epicatechin, low molecular weight phenolics	Murga et al. 2000 [75]
Olive leaf samples	CO_2/10% methanol	100 °C/334 bar	Phenols	Le Floch et al. 1998 [76]

[a] The conditions were found optimum

[b] The different compounds are separated by "/" to indicate that different extraction combinations have been applied

[c] The co-solvent or modifier was applied only with the combination 250 bar and 45 °C

3.4 Ultrasonic Extraction

Ultrasonic extraction (UE) or sonication-assisted extraction is an inexpensive, simple, and efficient method of extraction. The extraction method involves sound waves with frequencies higher than 20 kHz, which are mechanical vibrations in all the states of matter. The principle behind sonication-assisted extraction involves the expansion and compression cycles in the medium through which these mechanical waves travel. “Expansion pulls molecules apart and compression pushes them together” [42]. The expansion process creates bubbles in the liquid medium and produces negative pressure. These bubbles form, grow, and collapse. “Close to a solid boundary, cavity collapse is asymmetric, which produces high-speed jets of liquid” [42]; these jets lead to a “strong impact on the solid surface” [42]. Ultrasonic baths and closed extractors fitted with ultrasonic horn transducer are used for UE. The ultrasonic machines (in certain cases, ultrasonic cleaner is used as well) are also equipped with pressure, temperature, and time control [77]. There are several ultrasonic horns available which operate at different power levels and frequencies. Application of ultrasonics can be continuous or pulsed as well [78].

The cavitation effect of an ultrasonic process leads to superficial tissue or cell-wall disruption in a short period of time, increase in solvent transfer into the biological matrix, and release of cellular content, consequently leading to higher surface mass transfer [42, 79]. Scanning electron micrographs of the biological cells treated with ultrasound have showed evidences of mechanical stress created by ultrasound leading to cell destruction [42]. These cavitation bubbles present hydrophobic surfaces within the extraction liquid, thus increasing the net hydrophobic character of the extraction medium which makes possible the extraction of polar compounds into less polar extraction media or even hydrophilic extraction media [79]. Along with factors such as nature of the biological compound to be extracted, nature of the biological matrix, solvent properties, particle size, temperature level, and time of extraction, there are several other factors which affect the extraction efficiency, such as frequency, ultrasonic power, distribution of ultrasound in the extraction cavity, and pressure. All the solvents which are used for solvent extraction can be used in the case of ultrasonic extraction as well. Ultrasound wave distribution in the extractor is usually not uniform, and the maximum ultrasound power is observed in the vicinity of the radiating surface of the ultrasonic horn (source). Ultrasonic intensity decreases with increase in distance from radiating surface and also decreases in the presence of solid particles. To avoid standing waves and for a more uniform distribution of the solid particles, shaking or agitation can also be used. Ultrasound also produces heat, so the temperature level should be checked for prevention of excess heating (which might lead to temperatures higher than the range required for extraction and lead to the degradation of temperature-sensitive compounds). Use of ultrasound decreases the time required for extraction, with the same solvent used without ultrasound. Furthermore, the extraction time should be controlled to prevent spoilage of the extract quality. The use of ultrasound also decreases the requirement of a temperature increase for the extraction; hence, it is suitable for thermolabile compounds, and it has been reported to increase product quality [42].

Application of ultrasonics has evolved with time, and one of the first applications of ultrasonics can be dated back to 1980s when lignin was extracted from spruce wood, decreasing the extraction time from 14 days to 14 h [80]. Ultrasonics has been applied for different phenolic compounds such as tannins [81], flavonoids [82–84], anthocyanins [85–87], and many others [78, 88–90]. Also it has been applied to a variety of materials to extract their polyphenolic content, such as medicinal plants like *Selaginella doederleinii* [91] and *Citrus aurantium* [92], food industry wastes such as litchi [93] and longan pericarp [94], pomegranate [78] and orange peel [89], and grape by-products [86].

3.5 Microwave-Assisted Extraction (MAE)

Microwaves are nonionizing electromagnetic waves located between the radio-frequency range at the lower frequency and infrared at the higher frequency in the electromagnetic spectrum, within the frequency band of 0.3–300 GHz. A frequency of 2,450 MHz is generally used in domestic microwave and for microwave-assisted extraction as well. A microwave apparatus consists of "microwave generators, waveguide transmission, resonant cavities, mode stirrers (only for multi-mode cavities), and a power supply" [95]. The microwave generators are special oscillator tubes such as magnetrons and klystrons which generate the microwaves, which are transported to the resonant cavities (ovens) by means of waveguides. Waveguides can be used as applicators of microwave, where "the material to be heated is introduced by wall slots and the waveguide is terminated by a matched load" [29]. The field maxima keep changing with time in this case; hence, this configuration is called a traveling-wave device. The other kind of configuration is the "standing wave device where the microwaves irradiate the slot arrays (that cut the wall currents) or horn antennas (specially formed open ends) of waveguides" [29, 96]. "The mode stirrer ensures homogeneous heating by distributing the microwave radiation between the different resonant modes of the cavity" [95, 96]. There can be two types of microwave ovens: monomode or multimode cavities. Monomode cavity is used in case of focused microwave ovens (Fig. 65.5); it generates a frequency which excites only one mode of resonance. In this case, as the distribution of field is known, sample can be placed at the maximum of the electric field. MAE systems can be closed systems or open systems as well. One of the earliest studies on MAE was by Ganzler et al. in 1986, regarding extraction of organic compounds from biological and soil samples [97]. A method of MAE of natural products was patented by Paré et al. in 1991 [98]. Since then there have been many modifications in the parameters, modeling, and methodology of MAE, which have made possible the use of many different categories of biomaterials and extraction of many different kinds of compounds (Table 65.4).

The distribution of electromagnetic (EM) energy in MAE systems is determined by Maxwell's equations with suitable boundary conditions defined by the makeup

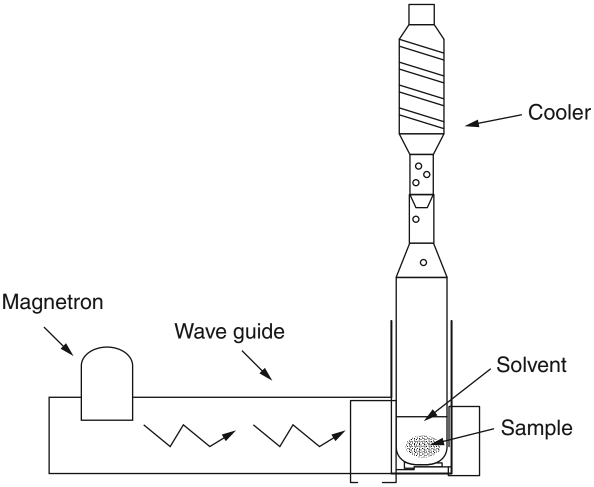


Fig. 65.5 Schematic diagram of an open focused-microwave system for extraction (Source: Kaufmann and Christen 2002 [119])

of the systems and the interfaces amid the treated biomaterial and remaining space. The dielectric properties of the biomaterial samples are the key property parameters of Maxwell's equations and therefore significantly affect the "efficiency of EM energy absorption into the materials, EM field distribution and conversion of EM energy into thermal energy within those biomaterials" [29]. The dielectric properties of a material can be explained in terms of the complex relative permittivity (ε* relative to that of free space) presented in the following equation:

$$\varepsilon^* = \varepsilon' - j\varepsilon'' \text{ where } \mathrm{j} = \sqrt{(-1)}$$

where ε′, the real part of the relationship, is the dielectric constant, reflecting the ability of the material to store electrical energy from an EM field, and ε″, the imaginary part, is the dielectric loss factor, influencing the conversion of the EM energy to thermal energy. The ratio of the real and imaginary parts of permittivity represents tangent of loss angle (δ), which is another important parameter expressed as

$$\tan \delta = \varepsilon''/\varepsilon'$$

which, in conjunction with the dielectric constant, determines the "attenuation of microwave power in a biological matrix" [29]. In the EM field, the amount of EM energy converted to thermal energy in biological material is proportional to the value of the loss factor ε″ (in case of biomaterial with similar dielectric constant) [109]. Hence, the dielectric properties, which depend on the characteristic properties of the biological sample such as composition of the biological matter, moisture content, and other physicochemical characters, determine the effectiveness of the microwave extraction method.

Table 65.4 Examples of application of MAE for extraction of polyphenolic compounds from biological matrices

Biomaterial	Solvent	Temperature/ power level	Time of application of microwave	Extract component	References
[a]Mandarin peels	Deionized water	400 W	3 min	Polyphenols	Ahmad and Langrish 2012 [99]
[a]*Agaricus blazei Murrill*	60% ethanol	500 W	3 extraction cycles (each 5 min)	Gallic acid, protocatechuic acid, catechin, myricetin, quercetin	Zhang et al. 2012 [100]
[a]Sweet potato (*Ipomoea batatas* (L.) Lam)	53% ethanol	302 W	123 s	Total phenolics	Song et al. 2011 [101]
[a]Fruit hulls of tea-oil tree (*Camellia oleifera*)	Water	76 °C	35 min	Total phenolics	Zhang et al. 2011 [102]
Parts of *Hippophae rhamnoides*	Ethanol	150 W	20 min	Phenolic components	Sharma et al. 2007 [103]
[a]*Radix puerariae*	65% ethanol	100 °C	2 min	14 phenolic compounds	Du et al. 2010 [104]
Peanut skin	30% ethanol	285 W	30 s	Total phenolics	Ballard et al. 2010 [105]
Mandarin peels	66% methanol	152 W	49 s	Phenolic acids	Hayat et al. 2009 [106]
[b]*Radix astragali*	90% ethanol	110 °C	25 min	Flavonoids	Xiao et al. 2008 [107]
Grape seed	Methanol or 90% methanol	150–300 W	20–200 s	Total phenolics	Hong et al. 2001 [108]

[a]The conditions are the optimum conditions
[b]The conditions mentioned yield the highest amount of extract component

In case of microwave heating, the energy transfer occurs via two mechanisms, specifically, by dipole rotation and ionic conduction, that is, by means of reversals of dipoles (which lead to molecular friction of the permanent dipoles within the material as they try to reorient themselves with the oscillating electric field of the microwave) and displacement of charged ions present in the solute as well as the solvent [110, 111]. The radiation frequency corresponds to the rotational motion of the molecules; in condensed matter, energy absorption immediately causes energy redistribution between molecules and heating of the surrounding medium [111]. During MAE, the disruption of weak hydrogen bonds initiated by the dipole rotation of the molecules also occurs with associated heat release [112].

Also there is migration of dissolved ions subsequently increasing penetration of the solvent into the biological matrix sample, thus leading to the collection of the target compounds [112]. To extract any biochemical component from a biological matrix, first it has to be removed from the matrix with cellular release into the surrounding solvent [113, 114]. During microwave application, a substantial amount of pressure builds up inside the biological matrix which modifies the physical properties of the matrix, improving the porosity and allowing better penetration of the solvent [115, 116]. Swelling occurs because of the pressure-building effect of the microwave, which generally forces the cells to split. During an image study of extraction of pectin from orange peel using microwave, it was observed that the destructive effect with microwave radiation was much more as compared with the traditional method. The final structure of pectin obtained after microwave treatment was particulate form similar to crystals and not cluster form as obtained in case with no microwave application [117]. Microwave application does not need any contact between the energy source and the target. MAE leads to rapid volumetric heating [110]. In case of uniform solvent composition (for extraction) and biomaterials with higher water content, MAE leads to uniform volumetric heating [110]. Along with lower solvent consumption and faster extraction, another major benefit of microwave-assisted extraction includes better extract quality [118].

"In general, the higher the dielectric constant and dielectric loss, the higher is the capacity of the solvent to absorb microwave energy" [29] and faster is the rate of heating of solvent with respect to the plant material, which eventually leads to a faster extraction rate. However, by combining different solvents, the solvent properties can be modified, and selectivity of the solvent for different compounds can be controlled or modified. A solvent combination with comparatively lower dielectric properties and higher dissolving power for the desirable extract components can be used for extraction of thermolabile compounds and ensuring that the solvent temperature remains low enough to cool down the solutes once they are extracted into the solvent combination. In this case, the microwave interacts more with the plant matrix with higher water content [119] leading to efficient release of the plant compounds in the cooler solvent. The extraction kinetics also depends on the solubility of the analytes in the surrounding solvent. In case of MAE, both polar [120] and nonpolar solvents [121] can be used; however, the rate of heating of polar solvents such as methanol and ethanol is generally higher than nonpolar solvents. Many polyphenolic compounds are soluble in polar solvents, though the solubility in different polar solvents depends on the number of hydroxyl groups and nature of the sugar molecule present [27]. Polyphenols vary in polarity, so solvents varying with level of polarities can also be used in MAE. Water can be used as a potential solvent because of its high polarity. Ionic solvents have also been tried for MAE of polyphenols. It has been observed that anions and cations of ionic liquids influence polyphenol extraction, and ionic liquids with electron-rich aromatic π-system enhance the yield [122]. Similar to the cases of other solvent extraction methods, MAE also depends on the concentration and nature of solvents, nature of the biochemical to be extracted, and temperature and pressure levels (MAE can be conducted at atmospheric pressure or under pressurized conditions), and the effect of various factors is the same as in other extraction methods. The microwave power level is another important factor which affects the extraction yield by affecting the rate of heating and the temperature attained depending on the nature of solvent (polarity) and time of application of microwave. Generally, the higher is the power level, the higher is the rate of heating of the material (solvent and biomaterial), leading to higher extraction yield with the higher rate of heating and subsequent increase of mass transfer and extraction kinetics. However, very high power levels should be avoided as they might lead to very high temperature level and loss of the desirable extract components and their properties. Hence, with the right combination of factors, MAE can be an efficient method of extraction of polyphenols.

All the extraction methods have their advantages and disadvantages when compared to one another. For specific compounds and the same extraction method, the selection of a particular solvent might be more effective than the other, and with a particular solvent in mind, a particular extraction method might be more helpful than the other. For example, it was observed that ASE at 85 °C with water as the solvent produced best results for flavonoid glycosides, whereas SFE using CO_2 and 10% ethanol as the modifier gave higher yields of flavonoid aglycones [123]. There are many other cases where different extraction methods when combined together become more efficient in terms of time and solvent consumption and extract quality. Methods such as high-pressure MAE [124] and vacuum MAE [125] have been successfully applied for extraction of different polyphenolic compounds from different biomaterials. However, some of the major drawbacks of the advanced methods of polyphenol extraction methods include high capital investment required for setting up the equipments (especially for methods such as ASE, SFE, and MAE) and energy costs as well.

4 Methods of Separation

Generally, extraction methods lead to production of crude extracts (non-phenolic components such as chlorophyll, terpenes, waxes, and other undesirable compounds are also extracted during the extraction process) from which the pure forms are further extracted or separated. Solvent extraction produces a mixture of many phenolic components which have to be separated from each other to be quantified or further utilized. Separation methods generally depend on the nature of extract and the end use of the desired separated component. It generally can be accounted as a purification method used to obtain a purer extract. It has typical application to characterize the compounds present in the extract using different analytical methods. It is also used for quantification of different components present in the extract or for the production of concentrated compounds to be marketed as a nutraceutical food. These can also be used as chemical standards or used for analysis of the biological effects during various animal tests or clinical trials. The separation and purification of different phenolic compounds is often required because some phenolic compounds interfere during the analytical procedures, thus affecting the characterization and quantification of different compounds present in an extract.

To separate the plant material from the extract, centrifugation for 15–20 min at 20,000–25,000 *g* is applied [126]. Ultrafiltration can also be used for separation of polyphenols; however, it depends on the particle size of the polyphenolic molecules. Generally, particles with a molecular weight of 3,000–100 kDa and higher can be separated using the regular ultrafiltration membranes [36].

The extraction solvents are then evaporated in the rotary evaporator under vacuum condition to concentrate the extract, to which other solvents can be added for purification and separation. Solubility of compounds in different solvents and pH are important criteria used for the separation and purification of polyphenolic compounds. Separation of phenolic compounds from the mixtures of compounds can be done using solvent separation methods and various chromatographic methods (including solid-phase extraction).

4.1 Liquid-Liquid Phase Separation

Liquid-liquid phase separation or solvent separation basically depends on the difference in solubility of the different compounds in different solvents. In case of crude extracts of flavonoids obtained after extraction using polar solvents such as methanol and water, many other impurities such as chlorophyll and lipids might be present. These can be removed by shaking with a small volume of petroleum ether or can be washed with hexane. The petroleum ether layer containing the green chlorophyll part is discarded, and the process is repeated until there is no green segment in the extract. The free sugars still present in the purified extract can be separated using ether (fraction containing glycosides) and ethyl acetate (fraction containing aglycones). However, if very polar glycosides are present in the aqueous residual part of the extract, they can be further purified using different methods of chromatography [22]. According to another liquid-liquid phase purification method, extraction of phenolic compounds from defatted/depigmented aqueous extract can be done with ethyl acetate to which ammonium sulfate (20%), metaphosphoric acid (2%), and ethanol (20%) are added, which are necessary for quantitative extraction of the phenolic compounds [126]. However, with this method, anthocyanins are not extracted as they are insoluble in ethyl acetate and remain in the aqueous phase. Acid and alkaline hydrolysis is also carried out for purification of polyphenolic compounds. The O-glycosides and esters of phenols and phenolic acids can be hydrolyzed by heating the concentrated aqueous-alcohol extract with reflux under N_2, using 2 M HCl; after cooling, the aglycones can be separated with suitable solvents such as diethyl ether and ethyl acetate. For alkaline hydrolysis, 2 M NaOH can be used under the similar conditions; however, before separation, acidification is applied where addition of borax to alkali prevents the oxidation of cinnamic acid and benzoic acid derivatives. The extract can be partitioned against different solvents of increasing polarity as well, such as petrol, n-hexane, chloroform, diethyl ether, ethyl acetate, and n-butanol, where generally most aglycones remain in the nonpolar fractions and glycosides and sugar esters are dissolved in more polar and aqueous fractions. In case of lignin analysis, different oxidation, reduction, and solvent-based methods have been summarized by Monteis in “Plant Phenolics” [22].

4.2 Chromatographic Separation/Purification Methods

Chromatographic methods are the separation methods where the constituents of a mobile phase (solvent or fluid carrying the extracted compounds) are separated using a stationary phase (a medium used in chromatography through which the solvent or fluid with extracted compounds is passed). There are many chromatographic methods which can be applied for separation of phenolic compounds; however, some are more prevalent than the others because of their convenience of use and efficiency and degree of utility in terms of end result. These methods can be preparative or analytical methods or both, and some methods are quantitative as well. The basic principles of certain methods and their utility in certain cases have been briefly discussed, which include paper chromatography, thin-layer chromatography, solid-phase extraction, high-performance liquid chromatography (HPLC), and gas chromatography (GC). Solid-phase extraction (SPE) is presented separately in the following subsection. It is one of the most widely used preparative separation methods.

Paper chromatography is used for separating and identifying the compounds present in the mobile phase depending on their solubility in the mobile phase and their adsorption capacity on the stationary phase. This technique is mostly used for phenolic compounds soluble in water (mainly flavonoid glycosides) [22]. It has been used for different phenolic compounds like gallic acid, vanillin, and hydroquinone [127]; and flavonoid pigments [128, 129]. Identification can be done using the tabular data on known compounds, by comparing the distance traveled by phenols on the stationary phase in a particular study with the tabular record on phenols summarized on the basis of previously observed interpretations. One of the greatest disadvantages of this method is the low resolving power and longer duration required for characterizing closely similar compounds [22]. Thin-layer chromatography (TLC) is another chromatographic method based on the difference in adsorption rate and movement up the plate due to capillary action of the phenolic components dissolved in different solvents. The plate (stationary phase) is generally glass, plastic, or aluminum coated with thin layers of silica gel or polyamide or microcrystalline cellulose. Less-hydrophilic phenolics have been reported to be successfully separated with TLC using silica gel and polyamide, and cellulose matrices have been found to be helpful for hydrophilic phenolic compounds. TLC has also been reported in many cases [130–132] for its flexibility in the choice of solvents, and TLC separation has been found to be more time efficient than paper chromatography [22]. However, SPE has been found to be a more helpful method than TLC for qualitative and quantitative separation of phenolic compounds and has the advantage of having the ability to be combined with HPLC for further analysis. For the successful characterization and quantification, more precise methods like HPLC have been found to be more helpful.

HPLC is a liquid chromatography method which has been applied for combined separation, quantification, and characterization of phenolic compounds. HPLC has been applied for different kinds of phenolic compounds [28, 133, 134], and one of the major advantages of HPLC is the simultaneous characterization and quantification of a variety of different compounds in a relatively short period of time based on their retention time and retention factor. Gas chromatography is another useful quantitative method used for simultaneous separation, detection, and quantification of many phenolic compounds, mostly restricted to volatile samples and for the phenolic compounds with low boiling point [22]. These methods are discussed in detail in ▶ Chap. 67, "Analytical Methods of Phenolic Compounds."

4.3 Solid-Phase Extraction (SPE)

SPE has been found to be an economical, easy preparative technique for separating the components from a mixture of different compounds. SPE is an efficient method which can be used for purification, concentration, and/or isolation. Polyphenols can be fractionated as neutral and acidic groups using methods based on solid-phase extraction or can be separated into anthocyanin-containing fraction and non-anthocyanin fraction, which makes the fraction easier to be analyzed [12]. Different protocols regarding use of SPE to separate different fractions of phenolic extract have been discussed in the "Handbook of Food Analytical Chemistry-Pigments, Colorants, Flavors, Texture, and Bioactive Food Components" [12]. During SPE, extracts are passed through different columns with different sorbents. Either the unwanted compounds from the extract get adsorbed on the sorbents which are removed by washing with different solvents in which they are soluble, or different desirable compounds are adsorbed which are fractionated by eluting using different solvents in definite sequences [135].

For SPE, different types of sorbents are used in the case of the separation of polyphenolic compounds such as alkylated silica gels principally C8 and C18 and also the combination of different cartridges with different sorbents such as C18 and quaternary amine [36]. The different cartridges have different characteristic features which make them more suitable for separation of certain phenolic compounds than others; however, the efficiency differs with the property of the different compounds as well. The potential of any SPE also depends on the solvents used for elution and the sequence of their application. Alkylated silica gel cartridges are hydrophobic in nature and have been suggested as a good method of separation for flavonoids and phenolic acids. With different C18 solid-phase support, the adsorption properties of the cartridges are modified [136], including physical properties, carbon loading, and pore size [126]. If the average pore size is high, then the retention capacity increases because of the "stronger interactions between the nonpolar surface and analyte" [126]. The capacity factor and carbon loading have been found to have a close linear relationship, which implies that higher carbon loading leads to greater retention of the ionized form of phenolic acids. Sep-Pak C18 cartridges have been used for the purification of phenolic compounds in many cases [12, 137–141]. Sephadex LH-20 is another liquid chromatography medium, which is beaded, cross-linked dextran, and has been hydroxypropylated for the matrix to be both hydrophilic and lipophilic in nature [142]. In this case, the separation is based on the extent of hydrogen bonding between the molecules and the matrix [126, 143], which also depends on the number of phenolic hydrogens per molecule. Sephadex LH-20 is useful for cleanup and had also been used for the separation of compounds such as condensed tannins [144], catechin [143], and fractionation of proanthocyanidin/condensed tannins [126] and also for other phenolic compounds [145]. Sephadex G-25 and G-50 which are generally used in gel filtration chromatography (which is a size-exclusion chromatography, based on size and/or shape of molecules) have also been used for purification of phenolic compounds and fractionation as well (especially proanthocyanidin or condensed tannins) [22, 146–148]. In this case, larger molecules which generally have less access to the pore volume elute before the smaller ones. Many other stationary phases are used for the separation, purification, and fractionation of phenolic compounds such as Amberlite XAD resins [149, 150], Serdolot PAD IV [149], and polyamide (for column chromatography) [151, 152]. In the case of polyamide column chromatography, hydrogen bonding between phenolic hydroxyl groups and amide has also been observed, because of which it has been applied for separating phenolic substances. During a comparative study regarding the applicability of different Amberlite XAD resins in flavonoid analysis, polystyrene resins were detected to be more appropriate for fractionation of flavonoids than polyacrylic resins. For fractionation and recovery of flavonoids from plant sources, Amberlite XAD-2 was reported to be more fitting [153].

Solvents used for elution in various cases are different, and the order of use also makes differences. For fractionation of polymeric proanthocyanidins, the solvent combination used is acetone with water which is applied for both Sephadex G-50 and LH-20 [22, 154]. The elution of solvents depends on the solubility of specific phenolic components in specific solvents. Anthocyanins are not soluble in ethyl acetate; however, many other polyphenolic compounds are soluble in ethyl acetate. Hence, in the protocol mentioned in the book "Handbook of Food Analytical Chemistry-Pigments, Colorants, Flavors, Texture, and Bioactive Food Components" for the separation of anthocyanins from the rest of the components in a polyphenolic extract, ethyl acetate is used for elution of the polyphenolic components other than anthocyanins, while the anthocyanins are eluted with acidic methanol [12]. pH is another important property affecting the separation of polyphenols through SPE. Phenolic acids have been reported to be "completely ionized at pH 7.0 and un-ionized at pH 2.0"; hence, this property is used as the basis for fractionation of neutral polyphenolics at pH 7.0 and acidic polyphenolics at pH 2.0, which helps in prevention of interference in-between polyphenolic compounds during HPLC [12]. In certain cases, combination of various SPE methods is used for the fractionation of the extracts, and then the purified extracts are further processed to be analyzed [140, 141, 150].

There are several analytical steps carried before SPE, and in some cases, SPE is followed by several analytical steps as well. Preconditioning of the matrices for achieving a certain pH level or a certain physical condition, washing of the cartridges with appropriate volumes of the solvent (e.g., 0.01 N aqueous HCl) to remove other unwanted components, and drying of cartridges with N_2 to prevent exposure to O_2 (leading to possible oxidation of polyphenols) are some of the important steps necessary to follow in a particular sequence [12]. After SPE with the proper solvents, the solvent containing the phenolic group of interest for further analysis is sometimes concentrated using rotary evaporator (preferably at temperature set at 40 °C or lower, keeping in mind the degradation temperature of different phenolic compounds). Overall success of SPE methods for the separation of polyphenols depends on the compound to be separated, nature of the other expected components of the extract, nature of solvent and the SPE matrix, and the overall order of the entire process, depending on the ultimate requirements.

5 Conclusion

Extraction of polyphenolic compounds is an important part of many analytical methods and in the production of phenolic concentrates for the nutraceutical industry. Standard solvent extraction method is still the most used extraction method; however, other advanced solvent-based extraction methods, such as ASE, SFE, UE, and MAE, are currently becoming more widely used extraction methods. With advancement of technologies and changing requirements, existing methods are improving, and new methods are being devised. Separation methods are an integrated part of the entire extraction process which contribute significantly in the improvement of the quality of the end product and increase in the rate of repeatability of the results. Because of the complexity of phenolic compounds, many separation methods have been applied, and for specific compounds, some protocols have been found to be more efficient. Solid-phase extraction method has been found to be the most applied separation method in case of phenolics and might retain its popularity for the time being, unless and until some other more efficient method of separation is devised. In future, we hope for the development of much more advanced methods which will contribute potentially in improving the polyphenolic compounds extraction further and increase the use of environmentally helpful processes, simultaneously making the extraction method simpler, less time consuming, and more accurate.

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Mass Spectrometric Detection of Phenolic Acids

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Contents

1 Introduction 2048
2 Plant Phenolic Compounds 2049
3 Isolation, Detection and Characterization of Phenolic Compounds 2051
4 Mass Spectrometry in Phenolic Acids Characterization 2052
4.1 GC-MS 2052
4.2 LC-MS/MS and LC-ESI-MS 2053
4.3 MALDI-TOF MS 2055
5 Conclusion 2057
References 2057

Abstract

Phenolics are an important class of natural products that have received immense interest for their remarkable biological activities. These are widely distributed in the plant kingdom. Nowadays, increasing attention is paid on rapid identification and characterization of phenolic acids from natural sources. This chapter particularly emphasizes on the diverse mass spectrometric application for the detection of phenolic acids, and several aspects related to fragmentation behavior of phenolic acids are discussed.

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Keywords
ESI • GC • HPLC • MALDI • MS • phenolic acids

Abbreviations

ESI	Electro spray ionization
GC	Gas chromatography
HPLC	High-performance liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry

1 Introduction

Crude plant extracts contain several hundreds of secondary metabolites of varying chemical nature and spectroscopic parameters. Several of these compounds are also present as conjugates within the cell and are difficult to separate from the tissue matrix. Extraction, chromatographic purification, or isolation is thus important for the detection, identification, and quantification of such compounds. Several chromatographic methods such as paper chromatography (PC), thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC) have been standardized for the purpose of isolation and quantification of secondary metabolites. These instruments when coupled to detectors, the most popular being the UV–vis detector for HPLC may detect a separated compound; however, identification depends on matching the retention time to that of a known standard compounds and pattern of UV–vis scan if a diode array detector is available largely limiting the process to previously known reported compounds. In addition to univariate identification, e.g., retention time in chromatography, and wavelength/frequency in spectrometry, the use of mass spectrometry is an excellent tool to define the chemical identification.

The first application of mass spectrometry to the analysis of biomolecules was reported in 1958 [1]. In 1989, Hans Dehmelt and Wolfgang Paul were awarded half of the Nobel Prize in Physics for the development of the ion trap technique in the 1950s and 1960s. Again, in 2002, the Nobel Prize in Chemistry was awarded to John Bennett Fenn for the development of electrospray ionization (ESI) and Koichi Tanaka for the development of soft laser desorption (SLD) and their application to biological macromolecules, especially proteins [2]. Mass spectrometry (MS) is an excellent analytical tool in modern techniques that measures the mass-to-charge (m/z) ratio of charged particles. Now, it is regularly used for determining masses of biomolecules, elemental composition of a sample or molecule, and elucidating the chemical structures of molecules, such as peptides and other chemical compounds [3].

The function of a mass spectrometry is divided into three major units: ionization source, mass analyzer, and detector. The method of sample introduction to the ionization source depends on the ionization procedure. Many ionization methods have been developed [4], and instruments are named accordingly. Each ionization procedure has its own advantages and disadvantages. The ionization techniques are atmospheric pressure chemical ionization (APCI), electron impact ionization (EI), chemical ionization (CI), thermospray ionization (TSP), field desorption ionization (FDI), fast atom bombardment (FAB), electrospray ionization (ESI), and matrix-assisted laser desorption ionization (MALDI). The ESI and MALDI ionization techniques have been widely used in biomolecules detection. The ionization procedure is involved in the formation of molecular ions; both positively and negatively charged ions are generated depending on the proton affinity of the sample. The ionization source chamber must be conducted in vacuum (atmospheric pressure 760 Torr) because ions are very reactive and short-lived. The pressure under which ions may be handled is roughly 10^{-5}–10^{-8} Torr (less than a billionth of an atmosphere). After ionization, it is separated by accelerating and focusing the ions in a beam, which is then bent by an external magnetic field. The ions are then detected electronically and analyzed in a computer-based algorithm.

The most intense ion is assigned an abundance of 100 and is referred as base peak. Most of the ions formed in a mass spectrometer have a single charge, so the m/z value is equivalent to mass itself. The highest-mass ion in a spectrum is normally considered to be the molecular ion, and lower-mass ions are fragments from the molecular ion. Fragmentation of molecular ion carries the significant message for structure and composition of a molecule. Taken into account, tandem mass spectrometry (MS/MS) is powerful tool than only MS to determine the accurate structural information and identification of biomolecules. In tandem mass spectrometry, the gas-phase ion is fragmented with different stages of mass analysis. There are different types of fragmentation and several methods of fragmentation of the ions have been reported [5]. The data acquisition in tandem mass spectrometry occur through different modes. Among them, product or daughter ion scan is particularly useful for providing structural information concerning phenolic acids. The first analyzer is used to select specified sample ions or molecular ions $[(M + H)^{+}$ or $(M-H)^{-}]$ arising from a particular component. Then, it passes into the collision cell and is bombarded by the gas molecules, resulting in generation of fragment ions. These fragment ions are separated according to their mass-to-charge ratios by the second analyzer. Fragment ions arise directly from the precursor ions and produce a fingerprint pattern specific to the compound.

2 Plant Phenolic Compounds

Phenolic compounds are widely distributed in the plant kingdom with approximately 8,000 characterized structures and represent a class of plant bioactive secondary metabolites having one or more hydroxyl groups attached to an aromatic ring (benzene), phenol being the structure on which the entire group is based (Fig. 66.1). They are weak acids due to the aromatic ring which renders the hydrogen of the phenolic hydroxyl labile. The phenolic compounds are generally found in the bound form as glycosides in the cytosol and lignins as attached to the cell wall.

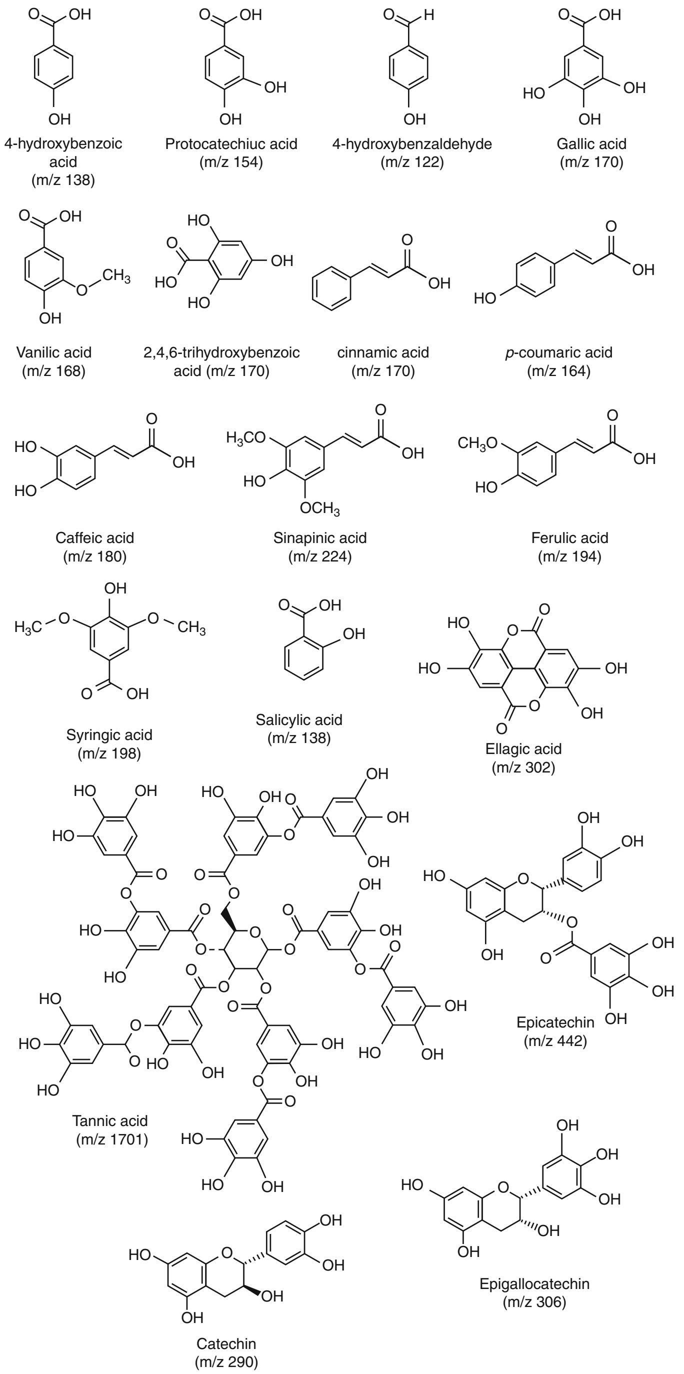
4-hydroxybenzoic acid (m/z 138)
Protocatechiuc acid (m/z 154)
4-hydroxybenzaldehyde (m/z 122)
Gallic acid (m/z 170)
Vanilic acid (m/z 168)
2,4,6-trihydroxybenzoic acid (m/z 170)
cinnamic acid (m/z 170)
p-coumaric acid (m/z 164)
Caffeic acid (m/z 180)
Sinapinic acid (m/z 224)
Ferulic acid (m/z 194)
Syringic acid (m/z 198)
Salicylic acid (m/z 138)
Ellagic acid (m/z 302)
Tannic acid (m/z 1701)
Epicatechin (m/z 442)
Epigallocatechin (m/z 306)
Catechin (m/z 290)

Fig. 66.1 Structural representation of some simple and compound phenolic acids

As the phenolics incorporate a diverse group of compounds, several classifications are available, namely:

- Swain and Bate-Smith [6] – "common" and "less common" categories.
- Harborne and Simmonds [7] – based on the number of carbon atoms in the molecule (C_6: simple phenolics; $C_6 - C_1$: phenolic acids and related compounds; $C_6 - C_2$ acetophenones and phenylacetic acid; $C_6 – C_3$: cinnamic acids, cinnamic aldehydes, cinnamyl alcohols, coumarins, isocoumarins and chormones; C_{15}: chalcones, aurones, dihydrochalcones; flavans, flavones, flavanonols, anthocyanidins, C_{30}: biflavonyls; $C_6 - C_1 - C_6$, $C_6 - C_2 - C_6$: benzophenones, xanthones, stilbene; $C_6 - C_6$, C_{10}, C_{14}: quinones, C_{18}: betacyanins; lignans, neolignans (dimmers and oligomers); lignin (polymers); tannins (oligomers, polymers).
- Ribereau-Gayon [8] grouped the phenolic compounds in three groups: widely distributed phenols, less widely distributed, polymers.

Three different metabolic pathways are known to be involved in the synthesis of different classes of phenolic compounds, namely, (1) ($C_6 - C_3$) phenylpropanoid derivatives produced by the shikimate/chorismate pathway; (2) side chain elongated phenylpropanoids, flavonoids ($C_6 - C_3 - C_6$), and few quinones synthesized by the acetate/malonate or polyketide pathway; and (3) the aromatic terpenoids synthesized through the acetate/mevalonate pathway.

Phenolic compounds play a major role in the physiology and biochemistry of plants and are involved in plant defense as phytoalexins, antifeedants, pollinator attractants, antioxidants, and protective agents against UV light and plant pigmentation to name a few. Exposure of the subepidermal layers of plant tissue to biotic and abiotic stress often triggers the production and accumulation of characteristic phenolic derivatives [9, 10]. Elicitations by pathogens or pathogenic determinants elevate the amount of phenolic compounds in plant tissues that are involved in defense signaling within plants and the development of systemic acquired resistance [11]. They are important contributors to plant growth and reproduction and determinants of sensory and nutrition quality in fruits and vegetables. Biosynthesis of phenolic compounds in chloroplasts is enhanced in the presence of light [12]. While, photoinhibition and several nutrition stress conditions may also trigger the accumulation of phenylpropanoids in some plants species [13, 14]. Simple phenolic acids are important determinants of nodule formation in plants [15, 16]. The antimicrobial [17, 18] and antioxidant properties of these compounds have been traditionally utilized in disease prevention and cure in humans and are active ingredients of several indigenous drugs. In the industry, polyphenols are utilized as food preservatives, natural colorants, and in the production of paints, paper, cosmetics, and nutraceuticals.

3 Isolation, Detection and Characterization of Phenolic Compounds

Chromatography techniques, most commonly TLC (thin layer chromatography) and HPLC (high-performance liquid chromatography), are the most popular methods for the separation and detection of phenolic compounds. In recent years, HPLC is coupled to DAD (diode array detector) and later mass spectrometers (MS) for better characterization. While DAD distinguishes the compounds based on their characteristic UV–vis spectrum, MS enables access to intact molecular as well as fragment ions enabling the determination of both molecular weight and structural features. However, with the increasing complexity and molecular mass of phenolic compounds and the presence several regio- and stereoisomers, there is a subsequent decrease in the resolution of chromatographic profiles.

4 Mass Spectrometry in Phenolic Acids Characterization

Several analytical tools coupled to mass spectrometers as GC-MS (gas chromatography) and LC-MS (liquid chromatography), including ICP-MS (inductively coupled argon plasma), SCF-MS (supercritical fluid), NMR-MS (nuclear magnetic resonance), and IR-MS (infrared-MS), are routinely used to determine the phenolic acids. With the development of soft ionization techniques, fast atom bombardment (FAB) [19] and plasma desorption (PD) [20], followed by the electrospray ionization (ESI) [21], have been used for phenolic acid analyses.

4.1 GC-MS

Gas chromatography (GC) has been widely utilized in the detection of phenolic compounds from plants. Most of the earlier detectors involved flame ionization, a major concern being the low volatility of phenolics [22–24]. Analysis of phenolic compounds by GC generally requires derivatization of the hydroxyl group (which increases the melting point by H-bond formation) to ethers or esters, although some reports are available on detection of underivatized acids [25]. Phenolic compounds are present in a complex matrix in the plant tissues and are moreover sometimes glycosylated increasing the difficulty of the derivatization process. However, the coupling of GC and MS increases the quantum of information that can be obtained from the analyte facilitating accurate identification [26]. Trialkylsilyl group is the most common derivative generated by covalently linking the alkyl-substituted silicon atom to the hydroxyl oxygen. The process is simple with both functional groups (acid and phenol) derivatized in the same step and is either mostly free of unwanted side products or the by-products are volatile and do not interfere with the analysis. Some derivatives may become unstable on exposure to moisture in air, which can be prevented by keeping them in an organic solvent after removal of solvent from the reaction step. Among the various commercially available silylating reagents are *N*,*O*-bis(trimethylsilyl)acetamide (BSA), *N*,*O*-bis (trimethylsilyl)trifluoroacetamide (BSTFA), and *N*-methyl-*N*-(trimethylsilyl) triflouroacetamide (MSTFA). Fused silica columns are popular for the analysis of phenolic compounds in GC with lengths varying from 25 to 30 m and inner dimensions from 0.25 to 0.5 mm. DB5 is the most common coating material used which contains 5 % phenyl silicon and 95 % methyl silicone. Gradient methods are

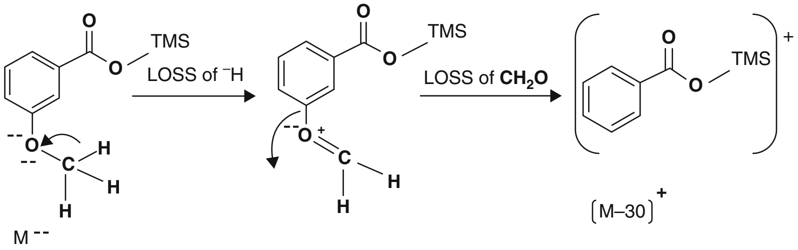

Fig. 66.2 Mechanism for the loss of formaldehyde and formation of ion [M-30]

preferred over isothermal methods with rate increments varying from 2 °C to 40 °C with the initial column temperature ranging from 40 °C to 140 °C.

In comparison to FID, MS performed in the electron ionization mode (EI), where electrons are removed from the analyte by an electron beam, has become the method of choice for GC separated samples. The high reproducibility of the method has made possible the construction of entire MA libraries such as NIST (www.nist.gov). The ionization voltage is normally set at 70 eV and spectra collected from *m*/*z* 39–650 in a continuous scanning mode. The cleavage patterns of several silylated phenolic isomers are dissimilar providing additional criteria of identification of complex compounds. Silylation of the hydroxyl groups generates distinct fragmentation patterns, and generally, the [$M^{+\bullet}$] is a prominent peak in the mass spectrum. Some of the phenolic compounds are exceptions such as gentisic acid which poses a substituent at the 2-position of the benzene ring. For these analytes, generation of the [M-15] fragment, loss of methyl group via alpha-cleavage is observed in both TMS ethers and esters and provides a base peak. In several analytes, like cinnamic acid (*m*/*z* 161), *p*-coumaric acid (*m*/*z* 249), and *p*-benzoic acid (*m*/*z* 223), TMS esters undergo subsequent fragmentation generating the [M-59] fragment. CO_2 is expelled subsequent to the loss of methyl group from the TMS group after rearrangement resulting in [M-59]. For derivatized carboxylic groups, loss of *O*TMS is also a common fragmentary pathway. The cleavage of the methoxy substituent of the phenyl ring yields the predominant [M-30] fragment (Fig. 66.2).

The base peak of sinapic acid and ferulic acid is produced by similar pattern of fragmentation. A [M-117] peak is generated for gallic, caffeic, and protocatechuic acids on fragmentation. Chemical ionization (CI) is also employed in some GC-MS systems where an electron beam is used to ionize a gas (methane or ammonia), which subsequently ionizes the analyte. This method gives more prominence to the molecular ion and results in less ionization.

4.2 LC-MS/MS and LC-ESI-MS

HPLC has been widely used for the separation and characterization of phenolic compounds, and reverse phase (RP) column is the most popular, and there are several reviews on the topic. RP-C_{18} is the preferred column with column diameter

Table 66.1 Identification of molecular ion and fragmented ions from different phenolic acids

Compounds	MW	$[M + H]^+$	MS/MS
4-Hydroxy benzoic acid	138	139	93, 97
3,5-Dihydroxybenzoic acid	154	155	80, 97, 108, 126, 136
4-Hydroxybenzaldehyde	122	123	57, 65, 71, 81, 92, 97, 107, 121
Gallic acid	170	171	85, 97, 101, 119, 131, 133, 147
Vanillic acid	168	179	
2,4,6-Trihydroxybenzoic acid	170	171	52, 69, 85, 126
Cinnamic acid	148	149	63, 73, 77, 103, 131, 135, 145
p-Coumaric acid	164	165	145, 119
Caffeic acid	180	181	61, 73, 117, 147
Sinapinic acid	224	225	112
Ferulic acid	194	195	91, 102, 117, 139, 145, 160, 175
Syringic acid	198	199	78, 89, 95, 106, 122
Salicylic acid	138	139	93, 137
Ellagic acid	302	303	78, 151, 190, 228, 246
Catechin	290	291	55, 68, 91, 111, 119, 123, 139, 147, 161
Epigallocatechin	306	307	289
Epicatechin gallate	442	443	289
Epigallocatechin gallate	458	459	289

ranging from 2.1 to 5 mm, particle size 3–5 μm, and column length 100–300 nm, although several smaller sized columns are also used currently. Acidic aqueous solvent systems comprising mainly methanol and acetonitrile with predominately linear gradients are employed. UV–vis with photodiode array (PDA) detection is a common method to detect phenolic acids which have characteristic spectrum at wavelengths from 190 to 380 nm.

Recently, a series of literature has been published on LC-MS/MS analysis of both simple/compound phenolic acids and flavonoids. The major advantage of LC-MS/MS is that simultaneously retention time and MS data could be consider for identification of natural phenolic acids. The common fragment for all simple phenolic acids in MS/MS spectra in negative mode is $[M–H–CO_2]^-$ formed by elimination of a carboxy group from deprotonated molecular ions. Several phenolic acids conjugate with glycosides that also produce some common fragmentation ions during MS/MS analysis. The cleavage of intact sugar and aglycon fragments in MS/MS revealed the nature of glycosides. LC-MS/MS analysis produces fragmented ions that are useful for correct chemical identity of the compounds. Identification is aided by comparisons with standard references or by previous reports. Table 66.1 lists a series of phenolic acids and their MS/MS fragmented patterns where few fragmented ions are specific to compound identity. LC-ESI-MS technique is straightforward than LC-MS/MS where fragmented ions are less in number. We have applied some standard phenolic acids in LC-ESI-MS, and their reference spectra are shown in Fig. 66.3.

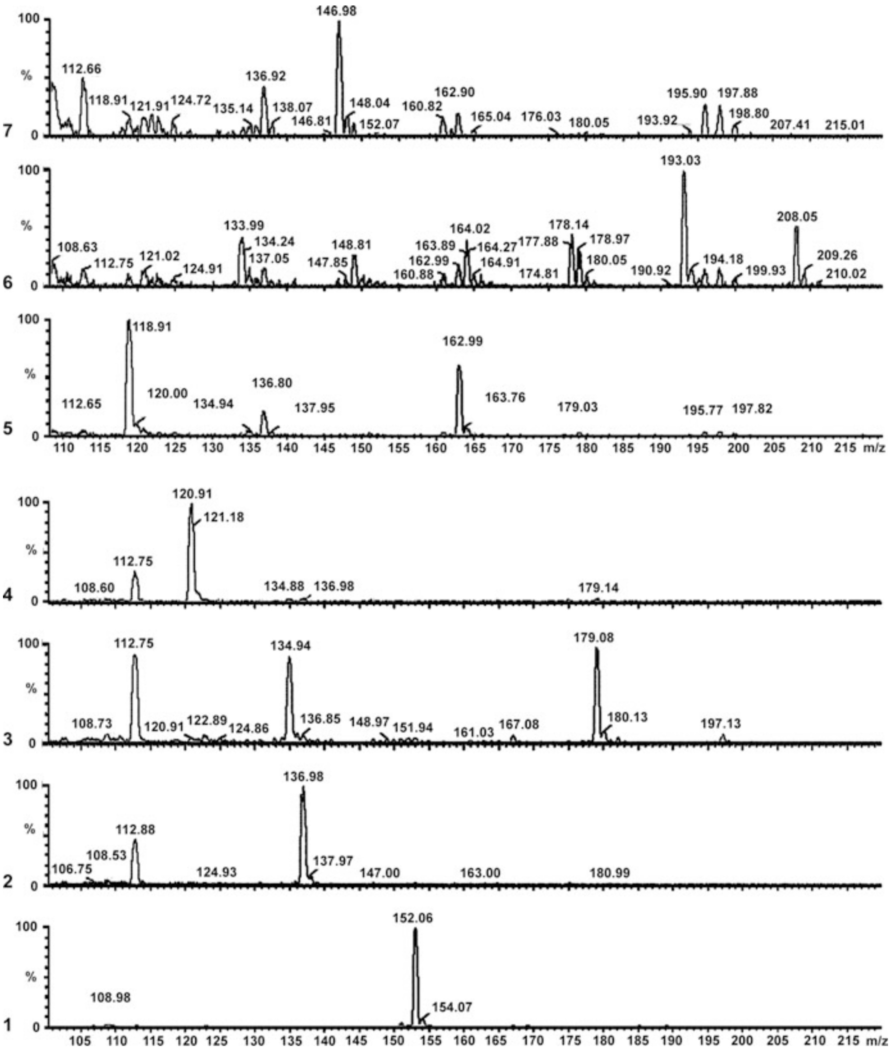

Fig. 66.3 ESI-MS spectrum of individual phenolic acids. Spectra of phenolic acids are marked as number *1* protocatechuic acid, *2* *p*-hydroxybenzoic acid, *3* caffeic acid, *4* *p*-hydroxybenzaldehyde, *5* *p*-coumaric acid, *6* ferulic acid, and *7* cinnamic acid

4.3 MALDI-TOF MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is also an efficient tool for large biomolecule analysis [27]. MALDI-TOF MS has several advantages over other methodologies, including speed of analysis, high sensitivity, and wide applicability with a good tolerance toward contaminants, and ability to analyze complex mixtures [28].

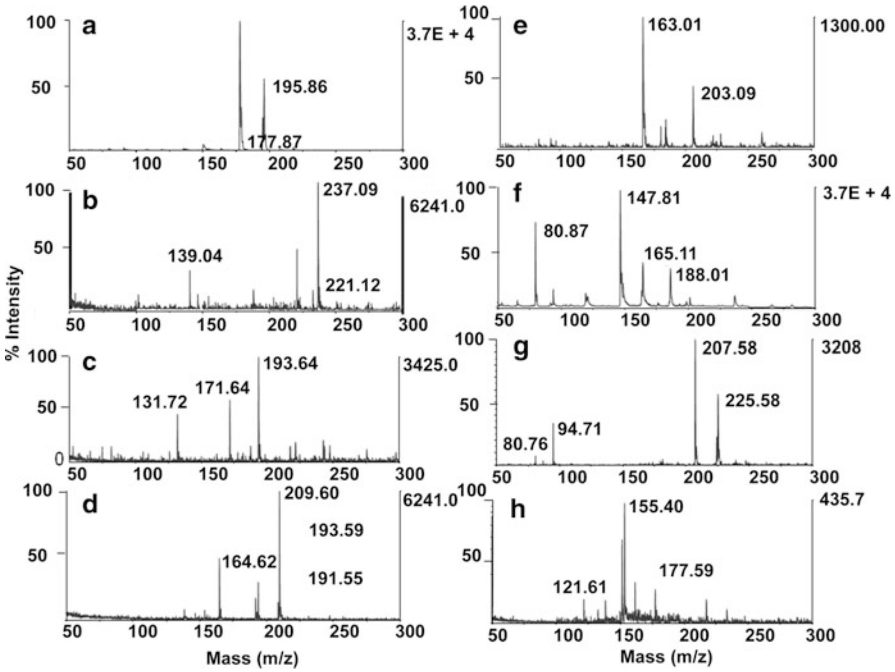

Fig. 66.4 Representative MALDI-TOF MS spectra of phenolic acids. MALDI-TOF MS spectra of phenolic acids are marked as number (**a**) ferulic acid, (**b**) syringic acid, (**c**) cinnamic acid, (**d**) gallic acid, (**e**) caffeic acid, (**f**) *p*-coumaric acid, (**g**) sinapic acid, (**h**) dihydroxybenzoic acid

Recently, the applications of MALDI-TOF MS have been extensively used in food analysis for oligomeric polyphenol and anthocyanin detection [29, 30]. Attempts have been made to characterize a series of phenolic acids using MALDI-TOF MS. Interestingly all the phenolic acids were ionized directly devoid of MALDI matrix. In general, matrices are used in MALDI technique to absorb the laser wavelength which cause codesorption of the analyte upon laser irradiation and promote analyte ionization. Phenolic acids absorb the nitrogen laser wavelength (337 nm) and cocrystallize on MALDI steel plate with solvent acetonitrile or methanol [31].

MALDI-TOF spectra of several standard phenolic acids were obtained in positive ion linear mode under a Voyager DE^{TM} Pro (Fig. 66.4). Spectra acquired from ferulic acid showed major ions at m/z 195 corresponding to the protonated $[M + H]^+$ mass of ferulic acid and with loss of one water molecule $[-H_2O]$ show a fragment ion of m/z 177. Direct MALDI-TOF analysis of syringic acid shows major ions at m/z 237 and m/z 221 which might be speculated in the addition of one potassium ion $[M + K]^+$ and one sodium ion $[M + Na]^+$, respectively. MALDI-TOF MS of polyflavons tend to favor an association with sodium $[M + Na]^+$ and potassium $[M + K]^+$ ions over the formation of a protonated molecular ion $[M + H]^+$ [32]. Another ion was observed at m/z 139 corresponding to the loss of $[CO_2 + CH_3]$ ion, i.e., [M- (CO_2 + CH_3)]$^+$. In case of cinnamic acid analysis, two major ions were observed as m/z 193 [M + 2Na] $^+$ and m/z 171 [M + Na]$^+$ indicating the successive addition of two sodium ions. One fragmented ion from cinnamic acid was observed at m/z 131 represents the loss of [OH] ion, [M-OH]$^+$. For gallic acid, the analysis is straightforward and it has the affinity to combine with both sodium and potassium ions, representing as m/z 193 [M + Na]$^+$ and m/z 209 [M + K]$^+$. Caffeic acid revealed only sodium ion addition as m/z 203 [M + Na]$^+$ and one fragmented ion, m/z 163, comes from the loss of one –OH ion. *Para*-coumaric acid is a naturally abundant simple phenolic acid which showed one major ion with addition to sodium, m/z 188 [M + Na]$^+$, and one molecular ion without addition of any metal ion, m/z 165 [M + H]$^+$. The fragmented ions from p-coumaric acid were m/z 147 and m/z 80 corresponding to the loss of one water molecule [M–H_2O]$^+$ and loss of [M–CO_2–C_3H_5]$^+$, respectively. Similarly, sinapic acid also showed ion, m/z 225 [M + H]$^+$, and fragmented ions were m/z 207 due to the loss of one water molecule. Dihydroxybenzoic acid represents the intact molecular ion m/z 155 [M + H]$^+$ and with addition with one sodium ion, m/z 178 [M + Na]$^+$. So, a general trend was observed for MALDI-TOF MS analysis of phenolic acids that addition of naturally occurring metal ions [Na or K] was universal. Moreover, the analysis is very simple and straightforward due to the absence of excessive fragmented ions.

5 Conclusion

Phenolic compounds are important bioactive constituents derived from plants and are active ingredients in traditional medicine, pharmaceuticals, and nutraceuticals. Extraction process for phenolic acids requires standardization as within plant tissue they can stay either in the soluble fraction or as conjugates with carbohydrates or linked to cell wall components. HPLC is by far the most popular method of separation. Mass spectrometers, especially LC-MS and MALDI-TOF, provide a viable rapid and accurate method of identification and characterization of the simple phenolic acids.

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Analytical Methods of Phenolic Compounds

67

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Contents

1 Introduction 2063
2 Sample Pretreatment 2063
3 Extraction of Polyphenols 2064
3.1 Extraction Conditions 2064
3.2 Extraction Methods 2068
4 Purification Methods 2071
5 Spectrophotometric Methods for Quantification of Polyphenols 2073
5.1 Determination of Total Phenolic Content 2074
5.2 Determination of Condensed Tannins 2074
5.3 Determination of the Total Content of Flavonoids and Anthocyanins 2075
6 Separation, Identification, and Analysis of Polyphenols 2075
6.1 Chromatographic Techniques 2076
6.2 Spectral Methods for Structure Elucidation 2080
References 2084

Abstract

Polyphenols are the most important phytochemicals found in a variety of fruits, vegetables, and plant-based foods. Currently there is an increasing interest for the separation, characterization, and valorification of these compounds due to their bioactive properties, health benefits, and potential use as natural antioxidants in foods, pharmaceuticals, and cosmetics.

The samples preparation and extraction of phenolics from source materials is the first step involved in their analysis. While colorimetric methods are used for determination of different classes of phenolics, chromatographic and

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spectrometric analyses are employed for the identification and quantification of individual compounds. This chapter provides a summary of background information and methodologies used for the analysis of phenolics in plant materials.

Key Words

Bioactive compounds • chromatographic techniques • extraction • purification • spectral techniques

Abbreviations

APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photo-ionization
ASE	Accelerated solvent extraction
BHT	Butylated hydroxytoluene
CE	Capillary electrophoresis
CI	Chemical ionization
DAD	Photodiode-array detector
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
EI	Electron impact
ESI	Electrospray ionization
FAB	Fast atom bombardment
GC	Gas chromatography
GPC	Gel permeation chromatography
HPLC	High-performance liquid chromatography
HSCCC	High-speed counter-current chromatography
IR	Infrared
LLE	Liquid–liquid extraction
MAE	Microwave-assisted extraction
MALDI–TOF	Matrix-assisted laser desorption/ionization time-of-flight
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion
MW	Molecular weight
NMR	Nuclear magnetic resonance spectroscopy
PLE	Pressurized liquid extraction
PVP	Polyvynil pyrrolidone
RP	Reverse phase
SCFs	Supercritical fluids
SEC	Size exclusion chromatography
SFE	Supercritical fluid extraction
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
THF	Tetrahydrofuran

TLC	Thin layer chromatography
UF	Ultrafiltration
UV	Ultraviolet

1 Introduction

Polyphenols are a widespread group of secondary metabolites found in plants derived from phenylalanine and tyrosine, and they are characterized by the presence of several phenol groups (i.e., aromatic rings with hydroxyls) [1]. Plant phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins [2, 3].

Polyphenols have received tremendous attention among nutritionists, food scientists, and consumers due to their biological activities and health-promoting benefits [2].

Phenolic compounds are important antioxidants, because of their high redox potentials. They act as reducing agents, hydrogen donors, singlet oxygen quenchers, and as metal-chelating agents [4].

These valuable properties of polyphenols are attracting a great deal of attention due to increasing evidence suggesting that they may prevent different chronic affections such as cancer, atherosclerosis, and neurological diseases [5].

On the other hand, polyphenols have many industrial applications, being used as natural colorants and preservatives in cosmetic or food industry [6].

Characterization of phenolic compounds requires methods that are comprehensive, rapid, and rich in spectral information.

The methodology used to analyze these compounds in plant-based materials, generally, includes a series of steps ranging from exhaustive solvent extraction, clean-up of extracts, and preconcentration procedures to simple filtration and centrifugation in liquid samples. After the extraction procedures, the phenolic compounds are characterized and quantified [7, 8]. Various complex analytical methods have been used for the determination of these compounds in natural samples and the most important of them are described in detail in this chapter.

2 Sample Pretreatment

Before the extraction and isolation of polyphenols, samples containing these compounds must be collected, preserved, and properly prepared.

According to their nature, the sources of phenolic compounds can be classified in liquid samples (beverages such as juice, tea, coffee, wine, biological fluids, etc.) and solid samples (fruits, vegetables, medicinal plants, by-products resulted from industrial processes, etc.).

The solid samples must be air-dried, frozen, sometimes with liquid nitrogen or freeze-dried [9–12], to remove the moisture that can significantly influence the extraction process. To improve the extraction process, the solid must be grounded and reduced to small particles to increase the contact surface between the raw material and the solvent and to reduce the extraction time because, theoretically, extraction time varies inversely with the square of the characteristic dimension of the solid particles.

Care must be taken to minimize the loss of compounds of interest during transportation, preparation, and preservation of the samples [2].

Heating and exposure to light and oxygen may affect the polyphenolic composition in many cases; therefore, high-temperature drying should be avoided as much as possible [13, 14].

The liquid samples requires less pretreatment than the solid ones, and in this case, they are usually freeze-dried, filtered, centrifuged, or directly submitted to the isolation step or sometimes directly submitted to the analysis [14, 15].

3 Extraction of Polyphenols

3.1 Extraction Conditions

The extraction of phenolic compounds from plant materials is considerably influenced by some factors such as: the nature of the sample, the chemical structure of polyphenols, the extraction method employed, the extraction agent involved, sample particle size, as well as the presence of interfering substances.

The chemical nature of plant phenolics varies from simple molecules (phenolic acids) to complex polyphenols such as flavonoids, anthocyanins, and highly polymerized substances that include varying proportions of phenolic acids, phenyl propanoids, anthocyanins, and tannins, among others [3, 16–18].

Therefore, natural extracts of plant materials are always a mixture of different classes of phenolics that are soluble in the solvent system used. Solubility of phenolic compounds is governed by the type of solvent (polarity) used, degree of polymerization of phenolics, as well as interaction of phenolics with other constituents and formation of insoluble complexes.

Thereby, there is no uniform or completely satisfactory procedure that is suitable for extraction of all phenolics or a specific class of phenolic substances in plant materials.

The chemical nature of polyphenols makes them relatively hydrophilic, thus free polyphenols, including aglycones, glycosides, and oligomers, are extracted using water, polar organic solvents such as methanol, ethanol, acetonitrile and acetone, or their mixtures with water [6, 19–21].

The most common extraction solvents, conditions, and sample pretreatments are reported in Table 67.1.

Also important is the pH of the extraction solvent. For polyphenols, most extractions are carried out under acidic conditions because they are generally

Table 67.1 Extraction conditions and pretreatment for different raw materials

			Sample pretreatment	
Matrix	Sample preparation	Extraction	Fractionation/Purification	References
Waste water olive oil	Stored at −20°C Filtration	Liquid–liquid microextraction with ethyl ether, *n*-hexane, dichloromethane, trichloromethane, and ethyl acetate	Directly submitted to analysis	[10]
Honey	Mixed with water, pH 2 Stirring Filtration	–	Directly submitted to analysis	[72]
Grapes	Grounded	Ethanol	Directly submitted to analysis	
Apples	Dried, grounded	Ethanol	Sephadex LH-20 column elution with aqueous ethanol 30% Solvent extraction: ethyl acetate, light petroleum Preparative HCCC elution with hexane/ethyl acetate/1% aqueous acetic acid	[73]
Propolis	None	Ethanol	Centrifugation	[74]
Cabbage leaves	Cut into small pieces	Accelerated solvent extraction with water/ethanol/formic acid	Filtration through a 0.45 mm Millex HV13 filter	[75]
Commercial juices	Filtered and diluted	–	Directly submitted to analysis	[7]
Mate leaves	None	Hydrochloric acid in 50% aqueous methanol acetone–water 70%	Centrifugation	[12]
Grapes skins	Flash frozen in liquid nitrogen ground	Acidified methanol	Centrifugation Filtration through a 0.45 μm membrane Dilution with 5% formic acid	[11]

(*continued*)

Table 67.1 (continued)

			Sample pretreatment	
Matrix	Sample preparation	Extraction	Fractionation/Purification	References
Blood orange	Squeezed in a centrifugal extractor	Macroporous adsorption resin	TSK gel Toyopearl HW-40S column eluted with 2 % formic acid acidified aqueous methanol	[76]
	Centrifugation	Weakly acidic-cation exchange resin		
	Stored at −18°C	Elution with acidic ethanol and ethyl acetate		
Eugenia myrtifolia Sims	Cut in small pieces	0.1% HCl in methanol	Filtration on a Buchner funnel, Concentrated, C-18 Sep-Pak cartridge eluted with ethyl acetate and methanol containing 0.01%HCl	[23]
Grape skins and seeds	Freeze-dried and grounded	Methanol/water/formic acid	Centrifugation, concentration, liquid–liquid extraction with ethyl acetate and diethyl ether	[9]
Hawthorn, common pine and skullcap	Grounded	Acetone	Fractogel Toyopearl HW-40S eluted with methanol	[54]
		Ethyl acetate	Sep-Pak C-18 eluted with acetonitrile and methanol	
		Methanol	Sephadex LH-20 eluted with 40 % isopropanol solution in 1% acetic acid	
Bark pine	Dried and grounded	98% ethanol under stirring	Liquid extraction with ethyl acetate	[13]
Tea	–	Acetone 75%	Polyamide column elution with methanol 2% acetic acid in methanol and *N*,*N*-dimethylformamide	[77]
Red and white grapes	Separation into skins and seeds	Acidic methanol	C18 Sep-Pak cartridges, AccuBond ENVI PS-DVB	[78]
		Stirring		
	Freeze-dried	Flushing with nitrogen	Elution with acidified MeOH (0.1 % Hcl, v/v) and ethyl acetate (pH 3, pH 7)	
	Grounded			

Almond skins	Milled	Acetone/water	Centrifugal UF membrane devices Amicon Ultra® from Millipore	[67]
	Lipid removal with hexane	Vortexed and sonicated		
Leaves of *Myrtus communis L.*	Quenched in liquid nitrogen stored at −80 °C, grounded in a mortar under liquid nitrogen.	70% EtOH for polyphenols	Liquid–solid extraction (LSE) on Extrelut cartridge	[79]
		Hexane for lipophilic compounds	Elution with hexane, ethyl acetate, acidic MeOH	
Mangosteen fruits	Lyophilized and stored in polyethylene bags at −20 °C	Aqueous methanol 80%	Diethyl ether	[27]
	Grounded			
Alcohol free beers	Acidified to pH 1.5	None	C18 Sep-Pak cartridge eluted by hexane, dichloromethane, ethyl acetate, acetonitrile, methanol, and acetone	[59]
Olive leaves	Dried	Acetone, ethanol, and their aqueous forms (10–90%,v/v)	Powdered silk fibroin elution with water and then with aqueous EtOH solutions	[80]
	Grounded			
Teucrium polium L.	Dried and powdered	Petroleum ether, chloroform, methanol, and water, by percolation successively	Silica gel 60 eluted with petroleum ether and petroleum ether: chloroform, chloroform, and chloroform: EtOAc, EtOAc, and EtOAc: MeOH	[81]
			Purified by TLC on silica gel 230–400 mesh and eluted with EtOAc: water: formic acid: glacial acetic acid	

more stable in low pH, and the acidic condition helps polyphenols to stay neutral, thus readily extracted into organic solvents [5, 6, 22, 23]. This is done using weak acid or low concentrations of a strong acid. High acid concentration can cause hydrolysis of glycosides or acylglycosides and thus may give different pictures of native polyphenol profiles [2].

Phenolic compounds may also exist as complexes with carbohydrates, proteins and other plant components, and some high-molecular weight phenolics, and their complexes may be quite insoluble [6].

For example, phenolic acids such as ferulic acid and lignans are often present as insoluble bound complexes, which are coupled to cell wall polymers through ester and glycosidic links and are not extractable by organic solvents. Bound phenolic acids are typically liberated by base hydrolysis, acid hydrolysis, or both [24]. The main step in most procedures involves base hydrolysis with NaOH ranging from 2 to 10 M, using incubation time up to 16 h, sometimes under nitrogen [25–27]. Following base hydrolysis, acid hydrolysis (2–4 M HCl) is sometimes performed to liberate bound phenolics that have not been previously hydrolyzed [19, 27–29].

Sometimes enzymatic hydrolysis-assisted extraction is performed to obtain aglycones using different types of commercial enzymes such as Ultraflo L, Viscozyme L, and α-Amylase or α-glucosidase [30, 31].

The hydrolysis step is also applied when the glycosylation patterns are extremely complex, and when standard reference materials of polyphenol glycosides are unavailable. The hydrolysis can simplify the chromatographic profile during separation, and aid quantification and structural identification of the polyphenols [1, 2, 22].

In conclusion, it must be mentioned that irrespective of the extraction method applied, the solvent selection is an important step based on several properties:

1. Solubility of the specific compounds in the solvent.
2. Recovery, if the solvent will be reused in subsequent extractions. If distillation or evaporation is used, the solvent should not form azeotropes and the latent heat of vaporization should be low.
3. Interfacial tension and viscosity. The solvent should be capable of wetting the solid matrix and penetrate through pores and capillaries. The viscosity should be sufficiently low so that it can flow easily. A low viscosity of the solvent also leads to low pressure drop and good heat and mass transfer.
4. Ideally, the solvent should have a good chemical and thermal stability and should be nontoxic, nonflammable, harmless to the environment, and cheap.
5. The solvent must have a high selectivity for the compounds that have to be extracted and the minimization of further substances recovery is required.

3.2 Extraction Methods

Many different extraction methods are available for different types of samples. For the majority of liquid samples, solvent extractions such as liquid–liquid partitioning and SPE (solid-phase extraction) are most frequently employed in the laboratory. The liquid extracts are sometimes partitioned with solvents such as ethyl acetate, diethyl ether, or chloroform, depending on the solubility of the target polyphenols [32–34].

The recovery of phenolic compounds from solid matrix is a more complex process where different methods can be applied, and it must be mentioned that in this case, the extraction of polyphenols requires special care, because they are easily oxidized and rapidly degraded by light and high temperatures. Antioxidants such as butylated hydroxytoluene (BHT) and ascorbic acid are often added to samples to avoid oxidation of the polyphenols when high temperatures are required [2, 8, 35].

Solid–liquid extraction is a multicomponent, multiphase, un-steady state mass transfer operation. It involves transfer of more than one chemical species – the solute – from a solid to a solvent. The solute is sometimes referred to as the extract, when the chemical species being recovered are ill defined, as occurs in the extraction of phytochemicals from plants [36, 37].

Conventional solvent extraction methods rely on conductive and convective processes to heat the product, and therefore, require longer extraction times. Longer extraction times increases the risk of degradation of thermolabile constituents.

Traditional Soxhlet extraction has been the most regarded among all the conventional extraction methods even though one of the major significant shortcomings of this method is the lengthy extraction time that can be 8, 16, 24 h, or more, which results in consumption of considerable time, solvent, and heat energy [8, 18, 22]. Additionally, the extended extraction times severely decrease sample throughput, which is a tremendous liability in terms of commercial applicability [38, 39].

Recently, different techniques such as ultrasound-assisted extraction, supercritical fluid extraction (SFE) – using either pure or modified CO_2 – and microwave-assisted extraction (MAE) have been applied. These techniques offer a better control over the extraction conditions and allow the extraction to be performed in shorter times and in a more selective way [40].

Thus, alternative novel extraction procedures are also able to reduce the solvent consumption, increase sample throughput, and improve analyte recovery.

Ultrasound-assisted extraction depends on the destructive effects of ultrasonic waves which accelerate the extraction process. The possible advantages of ultrasound in extraction are as follows:

- Intensification in mass transfer
- Cell disruption
- Enhanced penetration
- Capillary effects

Enhancement in mass transfer arises from creation of very high effective temperatures, which increase the solubility and diffusivity, and pressures that favor penetration and transport [41, 42].

During sonication, the cavitation process causes the swelling of cells or the breakdown of cell walls, which allow high diffusion rates across the cell wall in the first case or a simple washing out of the cell contents in the second [43, 44].

The extraction of bioactive compounds under microwaves irradiation is also one of the upcoming extraction techniques due to its inherent advantages (reduction in extraction time and solvent volume) over more traditional extraction techniques.

MAE uses microwave energy to directly heat the molecules within the material often in a matter of seconds. This procedure is suitable for the recovery of labile components such as polyphenols from different complex matrices. Using microwave irradiation, the degradative effects of high temperatures can be avoided. The energy of the microwaves facilitates rapid desorption of compounds from the matrices [45].

Recently, there has been an increasing interest in the use of supercritical fluid extraction (SFE) with carbon dioxide (CO_2) as a solvent. This process uses the properties of gases above their critical points to extract selective soluble components from a raw material. Carbon dioxide is an ideal solvent for the extraction of natural products because it is nontoxic, nonexplosive, readily available, and easy to remove from extracted products [3, 6]. SFE has the ability to use low temperatures, leading to less deterioration of the thermally labile components in the extract. In addition, SFE is typically carried out in the absence of air which also ensures minimal alteration of the active ingredients and preservation of the curative properties [46, 47]. SC CO_2 is generally efficient in the purification and fractionation of hydrophobic compounds, such as flavonoids and cinnamic acid derivatives from plant matrixes [49].

This process shows advantages over conventional ones, such as the possibility of continuous modulation of the solvent power/selectivity, elimination of polluting organic solvents, and the reduction of post-processing costs since there is no longer the need to eliminate solvents from the extracts [3, 6].

The only disadvantage of supercritical carbon dioxide is that it is often difficult to extract polar compounds, but this can be easily solved by using small amounts of organic modifiers [50].

SCFs have relatively low viscosity and high diffusivity, and they can penetrate into porous solid materials more effectively than liquid solvents and may render much faster mass transfer resulting in faster extractions. In SFE, a fresh fluid is continuously forced to flow through the samples; therefore, it can provide quantitative or complete extraction [38, 51]. Moreover, SFE may allow direct coupling with a chromatographic method, which can be a useful to extract and directly quantify the desired compounds.

In conclusion, to perform properly the extraction of polyphenols, the following important aspects must be kept in mind:

1. Sample pretreatment (drying, freeze-drying, grounding, etc.).
2. Solvent selection (selectivity, polarity, pH).
3. The use of an antioxidant if it is required.
4. The method employed must ensure a nearly complete recovery of phenolics, low consumption of time, solvent, and energy.
5. The removal of lipophilic fraction or other compounds that can interfere in the extraction process of the desired products.
6. The necessity to apply hydrolysis for the extraction of bound phenolics.

4 Purification Methods

The extraction methodologies described previously imply the co-extraction of nonphenolic substances such as sugars, organic acids, waxes, fats, terpenes, chlorophylls, and proteins [52, 53].

In this case, additional steps may be required to remove unwanted phenolics and nonphenolic substances.

The analytes isolation from liquid samples or liquid extracts obtained from a solid matrix is usually achieved by liquid–liquid extraction (LLE), SPE (solid-phase extraction), or size exclusion chromatography (SEC).

In the case of LLE, the most common solvents used are ethyl acetate and diethyl ether containing a small amount of an organic acid. LLE is usually directed at the isolation of aglycones. It must be mentioned that anthocyanins that are weakly soluble in ethyl acetate remain in the aqueous phase [6, 9, 10, 22, 54].

LLE can also be applied for the removal of lipophilic compounds using a suitable solvent, usually hexane [55].

Conventional LLE approach for sample pretreatment is tedious and time-consuming and requires great volumes of sample and organic solvents, which implies environmental contamination, risks for human health, and additional costs for residue treatment [14, 18].

Because of the importance of developing clean chemistry procedures, emerging methods for food matrices are based on solvent-free procedures. Newer sample preparation techniques offer automation and are clean, selective, rapid, and efficient.

Solid-phase extraction (SPE) is one of the most widely used technique for purification and preconcentration, mainly because of its ease of use and wide-ranging applicability and because it is a nonharmful environmentally preconcentration technique [56].

The most common stationary phases used for the separation of different classes of phenolics are silica gel, Sep-Pak C18, Toyopearl HW-40, Polyamide gel, Polystyrene resin, Fractogel TSK, Biogel P-2, Polyamide, Amberlite, and Sephadex LH-20 [57–64].

Solid-phase extraction with C18 cartridges has been extensively employed for the selective extraction of phenolics from complex mixtures. There are important differences between C18 solid-phase supports in relation to physical characteristics such as carbon loading and pore size. A higher average pore size increases the retention capacity due to stronger interactions between the nonpolar surface and the analyte. The SPE can be used for the isolation of neutral and acidic polyphenols. The cartridges must be preconditioned by sequentially passing through different solvents usually water/methanol adjusted to pH 7 for neutral phenolics and HCl for acidic phenolics. The samples are loaded onto the cartridge and washed with a suitable solvent in order to extract the desired compounds. The washing step is critical and should not provoke losses of compounds.

Extraction of phenolics can also be achieved using ion exchange cartridges. Phenolic acids, for example, as weakly acidic compounds are extracted most effectively by anion-exchange sorbents (quaternary amine columns) while polyvynil pyrrolidone (PVP) or PVP–silicagel has been employed to separate individual anthocyanins [65, 66].

In all cases, conditioning, washing, and elution of the cartridges or minicolumns have to be performed. Sequential elution with different solvents according to their polarity is useful for fractionation of different classes of phenolics. Solvents maybe slightly acidified to prevent ionization of the compounds.

SPME (Solid-phase microextraction) has recently been developed as a rapid, inexpensive, and solvent-free technique. This technique uses a fine fused silica fiber with a polymeric coating to extract organic compounds from their matrix. The main advantages of SPME are simplicity, high sensitivity, small sample volume, and lower cost per analysis. SPME techniques can be successfully applied for polar and nonpolar compounds in gas, liquid, and solid samples and can be easily coupled with various analytical instruments such as GC, GC–MS, HPLC, and LC–MS [22, 56].

Other significant aspects of SPME techniques are reproducibility, repeatability, fiber stability, and the possibility of quantitative determinations.

Prodanov et al. (2008) developed as an alternative to the above-mentioned purification methodologies, the membrane-based ultrafiltration (UF) technique which has not been much applied for the analytical purification of phenolics. Centrifugal membrane devices, suitable for the treatment of small volumes of samples, have shown good potential for separating high from low or middle size MM compounds in complex mixtures such as plant extracts. They permit fast and easy management of a large number of samples under mild operational conditions (low temperature and restricted exposure to oxygen and light). Complete recovery of both the permeate (filtrate) and the retentate (concentrate) from the centrifugal membranes is also possible [67].

Gel permeation chromatography using Sephadex LH-20 has been extensively used with good results for fractionating proanthocyanidins.

Generally, the GPC separation depends exclusively on the molecular size of the polymers, with no influence from other phenomena such as adsorption, partition, or ion exchange. It is, therefore, a fast, simple, and appropriate method for the study of the molecular weight (MW) distributions of polymeric molecules [68, 69].

On the other hand, the presence of hydroxyl groups in the tannin molecules can promote interactions by adsorption on the gel or intermolecular linkage by hydrogen bonding, which would lead to incorrect estimation of the MW. These phenomena can be minimized by the use of very polar mobile phases, such as dimethylsulfoxide (DMSO), dimethylformamide (DMF), or tetrahydrofuran (THF) [68–70]. Another procedure is to protect the hydroxyl groups as acetyl derivatives, methyl derivatives, or silyl derivatives [71].

The simultaneous use of an eluent such as THF and the acetyl derivatives provides better results for the GPC study of the MW distributions of tannins and other biopolymers, for example, lignins [68].

The methodologies used for the extraction, purification, separation, and analysis of phenolic compounds are briefly mentioned in Fig. 67.1.

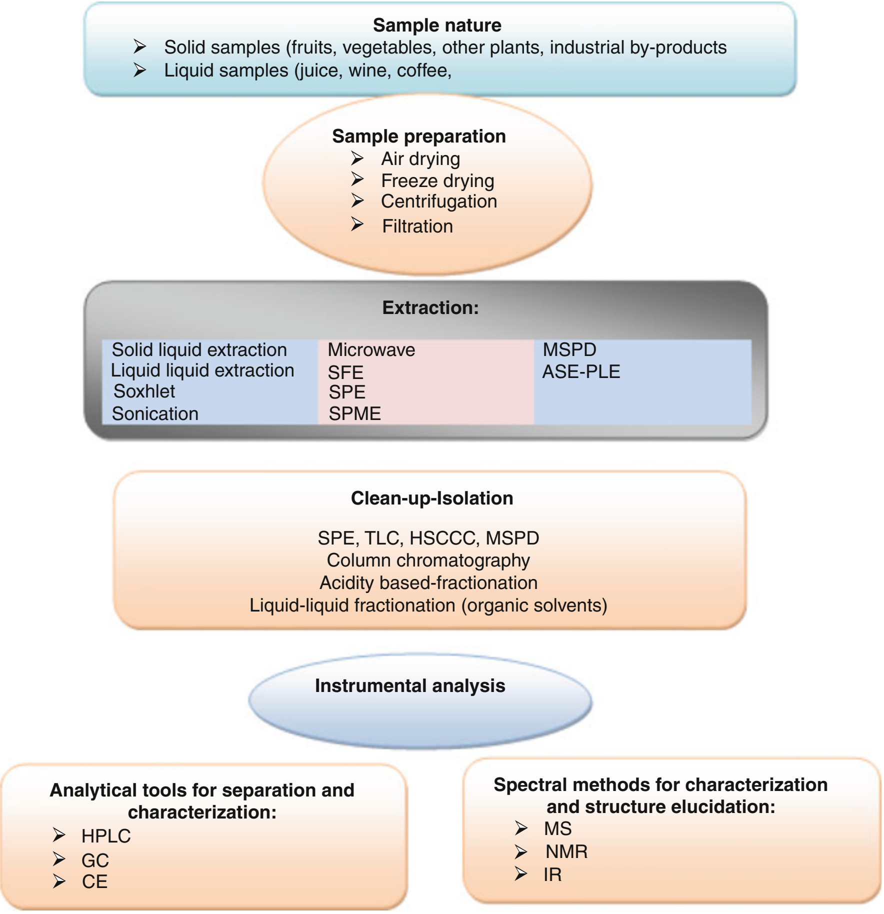
Sample nature
➢ Solid samples (fruits, vegetables, other plants, industrial by-products
➢ Liquid samples (juice, wine, coffee,
Sample preparation
➢ Air drying
➢ Freeze drying
➢ Centrifugation
➢ Filtration
Extraction:
Solid liquid extraction
Liquid liquid extraction
Soxhlet
Sonication
Microwave
SFE
SPE
SPME
MSPD
ASE-PLE
Clean-up-Isolation
SPE, TLC, HSCCC, MSPD
Column chromatography
Acidity based-fractionation
Liquid-liquid fractionation (organic solvents)
Instrumental analysis
Analytical tools for separation and characterization:
➢ HPLC
➢ GC
➢ CE
Spectral methods for characterization and structure elucidation:
➢ MS
➢ NMR
➢ IR

Fig. 67.1 Strategies for the isolation and characterization of phenolic compounds

5 Spectrophotometric Methods for Quantification of Polyphenols

Spectrophotometric methods especially colorimetric methods are nowadays intensively used for the quantification of different classes of polyphenols (total phenolic content, tannins content, flavonoids, and anthocyanins contents).

While these methods are rapid and simple, they lack the specificity for individual compounds. Interferences from nonpolyphenolic components of the sample can also cause false readings and thus lead to erroneous results. In order to accurately quantify and identify the individual polyphenols, these compounds must be firstly separated [2, 82].

5.1 Determination of Total Phenolic Content

The total phenolic content of plant extracts is usually determined by colorimetric method using Folin Ciocalteu reagent. This method entails oxidation of the phenolate ion coupled to the reduction of the phosphotungstic–phosphomolybdic reagent [6, 83, 84]. The chromophore produced is a blue phosphotungstic–phosphomolybdic complex which has a maximum absorption in the region of 750 nm [22, 85]. The total phenolic content is expressed as the number of equivalents of gallic acid, tannic acid, or caffeic acid used for the calibration curves.

The Prussian blue method is based on the reduction of Fe(III) to Fe(II) and the subsequent detection of Fe(II) by formation of the hexacyanoferrate(II) chelate (Prussian blue).

It is a popular method to quantify total phenolics because it is simple, rapid, and with little interferences by nonphenolic compounds. Critical for the successful use of this assay are temperature, pH, and order in which reagents are added, since this affects the formation of $Fe_4[Fe(CN)_6]_3$ (Prussian blue) [86, 87].

5.2 Determination of Condensed Tannins

Several methods have been described in the literature to quantify proanthocyanidins (condensed tannins); however, the most common are the acid-butanol assay and vanillin assay.

Acid-butanol assay uses an acid-catalyzed oxidative depolymerization of condensed tannins to yield red anthocyanidins. Proanthocyanidins depolymerize when treated in hot mineral acid to produce colored anthocyanidins with an absorbance maximum around 550 nm [88, 89]. The anthocyanidins result from the autoxidation of the carbocations initially formed by cleavage of the interflavonoid bond. Iron sulfate can be added to the reagent to accelerate the autoxidation reaction that increases color intensity and consistency of anthocyanidin yield [90].

Although the method is simple and gives good indication of the presence of condensed tannins, the color intensity can be affected by some factors such as the ratio of acid-butanol in the reaction mixture, chemical characteristics of tannins, and the amount of water in the samples [86, 91].

On the other hand, the choice of standards remains an unresolved issue because of the heterogeneity of condensed tannins and the lack of appropriate standards for their quantification. The use of internal standards obtained from the plant materials under study has been considered to minimize the problems derived from the use of inappropriate standards [87, 92, 93].

Despite the shortcomings outlined above, the acid-butanol assay remains the most commonly used method for determination of proanthocyanidins in foods [94].

The vanillin procedure involves reaction of an aromatic aldehyde, vanillin, with the meta-substituted ring of flavonols to yield a red adduct. Although the vanillin assay is used for the estimation of condensed tannins (proanthocyanidins), the reaction with vanillin is not specific particularly for tannins and any appropriately substituted flavonol reacts in this assay [22, 86, 93].

On the other hand, it must be taking into account that catechin, which is commonly used to standardize the vanillin reaction, also reacts with vanillin at different rates [95, 96]. Therefore, the use of internal standards is advisable.

Another method used for the estimation of total tannins content is based on the adsorption on casein. The total phenolic content of the sample is determined using Folin Ciocalteu reagent, before and after the adsorption process. The difference between absorbances of the initial and the final solution corresponds to the concentration of casein-adsorbed tannins in sample [97].

5.3 Determination of the Total Content of Flavonoids and Anthocyanins

The total content of flavonoids and flavones is usually determined using aluminum chloride as reagent. This method is based on the formation of a complex flavonoid-aluminum having the absorptivity maximum at 510 nm. Rutin or quercetin is used as a reference compound and total flavonoid content calculated from the calibration curve of the standard will be expressed as mg rutin equivalents [85, 98, 99].

The most common method employed for the determination of the anthocyanins content is the pH differential method which is generally accepted in international analytical practice [6, 100].

In an acidic medium, anthocyanins are found in the form of cation flavilia, which causes the bright red color of the solution. When the pH increases, the cation is transformed to carbinol and becomes colorless. It has been shown that the difference in absorption at the wavelengths 510 and 700 nm of solutions with $pH = 1$ and $pH = 4.5$ is proportionate to the anthocyanin content. Taking into consideration the small difference in the values of molar absorption of individual anthocyanins, it is possible to determine the total concentration of all the pigments relative to a single one, for example, cyanidin-3-glucoside.

6 Separation, Identification, and Analysis of Polyphenols

As discussed above, even though polyphenols share the common phenolic feature, due to the structural diversity, these phytochemicals vary significantly in their physicochemical properties [101–103]. Owing to the chemical complexity of polyphenols, the separation and quantification of these compounds remain as challenging as ever, despite the recent advances in new instrumentation. While it is nearly impossible to develop a protocol for all polyphenols, there are some advanced analytical techniques with large applicability in the analysis of phenolic compounds.

6.1 Chromatographic Techniques

6.1.1 Gas Chromatography (GC)

Gas liquid chromatography is a powerful technique designed to separate volatile compounds from a complex mixture. This technique uses the temperature of vaporization specific to each compound to separate them from a solution by passing the sample through a heated column where it is partitioned between an inert gas under pressure and a thin layer of nonvolatile liquid coated on an inert support inside the column [103].

The affinity of a particular molecule for the stationary phase determines the retention time of that constituent in the column. The molecules for each component of the sample will travel through the column at nearly the same rate and exit (elute) from the column within a narrow time band that is specific to that component. Thus, compounds with different retention times in the column are physically separated for presentation to a detector and analyzer. The instrument also includes a heated injection port to vaporize all volatile constituents of the sample and an oven to keep the constituents in gas form as they pass through the column.

Despite the numerous advantages of GC, its use in the separation of phenolic compounds is relatively restrictive because of the lack of volatility of most polyphenols.

For this reason, the vaporization capacity must be improved by replacing the hydroxyl groups by other chemical groups like trimethylsilyl groups before the injection onto GC [104, 105]. Therefore, before the injection onto GC, the ionic fraction obtained after polar extraction is derivatized by silylation in order to ensure a good vaporization of the sample and obtain volatile and thermostable derivatives [106, 107].

Although a very few studies based on gas chromatography (GC) have been proposed for the separation of different classes of phenolics, the use of GC with mass spectrometry (MS) detection provides important advantages because of the combination of the separation capabilities of GC and the power of MS as an identification and confirmation method [56].

6.1.2 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography is the most frequently used technique for the separation and characterization of polyphenols.

HPLC is a versatile and adaptable instrument with various advantages such as high selectivity, sensitivity, resolution, precision, and sample preservation [3, 108, 109].

HPLC is designed to separate compounds from complex mixtures based on polarity, solubility, and size properties of each compound. High-pressure liquid chromatography coupled with a photodiode-array detector (HPLC-DAD) provides extensive information on polyphenol structures [6, 110].

To obtain the identity of a polyphenol, the most common method is to compare the retention time of a particular compound with the standard. The photodiode-array detector DAD can collect UV/visible spectral data as the compounds are separated; thus, when a peak matches the retention time and the UV/Vis spectrum of a standard, it can be tentatively identified [2, 22, 111].

The quantitative determination involves analyzing a series of standards covering the concentration range of interest, and the sample analytes are quantified based on the calibration curves for each standard [112].

However, considering the lack of standards for some classes of polyphenols, especially, flavonoid glycosides and proanthocyanides, the HPLC analysis cannot be successfully applied.

To achieve a good separation, the flavonoid glycosides are usually hydrolyzed and converted to aglycones.

On the other hand, chiral separation of some flavonoids was also carried out by HPLC using a chemically bonded chiral stationary phase or more often by the addition of chiral additives in the mobile phase facilitating separation of enantiomers on conventional stationary phases [8, 22, 86]. For the separation of different polyphenols, chromatographic conditions involve almost exclusively the use of a RP C18 column and a binary solvent system containing acidified water using acetic acid, phosphoric acid, or sulfuric acid and a less polar solvent such as methanol or acetonitrile [113–121].

The separation of phenolic compounds can be achieved in isocratic mode when only one solvent is used or a mixture of solvents with constant composition or in gradient mode using two solvents whose composition varies throughout the analysis. According to the target polyphenols, different wavelengths' detection is required.

Several examples of HPLC conditions for polyphenols analysis are summarized in Table 67.2.

Although HPLC is one of the most used techniques, it presents some limitations in detection and quantification limit. Also in complex matrix such as crude plant extracts, identification based on the UV detection and the retention time of standards can lead to a wrong identification of the compounds.

In this context, hyphenation of LC with MS considerably improves the characterization of polyphenols, exploiting the power of separation of the HPLC and structure elucidation of MS.

Liquid chromatography–mass spectrometry (LC–MS) techniques are nowadays the best analytical approach to study polyphenols from different biological resources, and are the most effective tool in the study of the structure of phenolic compounds.

6.1.3 High-Speed Counter-Current Chromatography (HSCCC)

HSCCC is a continuous liquid–liquid partition chromatography with no solid support matrix, the stationary phase of which is retained in the separation columns by gravity and centrifugal force field. Therefore, HSCCC avoids the disadvantages arising from the interaction of samples with the solid support such as adsorption and denaturation of target products, tailing of solute peaks, and contamination [125–127].

In HSCCC, the stationary phase is immobilized by a centrifugal force and the solvent system used in the case of polyphenols is usually a mixture of ethyl acetate,

Table 67.2 HPLC condition for characterization of different classes of phenolic compounds

Compounds	Column	Solvent system	Detection	References
Benzenoid derivatives	Agilent	Methanol–water with 2 % acetic acid (1:1, v/v)	DAD 280, 360 nm	[73]
Flavonoids	Zorbax SB-C18 column (250 mm × 4.6 mm I.D., 5 µm)		MS-ESI	
Isoflavones	C8 reversed phase column	40 % acetonitrile–10 mM ammonium acetate	ESI-MRM	[113]
Flavonoids	150 mm × 4.6 mm stainless-steel column Synergi 4 µm Fusion-RP (C18)	0.25 % acetic acid and methanol	ESI/MS	[74]
Anthocyanins	1.8 lm Zorbax SB-C18, 100 × 2.1 mm	5 % formic acid aqueous solution – acetonitrile	ESI/MS and ESI/MS/MS	[75]
Phenolic acids, flavonoids, stilbenoids, procyanidins	Phenomenex Synergy Fusion RP100A 50 mm × 2 mm (2.5 µm) column	Water–acetonitrile, both with 0.1 % (v/v) formic acid	ESI-Qq-TOF	[7]
Hydroxycinnamoyl quinic acid esters and flavonol glycosides.	Nucleosil 120 C18 reversed phase column (250 × 4.6 mm, 5 µm	1% formic acid in deionized Water–acetonitrile	DAD 280, 360 nm MS-ESI	[12]
Anthocyanins	Agilent Zorbax SB-C18 analytical (5 µm, 250 mm × 3.6 mm)	5 % formic acid in water – methanol/ acetonitrile/water (33:60:7 v/v/v)	DAD 520 ESI	[11]
Polyphenols, flavonoids, phenolic acids	C18 SunFire ™ 4.6 mm × 150 mm, 5 µm	Methanol–acetic acid: water (1:99)	327–370	[122]
Hydroxybenzoic acid derivatives,flavon-3-ols, dihydrochalcones, hydroxycinnamic acid derivatives, flavonols, anthocyanins	C18 Phenomenex®Luna 250 mm × 4.6 mm, 5 µm	6 % acetic acid in 2 mM sodium acetate–acetonitrile	280, 320, 360, 520	[123]

Phenolic acids	C18	0.1 % formic acid–100 % methanol	DAD 270, 325	[25]
	Phenomenex Gemini			
	4.6 mm × 150 mm, 5 μm		ESI MS	
Anthocyanins, flavonols	Mediterranean Sea $_{18}$	5 % formic acid in water–methanol	520,360	[124]
	4.6 mm × 250 mm, 5 μm		ESI MS/MS	
Procyanidins	C18 Hypersil ODS Supelco	0.5 % acetic acid Milli-Q water solution – methanol	DAD 280	[13]
	250 mm × 4.6 mm, 5 μm			
Procyanidins, flavone	RP-18 column	2.5 % formic acid in water – 80 % solution of acetonitrile in 2.5 % formic acid	DAD 280	[54]
	Chromolith RP-18 4.6 mm × 100 mm			
	Polymer Laboratories	80 % solution of acetonitrilein 4.5 % formic acid – 4.5 % formic acid		
	PLRP-S 100 A°˜ (5 μm) column			
Gallotannins	NP Silica(Alltech Econosphere) 4.6 mm × 150 mm, 5 μm	Hexane–methanol/tetrahydrofuran/ trifluoroacetic acid	280	[86]
	RP	0.1 % aqueous trifluoroacetic acid	220	
	C18 ODS Backman Ultrasphere 4.6 mm × 250 mm, 5 μm	0.1 % trifluoroacetic acid in acetonitrile		

n-butanol, *n*-hexane, water, methanol, chloroform, or acetonitrile (Table 67.3). HSCCC has the unique features of high recovery, high efficiency, and the ease to scale-up. Industrial HSCCC is in the course of development. It has widely been used in the separation and purification of natural products from complex matrix [128–130].

6.2 Spectral Methods for Structure Elucidation

6.2.1 Mass Spectrometry

Using mass spectrometry to determine molecular weights and fragmentation patterns is a widely used method of identifying plant polyphenols because it has the potential to eliminate some of the ambiguities associated with other methods.

The high sensitivity and possibilities of hyphenation with chromatographic techniques (GC/MS, LC/MS) sets MS among the most appropriate physicochemical methods for the study of natural products from biological material. Mass spectrometry coupled with chromatography can provide selective molecular weight detection of biological active compounds in vegetal samples. Molecular ions and characteristic fragment ions obtained from the mass spectrometer are used to identify the desired constituents. These molecular ions, sometimes with or without reference to authentic standards, have been successfully used to identify polyphenols [112, 115, 118, 138].

Mass spectrometers use the difference in mass-to-charge ratio (m/z) of ionized molecules to separate one from another. This requires first that the various molecular species of interest have been charged (often by loss or addition of protons) and transferred into the gas phase, and that they are then separated as a function of their m/z values [106, 107, 139]. Various instruments based on different sources and analysers are available, and the various combinations made from them provide a large range of analytical possibilities more or less suitable for a given application according to the specificity of the source and analyser.

The main sources used to analyze phenolic compounds are: electron impact (EI), chemical ionization (CI), fast atom bombardment (FAB,) atmospheric pressure ionization (API) including atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo-ionization (APPI), electrospray ionization (ESI), and in parallel to the advent of electrospray ionization, matrix-assisted laser desorption ionization (MALDI) [104–107, 112].

The electron impact (EI) and chemical ionization (CI) are considered relatively harsh techniques involving the volatilization of the sample into gas phase by heating in a vacuum, then bombarded by a stream of electrons in order to cause ionization of the sample. The differences between EI and CI are that in the case of CI, the sample is ionized by a strong acid produced by the ionization of the reagent gas, usually ammonia or methane.

Polyphenols are thermally labile compounds and their evaporation without decomposition is impossible even in the ion source of mass spectrometer, where high vacuum exists. In this situation, soft ionization methods have to be applied for

Table 67.3 Counter-current chromatography: conditions for separation of different classes of phenolic compounds

Phenolic classes	Samples	CCC mode	Solvent system	Detection	References
Isomeric polyphenols	*P. laetevirens*	CCC	Light petroleum –ethyl acetate–methanol–water *n*-hexane–ethyl acetate–methanol–water	ESI-MS, ^{1}H-RMN, ^{13}C-RMN	[131]
Gallocatechins, alkaloids	*Camellia sinensis*	HSCCC	BME–CH3CN– TFA	UV-VIS	[129]
Flavonol glycosides, phenolic acids	Sea buckthorn fruits	HSCCC	*n*hexane– *n*-butanol–water	ESI-MS	[125]
Procyanidins	Grape seed	HSCCC	Ethyl acetate/2-propanol/water and ethyl acetate/1-butanol/water	UV-VIS	[132]
Ursolic acid and methyl ursolate	*Malus domestica*	HSCCC	n-hexane: AcOEt:MeOH:water	ESI- MS	[133]
Catechins	*Camellia sinensis*	HSCCC	Hexane, EtOAc, MeOH and water	UV -VIS	[134]
Flavanoids	*Patrinia villosa*	CCC	*n*-hexane–ethyl acetate–methanol–water	UV, IR, high resolution (HR)-electrospray ionization (ESI)-MS	[135]
Anthocyanins	*Vaccinium myrtillus*	HSCCC	Methyl *tert*-butyl ether–*n*-butanol -acetonitrile–water–trifluoroacetic acid	UV-VIS ESI-MS	[136]
Anthocyanins	Purple sweet potatoes	HSCCC	*n*-butanol/ethyl acetate/acetic acid Trifluoroacetic acid/n-butanol/methyl tertiary butyl ether/acetonitrile	UV-VIS, ESI-MS/MS, and NMR	[137]
Caffeic acid, chlorogenic acid,quercetin glucoside/quercetin-galactoside and phloridzin	Apple pomace	HSCCC	Hexane–ethyl acetate–aqueous acetic acid	UV, ESI -MS	[73]

analysis of this group of compounds, and the analyte molecules are ionized without evaporation in high vacuum (FAB, MALDI) or under atmospheric pressure (ESI, APCI) [2, 6, 8, 22].

APCI and ESI are the most widely used ionization methods for characterization of phenolic compounds, and they can be used to establish polyphenol fingerprints of complex extracts.

The advantage of these techniques is that they produce stable ions and low spontaneous fragmentation and allow being operated under both positive (molecular species $[M + H]^+$)and negative ion modes (molecular species $[M\text{-}H]^-$) [108].

Table 67.4 summarizes the fragment ions observed for several phenolic classes in negative and positive ionization modes.

ESI is more often used to ionize polar and nonvolatile molecules, like condensed tannins and anthocyanidins, and APCI is used for less polar and nonionic compounds especially flavonols, flavones, flavanones, and chalcones [67, 105, 139].

The matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) technique is suitable to determine the presence of molecules of higher molecular weight with high accuracy, and it has been applied with success to study procyanidin oligomers and other complex phenolic mixtures [67, 141].

Sensitivity and selectivity of detection can be increased using tandem mass spectrometry(MS/MS), that is, two (MS/MS) or more (MS^n) mass analyzers coupled in series. MS/MS and MS^n produce more fragmentation of the precursors and daughter ions, therefore providing additional structural information for the identification of flavonoids.

6.2.2 NMR Spectroscopy

NMR spectroscopy is nowadays one of the most promising spectroscopic techniques for the analysis of complex systems, such as food matrices.

Advantages such as simplicity of the sample preparation and measurement procedures, the instrumental stability, and the ease with which spectra can be interpreted have contributed to the growing popularity of the technique.

Another advantage of this technique is the simplicity of samples preparation. The solid samples can be extracted in a deuterated solvent while liquid or freeze-dried extracts are often prepared by adding 5–10 % of deuterated solvent D_2O [1, 6].

Deuterated solvents provide a signal for magnetic field stabilization and allow optimization of the resolution of the NMR peaks. Standard procedures should be followed to ensure repeatability and comparability when preparing a series of samples.

Although sensitivity reasons rendered the ^1H nucleus as the most exploited, other nuclei such as ^{13}C and ^{31}P gained popularity lately, because of their ability to attack specific problems in food science [142, 143].

^1H-NMR and ^{13}C NMR spectroscopy shows a number of advantages relative to other analytical techniques: They are fast (less than 5 min is required to record an one-dimensional spectrum); sometimes they need no calibration with internal standards or separation of the various components prior to the analysis; they show remarkable selectivity, it is possible to identify unknown compounds at

Table 67.4 Fragment ions detected for phenolic compounds

Compounds	MS mode	MW	Fragment ions m/z	References
Gallic acid	–	170	125, 79, 81	[72, 78]
Caffeic acid	–	180	135	[72, 78]
Syringic acid	–	198	182, 121, 153, 167, 138	[72, 78]
Chlorogenic acid	–	354	191, 85	[72, 78]
Ferulic acid	–	194	134, 178, 149	[72, 78]
P-cumaric acid	–	164	119, 93	[72, 78]
Vanillic acid	–	168	123, 108, 152	[72, 78]
Catechin	–	289	245, 205	[22, 72, 78]
Epicatechine	–	289	245, 205	[22, 72, 78]
Epigallocatechine	–	308	139	[22, 72, 78]
Epicatechin gallate	–	442	273, 289	[22]
Myrecitin	–	318	151, 179, 109, 107	[33, 78]
Quercetin	–	302	151, 179, 107, 121	[33, 78]
Apigenin	–	270	117, 151, 121, 107	[33, 72]
Kaempferol	–	286	151, 93, 117, 145	[22, 33]
Resveratrol		227	185	[22, 33]
Cyan-3,5-diglu	+	773	449, 287	[75]
Cyan-3-soph-5-glu	+	611	611, 449, 287	[75]
Procyanidin B1	–	577	451, 425, 407, 289	[78]
Procyanidin B2	–	577	451, 425, 407, 289	[78]
Procyanidin 3	–	577	451, 425, 407, 289	[78]

a molecular level; they also give a wealth of information in a single experiment and they provide quantitative results with excellent repeatability and reproducibility [14, 22, 141].

^{31}P NMR has a long history in food science and it is successfully applied for the structure elucidation of lignins [144].

This technique based on the derivatization of the samples by phosphytilation allows both quantitative and qualitative characterization of all the labile OH groups, namely, the aliphatic OH, different phenolic OH, and carboxylic OH. This method is based on the replacement of the acidic hydrogens of the hydroxyl and carboxyl groups with the phosphorus reagent 2-chloro-4,4,5,5-tetramethyphospholane and the use of ^{31}P-NMR spectroscopy to identify the phosphitylated compounds [142, 144, 145].

This facile magnetic resonance method supplements ^{1}H and ^{13}C NMR techniques, especially in cases where severe overlapped signals in ^{1}HNMR spectra or long relaxation times of the insensitive ^{13}C nuclei render the analysis a difficult task [145].

Disadvantages of NMR spectroscopy may be considered such as low sensitivity compared with chromatographic and mass spectrometry techniques and the high cost of the analysis, since the NMR spectrometers are quite expensive.

In the last few years, much effort was devoted to the development and improvement of LC-NMR.

The application of LC-NMR provides valuable structural information in studies of mixtures of natural phenolics and allows for a proper identification of compounds indistinguishable using other methods [146].

In LC-NMR, separation is usually performed on an RP C18 column and broad acetonitrile or methanol gradients are applied, using D_2O instead of water [14, 22].

The LC-NMR uses mainly the simple ^{1}H-NMR spectra or ^{1}H-^{1}H correlation experiments. Access to ^{13}C-NMR information is possible but is restricted only to a very limited number of cases where the concentration of the LC-peak of interest is very important and ^{13}C-NMR data can be deduced indirectly from inverse detection experiments. Indeed, due to the low natural abundance of the ^{13}C isotope (1.1%), the sensitivity for direct measurement in the LC-NMR mode is not sufficient [1, 2, 146].

6.2.3 Final Considerations

Plant phenolics are structurally diversified class of phytochemicals. This makes the development of standardized procedures for simultaneous analysis of all phenolics a very difficult task.

The discussion in this chapter focused on the developments of analytical techniques in the field of polyphenols separation and fractionation and provided at the same time an overview on the methods developed to enable identification of these compounds.

Selection of the extraction conditions (sample preparation, types of solvents, further purification steps, etc.) is crucial to ensure complete extraction of phenolics from vegetal material without losses or modifications of their chemical structure.

The analytical procedures outlined above have been applied during the last few years to enable further progress in the determination and characterization of important classes and subclasses of food phenolics.

Spectrophotometric methods provide valuable information concerning quantification of total phenolics and their classes even if they do not separate or give quantitative measurement of individual compounds.

HPLC with UV–Vis detection is the most widely utilized for separation of polyphenols, although other chromatographic methodologies such as GC, CCC, have also been used with success.

Spectral methods like MS and RMN are powerful and highly sensitive techniques successfully applied for structure elucidation of phenolics. Hyphenated techniques combine chromatographic and spectral methods to exploit the advantages of both, and they are the most extensively used to achieve good isolation and characterization of polyphenols from complex matrices.

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Phenolic Compounds in *Catharanthus roseus* 68

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Contents

1 Introduction 2094
2 Phenolics 2095
3 Extraction and Purification 2095
4 Detection and Identification 2096
4.1 LC-UV/LC-DAD 2096
4.2 LC-MS 2097
5 Benzoic Acids 2098
6 Phenylpropanoids (Hydroxycinnamic Acids) 2098
7 Flavonoids 2100
8 Antioxidant Activity 2104
9 Conclusion 2105
References 2105

Abstract

Catharanthus roseus (L.) G. Don is one of the most important medicinal plants, mainly due to the presence of anticancer alkaloids. For many years, the phenolic composition of this species remained largely unstudied. Recently, detailed

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phytochemical studies using the latest analytical techniques have helped understand the complex phenolic profile of this species. Several highly glycosylated flavonoids have been found, mainly quercetin, kaempferol, and isorhamnetin derivatives. Other classes of phenolic compounds, namely benzoic acids and phenylpropanoids have also been described. Additionally, the high content in phenolic compounds turned this plant an interesting source of antioxidants whose activity has already been shown.

In this chapter, the tissue-dependant distribution of phenolics, as well as their concentrations and antioxidant activity, will be presented.

Keywords

Catharanthus roseus (L.) G. Don • phenolics • flavonoids • benzoic acids • phenylpropanoids

Abbreviations

3-CQA	3-*O*-caffeoylquinic acid
4-CQA	4-*O*-caffeoylquinic acid
5-CQA	5-*O*-caffeoylquinic acid
CrPrx1	*Catharanthus roseus* peroxidase 1
DAD	Diode array detection
DPPH	2,2-diphenyl-1-picrylhydrazyl
LC-DAD	Liquid chromatography-diode array detector
LC-DAD-ESI-MS/MS	Liquid chromatography-diode array detector-electrospray ionization-mass spectrometry
LC-UV	Liquid chromatography-ultraviolet detector
SIM	Single ion monitoring

1 Introduction

Catharanthus roseus (L.) G. Don is a member of the Apocynaceae and was described for the first time in Madagascar. Although initially sorted in the *Vinca* genus (*Vinca rosea*), with time this species has been classified in a distinct one. The species was taken to France in the seventeenth century and, nowadays, it has acquired a pantropical distribution, mainly due to its easy cultivation and use as an ornamental plant [1].

In late 1940s, the plant received considerable attention mainly due to the antidiabetic properties claimed in its ethnopharmacological use. The discovery of the anticancer agents of *C. roseus* was made almost simultaneously by two independent groups – the group of Noble and collaborators in Canada [2, 3] and the group of Svoboda and colleagues from the Eli Lilly Company (Indianapolis) in the United States [4, 5].

In the 1950s, this species was included in a screening project to find new antidiabetic drugs by Professor Noble, University of Western Ontario, with little success. As a final resort, oral administration was abandoned and a concentrated extract was injected intraperitoneally in rats, which died in 5 days as a consequence of severe leukopenia. The attention was shifted from antidiabetic to anticancer activity, and subsequent fractionation of the extract rendered the isolation of vinblastine (vincaleukoblastine) [6].

At the same time, Svoboda and co-workers started a screening assay with 240 plant species to find new anticancer molecules. In the course of these experiments, the team realized that leukemic rats treated with *C. roseus* had an over-life quite superior to untreated subjects. With this, the interest was set on *C. roseus*, and vincristine (leurocristine) was isolated.

A few years later, two drugs based on these molecules entered the market: vinblastine, under the name Velban®, and vincristine as Oncovin®. The first anticancer molecules of natural origin were making their way.

2 Phenolics

Phenolic compounds are a class of metabolites that can be found in all plant species. These compounds can range from simple compounds bearing just one phenolic hydroxyl, to some more complex ones, like flavonoids, which are often polyphenols.

Given the chemical diversity of these compounds, they are usually classified in different groups. As so, the simplest phenolics are arranged in a C_6–C_1 skeleton, displaying at least one hydroxyl group attached to an aromatic ring. This class is frequently addressed as benzoic acids, given the fact that benzoic acid is the simplest member of this class.

Phenylpropanoids, which display a C_6–C_3 arrangement, are frequently, but not always, phenolic compounds. From a biosynthetic point of view, these compounds derive from phenylalanine and tyrosine and some examples are ferulic, cinnamic, caffeic acids, among others.

Finally, the third group of phenolics found in *C. roseus* is that of flavonoids, which have a C_6–C_3–C_6 skeleton and comprise several different classes, including flavonols, flavanols, flavones, anthocyanins, among others. It should be highlighted that, regarding flavonoids in *C. roseus*, only flavonols and anthocyanins have been described.

In this chapter, we will only focus on compounds found in plants, thus leaving studies performed in cell cultures out of the scope of this work. For a review on this subject, please see the work of Mustafa and Verpoorte [7].

3 Extraction and Purification

Sample preparation is a critical step for the analysis of phenolics in natural matrices and is far more critical for quantification than for identification.

The conditions employed should be as mild as possible to avoid chemical artifacts arising from hydrolysis, oxidation, and isomerization [8]. The addition of an antioxidant, such as *tert*-butyl-4-hydroxyanisole, *tert*-butylhydroquinone, or sodium fluoride, can prevent some of these interferences.

Hydrolysis of the extracts may be a useful approach for decreasing the number of compounds to be identified/quantified, thus resulting in a better resolution and better characterization of the matrix. Acid hydrolysis is employed to remove the sugar moiety, yielding the aglycones. Differently, alkaline hydrolysis is applied for deacylation of sugars and breaking of the covalent bond that phenolic acids may establish with the matrix. For an example, please see the work by Ferreres et al. [9].

4 Detection and Identification

4.1 LC-UV/LC-DAD

All flavonoid aglycones contain at least one aromatic ring and, therefore, absorb UV light. The A-ring is responsible for the first maximum, found in the 240–285-nm range, and the second maximum, observed at 300–550 nm, is due to the substitution pattern and conjugation of the C-ring [10].

When using an UV detector at a fixed wavelength, the obtained information is still very limited. In these situations, co-cromatography with authentic standards is recommended. Diode array detectors (DAD) are much more useful because they yield the full record of the UV-Vis spectrum of each molecule. Due to the fact that each class of phenolic compound has a characteristic spectrum, identification is facilitated, although a definitive characterization requires a standard to compare retention time and UV spectrum (see Fig. 68.2 for an example).

When two compounds with very close, or even superimposable, retention times and identical UV spectra are found in a chromatogram, more sophisticated detectors that can yield much more structural information are required. Liquid chromatography-diode array detection (LC-DAD) is still the main technique used for phenolics quantification, a parameter not usually determined by other techniques, such as liquid chromatography-mass spectrometry (LC-MS), given its poor sensibility.

Because of the great number of different flavonoid glycosides in nature, quantification using authentic standards is often impossible as they are not commercially available. However, given the fact that sugar moieties are poor chromophores, with small effect in the UV spectra, it is a common practice to quantify glycosides using standards of the corresponding aglycone or a similar one.

Regarding phenolic acids, most compounds are commercially available and can thus be positively identified by comparison of their retention time and UV spectra with those of reference compounds. Hydroxycinnamic acids absorb at two regions of the UV spectrum, presenting a maximum at 225–235 nm and two other, very near from each other, by 290–330 nm. The double absorption in this region arises from the presence of *cis* and *trans* isomers, and the ratio between these two forms contributes to the final spectrum.

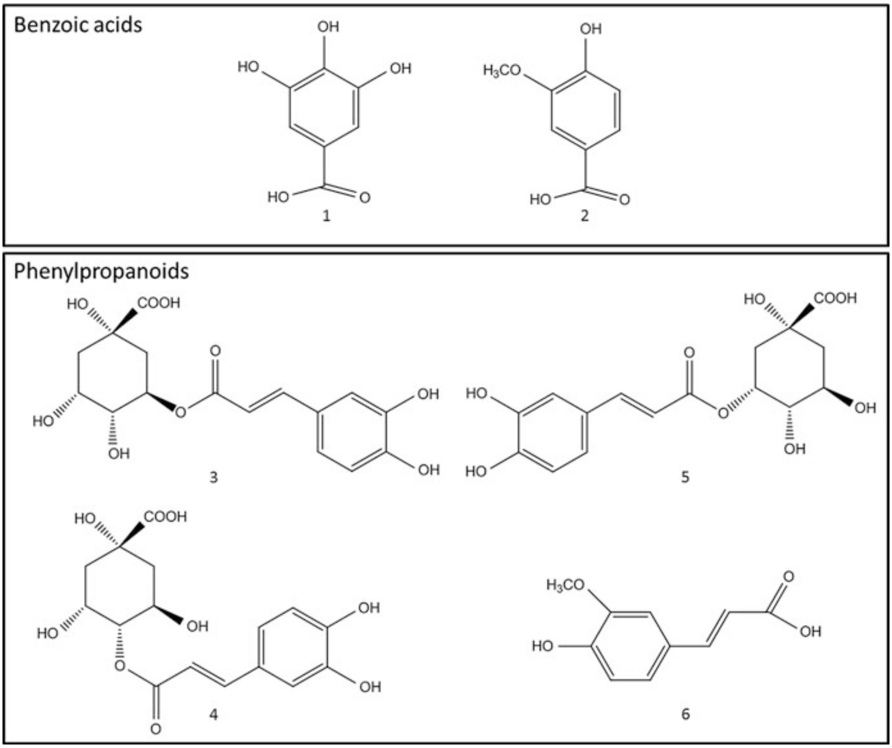


Fig. 68.1 Benzoic acids and phenylpropanoids described in *C. roseus*. Identity of compounds as in Table 68.1

In alkaline medium, all three maxima suffer a bathochromic shift. The different esters of the same acid present similar spectrum, regardless of the molecule conferring the alcohol function (quinic acid, sugar, or tartaric acid) [11].

The UV spectrum of benzoic acids is more influenced by the hydroxylation degree of benzenic moieties than that of hydroxycinnamic acids. Methylation of hydroxyl groups has almost no effect on the UV spectrum. Only dihydroxylated benzoic acids present two maxima [11].

4.2 LC-MS

MS is a powerful technique in the analysis of flavonoids and phenolic acids, mainly due to its high sensitivity and the possibility of coupling with different chromatographic techniques, especially with LC. In addition, the characteristic fragmentation pattern of some phenolics can aid in their identification [12].

The amount of information obtained by multisignal MS or MS/MS renders two levels for identification of compounds: positive and provisional. Positive identification can be achieved when reference compounds are available, thus allowing comparison of both retention time and spectra. When no standards are available, a common

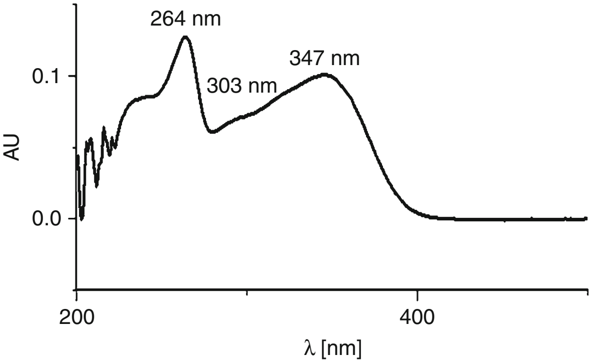


Fig. 68.2 UV spectrum of flavonol-3-*O*-glycosyl derivative present in the leaves of *C. roseus*

situation in natural product chemistry, provisional identification takes place. In this case, although the identity of subunits such as aglycones, sugars, and acyl moieties is elucidated, the positions of glycosidic and acyl linkages remain sometimes unknown, with the exception of some fragmentation patterns that allow a positive identification of the glycosidic bonds [13].

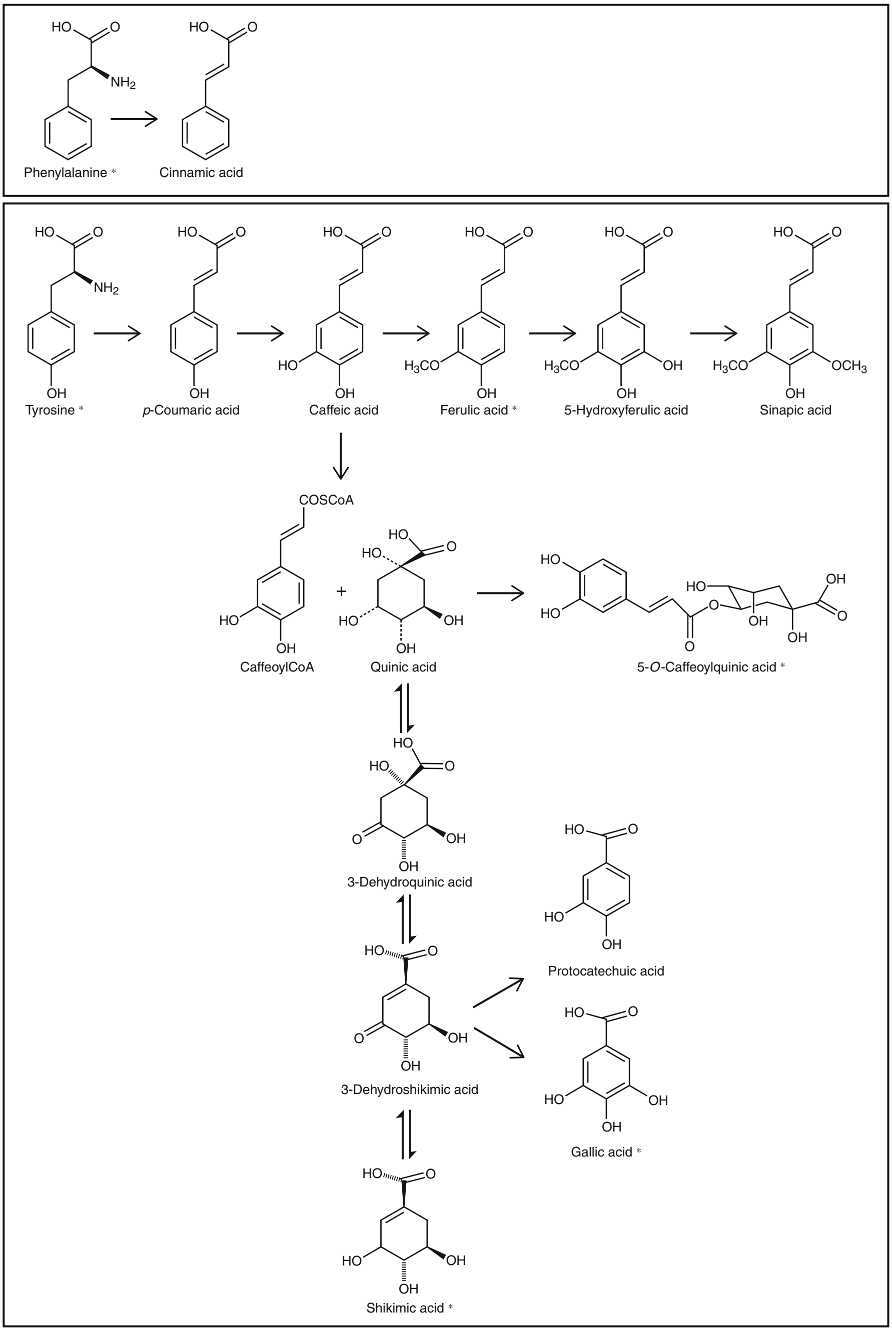
Spectroscopy by UV-Vis and MS in total ion mode can render detection limits for flavonoids in the range of 10 ng. When single ion monitoring (SIM) mode is used, MS analysis provides better detection limits, usually below 1 ng. SIM mode, however, causes loss of valuable information concerning fragmentation pattern, which is very important for the identification of many compounds. Among all ionization sources available, electrospray ionization (ESI) in negative ion mode is the one most suitable for the analysis of phenolics.

5 Benzoic Acids

Simple benzoic acids are synthesized in plants *via* the shikimate pathway, which is derived from shikimic acid, which is itself derived from quinic acid *via* 3-dehydroquinic and 3-dehydroshikimic acids (Scheme 68.1). In fact, shikimic, but not quinic, acid has been described in the leaves and stems of this species [14]. The simplest benzoic acids are protocatechuic acid (3,4-dihydroxybenzoic acid) and gallic acid (3,4,5-trihydroxybenzoic acid). The latter proved to be present in the tissues of *C. roseus*, in addition to vanillic acid (4-hydroxy-3-methoxybenzoic acid) [15]. The quantitative composition of *C. roseus* in benzoic acids can be seen in Table 68.1, and the structures of these compounds are represented in Fig. 68.1.

6 Phenylpropanoids (Hydroxycinnamic Acids)

Phenylpropanoids are a class of C_6–C_3 phenolics with a widespread distribution in nature. These compounds can be derived either from phenylalanine, like cinnamic acid,


Phenylalanine *
Cinnamic acid
Tyrosine *
p-Coumaric acid
Caffeic acid
Ferulic acid *
5-Hydroxyferulic acid
Sinapic acid
CaffeoylCoA
COSCoA
Quinic acid
5-*O*-Caffeoylquinic acid *
3-Dehydroquinic acid
Protocatechuic acid
3-Dehydroshikimic acid
Gallic acid *
Shikimic acid *

Scheme 68.1 Major biosynthetic steps involving benzoic acids and phenylpropanoids. *Indicates compounds described in *C. roseus*

Table 68.1 Benzoic acids (C_6–C_1) and phenylpropanoids (C_6–C_3) in *C. roseus* (mg/kg, dry basis)

Compound number	Compound	Seeds	Stems	Leaves	Petals	References
	Benzoic acids					
1	Gallic acid[a]	–	–	42 (0.03)	–	[15]
2	Vanillic acid[a]	–	–	1.3 (0.01)	–	[15]
	Phenylpropanoids					
3	3-*O*-Caffeoylquinic acid	–	769.9 (12.7)	2,971.6 (15.6)	–	[16, 17]
4	4-*O*-Caffeoylquinic acid	–	2,874.6 (151.6)	5,156.8 (137.2)	11,153.2 (126.4)	[16, 17]
5	5-*O*-Caffeoylquinic acid	–	22.5 (1.5)	187.7 (0.5)	–	[16, 17]
6	Ferulic acid[a]	–	–	250 (0.08)	–	[15]

[a] Expressed as mg/100 g, dry basis

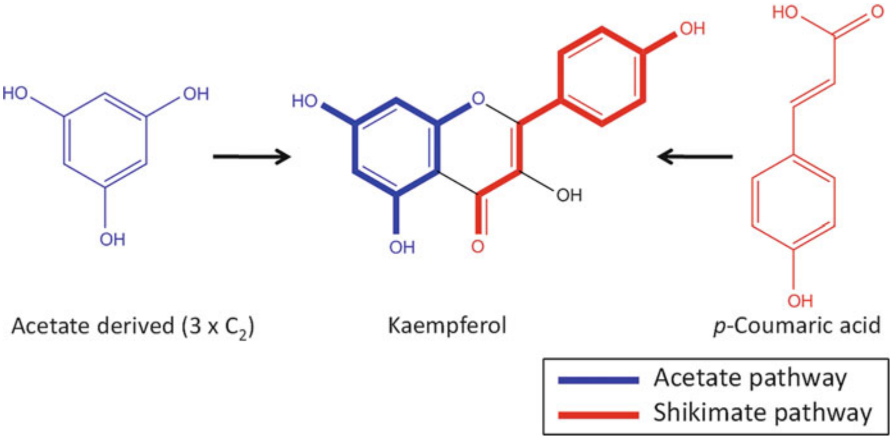
or from tyrosine, like *p*-coumaric acid (Scheme 68.1). In the particular case of hydroxycinnamic acids, the most common ones are ferulic and sinapic acids, which can be found in plants either in their free or esterified form. In *C. roseus*, several reports have described the presence of hydroxycinnamic acids esterified with quinic acid, namely 3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, and 5-*O*-caffeoylquinic acids (3-CQA, 4-CQA, and 5-CQA, respectively) [16]. The distribution of these compounds has been shown to be dependent on the tissue, as they can be found only in some plant parts, namely, in the stems and leaves. Seeds and petals were also investigated for the presence of these compounds, but they were not found. This tissue-dependent distribution of caffeoylquinic acids in leaves and stems can be related with their recognized action as protectors against herbivores and infection [18], a function particularly relevant for the organs involved in vegetative growth (stems and leaves). In what concerns compounds in their free form, hydroxytyrosol and ferulic acids have been described. The amount of these compounds in the plant can be found in Table 68.1, and their structures can be seen in Fig. 68.1.

7 Flavonoids

Among natural products, phenolics are one of the most studied classes due to their broad range of biological activities, which include antioxidant, anticancer, antiallergy, among others [19].

From a biosynthetic point of view, flavonoids derive from both the shikimate and the acetate pathways. A detailed discussion on the biosynthesis of these compounds is beyond the scope of this work; however, a simplified representation of the biosynthetic pathway can be seen in Scheme 68.2.

The phenolic composition of *C. roseus* has remained unknown for a long period of time. Early reports in the 1950s on the flavonoids of *C. roseus* described the presence



Scheme 68.2 Combination of acetate and shikimate pathways in flavonoids biosynthesis

of quercetin and kaempferol, by using paper chromatography, which are the only flavonoid aglycones found in this species to these days [20]. Although this study is remarkably important from a historical point of view, due to the fact that it was the first study on the phenolics of this species, the chromatographic methodology used is prone to some errors because of the lack of specificity and sensitivity. In 1996, Nishibe et al. isolated two flavonoids from *C. roseus* leaves: mauritianin (kaempferol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-rhamnopyranosyl(1 → 6)-β-D-galactopyranoside) and also quercetin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside [21]. In 1999, the same compounds were also isolated from stems, in addition to another one that was described in this species for the first time: syringetin-3-*O*-robinobioside [22]. As so, until early 2008, the only flavonoids reported in this species were reduced to five. Recently, an in-depth study of the phenolic composition of *C. roseus* was performed using high-performance liquid chromatography-diode array detection-electrospray ionization-mass spectrometry (LC-DAD-ESI-MS/MS) [23]. Leaves, stems, seeds, and flowers were studied in order to understand the distribution of these metabolites within the different plant tissues. Fifteen glycosides of quercetin, isorhamnetin, and kaempferol were found, with the latter constituting the majority. UV spectra characteristic of flavonol-3-*O*-glycosyl derivatives were found (Fig. 68.2). The identity and quantity of these compounds, as well as their tissue distribution, can be found in Table 68.2. It is worth mentioning that the above-referred syringetin-3-*O*-robinobioside [22] was not found in this latter study, which could mean that the presence of this compound may depend on the agricultural practices, climate, or the stadium of the plant's life cycle.

Anthocyanins in *C. roseus* are highly dependent on the cultivar under study, which can result in different petal color. As so, Little Bright Eye, a white variety, seems to be disposed of these compounds [16], while other varieties have been shown to contain this class of flavonoids. In particular, the 3-*O*-glucosides of malvidin, hirsutidin, and petunidin have been described, as well as their

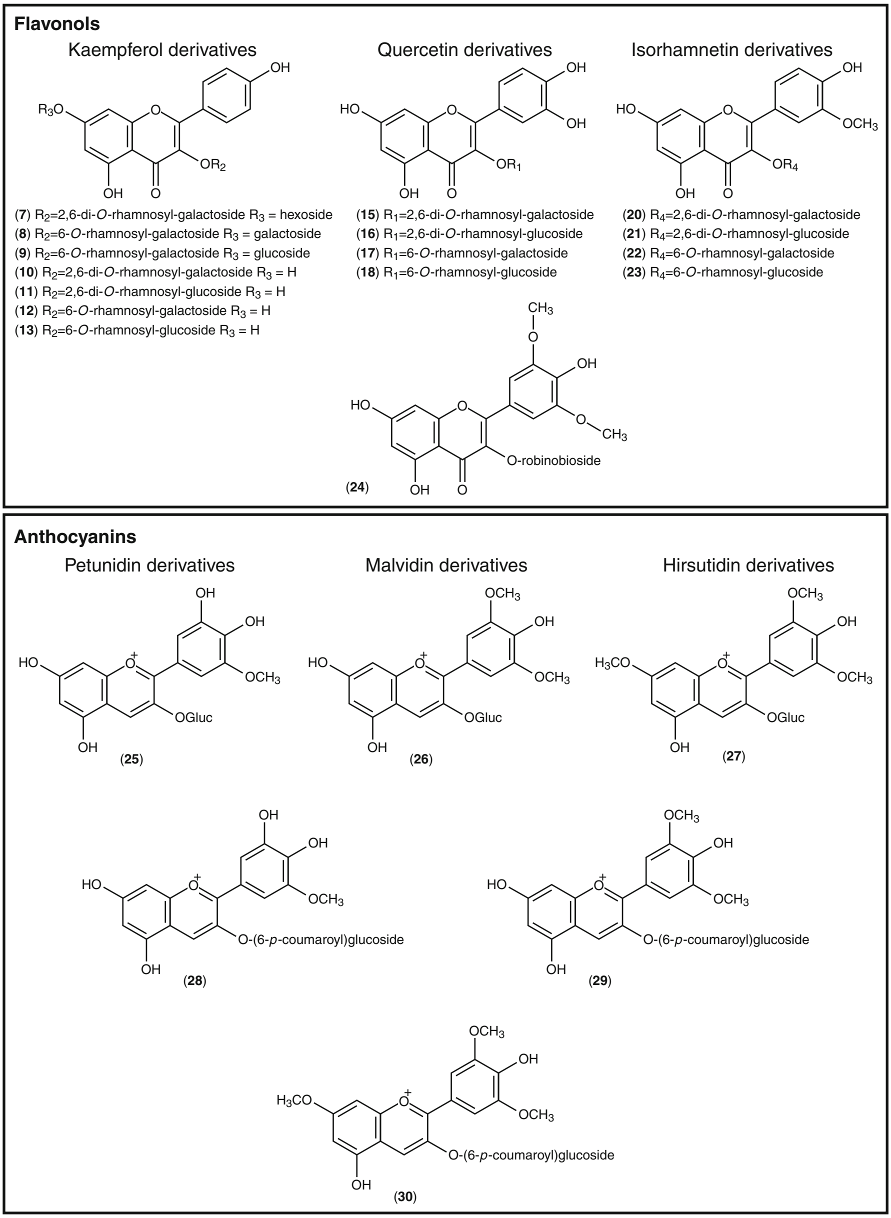

Flavonols
Kaempferol derivatives
Quercetin derivatives
Isorhamnetin derivatives
OH
R_3O
O
OR_2
OH O
HO
OH
OR_1
OCH_3
OR_4
(**7**) R_2=2,6-di-*O*-rhamnosyl-galactoside R_3 = hexoside
(**8**) R_2=6-*O*-rhamnosyl-galactoside R_3 = galactoside
(**9**) R_2=6-*O*-rhamnosyl-galactoside R_3 = glucoside
(**10**) R_2=2,6-di-*O*-rhamnosyl-galactoside R_3 = H
(**11**) R_2=2,6-di-*O*-rhamnosyl-glucoside R_3 = H
(**12**) R_2=6-*O*-rhamnosyl-galactoside R_3 = H
(**13**) R_2=6-*O*-rhamnosyl-glucoside R_3 = H
(**15**) R_1=2,6-di-*O*-rhamnosyl-galactoside
(**16**) R_1=2,6-di-*O*-rhamnosyl-glucoside
(**17**) R_1=6-*O*-rhamnosyl-galactoside
(**18**) R_1=6-*O*-rhamnosyl-glucoside
(**20**) R_4=2,6-di-*O*-rhamnosyl-galactoside
(**21**) R_4=2,6-di-*O*-rhamnosyl-glucoside
(**22**) R_4=6-*O*-rhamnosyl-galactoside
(**23**) R_4=6-*O*-rhamnosyl-glucoside
CH_3
O
OH
HO
O
CH_3
O-robinobioside
(**24**)
OH O
Anthocyanins
Petunidin derivatives
Malvidin derivatives
Hirsutidin derivatives
OH
OH
HO
$\overset{+}{O}$
OCH_3
OGluc
OH
(**25**)
OCH_3
OH
HO
$\overset{+}{O}$
OCH_3
OGluc
OH
(**26**)
OCH_3
OH
H_3CO
$\overset{+}{O}$
OCH_3
OGluc
OH
(**27**)
OH
OH
HO
$\overset{+}{O}$
OCH_3
O-(6-*p*-coumaroyl)glucoside
OH
(**28**)
OCH_3
OH
HO
$\overset{+}{O}$
OCH_3
O-(6-*p*-coumaroyl)glucoside
OH
(**29**)
OCH_3
OH
H_3CO
$\overset{+}{O}$
OCH_3
O-(6-*p*-coumaroyl)glucoside
OH
(**30**)

Fig. 68.3 Flavonols and anthocyanins described in *C. roseus*. Identity of compounds as in Tables 68.2 and 68.3

Table 68.2 Flavonols described in *C. roseus* (mg/kg, dry basis)

Compound number	Compound	Seeds	Stems	Leaves	Petals	References
	Kaempferol derivatives					
7	Kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside)-7-*O*-hexoside	292.3 (0.3)	–	52.7 (1.0)	–	[16, 17]
8	Kaempferol-3-*O*-(6-*O*-rhamnosyl-galactoside)-7-*O*-galactoside	nq	–	–	nq	[16, 17]
9	Kaempferol-3-*O*-(6-*O*-rhamnosyl-galactoside)-7-*O*-glucoside	–	–	–	nq	[16, 17]
10	Kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside)	2,714.2 (4.3)	190.8 (5.3)	8.5 (5.3)	8,120.8 (74.4)	[16, 17]
11	Kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside)	56.6 (0.4)	–	–	4,296.3 (34.4)	[16, 17]
12	Kaempferol-3-*O*-(6-*O*-rhamnosyl-galactoside)	112.1 (16.0)	–	–	9,567.2 (98.5)	[16, 17]
13	Kaempferol-3-*O*-(6-*O*-rhamnosyl-glucoside)	nq			4,639.8 (21.9)	[16, 17]
14	Mauritianin	–	[a]	[a]	–	[21]
	Quercetin derivatives					
15	Quercetin-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside)	582.7 (6.6)	190.5 (3.1)	310.9 (5.0)	1,027.9 (7.0)	[16, 17]
16	Quercetin-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside)[a]	–	–	–	nq	[16, 17]
17	Quercetin-3-*O*-(6-*O*-rhamnosyl-galactoside)[a]	–	–	–	nq	[16, 17]
18	Quercetin-3-*O*-(6-*O*-rhamnosyl-glucoside)[a]	–	–	–	nq	[16, 17]
19	Quercetin-3-*O*-α-L-rhamnosyl-(1 → 2)-α-L-rhamnosyl-(1 → 6)-β-D-galactoside	–	[a]	[a]	–	[21]
	Isorhamnetin derivatives					
20	Isorhamnetin-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside)	–	78.6 (3.9)	–	–	[16, 17]
21	Isorhamnetin-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside)	354.1 (8.2)	–	–	–	[16, 17]
22	Isorhamnetin-3-*O*-(6-*O*-rhamnosyl-galactoside)	–	–	–	989.2 (33.0)	[16, 17]
23	Isorhamnetin-3-*O*-(6-*O*-rhamnosyl-glucoside)	372.0 (65.2)	–	–	1,330.4 (10.8)	[16, 17]
	Others					
24	Syringetin-3-*O*-robinobioside	–	[a]	[a]	–	[22]

[a] Quantification not available in literature

Table 68.3 Anthocyanins described in *C. roseus* (μg/g, fresh weight)

Compound number	Compound	Seeds	Stems	Leaves	Petals	References
	Anthocyanins					
25	Petunidin-3-*O*-glucoside	–	–	3.5	–	[24]
26	Malvidin-3-*O*-glucoside	–	–	1.5	–	[24]
27	Hirsutidin-3-*O*-glucoside	–	–	28	–	[24]
28	Petunidin-3-*O*-(6-*O*-*p*-coumaroyl) glucoside	–	–	11.5	–	[24]
29	Malvidin-3-O-(6-*O*-*p*-coumaroyl) glucoside	–	–	6.2	–	[24]
30	Hirsutidin-3-O-(6-*O*-*p*-coumaroyl) glucoside	–	–	49.3	–	[24]

6-*O*-*p*-coumaroyl derivatives, totalizing six anthocyanins (Fig. 68.3, Table 68.3) [24]. As it can be seen, all flavonols are highly glycosylated, bearing no acylation, while anthocyanins display only one sugar moiety, being some of them acylated with *p*-coumaric acid.

Roots of *C. roseus* were also investigated for the presence of phenolic compounds, but no such metabolites were found [25].

Recently, the vacuoles of *C. roseus* leaves were isolated and analyzed by LC-DAD-ESI-MS/MS and the presence of three caffeoylquinic acids, a quercetin triglycoside, a kaempferol triglycoside, and two anthocyanins was demonstrated [26]. As expected for a class III peroxidase, CrPrx1 was capable of oxidizing the phenolic compounds tested, which included kaempferol, quercetin, ferulic acid, among others. By comparing the several standards, the highest apparent affinity was observed for kaempferol (0.018 mM) and the lowest for ferulic acid (1.606 mM). In fact, higher affinities were observed for kaempferol, 5-CQA, quercetin, and caffeic acid, while lower ones were noticed for ferulic acid and coniferyl aldehyde. 5-CQA, which is close to the major phenolic compound accumulated in the vacuoles of *C. roseus* leaves, presented one of the lowest Kms, indicating a high affinity of CrPrx1 for this compound.

In addition, it was concluded that glycosylation of the aglycone significantly lowers CrPrx1 affinity for the substrate since the Km increased from 0.045 mM for quercetin to 1.589 mM for quercetin-3-*O*-arabinoside.

8 Antioxidant Activity

The rich phenolic composition of *C. roseus* has led to the hypothesis that this species could present good antioxidant activity. Recently, aqueous extracts of leaves, stems, flowers, and seeds were tested for their antiradical capacity against the synthetic radical DPPH and the physiological radicals superoxide anion and nitric oxide. All tissues tested exhibited a concentration-dependent effect against superoxide radical, with the order of activity being seeds ($IC_{50} = 74$ μg/mL) > leaves ($IC_{50} = 90$ μg/mL) > stems ($IC_{50} = 202$ μg/mL) > petals ($IC_{50} = 260$ μg/mL) [23].

Regarding nitric oxide radical, the scavenging capacity was much lower and the order of activity was distinct from that found against superoxide anion: petals (IC_{25} = 232 μg/mL) > seeds (IC_{25} = 320 μg/mL) > leaves (IC_{25} = 505 μg/mL) > stems (IC_{25} = 546 μg/mL).

The order of antiradical activity against DPPH revealed to be similar to the one observed for nitric oxide, with the best material being petals (IC_{50} of 197 μg/mL) [23].

The antioxidant activity of *C. roseus* roots was also assessed in another work [25], and this material showed to have higher antioxidant potential than all of the previously studied plant materials. Although no phenolics were found in the roots, the organic acids present in the extract proved to contribute to a strong antioxidant activity against DPPH, superoxide, and nitric oxide radicals.

9 Conclusion

In the last few years, there has been a great evolution in the knowledge of the phenolic composition of *C. roseus*. While only five compounds were described in this species by 1996, today this number has risen to over 25, most of which are flavonoids. This is a consequence of recent studies that were undertaken, using more advanced analytical techniques like LC-MS, which is a powerful approach for chemically complex extracts. As *per* the state of the art, three classes of phenolics are known to occur in *C. roseus*: benzoic acids, phenylpropanoids, and flavonoids. Benzoic acids are limited to gallic and vanillic acids, and phenylpropanoids include ferulic acids and isomers of caffeoylquinic acid. The group of flavonoids represents the majority of the phenolics reported in *C. roseus* and is mostly constituted by quercetin, kaempferol, and isorhamnetin derivatives, all of them highly glycosylated and bearing no acylation. Anthocyanins deriving from malvidin, hirsutidin, and petunidin are also described. The rich composition in phenolics has turned *C. roseus* an interesting source of antioxidants.

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Flavonoids by HPLC

69

S. W. Annie Bligh, Olumuyiwa Ogegbo, and Zheng-Tao Wang

Contents

1 Introduction 2108
2 Structure and Physicochemical Properties 2108
3 Extraction 2111
4 Separation 2112
4.1 Stationary Phases 2112
4.2 Mobile Phases 2115
5 Identification 2118
5.1 Detection 2119
5.2 Combination of Detection 2123
6 Two-Dimensional (2D, LC × LC) HPLC 2124
7 Quantitation 2127
8 Selected Examples of Flavonoids Analysis by HPLC 2129
9 Conclusion 2129
References 2137

Abstract

Flavonoids are secondary plant metabolites that are synthesized via the shikimate pathway. HPLC has been an important tool for the separation of these metabolites in the last 4 decades. The coupling of HPLC with a number of detection technologies either online, in tandem, or off-line enables the

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identification of flavonoids in plant, food, and biological samples. This chapter provides an overview of flavonoid analysis by HPLC, including extraction of flavonoids in both aglycones and glycosides, separation by a selection of stationary and mobile phases, and finally, detection and identification by UV–VIS, fluorescence, electrochemical, mass spectrometry, and NMR spectroscopy.

Keywords
Flavonoids • Foods • HPLC • Medicinal plants • Quantitation

1 Introduction

Flavonoids are polyphenols and function as secondary metabolites in plants. Their biological importance in plants, animals, and microorganisms stems from their diversity in chemical substitution of the C6–C3–C6 framework (Fig. 69.1), giving over 10,000 known compounds [1, 2]. The rapid increase of new flavonoids reported in the last decade is partly due to the intense research on rationalizing of the molecular contribution of health benefits in traditional herbal medicine and food and on using flavonoid molecular entities in chemosystematics of plants. The improved bioassay-guided separation technology and the advance in the development of the HPLC system, especially the detection modality, have also contributed to the growth of identification of new flavonoids. A number of reviews have been published on analytical methods for flavonoids and polyphenols in plant, food, and biological samples [3–9].

In this chapter, we focus on the analysis of flavonoids strictly based on the C6–C3–C6 framework (i.e., excluding the isoflavonoids). The chemical diversity, size, three-dimensional shape, and physical properties of flavonoids are reviewed in recognition of their importance in determining the extraction and separation strategy. Various detectors coupled to the HPLC with different stationary and mobile phases are discussed for the feasibility of aiding the full identification of flavonoids. In addition, the challenges of using two-dimensional HPLC in the screening of flavonoids for their bioactivity in herbal medicine or food are highlighted.

2 Structure and Physicochemical Properties

Flavonoids are characterized by a C6–C3–C6 framework and typically with a phenylbenzopyran chemical structure. The heterocyclic benzopyran ring is known as the C ring. An aromatic ring (A ring) fused with the heterocyclic benzopyran ring (C ring) and linked to a phenyl moiety (B ring) as shown in Fig. 69.1. The A and B rings can be hydroxylated, and the hydroxyls can be *O*- and *C*-glycosylated, methylated, acetylated, pyrenylated, or sulphated. Sugar units can be D-glucose, L-rhamnose, D-galactose, L-arabinose, D-xylose, D-allose, D-apose, D-mannose, D-glactouronic acids, D-glucuronic acids, di- or trisaccharides.

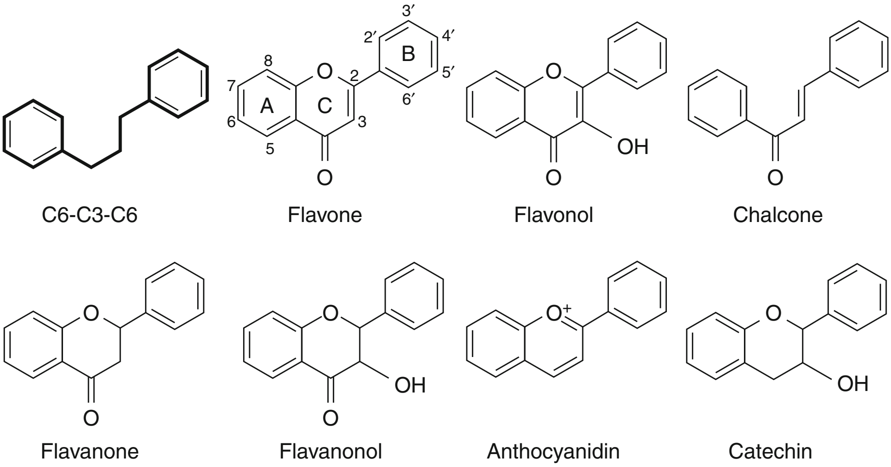


Fig. 69.1 Skeleton of a C6–C3–C6 defining the flavonoid class and structures of the main flavonoid subclasses

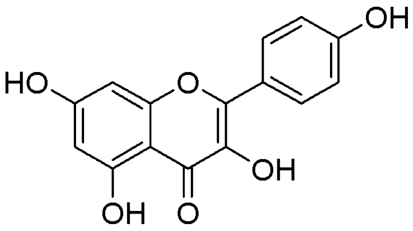
The center heterocycle is either pyran, pyrilium, or γ-pyrone. The main subclasses of flavonoids are flavone, flavonol, flavanonol, flavanone, anthocyanidin, and chalcone (Fig. 69.1). The latter subclass does not have a heterocycle B ring structure. Both flavanonol and flavanone have a chiral center at C2 position and they are stereoisomers.

The solubility of a flavonoid is a crucial factor in controlling its interaction with the mobile phase in HPLC. Therefore, the properties of these flavonoids such as hydrophobicity, dipole moment, hydrogen bonding, ionization, and steric effects are important to take into account when choosing a mobile phase for an effective separation. Flavonoids without sugar units attached tend to have low solubility in water and are pH dependent. The solubility of quercetin, isoquercitrin, rutin, chrysin, naringenin, and hesperetin was quantified in three different organic solvents (acetonitrile, acetone, and tert-amyl alcohol), and the data did not give a clear correlation between the solubility of flavonoids and their thermodynamic properties [10].

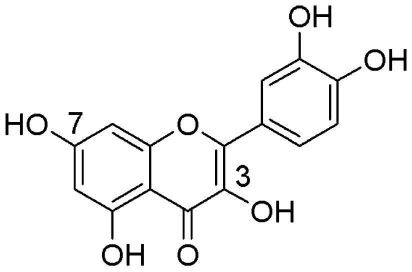
The number and the position of hydroxyl groups attached even in the same class of flavonoids can influence the lipophilicity of the flavonoids. Octanol–water partition coefficient (log P) values were reported for flavonoids from the flavone, flavonol, and flavanone [11], showing that aglycones are more lipophilic than any glycosylated or sulfated conjugates. However, there is not a trend of retention time of the flavonoid with respect to the log P (Table 69.1). Despite having the same number and position of hydroxyl substituents in luteolin (flavone) and eriodictyol (flavanone), they have different log P values but similar retention times.

The acidity of hydroxyl groups in flavonoids has been studied by theoretical calculations [12]. Interestingly the 4′-OH on the B ring and the 7-OH on the A ring

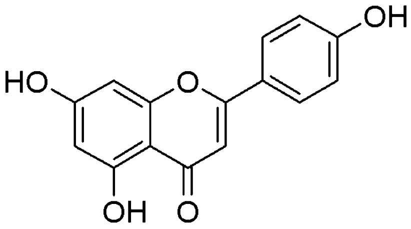
Table 69.1 Log P values and HPLC retention times [11]



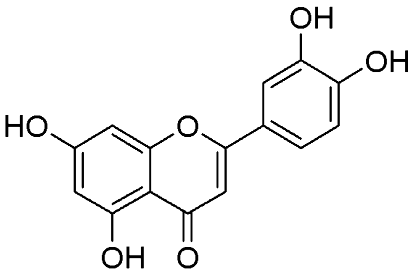
Kaempferol



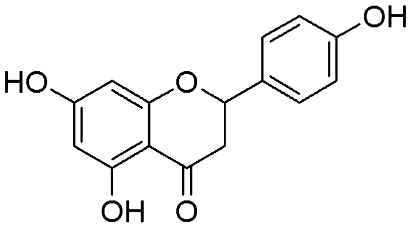
Quercetin



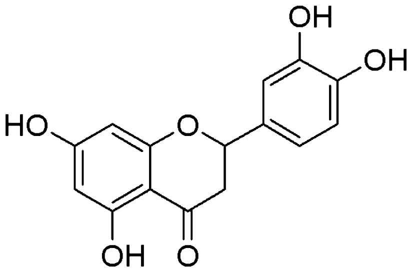
Apigenin



Luteolin



Naringenin



Eriodyctiol

Flavonoid	Log P ± SD	Retention time (min)
Luteolin	3.22 ± 0.08	20.08
Kaempferol	3.11 ± 0.54	23.72
Apigenin	2.92 ± 0.06	22.90
Naringenin	2.60 ± 0.03	22.30
Eriodictyol	2.27 ± 0.02	20.13
Quercetin	1.82 ± 0.31	20.50
Quercetin-3-glucoside	0.76 ± 0.01	12.21
Quercetin-7-sulfate	0.74 ± 0.02	14.72
Quercetin-3-rhamnoglucoside	−0.64 ± 0.05	10.88
Quercetin-3-sulfate	−1.11 ± 0.01	11.23

are identified as the most suitable deprotonation sites because of the favorable delocalization of the electron pair. Therefore, the most acidic flavonoids are those characterized by a high degree of π-electron delocalization, for which deprotonation gives anionic species that can be readily stabilized by resonance structure.

3 Extraction

The structural complexity of flavonoids has prohibited a single extraction method for all classes of flavonoids. Sample handling strategies (pretreatment, extraction, and clean-up) are important prior to the determination of flavonoids by HPLC. Sample pretreatment is required in most of the flavonoid-containing matrices, such as plant materials, food products, and biological samples. Work-up routines before extraction processes can involve freeze-drying, homogenization, centrifugation, and/or filtration. The pretreatment for liquid food products and biological samples is simply centrifugation and for solid plant materials and food products is homogenization.

Solvent extraction of flavonoids is commonly used after pretreatment. The factors that contribute to the efficiency of solvent extraction are polarity of solvent or solvent mixtures, pH, temperature, and particle size. The acidity of the extraction medium can influence the degree of solubility for soluble flavonoids and their conjugates, for example, glycones in plants and glucuronide and sulfate conjugates in biological samples. Solvent such as aqueous, methanol, ethanol, ethyl acetate, acetone, acetonitrile, or their mixture is commonly used to isolate flavonoids from powdered plant materials [13]. In anthocyanin extraction, acidified aqueous methanol or ethanol is used to denature the cell membrane and to solubilize the analyte. The use of weak organic acids and low concentrations of strong acids was reported to prevent the hydrolysis of anthocyanins to anthocyanidins [14–17]. Since flavonoids can exist as various conjugated forms, sample treatment with acid [18–20] or enzymatic [21, 22] hydrolysis is required to facilitate the identification of the aglycones.

Flavonoids can be extracted by solvent extraction through Soxhlet extraction, ultrasound-assisted extraction (USAE), microwave-assisted solvent extraction (MASE), accelerated solvent extraction (ASE), or supercritical fluid extraction (SFE) methods [23]. The latter two methods are based on using compressed fluids as extracting agents [24]. A comparative extraction study of flavonoids from dry cell cultures of *Saussurea medusa* Maxim by Soxhlet extraction, USAE, and MASE showed that MASE is more efficient in terms of yield and time for extraction. Another comparative study, using the compressed fluid techniques, accelerated solvent extraction (ASE) using water, and supercritical fluid extraction (SFE) using CO_2 and 10 % EtOH as modifier versus standard hot water or 70 % ethanol extraction of flavonoids from *Scutellaria lateriflora*, was reported [25]. The use of ASE at 85 °C with water as solvent gave the best results for flavonoid glycosides, whereas SFE gave higher yields of flavonoid aglycones.

Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are usually performed on liquid samples, such as beverages or biological fluids. These two extraction methods are used to concentrate flavonoid analytes based on their solubility in different solvents and their polarity. Unwanted lipids or lipophilic materials in crude extract can be eliminated by washing it with nonpolar solvents, such as hexane or dichloromethane.

Column chromatographic and SPE methods are used in the clean-up step before injection to the HPLC system. The polar nonphenolic compounds such as organic acids can be removed by SPE method using a preconditioned C_{18} cartridge. Apart from the commonly used C_{18} cartridge, a variety of adsorbent materials such as Amberlite, C_8, and HLB have also been used successfully for extracting flavonoid compounds from wine [26]. SPE method has been found useful in enhancing the extraction of glabridin from licorice (from 0.23 % to 35.2 % after SPE) [27].

4 Separation

Reversed-phase (RP) liquid chromatography is used for separation of analytes that dissolve in mixed aqueous–organic solvents. Separation of flavonoids is therefore commonly carried out in the reversed-phase mode, on C_8- or C_{18}-bonded silica columns with mixed aqueous–organic mobile phase. The aqueous mobile phase is usually acidified water using a mild organic acid such as formic or acetic acid. The organic mobile phase is typically either methanol or acetonitrile. Normal-phased liquid chromatography is seldom used for flavonoid analysis because the analyte often retains on the column. However, peracetylated flavonoids can be separated on a cyano-silica column using *n*-hexane-ethyl acetate mobile phase under isocratic conditions [28].

4.1 Stationary Phases

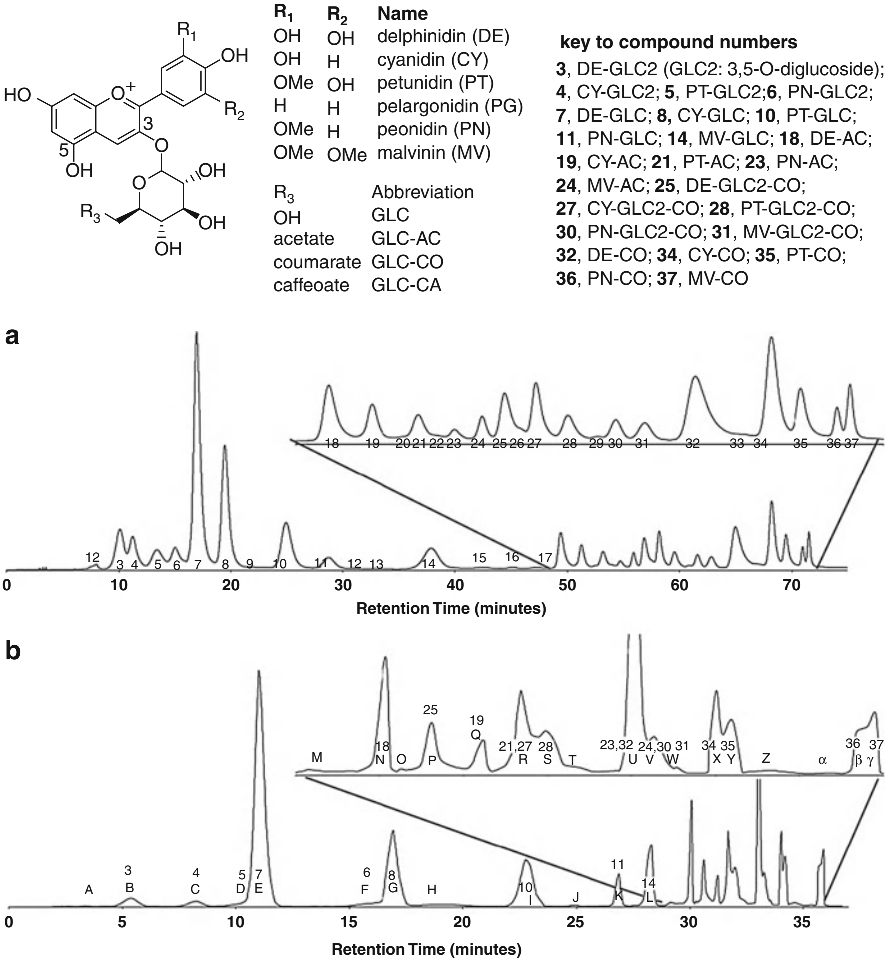
A complete separation of naturally occurring mixtures of flavonoids poses problems due to the wide range of polarities and the tendency for flavonoids of similar polarity to elute in groups. C_{18} is normally the stationary phase of choice. Typically columns have an internal diameter ranging from about 2–5 mm with particle sizes 3–5 μm with a length of 75–250 mm. C_8 stationary phase is also used for the separation of more polar flavonoids. A comparison of HPLC capacity factors of 27 flavonoids have been reported using Zorbax SB (250 × 4.6 mm) analytical columns containing C_{18}, C_8, and CN stationary phases [29]. The results showed that the hydrophobic flavonoids (usually aglycones) had similar capacity factors in C_{18} and C_8 columns and were much reduced in CN column (Table 69.2). However, for polar flavonoids, the capacity

Table 69.2 Capacity factors of flavonoids in chromatography columns with different stationary phases [29]

	Coefficients of retention		
Compound	C_{18}	C_8	CN
Apigenin (5,7,4′-trihydroxyflavone)	34.24	39.23	22.37
Herbacetin (5,7,8,4′-tetrahydroxyflavonol)	11.90	11.07	8.69
Quercetin (5,7,3′,4′-tetrahydroxyflavonol)	16.24	15.42	11.77
3-Methylquercetin (3-*O*-methyl-5,7,3′,4′-tetrahydroxyflavonol)	22.69	22.04	13.63
3-Methylkaempferol (3-*O*-methyl-5,7,4′-trihydroxyflavonol)	51.38	51.14	24.33
Myricetin (5,7,3′,4′,5′-pentahydroxyflavonol)	6.24	6.03	5.81
Patuletin (5,7,3′,4′-tetrahydroxy-6-methoxyflavonol)	16.78	15.02	10.76
Chrysoeriol (5,7,4′-trihydroxy-3′-methoxyflavone)	40.97	42.88	25.47
Mono- and diglycosides			
Guaiaverin (quercetin-3-arabinoside)	3.17	3.32	3.16
Genistin (5,4′-dihydroxyflavone-7-*O*-β-L-glucoside)	2.79	3.08	2.70
Herbacetin–8-*O*-glucoside	8.31	7.13	5.30
Hyperoside (quercetin-3-*O*-galactoside)	3.54	3.49	3.57
Quercetin-3′-*O*-glucoside	4.49	4.37	3.95
Rutin (5,7,3′,4′-tetrahydroxyflavonol-3-*O*-β-D-rutinoside)	1.51	1.47	1.69
Linarin (5-hydroxy-4′-methoxyflavone-7-*O*-α-L-rhamno-β-D-glucoside)	15.72	14.52	7.23
Luteolin-3-glucoside (5,7,4′-trihydroxyflavonol-3-*O*-glucoside)	2.21	2.46	2.73
Luteolin-7-*O*-glucoside	2.25	2.50	2.82
3-Methylkaempferol-7-*O*-glucoside	7.23	7.03	4.65
Myricetin-3-*O*-galactoside	1.12	1.17	1.44
Myricetin-3-*O*-rhamnoside	1.96	2.01	2.12
Patuletin (patuletin-7-*O*-glucoside)	2.30	2.23	2.23
Pectolinarin (5-hydroxy-6,4′-dimethoxyflavone-7-*O*-(6-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside)	17.97	16.42	7.59
Scoparin (chrysoeriol-8-C-β-D-glucopyranoside)	2.46	2.44	2.52
Scutellarein-7-rhamnoxyloside (5,6,4′-trihydroxyflavone-7-*O*-β-rhamnoxyloside)	2.74	3.12	2.74
Hirsutrin (quercetin-3-*O*-glucoside)	2.19	2.22	2.31
Eriodictyol (5,3′-dihydroxy-4′-methoxyflavone-7-*O*-glucoside)	2.24	2.24	1.85

factors were similar in all columns. Optimum separation of flavonoids also depends on column types of C_{18}; for example, the separation of 51 flavonoids in a Chinese herbal prescription of Longdan Xiegan Decoction using a Symmetry column from Waters was better than that of Zorbax (Agilent) and LiChroCART (Merck) [30].

The anthocyanins are most often separated by HPLC on a C_{18} column with long gradients to achieve the best chromatographic resolution [31–33]. A new approach using a HPLC column that combines both ion-exchange and reversed-phase (RP) separation mechanisms showed significant improvement in chromatographic



R_1	R_2	Name
OH	OH	delphinidin (DE)
OH	H	cyanidin (CY)
OMe	OH	petunidin (PT)
H	H	pelargonidin (PG)
OMe	H	peonidin (PN)
OMe	OMe	malvinin (MV)

R_3	Abbreviation
OH	GLC
acetate	GLC-AC
coumarate	GLC-CO
caffeoate	GLC-CA

key to compound numbers

3, DE-GLC2 (GLC2: 3,5-O-diglucoside); **4**, CY-GLC2; **5**, PT-GLC2;**6**, PN-GLC2; **7**, DE-GLC; **8,** CY-GLC; **10**, PT-GLC; **11**, PN-GLC; **14**, MV-GLC; **18**, DE-AC; **19**, CY-AC; **21**, PT-AC; **23**, PN-AC; **24**, MV-AC; **25**, DE-GLC2-CO; **27**, CY-GLC2-CO; **28**, PT-GLC2-CO; **30**, PN-GLC2-CO; **31**, MV-GLC2-CO; **32**, DE-CO; **34**, CY-CO; **35**, PT-CO; **36**, PN-CO; **37**, MV-CO

R1
OH
HO
O+
3
R2
O
5
OH
O
OH
R3
OH
OH
a
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
12 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0 10 20 30 40 50 60 70
Retention Time (minutes)
b
M 18 N O 25 P 19 Q 21,27 R 28 S T 23,32 U 24,30 V 31 W 34 35 X Y Z α 36 β 37 γ
A 3 B 4 C 5 D 7 E 6 F 8 G H 10 I J 11 K 14 L
0 5 10 15 20 25 30 35
Retention Time (minutes)

Fig. 69.2 HPLC anthocyanin profiles (520 nm) of Concord (*Vitis labrusca*) skin extracts (MeOH–H_2O–HCOOH = 70:28:2). Magnified regions focus on acylated anthocyanins. (**a**) Mixed-mode column (Primesep, SIELC). (**b**) C_{18} RP column (Zorbax SB-C_{18}, Agilent) [34]

performance, especially for the separation of 3,5-diglucoside anthocyanins from 3-monoglucoside anthocyanins in analyzing grape anthocyanins. A total of 37 anthocyanin peaks were detected in the Concord skin extract using a Primesep column, i.e., a mixed ion-exchange and reversed-phase mode column [34]. In Fig. 69.2a, the separation of different anthocyanin subgroups using a Primesep mixed mode column is achieved avoiding overlaps found with a C_{18} column (Fig. 69.2b). A total of 25 compounds were clearly identified. Other column such as monolithic or rod column has been used to separate 24 anthocyanins in a red cabbage sample in 18 min [35]. The advantages of using monolithic columns over the conventional particulated columns are shorter run times, higher flow rates, and faster column equilibration [36, 37].

A porous polyamide resin is shown to possess hydrogen bond acceptor properties suitable for the separation of polyphenolic solutes such as phenolic acids, flavonols, and flavonoids. The separation is achieved in the presence of solvent mixtures of acetic acid and ethanol. The extent of hydrogen bond adsorption is reviewed based on data obtained from the elution behavior of a variety of simple polyphenolic solutes. Polyamide adsorption chromatography was applied for the purification of resveratrol and polydatin from *Polygonum cuspidatum* Sieb. & Zucc [38].

The highly cross-linked 12 % agarose gel, Superose® 12 HR 10/30, possesses hydrogen bond acceptor properties suitable for the separation of polyphenolic solutes such as phenolic acids, flavonols, and flavonoids. The separation is achieved isocratically in the presence of solvent mixtures of acetic acid and ethanol. The extent of hydrogen bond adsorption is reviewed based on data obtained from the elution behavior of a variety of simple polyphenolic solutes including dihydroxybenzoic acids [39, 40].

Columns of HPLC with monolithic supports generally enable faster separations, for example, a 4 mL/min elution flow could be utilized achieving an HPLC analysis [35]. However, the high flow rate makes this type of column not suitable for mass spectrometry detection. Alternatively, smaller dimension columns packed with smaller particle sizes than the conventional ones achieve a faster separation while maintaining resolution. A Zorbax SB C_{18} column (1.8 μm particle size) has been used for the determination and identification of flavonoids and isoflavonoids (genistin, genistein, daidzein, daidzin, glycitin, glycitein, ononin, formononetin, sissotrin, and biochanin A) in fmol quantities in submicroliter sample volumes by HPLC/UV–VIS DAD separation method (which takes <1 min) [41].

Immobilized artificial membrane (IAM) stationary phase consists of a monolayer of phospholipid covalently immobilized on an inert silica support. The IAM stationary phase mimics the lipid environment found in cell membranes, and it can be used for elucidating drug-membrane interactions. The interaction of catechins, flavones, flavonols, anthocyanidins, and anthocyanins with phosphatidylcholine was investigated by HPLC with an IAM column. The IAM partition coefficients of the flavonoids correlated well with the amounts flavonoids incorporation into the liposomes [42].

4.2 Mobile Phases

One of the most important parameters for well separation of flavonoids is the composition of the eluent. Controlling the solubility of the flavonoids in the eluent is a crucial factor for determining the combination of solvents used. In RP-HPLC, analytes are retained on the stationary phase based on their hydrophobicity. Elution of flavonoids in RP-HPLC is therefore in the order of decreasing polarity. Polarity increases most by hydroxyls at the fourth position, followed by those at the second and third positions. Loss of polar hydroxyl groups or additions of methoxy groups reduce polarity and hence increase retention times.

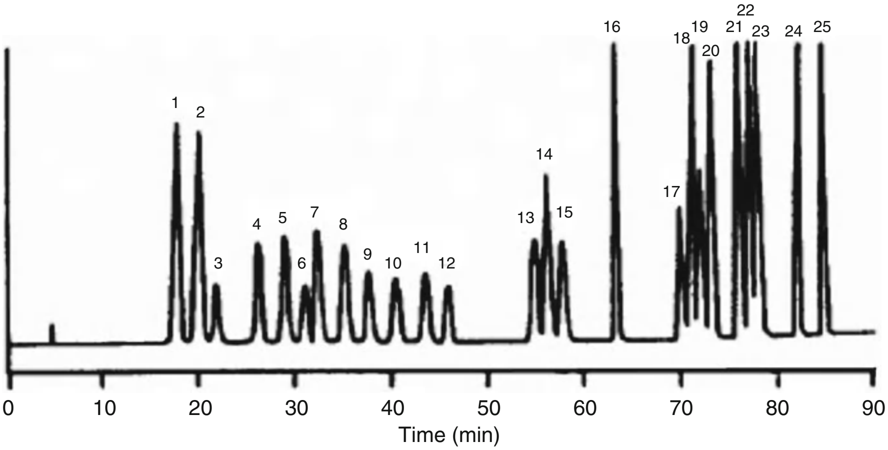
In 1974, the first application of HPLC to flavonoid analysis was published [43], and 2 years later, 12 flavonoids were separated by RP-HPLC in a methanol–water–acetic acid (30:65:5) mobile phase system [44]. In 1994, Nogata et al. reported a separation of 25 naturally occurring *Citrus* flavonoids (flavones, flavonols, and flavanones) simultaneously with a gradient system of 0.01 M phosphoric acid (A) and methanol (B), in three steps: (1) 0–55 min, 70–55 % (v/v) A in B, (2) 55–95 min, 55-0% A in B, and (3) 95–100 min, isocratic, 100 % B, measured at 285 nm, Fig. 69.3 [45].

Both isocratic and gradient elution methods have been successful in separating flavonoids from extracts. However, isocratic elution is less used than that of gradient because it tends to resolve better of members of the same class of flavonoids but works well with monolithic columns. An RP-HPLC method using a monolithic column was developed and validated for the separation and quantification of three flavonols, myricetin, quercetin, and kaempferol, in Rhus coriaria L. The method employed the isocratic mobile phase acetonitrile-10 mM potassium dihydrogen orthophosphate buffer adjusted to pH 3.0 using orthophosphoric acid at a flow rate of 4.0 mL/min, a Chromolith Performance RP-18e (100 × 4.6 mm) monolithic column kept at 40 °C, and UV detection at 370 nm [46]. Successful attempts in simultaneous determination of different classes of flavonoids (quercetin, naringenin, naringin, myricetin, rutin, and kaempferol) using a commercially available monolithic column and isocratic elution were also achieved [36].

Gradient elution is more often employed in recognizing the complex flavonoid profiles of plants, food, and drinks. Rutin, quercetin-3-arabinoside, naringin, myricetin, quercetin, apigenin, and quercetin dimethyl ether in beer samples were separated by gradient elution using a multichannel electrochemical detection with a CoulArray detector [47]. A step linear gradient method using a mixture of methanol and 0.1 % formic acid as a mobile phase was validated for the simultaneous determination of five flavonoids (rutin, quercitrin, quercetin, kaempferol, and isorhamnetin) in rat plasma [48]. Another example is the analysis of rat urine, bile, and plasma after the oral dose administration of rhubarb extract using a gradient of 0.1 % formic acid (A) and methanol (B) starting with 5 % B at 0–10 min, 5–20 % B at 10–30 min, 20–25 % B at 30–40 min, 25–45 % B at 40–160 min, 45–60 % B at 160–180 min, 60–80 % B at 180–200 min, and 80 % at 200–220 min (Fig. 69.4) [49].

Multisolvent gradient elution conditions have been suggested to tackle materials that are difficult to separate. Three-component solvent system, methanol–acetonitrile–water, is commonly used in separation of natural products [50, 51]. Recently, a detailed study on the ratio of acetonitrile to methanol in a three-component solvent system for achieving improved separation capabilities of 11 flavonoids (flavanols, biflavanol, triflavanol, and flavanones) was made [52].

Ionic strength and pH of mobile phase is known to influence the retention of flavonoids on the column depending on if there is protonation dissociation or a partial dissociation. An increase of pH enhances the ionization of flavonoids and could reduce the retention in a reversed-phase separation. Thus, small amounts of HOAc (2–5 %), H_3PO_4, or TFA (0.1–1 %) are normally included in the solvent to suppress ionization of phenolic or carboxylic groups and hence improve resolution



No.	Name	subclass	OH	OMe	O-glycoside
1	Eriocitrin	flavanone	5,3′,4′	–	7-O-rutinoside
2	Neoeriocitrin	flavanone	5,3′,4′	–	7-O-neohesperidoside
3	Robinetin	flavonol	7,3′,4′,5′	–	–
4	Narirutin	flavanone	5,4′	–	7-O-rutinoside
5	Naringin	flavanone	5,4′	–	7-O-neohesperidoside
6	Rutin	flavonol	5,7,3′4′	–	3-O-rutinoside
7	Hesperidin	flavanone	5,3′	4′	7-O-rutinoside
8	Neohesperidin	flavanone	5,3′	4′	7-O-neohesperidoside
9	Isorhoifolin	flavone	5,4′	–	7-O-rutinoside
10	Rhoifolin	flavone	5,4′	–	7-O-neohesperidoside
11	Diosmin	flavone	5,3′	4′	7-O-rutinoside
12	Neodiosmin	flavone	5,3′	4′	7-O-neohesperidoside
13	Neoponcirin	flavanone	5	4′	7-O-rutinoside
14	Quercetin	flavonol	5,7,3′,4′	–	–
15	Poncirin	flavanone	5	4′	7-O-neohesperidoside
16	Luteolin	flavone	5,7,3′,4′	–	–
17	Kaempferol	flavonol	5,7,4′	–	–
18	Apigenin	flavone	5,7,4′	–	–
19	Isorhamnetin	flavonol	5,7,4′	3′	–
20	Diosmetin	flavone	5,7,3′	4′	–
21	Rhamnetin	flavonol	5,3′,4′	7	–
22	Isosakuranetin	flavanone	5,7	4′	–
23	Sinensetin	flavone	5,6,7,3′,4′	–	–
24	Acacetin	flavone	5,7	4′	–
25	Tangeretin	flavone	5,6,7,8,4′	–	–

Fig. 69.3 Separation of 25 flavonoid standards. The detector monitored the eluent at 285 nm and measured spectra from 200 to 360 nm. A two-solvent gradient system: (1) 0–55 min, 70–55 % (v/v) A (0.01 M phosphoric acid) in B (methanol), (2) 55–95 min, 55-0 % A in B, and (3) 95–100 min, isocratic, 100 % B [45]

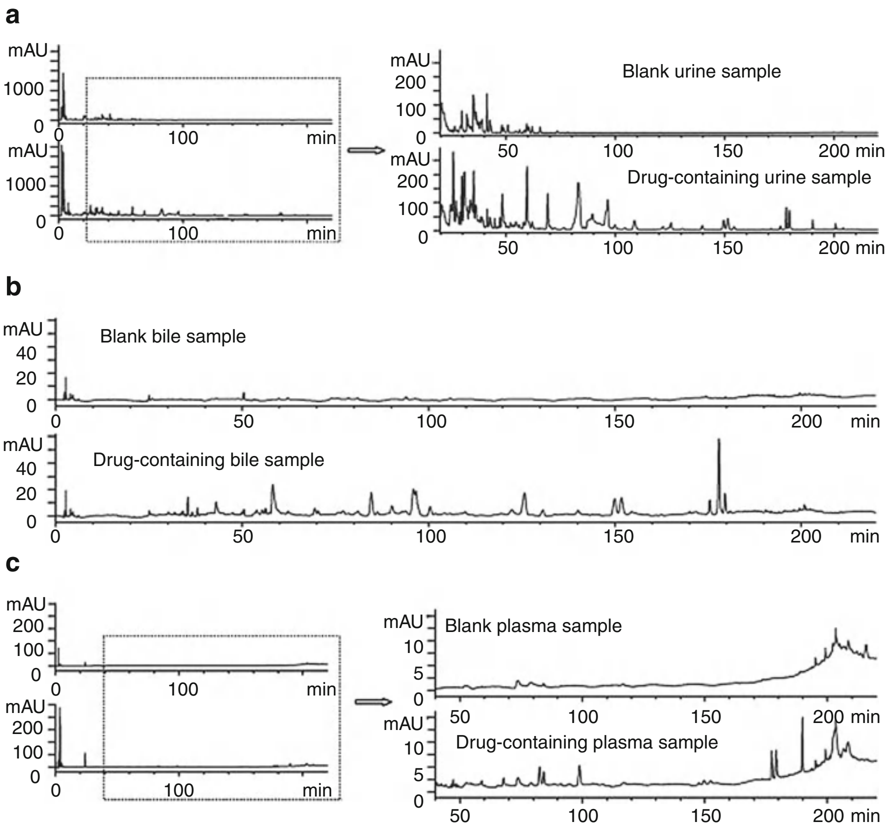


Fig. 69.4 HPLC–DAD chromatograms monitored at 280 nm of rat (**a**) urine, (**b**) bile, and (**c**) plasma before and after administration of rhubarb decoction [49]

and reproducibility of each separation [53]. Both acetate and phosphate buffers have been used as part of the mobile phase for optimizing the analysis time and enhancing separation [29, 54].

5 Identification

Choosing an appropriate detector in an HPLC analysis of flavonoids is as crucial as the stationary and mobile phase. The detector reports the chemical composition of the column effluent via a recorded or digitized signal. The chemical information can be processed differently depending on the type of detectors used. The selection of a detector in flavonoid analysis is normally based on the chemical properties and the sensitivity of the analytes. The two detection techniques widely used in flavonoid analysis are UV–VIS spectrophotometry and mass spectrometry. Multiple-wavelength detection, such as diode array detection (DAD), can be used for positive identification by comparing the retention time and UV spectrum with authentic standards. However, if no reference standard is available, detections such as tandem mass spectrometry and NMR spectroscopy have proved useful in the identification of flavonoids.

5.1 Detection

5.1.1 UV–VIS and Photodiode Array Detection (UV-DAD)

UV–VIS spectrophotometry offers a routine detection and quantitation of flavonoids in HPLC. The two common solvents used as mobile phase in flavonoid analysis are acetonitrile and methanol, and their UV cut-off λ_{max} are 190 and 205 nm, respectively. They do not interfere with the two UV–VIS absorption bands at 240–285 nm and 300–560 nm corresponding to two aromatic rings (A and B) of the flavonoid aglycones [55]. For flavones, the substitution of OH or OMe positions in aglycones and the type of glycosides (either *C*- or *O*- glycosides) give a slight change of the λ_{max} of both bands [30]. The hydrolyzed anthocyanins, anthocyanidins, show a characteristic absorbance in the visible region between 515 and 540 nm [3, 56]. On the other hand, there is little or no conjugation between the A- and B-rings of flavanones and isoflavaones, and hence they only exhibit a low intensity in band I which often appears as a shoulder to the peak of band II [57].

Multiple-wavelength absorbance detection offers advantages over single-wavelength absorbance detection in flavonoid analysis of plant and food products by HPLC. These products normally contain flavonoids of different subclasses and variable substitutions in the same subclass. Two compounds may elute very close together within one peak, but they may be identified by the differences in their spectra. For example, catechins in tea infusions were identified by comparing peak retention times and online DAD spectra of authentic standards, (−)-epigallocatechin, (−)-epigallocatechin gallate, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin 3-*O*-(3-*O*-methyl) gallate, and (−)-3-*O*-methyl epicatechin gallate [58]. The total flavonoid content of leaves of *Passiflora incarnata* L., Passifloraceae harvested from plants cultivated or collected under different conditions was evaluated by high-performance liquid chromatography with ultraviolet detection (HPLC-UV-DAD) [59]. Figure 69.5 shows an HPLC-UV-DAD chromatogram measured at $\lambda = 337$ nm of leaves and the UV-DAD spectra of flavonoids: orientin, homoorientin, vitexin, and luteolin.

5.1.2 Electrochemical Detection (ECD)

Electrochemical detectors measure chemical properties of a compound and rely on chemical reactions in which electrons are transferred from one compound to another. There are two types of electrochemical detectors, amperometric or coulometric detectors. The latter one is commonly used because of its high surface of contact with a structure of porous graphite working electrodes giving 100 % of the analyte. The magnitude of the current is therefore directly proportional to the injected compounds, and conveniently the peak areas in an HPLC chromatogram represent the total current as a function of time.

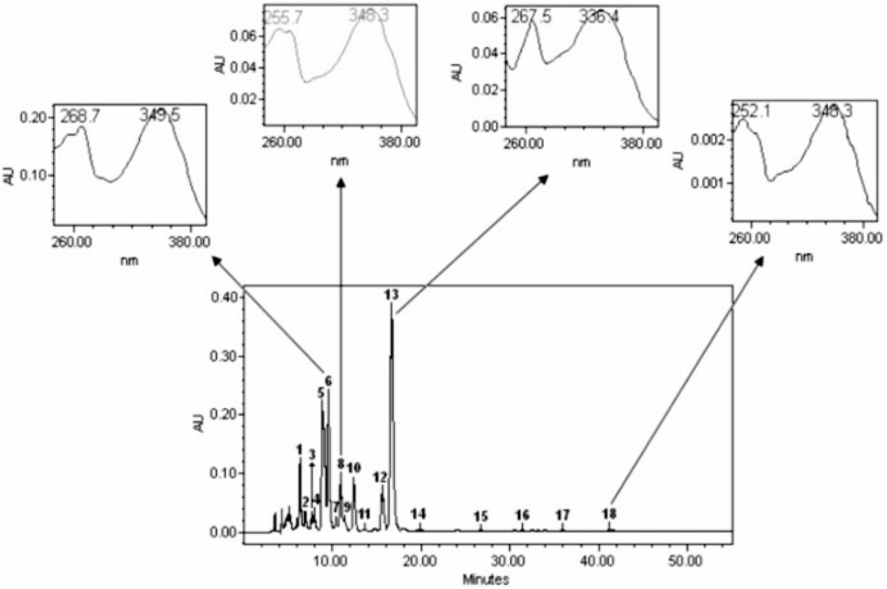


Fig. 69.5 Representative HPLC-UV-DAD ($\lambda = 337$ nm) chromatogram of leaves of *Passiflora incarnata* L. and UV-DAD spectra of flavonoids peaks identified as (**6**) orientin, (**8**) homoorientin, (**12**) vitexin, and (**18**) luteolin [59]

Coulometric detectors are particularly suited to the analysis of flavonoids since the electroactive hydroxyl group present in rings A and B often has a low potential of oxidation. The capabilities of electrochemical detection techniques were demonstrated on 11 compounds belonging to three different classes of flavonoids: flavanone glycosides, flavone and flavonol aglycones. Separation of all compounds examined has been carried out under reversed-phase conditions on a C_{18} standard-bore column and using a porous graphite electrode for electrochemical detection. Instrumental precision in terms of relative standard deviation was found to be between 0.6 % and 10 % [60]. Another example of HPLC-ECD using a microbore column analyzing 15 flavonoids in bottled Japanese green tea samples were reported. The flavonoids were divided into two groups according to their hydrophobicity and were resolved by two isocratic systems: methanol–water (1:1 and 3:7, v/v) containing 0.5 % phosphoric acid. The retention factor (k) of each flavonoid linearly correlated with the log P values. The detection limits (S/N = 3) of the flavonoids tested were in the range of 2–25 fmol, that is, 600 times more sensitive than conventional HPLC with UV detection [61].

Multichannel electrochemical coulometric detection or coulometric array detection has been developed so that different potentials are applied on the electrodes. A number of chromatograms (8, 12, or 16) can be recorded simultaneously. Flavonoids can have several oxidation processes across the array of potentials, giving characteristic profiles for identification. Methods were developed for the analysis of flavonoids in beverages and plant extracts using gradient HPLC with multichannel electrochemical coulometric detection. Eight-channel CoulArray detection offers high selectivity and sensitivity with limits of detection in the low $\mu g\ L^{-1}$ range, at least an order of magnitude lower than single-channel coulometric detection using the Coulochem detector [62]. An example is given in Fig. 69.6 showing the chromatogram of a mixture of standard phenolic and flavonoid compounds, at 0.25 $mg\ L^{-1}$ each, at the optimized HPLC separation selectivity and CoulArray sensitivity under gradient conditions on a Purospher Star column.

5.1.3 Fluorescence Detection (FD)

Fluorescence detection in conjunction with HPLC post-column treatment is commonly used to fulfill the requirements of sensitivity and specificity needed for the study of flavonoids in body fluid. The number of flavonoids that exhibit native fluorescence is limited, and derivatization of flavonoids with reagents such as Al^{3+} [63] and Tb^{3+} [64] is needed before detection. If a hydroxyl group is replaced by a methoxy group, fluorescence becomes considerably more intense as demonstrated by a study using luteolin flavones [65].

Another example of post-column liquid chromatographic reaction system for the determination of flavonoids in orange juices is based on the use of the long-wavelength fluorophore cresyl violet and cerium (IV) in a cetyltrimethylammonium bromide micellar medium [66]. Two flavone aglycones (quercetin and kaempferol), a flavanone aglycone (naringenin), one flavone-*O*-glycoside (rutin), and two flavanone-*O*-glycosides (hesperidin and naringin) were used as analyte models. The reaction process involves the interaction between the analyte, cerium(IV), and cresyl violet giving rise to a decrease in the fluorescence, measured at λ_{ex} 585, λ_{em} 625 nm, which is proportional to the analyte concentration.

5.1.4 Mass Spectrometry Detection

Improvements in the instrumentation, ionization sources, high-resolution mass analyzers, and detectors [67–69], in recent years have taken mass spectrometry to a different level of HPLC-MS for natural product analysis. Mass spectrometry detection offers excellent sensitivity and selectivity, combined with the ability to elucidate or confirm chemical structures of flavonoids [70–72]. Both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are most commonly used as ionization sources for flavonoid detection [73–76]. Both negative and positive ionization sources are applied. These sources do not produce many fragments, and the subsequent collision-induced dissociation energy can be applied to detect more fragments. Tandem mass spectrometry (MS^n, $n \geq 2$) provides information about the relationship of parent and daughter ions, which enables the confirmation of proposed reaction pathways for fragment ions and is key to identify types of flavonoids (e.g., flavones, flavonols, flavanones, or chalcones) [77–80].

Anthocyanins are in glycosylated forms, and their aglycones are known as anthocyanidins. The positive charge in the tetravalent oxygen makes anthocyanidins more suitable for MS analysis in positive mode at low voltages [81]. MS detection of catechins and gallocatechins, which are proanthocyanidins,

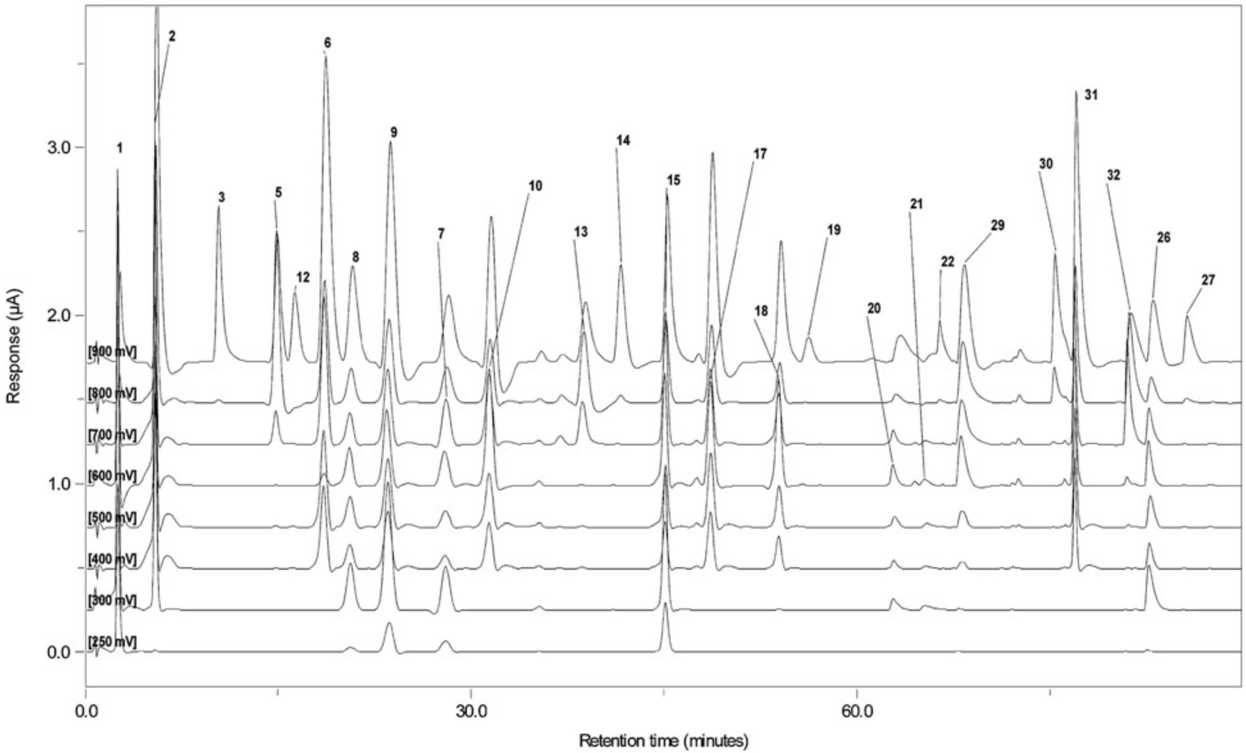


Fig. 69.6 Chromatogram of a mixture of phenolic and flavonoid antioxidant standards. Column (Purospher STAR, RP-18e,150 × 2.9 mm, 5 µm), gradient condition, 0 min: 2 % MeCN; 20 min: 2 % MeCN; 50 min: 9 % MeCN; 65 min: 19 % MeCN; 90 min: 50 % MeCN, pH = 3.14, flow rate 0.4 mL min^{-1}. (Flavonoids: **7**, (+)-catechin; **15**, (−)-epicatechin; **20**, rutin; **21**, quercetin-3-arabinoside; **22**, naringin; **23**, myricetin; **24**, quercetin; **25**, apigenin; **26**, quercetin dimethyl ether; **30**, naringenin; **31**, hesperetin) [62]

can solve the problem suffered from the interferences caused by co-eluting phenolics in UV detection [82]. In the fragmentation of catechins, a loss of 152 mass unit (168 mass unit for gallocatechins) is produced due to their retro-Diels–Alder fission. The characteristic signals in mass spectra of catechins and gallocatechins enable identification of their polymerization [83].

Further details on mass spectrometric analysis of flavonoids are discussed in ► Chap. 66, "Mass Spectrometric Detection of Phenolic Acids."

5.2 Combination of Detection

Multiple detections, such as HPLC-UV-MS^n, HPLC-UV-NMR, HPLC-DAD-FD-UV, and HPLC-DAD-FD-MS, have been adopted to identify and characterize the structure of flavonoids and also used to evaluate the bioactivity of components [72, 84–87]. In most cases, single-stage MS is used in combination with UV detection to facilitate the confirmation of the identity of flavonoids in a sample with the help of standards and reference data (an example is given in Fig. 69.7, [88]). For the identification of unknowns, tandem mass spectrometry is used.

A recent example of using the multiple detection system elegantly by incorporating a simultaneous bioactivity screening into a three-detection system (photodiode array and fluorescence detectors and an electrospray ionization tandem mass spectrometer, DAD-FD-MS^2) [87] demonstrated that 25 flavonoids could be characterized and/or tentatively identified in an aqueous infusion of leaves of *Ficus deltoidea* (Moraceae). The main constituents are flavan-3-ol monomers, proanthocyanidins, and C-linked flavone glycosides. The proanthocyanidins were dimers and trimers comprising (epi)catechin and (epi)afzelechin units. The antioxidant activity of *F. deltoidea* extract was analyzed using HPLC-DAD-FD-UV$_{\text{antioxidant}}$ detection, showing 85 % of the total antioxidant activity of the aqueous *F. deltoidea* infusion was attributable to the flavan-3-ol monomers and the proanthocyanidins. The data obtained from the online HPLC-ABTS antioxidant detection system are shown in Fig. 69.8 along with absorbance traces at 280 and 365 nm. The chromatographic profiles after 34 min did not exhibit antioxidant activity. The peaks contributing the main antioxidant activity were the flavanonol monomers gallocatechin (peak 1), catechin (peak 3), and epicatechin (peak 9), and the flavone apigenin-6,8-*C*-diglucoside (peak 11).

HPLC-UV-NMR is a powerful technique for the identification and characterization of flavonoids. However, there are drawbacks, as NMR remains rather insensitive because of the need for solvent suppression, which has restricted the observable NMR range. Recently, two major research developments in HPLC-UV-NMR are post-column solid-phase extraction (HPLC-UV-SPE-NMR) and combination of HPLC-UV-SPE with capillary separations and NMR detection [89]. A post-column treatment of analyte focusing and multiple trapping through a SPE has solved the problem of sensitivity and solvent suppression. The separation and elucidation of three *C*-methylated flavanones and five dihydrochalcones from *Myrica gale* seeds have been achieved by HPLC-DAD-SPE-NMR and

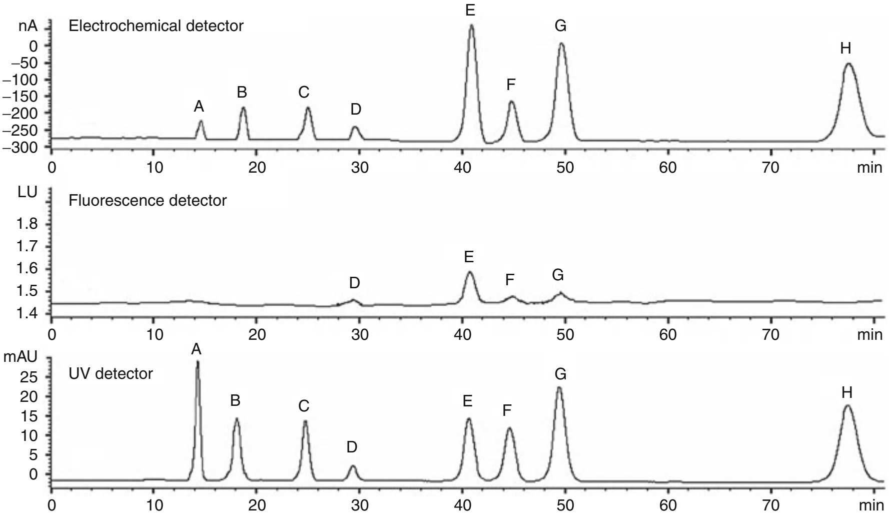


Fig. 69.7 Chromatograms of the flavonoid standards; rutin (*A*), isoquercitrin (*B*), luteolin-40-glucoside (*C*), quercetin-40-glucoside (*D*), quercetin (*E*), naringenin (*F*), luteolin (*G*), and apigenin (*H*) with electrochemical, fluorescence, and UV detectors [88]

HPLC-DAD-MS [90]. Analysis of flavonoids in Wormwood (*Artemisia absinthium* L.) and in the leaves of 12 Litsea and Neolitsea plants has been also achieved by HPLC-DAD-SPE-NMR and HPLC-DAD-MS [91, 92].

6 Two-Dimensional (2D, LC × LC) HPLC

Online 2D LC × LC separation is achieved by a direct coupling of primary and secondary columns through switching valves. Two approaches are used. In the first, eluent containing peaks of interest and monitored during the first dimension of separation is redirected to the second dimension of separation. In the second, a comprehensive 2D setup, the whole sample is subjected to both separations. The advantage of 2D chromatographic techniques over 1D methods is the increase in peak capacity (resolving power) but the timescale for achieving it is comparatively long [93]. Separation of flavonoids in plants and foods requires comprehensive 2D LC × LC approach for full separations. RP-HPLC is normally used in the 1D separation, and hence RP × RP systems, perhaps with different selectivity stationary phases, is selected for 2D analysis.

A review on the use of different stationary phases in polyphenols, polycarboxylic acids, and flavonoids has highlighted the differences in selectivity of these classes of polar or possibly ionized compounds [94, 95]. Figure 69.9 shows the contour plot and the elution conditions of the comprehensive 2D separation of phenolic acids and flavones using parallel gradients of acetonitrile in a 5 mM ammonium acetate buffer on a PEG microcolumn in the first dimension and

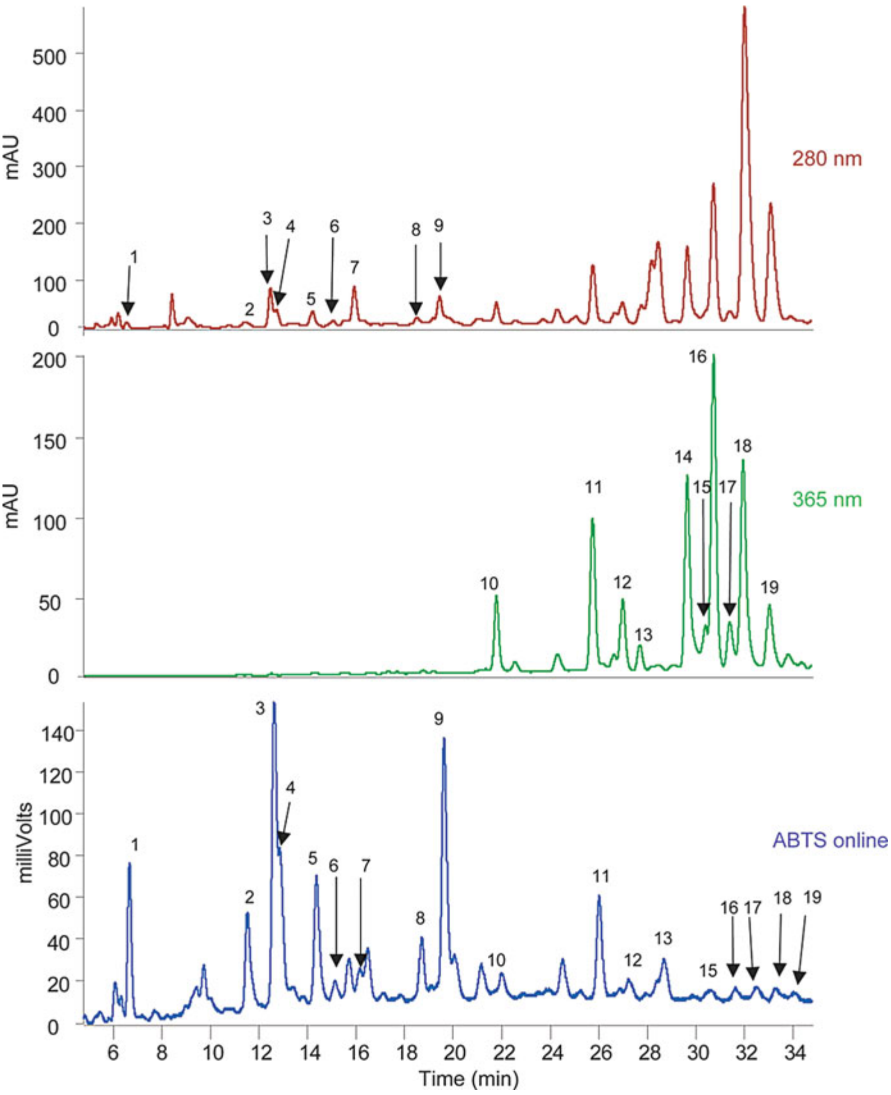


Fig. 69.8 Reversed-phase HPLC of an aqueous infusion of *F. deltoidea* leaves with absorbance detection at 280 and 365 nm and online ABTS$^+$ antioxidant detection at 720 nm [87]

a short monolithic C_{18} column in the second [95]. In this study, flavonoids included for separation are **19** (+)-catechin, **20** (−)-epicatechin, **21** rutin, **22** naringin, **23** myricetin, **24** quercetin, **25** apigenin, **27** luteolin, **28** naringenin, **29** 7-hydroxyflavone, **30** hesperidin, **31** morin, **32** hesperetin, and **33** flavone. There is a clear separation of compounds **19** and **33**.

In addition, from the same group has developed a comprehensive 2-D LC × LC system for the separation of phenolic and flavone antioxidants, using a PEG-silica

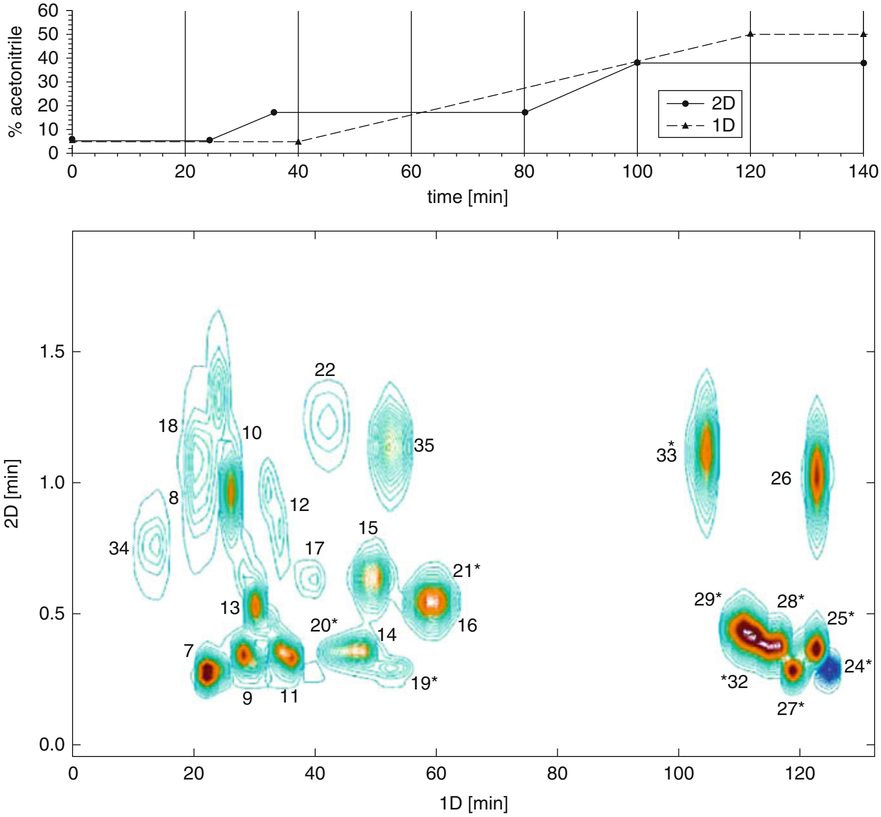


Fig. 69.9 Contour plot and elution conditions (*top*) showing comprehensive LC × LC separations of phenolic acids and flavones on a PEG column in the first dimension and on a Chromolith RP-18e column in the second dimension with parallel gradients of acetonitrile in the two dimensions. Compounds that are flavonoids in this figure (*) are **19** (+)-catechin, **20** (−)-epicatechin, **21** rutin, **22** naringin, **24** quercetin, **25** apigenin, **27** luteolin, **28** naringenin, **29** 7-hydroxyflavone, **32** hesperetin, and **33** flavone [95]

column in the first dimension and a C-18 column with porous-shell particles in the second dimension and the use of electrochemical coulometric detection to compensate the effects of the baseline drift observed in UV during the gradient elution [96]. Superficially porous columns with fused core particles improve the resolution and speed of second dimension separation in comparison to a fully porous particle C_{18} column. The developed system has been applied to the analysis of flavonoids and phenolic acids in beer samples.

A comprehensive two-dimensional HPLC system, with an RP column as a primary column and an immobilized liposome chromatography (ILC) column as a secondary column, was developed for the screening and analysis of the membrane-permeable compounds in the traditional Chinese medicine

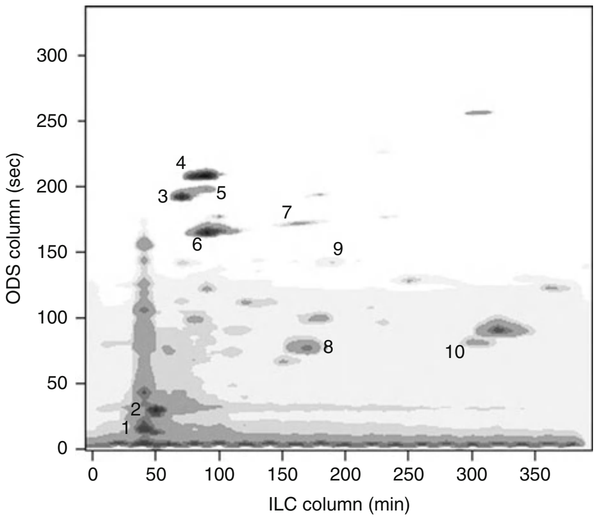


Fig. 69.10 2D chromatogram of Longdan Xiegan Decoction. Chromatographic conditions for the ILC column: isocratic elution with 10 mM ammonium acetate solution (pH 6.8); flow rate, 0.05 mL/min. Chromatographic conditions for the ODS column: linear gradient elution from 10 % MeCN to 70 % MeCN in 7 min, and then returning to the initial mobile phase and holding for 3 min for re-equilibration; flow rate, 2.0 mL/min; injection volume, 5 μL; detection wavelength, 210 nm. Cycle time for the second dimension is 10 min. Compounds (1–10): geniposide, gentiopicroside, oroxylin A-7- *O*-glucuronide, wogonoside, 7-*O*-β-D-glucuronopyranosylchrysin, baicalin, ononin, liquiritin apioside, 3′,4′-dihydroxy-5,6-dimethoxy-7-*O*-glucosideflavone, liquiritin [97]

prescription Longdan Xiegan Decoction (LXD) [97]. More than 50 components in LXD were resolved using the developed separation system. Eight flavonoids and two iridoids were identified interacting with the ILC column, a system that mimics biomembranes (Fig. 69.10). The results show that the developed comprehensive two-dimensional chromatography system can be used for identifying membrane permeable flavonoids in complex matrixes such as extracts of traditional Chinese medicine prescriptions. A similar system with an RP column and a silica-bonded human serum albumin (HSA) column was developed for the biological fingerprinting analysis of bioactive components in LXD [98].

7 Quantitation

Quantitative analysis of flavonoids in plant, food, and biological samples is important because these compounds are partially responsible for the biological activity and medical benefits in these products. Flavonoids are commonly used as chemical markers for quality control purpose of plant and food products.

Flavonoids can be determined quantitatively by direct (in glycoside or conjugated form) or indirect (after hydrolysis) analysis. However, sample preparation (e.g., particle size) and solvents used in extraction steps can significantly affect the results [99]. Method development for quantitation is often validated in terms of selectivity, accuracy, precision, recovery, calibration curve, and reproducibility. Biological sample methods have to comply with the Food and Drug Administration (FDA) guidelines for validation of bioanalytical method [100].

With the coupling of HPLC to different sensitive detection techniques, quantitation of flavonoids in all types of samples has been explored whenever the reference standards are available for calibration [101–103]. Furthermore, the quantitation potentials of analytes (regardless of the type of compounds) rely mainly on the sensitivity limits of the coupled detection system. To date, HPLC coupled to a UV–VIS detector is the most popular quantitation technique used in flavonoid analysis especially for samples with high flavonoid concentrations. The wavelengths used to quantify anthocyanins are at the range 510–520 nm, flavanonols at 280 nm, flavones and flavanols at 270 and 360–370 nm. However, DAD is the other detection mode for quantitation but only slightly more sensitive than UV, and it is still not as sensitive as MS. The detection limits of LC-UV/DAD are usually in the region of μg/mL to the ng/mL levels, while for LC-MS, it is in the region of ng/mL to the pg/mL levels. The ability for the analysis to attain the lowest possible limit of detection, characteristic of the detector, depends on the chromatographic and detection method development, as well as the sample preparation/clean-up method. These are important factors to consider in order to prevent interferences, which could cause inaccurate and misleading measurements. A good review on quantitative analysis of flavonol glycosides, biflavones, and proanthocyanidins in *Ginkgo biloba* leaves, extracts, and phytopharmaceuticals has been published highlighting the different factors from extraction, separation to detection on the quantitative analysis of one subclass of flavonoid [104].

Quantitative methods using the HPLC-electrospray ionization-tandem mass spectrometry method (HPLC–MS^2) facilitates the achievement of adequate sensitivity for pharmacokinetic and metabonomic studies with flavonoids. Matrix effects on signal intensity are important in biological samples, especially during the preparation of calibration curves, to avoid errors from nonlinear range at high concentration [50]. Flavonoid kaempferol, for example, is mainly present as glucuronides and sulfates, and small amounts of the intact aglycone in rat plasma. A validated HPLC–MS^2 method following FDA guidelines has been reported for determination of kaempferol and its major metabolite glucuronidated kaempferol in rat plasma in a study of the pharmacokinetics after oral administration of kaempferol with different doses [103]. The separation of kaempferol and its metabolites was carried out on a C_{18} column (150 × 2.1 mm, 4.5 μm, Waters Corp.) with isocratic elution at a flow rate of 0.3 mL min^{-1}, and a mobile phase consisting of 0.5 % formic acid and acetonitrile (50:50, v/v). The quantitative determination was from a Quattro Premier mass spectrometer operating under a multiple-reaction monitoring mode (MRM), using the electrospray ionization technique.

8 Selected Examples of Flavonoids Analysis by HPLC

Some examples of more flavonoid analyses by HPLC have been selected and detailed as presented in Tables 69.3–69.5. These examples are divided into plant (Table 69.3), food (Table 69.4), and biological (Table 69.5) samples. Notably, these examples are mainly from work published from 2008 onward, except for three papers in 2006 and 2007. These examples have been the selected ones due to the research article details including good representation(s) of chromatogram(s) to assist other researchers to easily validate their studies. These examples show the application of this chapter's aforementioned types of extractions, separations (in terms of column chemistry, dimensions), and the detection systems.

9 Conclusion

The number of flavonoids (9,000 in 2004 [105], 9,600 in 2007 [1], 10,380 in 2009 [2]) identified from 2004 to 2009 indicates the high level of interest of this class of secondary metabolites in plant. The achievements are a result of the advance in the technology of detection. In 1994, the separation of 25 flavonoids reference standards was made comfortably relying on the stationary phase technology and the knowledge of the interaction of mobile phase and analytes. The improvement of sensitivity and target-specific detection, for example, tandem mass spectrometry detection, has compensated the inability of complete resolution of peaks in a chromatogram and assisted the identification of sugar units, which partly contributed to the boom of newly identified flavonoids in recent years. In certain ambiguous circumstances, for example, the position of substitutions, NMR spectroscopy can provide a fuller picture of the identity of the flavonoids. HPLC-MS has been used to screen compounds for drug discovery programs [106]. In flavonoids, HPLC-UV-MS has been used for screening the antioxidant activities in teas [87] and using the 2D (LC × LC) system in an herbal decoction [97, 98].

In many examples, with little regard of the sample type, the analyses of flavonoids are usually done in the reversed-phase HPLC mode using nonpolar C_{18} (in few cases, C_8) columns and polar mobile phase (mixed aqueous–organic solvents) due to their structural and physicochemical properties. Conversely, with more regard of the sample type, different extractions and sample pretreatments including Soxhlet, LLE, SPE, USAE, ASE, and SFE have been used prior to HPLC flavonoid analysis.

Table 69.3 Selected examples of flavonoid analysis by HPLC in plant (*SLE* solid liquid extraction, *aq*. aqueous, *Q-TOF* quadrupole-time of flight, *TQ* triple quadrupole)

Plant	Flavonoid sub-class	Extraction	Stationary phase	Mobile phase	Detector(s)	References
Glycyrrhiza L. (Leguminosae family): licorice	6 chalcones	SLE (ultrasonification, 70 % aq. MeOH)	Agilent Zorbax SB-C_{18} column (50 × 4.6 mm, 1.8 μm)	Gradient: 0.2 % formic acid (aq.) and MeCN	UV-MS (+ve, Q-TOF MS^2)	[107]
Bupleurum species	1 catechin, 2 flavones, 8 flavonols	SLE (ultrasonification, MeOH)	Shimpack ODS C_{18} column (150 × 4.6 mm, 5 μm)	Gradient: 0.1 % formic acid (aq.) and MeCN	UV	[108]
Orostachys japonicus	7 flavonols, 7 catechins	SLE (reflux, 70 % aq. MeOH; hexane; EA)	Agilent Zorbax SB-C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: 0.05 M ammonium formate (aq.) and MeOH	UV-MS (+ve, Q TRAP)	[71]
Hypericum japonicum	1 flavanonol, 4 flavonols	SLE (ultrasonification, 70 % aq. MeOH)	Agilent Zorbax SB-C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: 0.5 % formic acid (aq.) and MeCN	UV-MS (+ve, Q-TOF MS^2)	[109]
Iris tectorum Maxim. (Iridaceae)	2 flavanones, 1 flavonol, 1 flavanonol	SLE (ultrasonification, MeOH)	Agilent Eclipse Plus™ C_{18} column (150 × 3.0 mm, 3.5 μm)	Gradient: 0.05 % acetic acid (aq.) and MeCN	DAD-MS (−ve and +ve MS^2)	[110]
Murraya paniculata (L.) Jack	14 flavones, 2 chalcones	SLE (ultrasonification, 70 % aq. MeOH)	Agilent Zorbax Eclipse Plus C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: 0.1 % formic acid (aq.) and MeCN	DAD-MS (+ve MS^2)	[111]
Ziziphus jujuba Mill. and *Z. jujuba* var. spinosa (Bunge) Hu ex H.F	3 flavonols	SLE (ultrasonification, 80 % aq. MeOH)	Waters Sunfire C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: 0.2 % acetic acid (aq.) and MeCN	DAD-MS (−ve, Q-TOF MS^2)	[112]

Scutellaria baicalensis Georgi (*S. baicalensis*)	27 flavones, 1 flavanol, 2 flavanones, 1 flavanonol, 1 biflavone	SLE (reflux, 60 % aq. MeCN; CH_2Cl_2)	Welch Materials Ultimate XB C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: 0.06 % acetic acid (aq.) and MeCN	UV-MS (−ve LCQ Ion trap MS^n)	[113]
Chrysosplenium (Turn.) L. (aerial parts) of flowering *C. alternifolium*	4 flavonols	SLE (reflux, MeOH)	Agilent Hypersil C_{18} column (125 × 4 mm, 5 μm)	Gradient: 0.5 % phosphoric acid (aq.) and MeCN	DAD	[114]
Glycin tomentella Hayata (leaves and roots)	3 flavones, 7 flavonols, 6 flavanones	SLE (reflux, 95 % aq. EtOH)	Thermo Hypersil GOLD C_{18} column (250 × 4.6 mm, 5 μm).	Gradient: 9 % acetic acid (aq.) and MeOH	DAD	[115]
Houttuynia cordata Thunb	4 flavonols	Pressurized liquid extraction or hot soaking with shaking (70 % EtOH)	Kromasil Turner YWG C_{18} column (250 × 4.6 mm, 10 μm)	Gradient: water–MeCN–phosphoric acid (400:100:0.2) and MeCN–MeOH–water–phosphoric acid (375:75:50:0.1)	UV	[116]
Artemisia annua L.	5 flavonols, 1 flavone	SLE (Maceration, DCM or hexane)	Merck Eurosphers Star RP-18 column (200 × 4.6 mm, 5 μm)	Gradient: formic acid aq. (pH 3.2) and MeCN	DAD-MS (+ve, ion trap, MS)	[117]

Table 69.4 Selected examples of flavonoid analysis by HPLC in foods (*SLE* solid liquid extraction, *LLE* liquid–liquid extraction, *aq*. aqueous, *Q-TOF* quadrupole-time of flight, *TQ* triple quadrupole)

Food	Flavonoid subclass	Extraction	Stationary phase	Mobile phase	Detector(s)	References
Black currant juice	3 flavones, 2 flavonols, 3 flavanones	None (direct injection)	Agilent Zorbax Rapid Resolution C_{18} column (50 × 2.1 mm, 1.8 μm)	Gradient: 0.1 % formic acid (aq.) and 0.1 % formic acid in MeCN	MS (−ve, LTQ-Orbitrap, MS^2)	[118]
Tomato (*Lycopersicon esculentum* Mill.)	8 flavonols, 11 flavanone, 2 dihydrochalcones	SLE (Homogenization, sonication, and centrifugation); SPE	Phenomenex Luna C_{18} column (50 × 2.0 mm, 5 μm)	Gradient: 0.1 % formic acid (aq.) and 0.1 % formic acid in MeCN	DAD-MS (−ve, LTQ-Orbitrap and TQ, MS^n)	[119]
Passiflora edulis fruit pulp	2 flavones, 1 flavonol	LLE (sonication 60 % or 100 % MeOH or EtOH); SPE	Waters Symmetry C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: 0.2 % formic acid (aq.) and 0.2 % formic acid in MeCN	DAD-MS (−ve, TQ, MS^2)	[120]
Ripe red paprika (*C. annuum*) and yellow habanero (*C. chinense*) peppers	3 flavonols, 2 flavones	LLE (homogenization, EtOH); 3 M HCl	Phenomenex Gemini C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: 0.03 M phosphoric acid (aq.) and MeOH.	DAD-MS (−ve or +ve, Q-TOF, MS)	[121]
Citrus grandis, Citrus paradise (flavedos "external layer of peel" and juices)	15 flavonols, 13 flavanones	LLE (sonication, MeOH)	Agilent Zorbax SB C_{18} column (250 × 4.0 mm, 5 μm)	Gradient: 1 % acetic acid (aq.) and 1 % acetic acid in MeCN	DAD-MS (−ve, Ion trap, MS^2)	[122]
Rooibos tea from *Aspalathus linearis*	4 flavones, 8 flavonols, 2 dihydrochalcones	SLE (boiled, water)	Phenomenex Luna Phenyl–Hexyl (250 × 4.6 mm, 5 μm)	Gradient: 2 % acetic acid (aq.) and MeCN	DAD	[123]

Ocimum gratisimum L., *Vernonia amygdalina* L., *Corchorus olitorius* L., *Manihot utilissima* Pohl.	6 flavonols, 10 flavones	SLE (MeOH; 70 % aq. EtOH, pH 2.5)	Phenomenex Synergi max C_{12} column (150 × 4.0 mm, 4 μm)	Gradient: formic acid aq. (pH 3.2) and MeCN	DAD-MS (−ve, Ion trap, MS)	[124]
Sugarcane raw juice (*Saccharum sinense* Roxb.)	1 flavone, 1 anthocyanin	LLE (n-butanol (1:1 v/v); MeOH)	Waters Symmetry C_{12} column (150 × 4.6 mm, 4.6 μm)	Gradient: 0.1 % formic acid (aq.) and MeOH	DAD	[125]
Slovenian honeys: *Robinia pseudoacacia*, *Tilia spp.*, *Castanea sativa*, *Abies alba* Mill., *Picea abies* (L.) Karst	4 flavonols, 3 flavones, 3 flavanones, 1 flavanonol	LLE (acidified water pH 2); SPE, MeOH–MeCN (2:1, v:v).	Phenomenex Luna C_{18} column (150 × 2.0 mm, 3 μm)	Gradient: 1 % formic acid (aq.) and MeCN	DAD-MS (−ve, TQ, MS^2)	[126]
Red grape skin (Grapes from four varieties of *Vitis vinifera* L.)	5 anthocyanins, 5 flavonols, 1 catechin	Ultrasonification; SLE (ultrasonification, MeOH/HCl 99/1)	Waters Xbridge C_{18} column (150 × 4.6 mm, 5 μm)	Gradient: 2 mM KCl, water/MeOH/formic acid (83/16/1) or Water/MeOH/formic acid (68.5/30/1.5)	ECD	[127]
Buckwheat (*Fagopyrum esculentum* Möench)	4 flavones, 5 flavonols, 19 catechins	SLE (ultrasonification, 80 % EtOH)	Agilent Zorbax Eclipse plus C_{18} column (150 × 4.6 mm, 1.8 μm)	Gradient: 1 % acetic acid (aq.) and a mixture of 1 % acetic acid (aq.) in MeCN (60:40)	MS (−ve, Q-TOF MS^2)	[128]
Rosmarinus officinalis L. (Lamiaceae)	9 flavones, 1 flavonol	SLE (stirring and ultrasonification, EtOH)	Phenomenex Fusion C_{18} column (150 × 3.9 mm, 4 μm)	Gradient: 0.1 % formic acid (aq.) and MeCN	DAD-MS (+ve and −ve, Q, MS)	[129]
Sugarcane (*Saccharum officinarum* L., Gramineae)	9 flavones	SLE (ultrasonification, 50 % MeOH)	Waters Symmetry C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: 0.2 % formic acid (aq.) and MeCN	UV/DAD	[130]

(continued)

Table 69.4 (continued)

Food	Flavonoid subclass	Extraction	Stationary phase	Mobile phase	Detector(s)	References
Concord grape juice	12 procyanidins, 25 anthocyanins, 5 flavanonols	SLE (vortexing, ultrasonification, acetone/water/acetic acid, 70:29.5:0.5, v/v/v)	Phenomenex Luna C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: ACN/EtOAc (7:1, v/v) and 0.05 % acetic acid (aq.).	FD	[131]
Chocolate and cocoa-containing food products	12 procyanidins, 25 anthocyanins, 5 flavanonols	SLE (vortexing, ultrasonification, acetone/water/acetic acid, 70:29.5:0.5, v/v/v)	Phenomenex Luna C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: ACN/EtOAc (7:1, v/v) and 0.05 % acetic acid (aq.)	FD	[131]
Vaccinium macrocarpon cranberry concentrate	7 anthocyanins, 2 proanthocyanidins, 10 flavonols	LLE (EtOAc)	Waters Acquity C_{18} column (100 × 2.1 mm, 1.8 μm)	Gradient: 5 % formic acid (aq.) and MeOH.	DAD-MS (+ve and −ve, Q, MS^2)	[132]

Table 69.5 Selected examples of flavonoid analysis by HPLC in biological samples (*SLE* solid liquid extraction, *LLE* liquid–liquid extraction, *aq*. aqueous, *Q-TOF* quadrupole-time of flight, *TQ* triple quadrupole)

Biological sample	Flavonoid subclass	Extraction	Stationary phase	Mobile phase	Detector(s)	References
Ginkgo biloba in rat plasma	3 flavonols	Acid hydrolysis (sample: 10 M HCl: MeOH 2:1:2 v:v: v); neutralized with 15 M NH_3; LL (MeOH)	Waters C_{18} column (150 × 4.6 mm, 5 μm)	Isocratic: MeCN-0.02 M NaH_2PO_4 (0.2 % H_3PO_4), pH = 2.0, (35:65)	DAD	[133]
Aspalathus linearis in human urine and blood plasma	8 flavones, 4 flavonols, 2 dihydrochalcones	Urine: SPE (Oasis WCX cartridges) Blood: centrifugation (2,000 g for 10 min at 4 C) – plasma: (LLE, EtOAc)	Phenomenex Luna Phenyl–Hexyl (250 × 4.6 mm, 5 μm)	Gradient: 2 % acetic acid (aq.) and MeCN	UV-MS (−ve, ion trap, MS^2)	[123]
Herba Epimedii in dog plasma	7 flavonols	SLE (Vortex, centrifugation MeOH, 70 % EtOH aq.)	Agilent Zorbax Eclipse SB-C_{18} column (50 × 2.1 mm, 1.8 μm)	Gradient: 0.3 % acetic acid (aq.) and 0.3 % acetic acid in MeCN	MS (+ve, TQ, MS^2)	[134]
Hawthorn leaves in rat plasma	2 flavones	SLE (Vortex, centrifugation MeOH)	Dikma Diamonsil TM C18 column (200 × 4.6 mm, 5 μm)	Isocratic: MeOH–MeCN–THF–0.5 % acetic acid (1:1:19.4:78.6)	UV	[135]
Vitis labrusca vines (Concord grapes) in human urine and blood	25 anthocyanins	Acidify with 50 % formic acid aq., SPE (Phenomenex Strata C_{18} (6 mL/500 mg), 1 % formic acid containing 10 % MeOH)	Phenomenex Synergi (250 × 4.6 mm, 4 μm)	Gradient: 1 % formic acid (aq.) and MeOH	DAD-MS (+ve or −ve, ion trap, MS^n)	[131]

(*continued*)

Table 69.5 (continued)

Biological sample	Flavonoid subclass	Extraction	Stationary phase	Mobile phase	Detector(s)	References
Dalbergia odorifera in rat urine	4 neoflavones, 2 flavanones, 2 chalcones	LLE (Vortex, centrifugation EtOAc)	Agilent Zorbax SB C_{18} column (250 × 4.6 mm, 5 μm)	Gradient: 0.3 % acetic acid (aq.) and MeCN	UV-MS (−ve, MS)	[136]
Eriobotrya japonica (Thunb.) Lindl.	9 flavonols	SLE (EtOH)	Hanbon Kromasil C_{18} column (200 × 4.6 mm, 5 μm)	Gradient: 1 % acetic acid (aq.) and 1 % acetic acid in MeOH	DAD-MS (−ve, Ion Trap, MS)	[137]
Vaccinium macrocarpon cranberry concentrate in rats urine and blood	2 flavonols, 7 anthocyanins	Tissue: LLE (homogenization, 80 % MeOH containing 0.1 % acetic acid; vortex, centrifugation 80 % MeOH aq.)	Phenomenex Fusion (150 × 2.0 mm, 1.8 μm)	Gradient: 0.1 % formic acid (aq.) and 0.1 % formic acid in MeCN	MS (−ve, TQ, MS^2)	[132]
		Urine, plasma: Hydrolysis, LLE (diethyl ether)				

Indeed, with the development of separation techniques for HPLC to the ultra-HPLC leading to faster analyses and high throughput, coupled with the advancement of the detection technique, even more flavonoids would both be identified and quantified quicker in the future.

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Polyphenol Purification by Solid Support-Free Liquid–Liquid Chromatography (CCC, CPC)

70

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Contents

1 Introduction 2146
2 The Technology of Solid Support-Free Liquid–Liquid Chromatographic Systems (HSCCC and CPC) 2147
2.1 General Principles 2147
2.2 Crucial Parameters for Efficient Separations in HSCCC and CPC 2148
3 Applications of HSCCC and CPC for the Purification of Polyphenols 2151
3.1 Direct Purification, Orthogonal Purification, or Bioassay-Guided Fractionation? 2151
3.2 Direct Purification or Orthogonal Purification 2151
3.3 Bioassay-Guided Fractionation and Characterization of Plant-Derived Polyphenols 2160
3.4 Solvent System Selection 2161
3.5 HSCCC and CPC Chromatographic Development Modes 2163
4 Conclusion 2165
References 2166

Abstract

In recent years, there has been an increased interest in the use of solid support-free liquid–liquid chromatographic techniques for the purification of polyphenols from natural plant sources. These methods include mainly high-speed countercurrent chromatography (HSCCC) and centrifugal partition chromatography (CPC). This chapter presents a summary of all recent HSCCC and CPC studies investigating the fractionation or purification of phenolic compounds from complex plant extracts. This synopsis, covering the last 5 years and comparing over 90 studies, demonstrates that solid support-free liquid–liquid

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separation techniques offer distinct advantages for the isolation of polyphenols in terms of analyte preservation, solvent consumption, and run duration while the purity and recovery values remain equivalent to or even higher than those obtained with conventional chromatographic methods using solid supports. Crucial parameters affecting the separation of different polyphenol classes will be discussed, particularly the nature of the solvent system, the chromatographic development modes, the flow rate, and the column capacity.

Keywords

Centrifugal partition chromatography • Countercurrent chromatography • Flavonoids • Phenolic acids • Polyphenols

Abbreviations

α	Selectivity
AA	Acetic acid
ACN	Acetonitrile
AM	Ascending mode
BuOH	*n*-Butanol
$CHCl_3$	Chloroform
CPC	Centrifugal partition chromatography
DM	Descending mode
EtOAc	Ethyl acetate
EtOH	Ethanol
FA	Formic acid
Hept	*n*-Heptane
Hex	*n*-Hexane
H_2O	Water
HPCCC	High-performance countercurrent chromatography
HSCCC	High-speed countercurrent chromatography
K_D	Distribution coefficient = analyte concentration in the stationary phase/analyte concentration in the mobile phase
MAE	Microwave-assisted extraction
MeOH	Methanol
M*t*BE	Methyl tert-butyl ether
PA	Phosphoric acid
PE	Petroleum ether
Prep-HPLC	Preparative high-performance liquid chromatography

1 Introduction

A wide range of methods can be used for the purification of phenolic compounds from natural sources. The most common procedures are based on “solvent extraction,” a simple and efficient process applicable to a wide range of raw materials. The starting material is partitioned between at least two liquid phases in order to obtain fractions enriched in the target compound(s). This step is generally followed by macroporous resin separation, silica gel, Sephadex or polyamide chromatography, and preparative thin layer chromatography or preparative high-performance liquid chromatography [1, 2]. These procedures are usually tedious as well as solvent- and time-consuming because of multiple chromatographic steps. In addition, low recoveries are usually obtained, and a large amount of organic solvent waste is often generated [3].

In recent years, there has been an increased interest in the use of solid support-free liquid–liquid chromatographic methods for the separation and purification of flavonoids and other phenolic compounds from natural sources [4, 5]. High-speed countercurrent chromatography (HSCCC) and centrifugal partition chromatography (CPC) are the two main variants of support-free liquid–liquid chromatographic systems being used today. In the development of a HSCCC or CPC separation method, a least two immiscible liquid phases (solvent system) are required. One phase is maintained into the column (stationary phase) by a centrifugal force field, while the other phase (mobile phase) is pumped through it. The principle of both methods relies on the partition of solutes between the two immiscible liquid phases of the solvent system according to their distribution coefficient K_D. Due to the liquid nature of the stationary phase, these techniques are an excellent alternative to more traditional solid support chromatography. Irreversible adsorption of samples on the solid support is avoided, and the chemical integrity of mixtures subjected to fractionation or purification is preserved. Additional benefits include total sample recovery, high sample mass loading capacity, good selectivity, and versatility while consuming low solvent quantities.

This chapter presents an overview reporting over than 90 studies conducted between 2007 and 2012 and dedicated to the fractionation or purification of polyphenols from natural sources using HSCCC or CPC. It is clear that solid support-free liquid–liquid separation techniques have acquired a significant place among the chromatographic techniques commonly used for the purification of natural products. In particular, HSCCC and CPC are extremely promising for the purification of polyphenols. Efficient procedures are currently being developed to allow either the direct purification of phenolic compounds from complex plant extracts, their partial purification for a further combination with an orthogonal solid support-based technique, or for bioassay-guided fractionation strategies investigating the bioactivity of particular polyphenol classes.

2 The Technology of Solid Support-Free Liquid–Liquid Chromatographic Systems (HSCCC and CPC)

2.1 General Principles

High-speed countercurrent chromatography (HSCCC) and centrifugal partition chromatography (CPC) refer to particular types of hydrodynamic and hydrostatic solid support-free chromatographic systems, respectively. Countercurrent chromatography was firstly introduced by Ito, Harada, and Aoki in 1966 [6], while centrifugal partition chromatography was introduced by Nunogaki in 1982 [7]. In HSCCC, hydrodynamic instruments are used where the separation takes place in a so-called "multilayer coil" made of a long piece of continuous tubing wrapped around a holder in multiple layers (Fig. 70.1). The resulting multiple coils can be connected in series to increase the total volume of the instrument. The coil is submitted to a centrifugal force field and rotates around its own axis while simultaneously rotating around the central axis of the system. This results in stationary phase retention and partitioning of the analytes between the two liquid phases. In contrast, CPC is hydrostatic and uses a spinning rotor, comprised of a series of interconnected cells circumferentially engraved on stacked disks in rotation around a single axis. In CPC, the centrifugal force field applied to maintain one phase stationary inside the column remains constant in intensity and direction. In both systems, the mobile phase is pumped through the stationary phase, and the hydrodynamic equilibrium between phases depends on the intensity of the centrifugal force (generated by the rotation speed), on the flow rate of the mobile phase, and on the physicochemical properties of the biphasic solvent system (density, viscosity, interfacial tension, *etc*....). HSCCC and CPC are very interesting in terms of selectivity, sample loading capacity, and scale-up ability [8, 9] and have both been successfully applied to the purification of natural products [4, 5]. They also allow total recovery of injected samples (up to 100 %) and consume low solvent quantities.

The column capacity of HSCCC or CPC instruments ranges from 200 to 1,000 mL for semi-preparative or preparative-scale separation processes. Analytical columns have also been recently developed (25 mL for CPC and 18 mL for HSCCC). Pilot and industrial instruments for large-scale applications are progressively appearing on the market (5–15 L for CPC and up to 18 L for HSCCC). Development of solid support-free liquid–liquid separation instruments is currently focused on the improvement of coil and column design, scale-up capacities, and operating conditions to guarantee industrial applications with high productivity.

2.2 Crucial Parameters for Efficient Separations in HSCCC and CPC

One of the key factors for a successful HSCCC or CPC separation relies on the choice of a suitable biphasic solvent system. A good solvent system should ideally both dissolve as well as efficiently partition the target compounds between the two immiscible phases while displaying a sufficient stability at the hydrodynamic equilibrium of the two liquid phases during the experiment. Generally, the distribution coefficient (K_D) of a compound in a biphasic solvent system should range between 0.5 and 2 [10], and the selectivity ($\alpha = K_b/K_a$) between any pair of compounds to be separated should be greater than 1.5 (with $K_b > K_a$). It should be noted that a high K_D value results in broad chromatographic peaks and long elution duration, while a low K_D value results in poor separation resolution. The stability of a solvent system is reflected by its settling time, which should ideally be shorter than 30 s when measured in a test tube, in order to ensure a sufficient retention of the stationary phase. Finally,

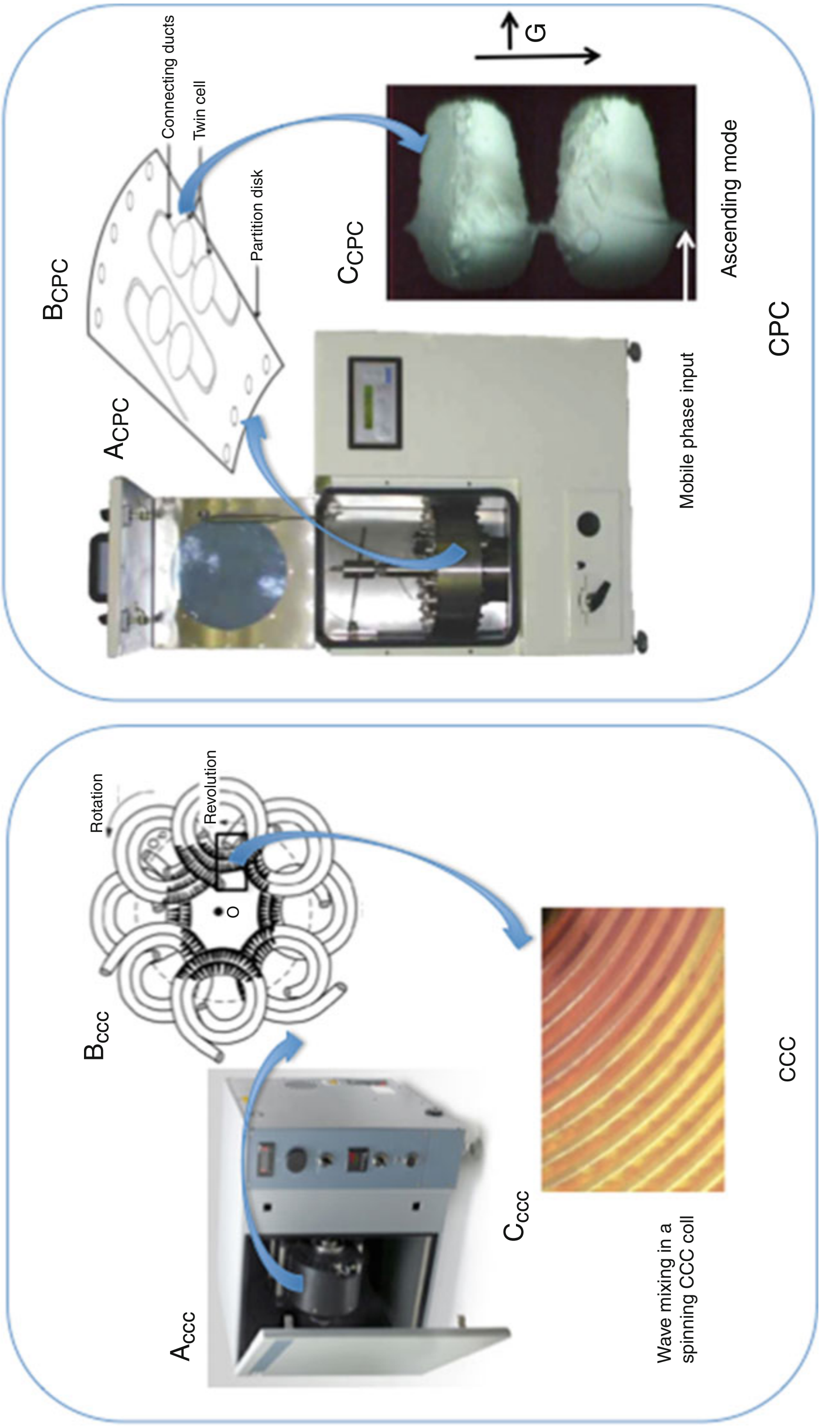
A_{CPC}
B_{CPC}
Connecting ducts
Twin cell
Partition disk
C_{CPC}
G
Mobile phase input
Ascending mode
CPC
A_{CCC}
B_{CCC}
Rotation
Revolution
O
C_{CCC}
Wave mixing in a
spinning CCC coil
CCC

Fig. 70.1 CCC and CPC support-free liquid-liquid separation systems

the selected solvent system should result in similar volumes of each immiscible phase in order to optimize their use and avoid solvent waste.

A large choice of biphasic solvent systems is available in the literature, and sometimes several systems can be suitable for the purification of the same compound. Solvent systems composed of an organic and an aqueous phase are the most popular in conventional HSCCC or CPC processes [10]. For instance, the Arizona liquid system, which combines *n*-heptane/ethyl acetate/methanol/water in different possible proportions, is widely used for the purification of natural products and especially of phenolic compounds [11]. Other solvent systems composed of chloroform/methanol/water or ethyl acetate/*n*-butanol/water have also been widely used for HSCCC and CPC applications. More recently, another kind of solvent system composed of water-soluble hydrophilic organic solvent and inorganic salt solution, first designed for peptide and protein separation, has been used for the purification of natural products including salvianolic acid [12]. These aqueous two-phase systems present the advantage of being low cost and environmentally safe, providing higher polarity compared to the conventional organic/aqueous systems and being suitable for the separation of highly polar natural products. Another original solution consists in the use of salting-out gradients in CPC (chloroform/*n*-butanol/LiCl solution and/or phosphate buffer) for the isolation of chlorogenic acid derivatives [13].

After optimization of the biphasic solvent system, a suitable pumping mode must be selected. When the lower phase of the biphasic solvent system is used as the stationary phase, the experiment is performed in the “ascending mode” in CPC or “tail to head” in HSCCC. When the lower phase is used as the mobile phase, the experiment is performed in the “descending mode” in CPC or “head to tail” in HSCCC. Another interesting aspect of these techniques is the ability to change the role of the two phases during the run, the stationary phase becoming mobile and *vice versa*, to operate in dual mode or in multiple dual mode [14].

In addition to the classical elution mode (isocratic or gradient) where the selectivity depends only on the affinity of the analytes for the two liquid phases, other development strategies based on displacement methods can be applied. Basically, the displacement mode is performed by adding a retaining agent or ion exchanger in the stationary phase and a displacer in the mobile phase. In the pH-zone-refining displacement mode, an acid or a base is dissolved in the stationary phase as retainer. Depending on the nature of the target compound, displacement by pH-zone-refining can be suitable for the separation and purification of ionizable compounds whose electric charge and solubility properties depends on the pH value [15]. This is the case for some polyphenols. In the ion-exchange displacement mode, a cationic exchanger is, for instance, dissolved in the stationary phase in order to capture all anionic analytes present initially in the crude sample via the formation of ion pairs. This method mainly concerns ionic compounds whose charge is invariant and independent of the pH in aqueous solution. Then a displacer agent presenting a stronger affinity for the exchanger is added to the mobile phase, resulting in a step-by-step selective transfer of the analytes from the stationary to the mobile phase [16]. This ion-exchange displacement mode has been previously developed in combination with CPC for the purification of glucosinolates from white mustard and broccoli seeds [17] and rosmarinic acid from *Lavandula vera* cell cultures [18].

3 Applications of HSCCC and CPC for the Purification of Polyphenols

3.1 Direct Purification, Orthogonal Purification, or Bioassay-Guided Fractionation?

Due to their wide injection capacity and absence of solid support, crude samples from several milligrams to several grams can be injected in HSCCC and CPC without extensive preparation. This overview reports a large panel of studies carried out from 2007 to 2012 applying solid support-free liquid–liquid separation methods for the direct purification of polyphenols starting from crude plant extracts. In this literature survey, the purification of phenolic compounds from complex plant extracts was achieved in three ways: either totally by HSCCC or CPC without need for another complementary technique (Table 70.1-A), partially with a further orthogonal solid support-based purification procedure required (Table 70.1-B), or in a bioassay-guided fractionation perspective for the biological investigation of particular polyphenol classes (Table 70.1-C).

3.2 Direct Purification or Orthogonal Purification

Among these studies, HSCCC has been by far the most widely used technique for the purification of polyphenols as compared to CPC. This is probably because HSCCC is becoming a popular and very useful technique for people working in pharmacognosy, particularly in Asian and North American laboratories. Most of the studies presented in Table 70.1 have been performed in Asia and concern the preparative isolation of active compounds from traditional Chinese medicinal herbs [19–22]. For instance, several isoflavones were recently isolated with high purity by HSCCC from the rhizomes of *Belamcanda chinensis*, a plant traditionally used in Eastern Asia to treat inflammation, asthma, or pharyngitis [23]. The phenolic compounds of *Halimodendron halodendron*, a Fabaceae species distributed in Northwest China [24]; the prenylflavonoids of *Artocarpus altilis* whose leaves are used in Indonesia for the treatment of liver cirrhosis, hypertension, and diabetes [25]; the flavanones of *Poncirus trifoliata*, a fruit widely used in oriental traditional medicine to treat digestive ulcers, gastritis, or inflammation [26]; or the rotenoids of *Millettia pachycarpa*, a famous Chinese herb used as an antihelminthic [21] are only a few examples of phenolic compounds which have been efficiently purified by HSCCC starting from crude extracts of Asian medicinal plants.

High-performance countercurrent chromatography (HPCCC) is another recent development of HSCCC enabling higher flow rates and thus shortening the separation time while maintaining good resolution [27]. HPCCC was used for the purification of several flavonol glycosides and flavones from the Chinese herb *Flaveria bidentis* at the preparative scale [28].

Table 70.1 Recent applications of HSCCC and CPC in the purification of polyphenols from complex plant extracts

Targeted compound(s) (purity %)	General method column capacity	Starting material	Solvent system	Flow rate, Rotation speed, run duration	Refs
A – Direct isolation of highly pure polyphenols					
Phloroglucinol: hyperforin (98 %)	HSCCC (220 mL) gradient elution	*Hypericum perforatum* (1 g)	Hept:MeOH:ACN (1.5:0.5:0.5 v/v), Hept:MeOH (1.5:1 v/v)	2 mL/min; 800 rpm; DM; 480 min	[48]
Naphthodianthrone: hypericin (95 % purity)	HSCCC (260 mL) gradient elution	*Hypericum perforatum* (104 mg)	From Hex:EtOAc:MeOH:H_2O (1:3:1:3 v/v) to (1:1:1:1 v/v)	2 mL/min 850 rpm; DM; 300 min	[48]
Flavones: Rutin (>95 %), hyperoside (>95 %)	HSCCC (260 mL) elution-extrusion	*Hypericum perforatum* (110 mg)	BuOH:EtOAc:H_2O (1:4:5 and 1.5:3.5:5 v/v)	2 mL/min 850 rpm DM; 250 min	[48]
Chlorogenic acid (94.8 %)	HSCCC (280 mL) elution	*Crataegus laevigata* (500 mg)	BuOH:EtOAc:H_2O (1:1:2 v/v)	1.5 mL/min 850 rpm DM; 260 min	[55]
Ellagic acid derivatives (>95 %)	HSCCC (*n.i.*) elution	*Oiltea camellia* (300 mg)	$CHCl_3$:EtOH:H_2O:AA (4:3:2:0.01 v/v)	3 mL/min; 950 rpm; DM; 240 min	[46]
Glabrone and glabridin derivatives (*n.i.*)	CPC (1 L) elution	*Glycyrrhiza glabra* (750 mg)	Hex:acetone:H_2O (5:9:1 v/v)	25 mL/min 1,100 rpm AM; 102 min	[54]
Flavonols, flavonols glycosides (>93 %)	HPCCC (912.5 mL) elution	*Flaveria bidentis* (1.5 g)	EtOAc:MeOH:H_2O (10:1:10 v/v)	50 mL/min; 1,200 rpm; DM; 90 min	[28]
Dihydrochalcone and flavanone glycosides (>89 %)	CCC (95 mL) 2 steps Instrument comparison	*Sparattosperma leucanthum* (150 mg)	Hex:EtOAc:MeOH:H_2O (4:10:4:10 v/v), EtOAc:BuOH:H_2O (8:2:10 v/v), BuCN:ACN:H_2O (5:10:10 v/v)	2 mL/min 850 rpm AM	[56]
Hydroxyflavone glucuronide derivatives	Stop-and-go 2D CCC*LC (260 mL)	*Medicago sativa* (126.8 mg)	Isopropanol:20 % NaCl (1:1 v/v)	1.5 mL/min 850 rpm DM; 336 min	[57]
Flavonoids and diarylheptanoids (>93 %)	HSCCC (240 mL)	*Alpinia katsumadaihayata*	Hex:EtOAc:MeOH:H_2O (3:7:6:4 v/v)	2.5 mL/min 800 rpm AM; 240 min	[58]
Flavonol glycosides (>97 %)	HSCCC (230 mL)	*Flaveria bidentis* (400 mg)	EtOAc:MeOH:H_2O (10:0.4:10 v/v)	2 mL/min 800 rpm DM; 300 min	[42]

Isorhamnetin-3-sulphate (93.4 %)	HSCCC (230 mL)	*Flaveria bidentis* (83 mg)	BuOH:EtOAc:H_2O (4:1:5 v/v)	2 mL/min 800 rpm DM; 240 min	[59]
Isorhamnetin-3-sulphate (97 %)	HSCCC (230 mL)	*Flaveria bidentis* (553 mg)	BuOH:0.25 % NaCl (1 :1 v/v)	2 mL/min 800 rpm DM; 240 min	[59]
Cajaflavanone (86.9 %)	CPC (275 mL)	*Derris ferruginea* (5 g)	Hept:EtOAc:MeOH:H_2O (4 :1 :4 :1 v/v)	10 mL/min 900 rpm AM, 80 min	[40]
Flavonoids Eupatilin (>97 %) and jaceosidin (>97 %)	HSCCC (300 mL)	*Artemisia princeps*	Hex:$CHCl_3$:MeOH:H_2O (2:5:5:2 v/v)	1.3 mL/min; 800 rpm; DM; 250 min	[60]
Stilbenoids (>80 %)	CPC (250 mL)	*Vitis vinifera* (1.5 g)	Hept:EtOAc:MeOH:H_2O (1:2:1:2 and 5:6:5:6 v/v)	3 mL/min 1,000 rpm AM; 130 min	[61]
Polyphenols: quadrangularin A, parthenocissin A (>97 %)	CCC (1,600 mL)	*Parthenocissus laetevirens* (500 mg)	Hex:EtOAc:MeOH:H_2O (1:2:1:2 v/v)	4 mL/min 500 rpm DM; 300 min	[41]
Flavone glycosides (>98 %)	HSCCC (260 mL) Ionicliquid	*Oroxylum indicum* (120 mg)	EtOAc:H_2O:[C4mim][PF6] (5:5:0.2 v/v)	1 mL/min 700 rpm DM; 350 min	[62]
Flavonols: hyperoside, quercitrin (>98 %)	HSCCC (260 mL)	*Osteomeles schwerinae* (160 mg)	Hex:EtOAc:MeOH:H_2O (0.5:5.5:1.5:4.5 v/v)	1.5 mL/min 800 rpm DM; 300 min	[63]
Flavanones (>97 %)	HSCCC (300 mL) 2 steps	*Citrus aurantium* (1.42 g)	EtOAc:BuOH:H_2O (2 :1 :3 v/v) and $CHCl_3$:MeOH:BuOH:H_2O (4:3:0.5:2 v/v)	2 mL/min 800 rpm DM; 24 h (continuous injection mode) + 7 h	[64]
Phloretin (98.2 %)	HSCCC (260 mL)	phloretin extract of apple tree (767.3 mg)	Hex:EtOAc:EtOH:H_2O (2:2:1:2 v/v)	2 mL/min; 850 rpm; DM; 500 min	[47]
Flavonols, biflavonoids	HPCCC (146 mL) Gradient elution	*Apocynum venetum* (*n.i.*)	Hex:EtOAc:ACN:H_2O (1.5:3.5:2:5 and 0:5:3:7 v/v), EtOAC:MeOH:H_2O (5:2:5 v/v) and BuOH:MeOH:H_2O (5:1:5 v/v)	2 mL/min 1,000 rpm AM; 130 min	[65]

(*continued*)

Table 70.1 (continued)

Targeted compound(s) (purity %)	General method column capacity	Starting material	Solvent system	Flow rate, Rotation speed, run duration	Refs
Prenylflavanones (>94 %)	HSCCC (300 mL): stepwiseelution	Biotransformed extract of kurarinone (520 mg)	Hex:EtOAc:MeOH:H_2O (1:1:0.7:1 v/v) and (1:1:1.2:1 v/v)	1.5 mL/min; 900 rpm; DM; 700 min	[66]
Flavonoids: Spinosin derivatives (>90 %)	HSCCC (80 mL)	*Ziziphus jujubam* (50 mg)	EtOAc:BuOH:H_2O (3:2:5 v/v)	1.5 mL/min; 1,500 rpm; DM; 240 min	[43]
Kaempferol-3,7-dirhamnopyranoside	HSCCC (300 mL)	*Siraitiagros venori* (90 mg)	EtOAC:BuOH:H_2O (4:1:5 v/v)	1.5 mL/min; 850 rpm; DM; 5.5 h	[67]
Homoisoflavones (>85 %)	HSCCC (300 mL)	*Caesalpinia sappan* (120 mg)	$CHCl_3$:MeOH:H_2O (4:3:2 v/v)	1 mL/min; 900 rpm; DM; 550 min	[45]
Senkyunolide I and H (>93 %) and ferulic acid (99 %)	CCC (230 mL)	*Ligusticum chumanxiong* (400 mg)	Hex:EtOAc:MeOH:H_2O (3:7:4:6 v/v)	2 mL/min; 800 rpm; DM; 300 min	[68]
Prenylflavonoid: xanthohumol (>95 %)	HSCCC (280 mL)	*Humuluslupulus* (607 mg)	Hex:EtOAc:MeOH:H_2O (5:5:4:3 v/v)	2 mL/min; 850 rpm; DM; 300 min	[39]
Chromones (>90 %)	HPCCC (132 mL)	*Saposhnikoviadivaricata* (300 mg)	EtOAc:BuOH:EtOH:H_2O (1:1:0.1:2 v/v)	6 mL/min; 1,600 rpm; DM; 150 min	[69]
Punicalagin (>92 %), gallic acid (75 %)	HSCCC (250 mL)	*Punica granatum* (350 mg)	BuOH:TFA:H_2O (100:1:100 v/v)	2 mL/min; 800 rpm; AM; 360 min	[70]
Phenolic acids (>94 %)	HSCCC (200 mL)	*Salvia miltiorrhiza* (*n.i.*)	Hex:EtOAc:MeOH:AA:H_2O (1 :6 :1.5 :1.5 :8 v/v)	1.5 mL/min; 850 rpm; DM; 350 min	[44]
Luteolin (91.2 %), apigenin (97.4 %), 3′-hydroxygenkwanin (94.3 %), genkwanin (95.8 %)	HSCCC (240 mL) elution	*Daphne genkwa* (*n.i.*)	Hex:EtOAc:MeOH:H_2O (5:7:5:5 v/v)	1.2 and 2 mL/min; 850 rpm; DM; 450 min	[20]
Flavonols (>97 %)	HSCCC (200 mL)	*Flos Gossypii* (80 mg)	Hex:EtOAc:MeOH:H_2O (1:2:0.8:0.9 v/v)	1.5 mL/min; 800 rpm; DM; 300 min	[71]
Flavones glycosides: Camellianins A and B (*n.i.*)	HSCCC (260 mL) elution	*Adinandra nitida* (200 mg)	EtOAC:EtOH :H_2O (5:1:5 v/v)	2 mL/min; 900 rpm; DM; 150 min	[72]

Flavonoids, biflavonoids (>85 %)	HPCCC (Midi-DE 915.5 mL)	*Selaginella tamariscina* (400 mg)	Hept:EtOAc:MeOH:H_2O (2:3:2:3 v/v)	50 mL/min; 1,250 rpm; DM; 75 min	[27]
Rotenoids and isoflavones (>93 %)	HSCCC (290 mL) elution	*Millettia pachycarpa* (400 mg)	Hex:EtOAc:MeOH:H_2O (1:0.8:1:0.6 v/v)	2 mL/min; 850 rpm; DM; 400 min	[21]
Flavonoid glycosides (>97 %)	HSCCC (230 mL) elution	Sugarcane juice (250 mg)	EtOAc:BuOH:H_2O (9:1:10 v/v)	1.5 mL/min; 900 rpm; DM; 400 min	[73]
Phenolicacids (96 %) and flavonoids (>82 %)	HSCCC (230 mL) Stepwise elution	*Eupatorium denophorum* (378.5 mg)	EtOAc:MeOH:H_2O (10:1:10 and 5:1:5 v/v)	2 mL/min; 800 rpm DM; 720 min	[50]
Flavonols glycosides (>90 %)	HSCCC (40 mL)	*Ginkgo biloba* (40 mg)	Hex:BuOH:EtOAc:MeOH-0.5 % AA (1:0.5:3.5:1:4 v/v)	1 mL/min; 1,600 rpm; AM; 250 min	[74]
Rutin (61.4 %), quercitrin (76.7 %) [75]	CPC (200 mL)	*Panicum virgatum* (600 mg)	EtOAc:EtOH:H_2O (2:1:2 v/v)	3 mL/min; 1,100 rpm; DM; 140 min	[75]
Flavonol glycosides and (+)-catechin (>92 %)	HSCCC (240 mL)	*Byrsonima basiloba* (1 g)	EtOAc:BuOH:H_2O (2:1:3 v/v)	1 mL/min; 850 rpm; DM; 240 min	[76]
Flavonol glycosides (>95 %)	HSCCC	*Hedyotis diffusa* (200 mg)	Hex:EtOAc:BuOH:MeOH:1% AA (1:1:3.5:1:4.5 v/v)	2 mL/min; DM; 130 min	[77]
Myricetin, quercetin, kaempferol (>93 %)	CPC in 2 steps	*Rhododendron mucronulatum* (500 mg)	Hex:EtOAc:MeOH:H_2O (3:5:3:5 and 4:5:4:5 v/v)		[78]
Isorhamnetin (94 %), irigenin (95 %), hispidulin (90 %)	HSCCC (230 mL)	*Belamcanda chinensis* (100 mg)	Hex:EtOAc:MeOH:H_2O (4:5:5:5 v/v)	1.2 mL/min; 800 rpm; DM; 400 min	[22]
Quinines, phenolic acids and flavonols (>85 %)	CPC (275 mL)	*Senecio giganteus* (500 mg to 1 g)	EtOAc:ACN:H_2O (5:1:4 v/v)	10 mL/min; 900 rpm; AM	[79]
Flavonols (>95 %)	HSCCC (300 mL)	*Nelumbo nucifera* (80 mg)	Hex:EtOAc:MeOH:H2O (1:5:1:5 v/v)	1.8 mL/min; 800 rpm; DM; 350 min	[80]
Flavonoids: Epimedin A, B and C (>90 %), icariin (96.8 %)	HSCCC (320 mL) dual mode	*Epimedium brevicornum* (300 mg)	BuOH:EtOAc:H_2O (3:7:10 v/v)	1 and 2 mL/min; 1,000 rpm; DM; 660 min	[81]

(*continued*)

Table 70.1 (continued)

Targeted compound(s) (purity %)	General method column capacity	Starting material	Solvent system	Flow rate, Rotation speed, run duration	Refs
Flavanone glycosides: naringin, neoponcirin, poncirin (>90 %)	CPC (230 mL)	*Poncirus trifoliata* (524 mg)	EtOAC:ACN:H_2O (3:2:5 v/v)	2 mL/min; 1,200 rpm; DM; 250 min	[26]
Prenylflavonoids (>95 %)	2D-CCC (1,600 and 210 mL)	*Artocarpus altilis* (500 mg)	Hex:EtOAc:MeOH:H_2O (5:5:7:3 and 5:5:6.5:3.5 v/v)	5 and 1.5 mL/min; 500 and 800 rpm; DM; 500 min	[25]
Phenolic acids and flavonols	HSCCC (280 mL)	*Halimodendron halodendron* (700 mg)	$CHCl_3$:MeOH:H_2O:AA (4:3:2:0,05 v/v)	3 mL/min; 850 rpm; DM; 200 min	[24]
Didymin, narirutin, clinopodiside A (>96 %)	HSCCC 2 steps	*Clinopodium chinensis*	EtOAc:BuOH:H_2O (5:0.8:5 and 5:1:5 v/v)		[82]
B- HSCCC or CPC used as orthogonal technique for the purification of polyphenols					
Nevadensin (98 %)	MAE + HSCCC elution	*Lyeicnotuspauciflorus* (3 g)	Hex:EtOAc:MeOH:H_2O (7 :3 :5 :5 v/v)	2 mL/min; 800 rpm; DM; 720 min	[83]
Erionic acids (*n.i.*)	CPC + preparative HPLC	*Eryodictyon augustifolium* (60 mg)	Hept:EtOAc:MeOH:H_2O (5:4:4:5 v/v)	8 mL/min AM, 10 mL/min DM; 1,100 rpm; 70 min	[84]
Neohesperidin (97.5 %)	Macroporous resin + HSCCC (260 mL)	*Citrus reticulata* (60 mg)	EtOAC:BuOH:H_2O (4:1:5 v/v)	2 mL/min; 800 rpm; DM; 300 min	[85]
Naringenin-7-*O*-β-*D*-glycoside, isoquercitrin, astragalin (>93.6 %)	Sephadex LH-20 + HSCCC (200 mL)	*Helichrysum arenarium* (160 mg)	EtOAc:H_2O (1 :1 v/v)	1.5 mL/min; 800 rpm; DM; 350 min	[86]
6-hydroxyluteolin-7-O-β-glucoside and quercetagetin-7-O-β-glucoside (*n.i.*)	pH-ZR HSCCC (144 mL) + semiprep-HPLC	*Athrixia phylicoides* (100 mg)	M*t*BE:ACN:H_2O (2:2:3 v/v) (formic acid, NH_3)	5 mL/min; 1,600 rpm; DM; 40 min	[87]
Flavonols : isoquercitrin, quercetin glycosides (>98.7 %)	HSCCC (260 mL) + Sephadex LH-20	*Poacynum hendersonii* (240 mg)	BuOH:petroleum ether:H_2O + 0.5 % AA (5:3:5 v/v)	1.5 mL/min; 850 rpm; DM	[88]
Anthraquinones (>96 %)	HSCCC (230 mL) + prep-HPLC	*Morinda officinalis* (300 mg)	Hex:EtOAc:MeOH:H_2O (6 :4 :5 :5 v/v)	1.5 mL/min; 800 rpm; DM; 300 min	[29]

Polymethoxyflavones : tetramethyl-o-isoscutellarein and nobiletin (>97 %)	HSCCC (1,200 mL) + prep-hplc	*Citrus reticulata Blanco* (5 g)	Hex:EtOAc:MeOH:H_2O (1:1:1:1.5 v/v)	5 mL/min 700 rpm DM; 330 min	[52]
Silydianin (95 %), silychristin (99 %), taxifolin (98 %)	HSCCC (260 mL) after removal of silybin	Silymarin extract (1,463 mg)	Hex:$CHCl_3$:MeOH:H_2O-0.5 % AA (0.5 :11:10:6 v/v)	2 mL/min; 850 rpm; DM; 600 min	[89]
Flavonol glycosides (*n.i.*)	polyamide chromatography + HSCCC (230 mL)	*Nelumbo nucifera* (125 mg)	EtOAc:MeOH:H_2O:AA (4:1:5:0.1 v/v)	1 mL/min; 800 rpm; DM; 480 min	[90]
Flavonoids: apigemin (98.8 %), 3-hydroxyl-genkwanin (97.7 %) and genkwanin (93.5 %)	macroporous resin + CCC (4,800 mL)	*Daphne genkwa* (3 g)	Hex:EtOAc:EtOH:H_2O (4 :5 :4 :5 v/v)	30 mL/min; 480 rpm; DM; 320 min	[53]
Flavonol glycosides (>98 %)	HSCCC (260 mL) + Sephadex LH-20	*Poacynum hendersonii* (240 mg)	BuOH:petroleum ether:0.5 % AA (5:3:5 v/v)	1.5 mL/min; 850 rpm; DM; 400 min	[88]
Kaempferol triglycosides (>95 %)	macroporous resin + HSCCC (260 mL)	*Actinidia valvata* (*n.i.*)	EtOAc:BuOH:H_2O (2:1:3 v/v) and (4:1:5 v/v)	1.5 mL/min; 800 rpm; DM; 400 + 450 min	[91]
Isorhamnetin-3-gentiobioside, rutin and narcissin (>95 %)	silica gel column + HSCCC (290 mL)	*Astragalus altaicus* (200 mg)	EtOAc:BuOH:H_2O (2:1:6 v/v)	1.5 mL/min; 800 rpm; DM; 500 min	[92]
Chlorogenicacid (85 %), flavonol glycosides (>70 %)	HSCCC (220 mL) + gel chromatography	apple pomace (2.1 g)	Hex:EtOAc:1 % AA (1:9:10 v/v)	2 mL/min; 800 rpm; DM; 420 min	[93]
Hydroxytyrosol (90 %)	CPC (1 L) after filtration and resin adsorption	Olive oil mills waste water residue (30 g)	CycloHex:EtOAc:MeOH:H_2O (4:6:4:6 v/v)	18 mL/min; 900 rpm; AM; 450 min	[94]
Flavonoid glycosides (*n.i.*)	HSCCC (325 mL) + resin chromatography	*Radix astragali* (100 mg)	Hex:EtOAc:BuOH:MeOH:H_2O-0.5 % TFA (1:2:1:1:5 v/v)	2 mL/min 800 rpm DM; 250 min	[95]
Naringin (98.3 %)	macroporous resin + HSCCC (260 mL)	*Fructus aurantii* (600 mg)	EtOAc:BuOH:H_2O (2:0.8:3.2 v/v)	1.5 mL/min 850 rpm DM; 500 min	[96]
Alquds (98.8 %), hesperidin (98.1 %)	C18 column + HSCCC (420 mL)	*Taraxacum mongolicum* (600 mg)	Hex:BuOH:H_2O (1:1:2 v/v)	1.5 mL/min; 800 rpm; DM; 350 min	[97]

(*continued*)

Table 70.1 (continued)

Targeted compound(s) (purity %)	General method column capacity	Starting material	Solvent system	Flow rate, Rotation speed, run duration	Refs
Polymethoxyflavanones (>96 %)	HSCCC (230 mL) + prep-HPLC	*Pogostemon cablin* (300 mg)	Hex:EtOAc:MeOH:H_2O (11:5:11:5 v/v)	1.5 mL/min; 800 rpm; DM; 330 min	[98]
Quercitrin (97 %), quercetin (93 %)	HSCCC (230 mL) + prep-HPLC	*Hypericum Perforatum* (900 mg)	EtOAc:MeOH:H_2O (10:1:10 v/v)	2 mL/min; 800 rpm; DM; 360 min	[99]
Flavonols and flavonol glycosides (>97 %)	HSCCC (290 mL) + prep-HPLC	black currant (100 mg)	Hex:EtOAc:MeOH:H_2O (1:10:1:10 v/v)	1.5 mL/min; 800 rpm; DM; 250 min	[100]
Flavones (*n.i.*)	HSCCC (310 mL) + Sephadex, silica gel	*Lantana trifolia* (3 g)	Hex:EtOAc:BuOH:H_2O (0.4:1:X:1 v/v) where X=0.1, 0.3, 0.5, and 0.7	3 mL/min 850 rpm AM	[101]
Isoflavones (>92 %)	HSCCC (1 L) 2 steps + Flash column	*Belamcanda chinensis* (2 g)	Hex:EtOAc:isopropanol:MeOH:H_2O (5:6:2:3.5:6 v/v) and (0:10:0:2:9 v/v)	5 mL/min 400 rpm DM; 350 min	[23]
Flavonoid glycosides (>98 %)	polyamide resin + HSCCC (420 mL)	*Taraxacum mongolicum* (500 mg)	EtOAC:BuOH:H_2O (2:1:3 v/v)	1.5 mL/min 800 rpm DM 350 min	[102]
C- Bioassay-guided fractionation and rapid characterization of plant-derived polyphenols by HSCCC or CPC					
Flavonols	CPC (200 mL) antimicrobialactivity	*Kalanchoepinnata* (350 mg)	EtOAc:EtOH:H_2O (4.5:1.5:4.5 v/v)	4 mL/min; 1,200 rpm; DM; 125 min	[30]
Isoflavones	HSCCC (260 mL) antioxidant activity	*Puerarialobata* (210 mg)	Petroleum ether:EtOAc:MeOH:H_2O (2:3:2:3, 2:3:5:4, 1:5:2:5 and 1:1.3:1:1.3 v/v)	1.2 mL/min; 850 rpm; DM; 625 min	[31]
Flavonoids : Butrin, isobutrin	CPC (200 mL) antioxidant activity	*Butea monosperma* (*n.i.*)	Hept:EtOAc:MeOH:H_2O (1:4:1:4 v/v) and BuOH:H_2O (1:1 v/v)	3 mL/min; 1,300 rpm; AM; 280 min	[32]
Phenolicacids, anthocyanins	HSCCC (100 mL) antioxidant activity	Red wine extract (1 g)	MtBE:BuOH:ACN:H_2O + 0.1 % TFA (2:2:1:5 v/v)	3 mL/min; DM	[103]

Isoflavones, flavonols	HSCCC (500 mL) antioxidant activity	*Mucunasempervirens* (600 mg)	Stationary phase: H_2O saturated with BuOH and EtOAC. Mobile phase: *n*-Hex:EtOAc (1:1 to 1:4 v/v) and BuOH:EtOAc (1:4 to 2:1 v/v)	3 mL/min; 1,000 rpm; AM; 640 min	[104]
Flavonoids and coumarins	HSCCC (300 mL) antioxidant activity	*Psoralea coryfolia* (250 mg)	Hex:EtOAc:MeOH:H_2O (1:1.1:1.3:1 v/v)	1.3 mL/min; 1,000 rpm; DM; 600 min	[105]
Flavonoids and biflavonoids	HSCCC (420 mL) antioxidant activity	*Selaginella sinensis* (*n.i.*)	Hex:EtOAc:MeOH:H_2O (8:8:9:7 v/v)	1.2 mL/min; 1,000 rpm; DM; 400 min	[106]
Prenylated flavones	HSCCC (1,000 mL) antibacterialactivity	*Sophora flavescens* (8 g)	Hex:EtOAc:MeOH:H_2O (1:1:1:1 v/v)	8 mL/min; 500 rpm; DM; 270 min	[35]
Flavonols	CPC (200 mL) hyphenated with HPLC	*Hippophaërhamnoides* (1.5 g)	Hept:EtOAc:MeOH:H_2O (1:4:1:4 v/v)	3 mL/min; 1,300 rpm; AM; 180 min	[36]
Flavonol glycosides	CPC (200 mL) hyphenated with ESI-MS	Apple peel extract (350 mg)	EtOAC:EtOH:H_2O (4.5:1:4.5 v/v)	4 mL/min; 2,000 rpm; DM; 400 min	[107]
Flavonol glycosides	HSCCC (850 mL) combined to ESI-MS	Sea buckthorn juice concentrate (4.1 g)	Hex:BuOH:H_2O (1:1:2 v/v)	3 mL/min; 800 rpm; DM; 600 min	[108]
betacyanins	Ion-pair HSCCC (850 mL) combined to HPLC/ESI-MS	*Bougainvilleaglabra* (755 mg)	M*t*BE:BuOH:ACN:H_2O-0.7 % TFA (2:2:1:5 v/v)	3 mL/min; 850 rpm; DM; 360 min	[37]
Phenolic acids and flavonoids	HSCCC (850 mL) combined to HPLC/ESI-MS	Rum aged in oak barrels (800 mg)	Hex:EtOAc:MeOH:H_2O + 0.1 % FA (1:1:1:1 v/v)	3 mL/min; 900 rpm; DM	[51]

n.i. non indicated

In general, the objective when isolating natural polyphenols from complex plant extracts is to obtain compounds in sufficient quantities and with high purities for structural elucidation, for further use in bioactivity studies, or as standard reference substances for chromatography. The studies reported in Table 70.1 clearly demonstrate that HSCCC and CPC are efficient in directly purifying polyphenols from crude plant extracts without additional clean-up. Other studies have concentrated their efforts on the rapid but partial purification of polyphenols by HSCCC or CPC. In these cases, an additional orthogonal technique such as preparative HPLC, Sephadex, or open silica gel column chromatography was required to achieve the full purification of the target compound(s). This strategy combines the advantages of HSCCC or CPC and those of solid support-based chromatographic techniques to improve purification efficiency. This can be particularly appropriate for the rapid preparative isolation of complex polyphenolic structures. For example, HSCCC was combined with preparative HPLC for the rapid separation and purification of anthraquinones from *Morinda officinalis* [29].

3.3 Bioassay-Guided Fractionation and Characterization of Plant-Derived Polyphenols

Plant extracts normally contain a large number of chemical components among which polyphenols can be present in various amounts. These complex mixtures are not easily characterized, and sometimes bioassay-guided fractionation is preferentially used in order to focus only on the compounds providing a biological or pharmacological interest. In this field, HSCCC and CPC have a high potential due to their inherent characteristics. Classical bioassay-guided fractionation methods based on solid support materials are generally time-consuming. In addition, multistep purification procedures sometimes result in drastic sample or activity loss during the isolation of target compounds because of sample degradation, irreversible adsorption on solid support, or dilution.

Due to the absence of solid support, both HSCCC and CPC circumvent these problems by maintaining the integrity of all compounds present in the initial crude sample during the separation process. They also provide high sample recovery and thus offer an excellent alternative for the rapid screening of bioactive compounds in complex plant extracts.

As presented in Table 70.1-C, several studies investigating the biological effects of polyphenols have developed bioassay-guided fractionation methods using HSCCC or CPC. Generally in a first step, the initial crude extract is fractionated into individual polyphenols or into highly simplified polyphenol-enriched fractions. The biological activity of each collected fraction is then evaluated on a specific model. This strategy allows the rapid identification of structures responsible for the observed activity. For example, a *Kalanchoe pinnata* extract was fractionated by CPC, and each fraction was evaluated for its antimicrobial activity and cytotoxicity [30]. In another work, HSCCC was combined with DPPH-chemical tests in order to screen and separate several antioxidant isoflavones from a *Pueraria lobata* extract [31]. In a similar way, the different flavonoids present in a crude extract of *Butea monosperma* flowers were fractionated by CPC according to their molecular family and polarity, and each fraction was evaluated for its antioxidant activity [32].

Antioxidant activity tests are by far the most commonly used models for bioassay-guided fractionation of plant-derived polyphenols. This is mainly because there has been considerable research on natural antioxidants, particularly for their potential use as additives in pharmaceutical or cosmetic formulations. Polyphenols are the major well-recognized natural antioxidants consumed in the human diet [33]. A range of in vitro and in vivo methods have been developed to evaluate their antioxidant properties and mechanisms of action on human metabolic pathways [34]. However, antioxidant screening in complex mixtures of plant origin requires simple and rapid in vitro models for a possible combination with chromatographic techniques such as TLC, HPLC, HSCCC, or CPC. The reduction of the stable free-radical DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) by antioxidant substances is currently the most widely used chemical test for the screening of plant extracts. Only a few bioassay-guided fractionation processes have also evaluated the antimicrobial, antibacterial activity, or cytotoxicity of fractions enriched in specific flavonoids by using HSCCC or CPC liquid–liquid systems [30, 35].

HSCCC or CPC fractionation of complex plant extracts containing polyphenols has also been employed in a number of studies in order to rapidly obtain information on sample composition without purifying each individual compound (Table 70.1-C). In these cases, each simplified fraction obtained from the HSCCC or CPC separation procedure is usually monitored by high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), or mass spectrometry (MS), either by off-line or direct coupling with the appropriate detectors. In a recent study, CPC was directly associated with HPLC to separate and on-line guide the fractionation of flavonols (quercetin and isorhamnetin derivatives) from *Hippophaë rhamnoides* berries [36]. In another study, HSCCC was coupled to HPLC-MS/MS in order to fractionate and resolve the highly complex mixture of betacyanins present in purple bracts of *Bougainvillea glabra* [37].

3.4 Solvent System Selection

Due to the versatility of HSCCC and CPC instruments, various solvent systems have been tested for the purification of polyphenols according to their structure, polarity, or other physicochemical properties. As illustrated in Fig. 70.2, the solvent system composed of *n*-hexane, ethyl acetate, methanol, and water is by far the most commonly used solvent system for the purification of polyphenols despite the large number of other possibilities. Generally, this solvent system (related to the Arizona liquid system) is particularly adapted for the purification of polyphenols with moderate polarity [38]. As shown in Table 70.1 and Fig. 70.2, *n*-hexane can be replaced by *n*-heptane or petroleum ether, and methanol can be replaced by acetonitrile or ethanol. The proportions of each solvent can vary greatly with

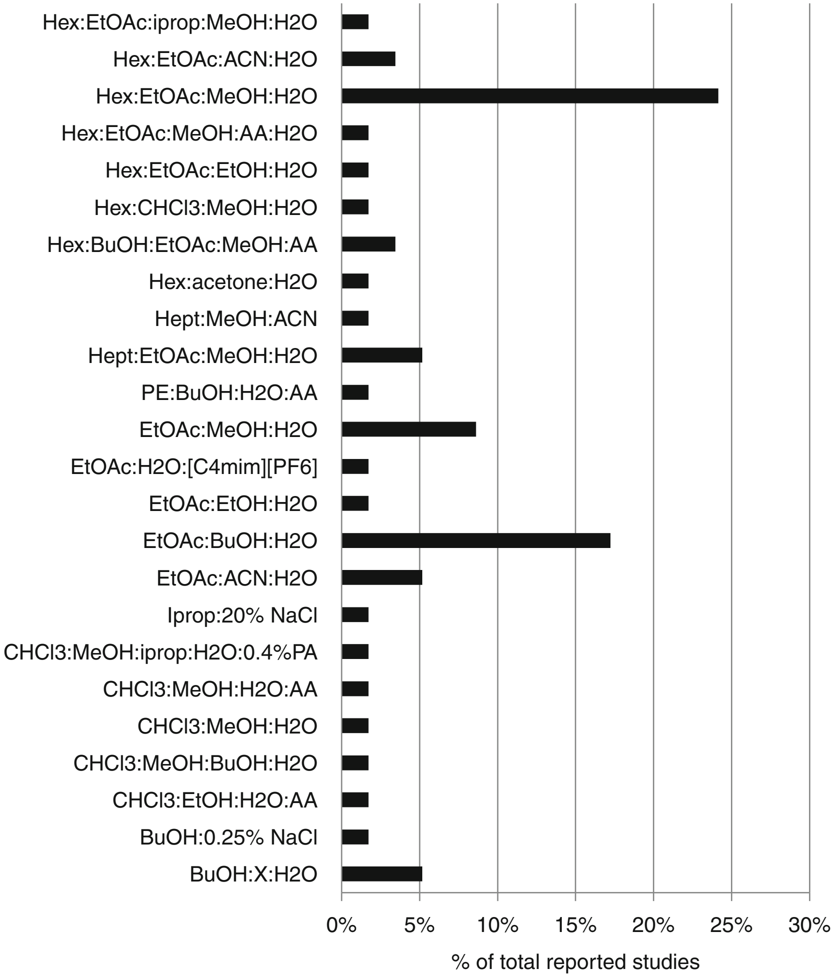

Fig. 70.2 Solvent systems commonly used for the purification of polyphenols from crude plant extracts (55 studies reported over the five last years)

respect to the structures to be purified, but globally, all these variants have also proven to be efficient in the purification of moderately polar phenolic compounds such as flavonoid aglycones. For example, xanthohumol and related prenylflavonoids have been successfully isolated from a hops extract in only one step by HSCCC using the quaternary biphasic solvent system Hex/EtOAc/MeOH/H_2O in the proportions 5:5:4:3 (v/v) [39]; bioactive cajaflavanones have been purified from *Derris ferruginea* by CPC using the biphasic solvent system Hept/EtOAc/MeOH/H_2O in the proportions 4:1:4:1 (v/v) [40], while resveratrol dimers (quadrangularin A and parthenocissin A) have been purified from the roots of *Parthenocissus laetevirens* by HSCCC in the elution mode using Hex/EtOAc/MeOH/H_2O as biphasic solvent system in the proportions 1:2:1:2 (v/v) [41].

Ternary biphasic solvent systems composed of EtOAc/BuOH/H_2O have also been used to purify polyphenols by solid support-free liquid–liquid chromatography. Because they do not contain apolar solvents like *n*-heptane, *n*-hexane, or petroleum ether, these systems are more appropriate for the separation of compounds exhibiting higher polarities such as flavonoid glycosides. For instance, several flavonol glycosides have been isolated with purities over 97 % from a *Flaveria bidentis* extract by HSCCC with a two-phase solvent system composed of EtOAc/MeOH/H_2O in the proportions 10:0.4:10 (v/v) [42]. Similarly, three spinosin derivatives have been isolated with purities over 90 % from *Ziziphus jujuba* by HSCCC using a biphasic solvent system composed of EtOAc/BuOH/H_2O in the proportions 3:2:5 (v/v) [43].

Different variants of these systems have also been tested by adding a small amount of organic acid. The presence of an acid in the solvent system can significantly influence the distribution coefficient of negatively charged analytes such as phenolic acids. They become more hydrophobic in acid medium and thus provide a better affinity for the organic phase. Three polyphenols (3,4-dihydroxyphenyllactic acid, salvianolic acid B, and protocatechualdehyde) were, for example, efficiently purified from *Salvia miltiorrhiza* in a one-step HSCCC experiment using the biphasic solvent system Hex/EtOAc/MeOH/AA/H_2O in the proportions 1:6:1.5:1.5:8 (v/v) [44]. In addition, the presence of an acid in the solvent system can reduce its settling time and thus improve the hydrodynamic properties of the stationary and mobile phases during the separation experiment. A reduction of the settling time induces an increase of the stationary phase retention volume inside the HSCCC or CPC column. This results in an improvement of separation resolution.

Other solvent systems composed of $CHCl_3$/MeOH/H_2O have been successfully tested and proposed for the separation of free and glycosylated flavonoids. The use of chloroform instead of ethyl acetate can be helpful when the initial crude sample is more soluble in chlorinated solvents [38]. Homoisoflavonoids were purified from *Caesalpinia sappan* by HSCCC in the descending mode using the biphasic solvent system composed of $CHCl_3$/MeOH/H_2O in the proportions 4:3:2 (v/v) for a total elution duration of 550 min [45]. Solvent systems containing methyl *tert*-butyl ether (M*t*BE) have also been suggested for the purification of anthocyanin derivatives [37].

3.5 HSCCC and CPC Chromatographic Development Modes

Most of the purification procedures presented in our literature survey were conducted in the classical elution mode. In this case, the column is first filled by one of the two liquid phases of the biphasic solvent system, playing the role of stationary phase, while the other is used as the mobile phase and pumped through it. This classical elution mode can be effective if the number of target compounds is not too high or if the starting plant extract is not too complicated. A simple elution mode was used in HSCCC for the efficient purification of ellagic acid and

3-*O*-methylellagic acid 4′-*O*-β-D-glucopyranoside from an *oil tea camellia* extract. The purification was achieved in 240 min in the descending mode by using a biphasic solvent system composed of $CHCl_3$/EtOH/H_2O/AA in the proportions 4:3:2:0.01 (v/v) [46]. Similarly, phloretin was isolated with high purity from an apple tree bark extract by an elution HSCCC procedure using the solvent system Hex/EtOAc/MeOH/H_2O in the proportions 2:2:1:2 (v/v) [47]. When the target polyphenolic compounds present in the initial crude extract are numerous, structurally highly different or if they exhibit a broad range of polarities, it can be difficult to purify each of them in a single elution-step procedure by HSCCC or CPC. To circumvent this problem, a stepwise elution or a gradient elution method can be performed to achieve a more efficient separation. The gradient elution mode, mostly applied with ternary solvent systems, has been often used to cover a large polarity range of flavonoids [48, 49].

The combined use of two different solvent systems in stepwise elution can also be useful to obtain an excellent purification of polyphenols within a short period of time. For example, a stepwise elution was applied to the preparative purification of caffeic acid and quercetagetin glycosides from a crude extract of *Eupatorium adenophorum* by using a pair of solvent systems composed of EtOAc/MeOH/H_2O in the proportions 10:1:10 (v/v) and 5:1:5 (v/v) [50].

It should also be mentioned that the flow rate of the mobile phase plays a significant role in the efficiency of HSCCC or CPC separations. As illustrated in Fig. 70.3, the flow rate values used in the HSCCC studies reported in our literature survey were globally comprised between 1 and 5 mL/min for column capacities up to 500 mL. In CPC-based studies, the flow rates were slightly higher, ranging from 3 to 10 mL/min for column capacities up to 275 mL. Increasing the flow rate usually allows a significant productivity gain. However, reducing the flow rate can sometimes improve resolution. Recent developments in HSCCC and CPC column designs have particularly focused on the scale-up of separation processes. For the separation of polyphenols from crude plant extracts, only a few studies have used pilot-scale HSCCC coils of 850-mL [37, 51], 1,000-mL [23, 35], 1,200-mL [52], 1,600-mL [25, 41], and 4,800-mL [53] capacities. A 1,000-mL CPC rotor was used for the fractionation of prenylated flavonoids from a licorice root extract [54]. In this study, the flow rate was set at 25 mL/min, resulting in separation duration of only 55 min. Increasing the column capacity allows not only the application of higher flow rates but also the injection of higher crude sample mass. As shown in Fig. 70.3b, however, there is no clear linear relationship between the sample mass and the column capacities used in studies investigating polyphenol purification from plant extracts by HSCCC or CPC. Globally the quantities of crude samples were lower than 2 g for column capacities up to 500 mL. In only one study, 5 g of a crude *Derris ferruginea* stem extract were injected into a 275-mL CPC rotor for the fractionation of bioactive cajaflavanones [40]. Such multi-gram quantities of crude sample are normally loaded into HSCCC or CPC column capacities higher than 800 mL. Nevertheless, it should be noted that the amount of injected sample is generally small compared with the column capacities used. This can be explained by the negative effects of mass overloading when purifying phenolic compounds by solid support-free liquid–liquid chromatographic techniques. Indeed, the π-stacking

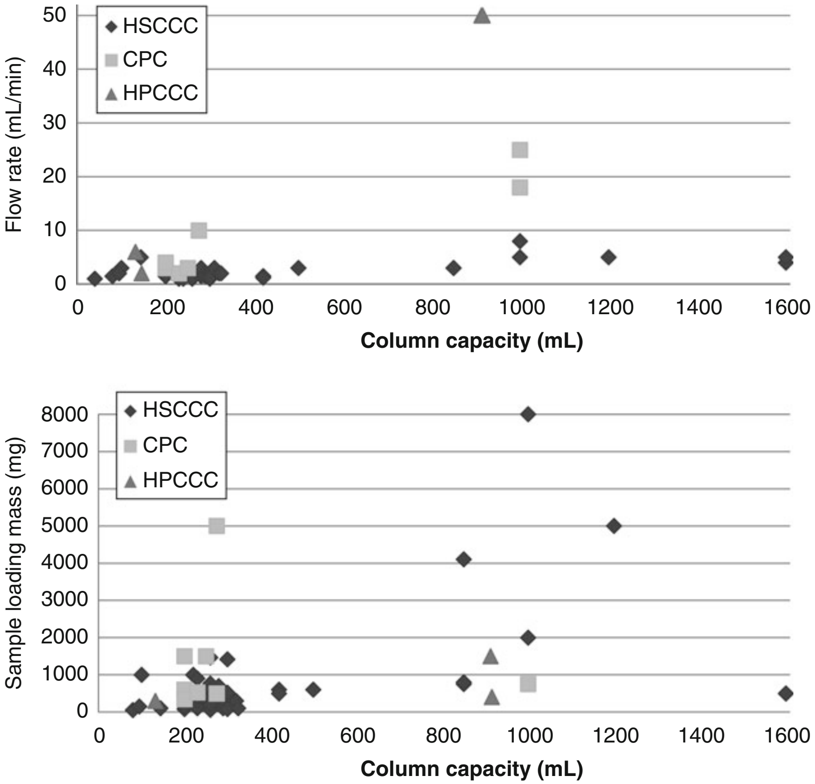

Fig. 70.3 Flow rate application and sample loading as a function of HSCCC, HPCCC or CPC column capacities for the separation of polyphenols from crude plant extracts

phenomena occurring in the aqueous phase can disturb the polyphenol behavior in the biphasic system, thus decreasing the resolution of the separation. This phenomenon has been observed, for example, in the case of the anthocyanins purification by gradient elution CPC on a column of 5 L. Thus, if the absence of solid support often permits very high selectivities and limited adsorption phenomena or irreversible degradations, these advantages are partially counterbalanced by the phenomena of aromatic ring associations. One solution would be to use the displacement mode (ion exchange or pH-zone refining), exchange or acid–base reactions generating interactions of higher energy than those governing the π-stacking phenomena.

4 Conclusion

High-speed countercurrent chromatography (HSCCC) and centrifugal partition chromatography (CPC) are two very promising chromatographic techniques which have been successfully applied to the purification of natural products.

The number of studies presenting HSCCC or CPC applications for the purification of phenolic compounds remains low as compared to other classes of secondary metabolites like alkaloids. However, the increasing number of papers published the last 5 years confirms the potential of these techniques for the separation and purification of different polyphenol classes including flavonoids and phenolic acids.

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Part VIII

Phenolics: Biological Activity and Bioavailability

Cellular, Molecular, and Biological Perspective of Polyphenols in Chemoprevention and Therapeutic Adjunct in Cancer

71

Sanjeev Banerjee and Paulraj Rajamani

Contents

1 Introduction 2177
2 Chemistry and Classification of Polyphenols in Food 2178
3 Polyphenols and Modulation of Phase I and II Biotransformation Enzymes 2178
4 Anti-inflammatory Effect 2181
4.1 Cyclooxygenase 2182
4.2 Lipoxygenase 2183
4.3 Inducible Nitric Oxide Synthase (iNOS) 2183
5 Antioxidant Potential of Polyphenols 2184
6 Polyphenols and Cell Cycle 2186
7 Polyphenols, Transcription Factors, and Cell Signaling 2187
7.1 Polyphenols and Nuclear Factor-κB 2187
7.2 Aryl Hydrocarbon Receptor (AhR) 2189
7.3 Nuclear Factor E2 p45-Related Factor (Nrf2) 2190
7.4 Regulation of the Akt Pathway 2191
7.5 Polyphenols and MAP Kinase 2192
7.6 Wnt and Hedgehog Signaling and Polyphenol 2194
7.7 Regulation of the STAT Pathway 2195
7.8 Androgen Receptor (AR) 2195
7.9 Mammalian Target of Rapamycin (mTOR) Signaling 2196
7.10 Regulation of the Growth Factor Signaling Pathway 2197
8 Apoptosis 2198
9 Invasion and Metastasis 2201
10 Angiogenesis 2203
11 Polyphenols and MicroRNA 2205
12 Polyphenol and Cancer Chemoprevention 2206
12.1 Gastointestinal Cancers 2207

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12.2 Hepatocellular Cancer 2208
12.3 Prostate Cancer 2209
12.4 Lung Cancer 2210
12.5 Breast Cancer 2212
12.6 Oral Cancer 2213
12.7 Bladder Cancer 2214
13 Polyphenols and Chemosensitization of Cancer Therapy 2214
14 Clinical Trials for Cancer Therapies by Polyphenols 2218
15 Prologue on Select Polyphenol Compounds from Natural Dietary Sources 2222
15.1 Genistein 2222
15.2 Resveratrol 2223
15.3 (−)-Epigallocatechin Gallate 2223
15.4 Silibinin 2224
15.5 Quercetin 2224
15.6 Curcumin 2225
16 Conclusion and Perspective 2225
References 2226

Abstract

Emerging evidences from extensive laboratory research backed by epidemiological data lead credence to a family of natural origin compounds with putative chemopreventive potential to intervene ontogeny and progression of cancer. This family of natural compounds called “polyphenols” has more than one phenol unit or building block per molecule and considered beneficial if not critical to human health. Major dietary sources contributing to total polyphenol intake include fruits, vegetables, cereals, chocolates, dry legumes and beverages such as fruit juice, tea, coffee and red wine. Polyphenols have captivated great attention because of their pleiotropic actions including antioxidative, xenobiotic detoxification, anti-inflammatory, and therapeutic benefits including antitumor and chemosensitizing potential to several common cytotoxic chemotherapeutic agents. Mechanistically, polyphenol-rich diet has shown to modulate transcription factors and interrupt the complex cell signaling pathways and their component enzymes, microRNAs, with common aim to thwart tumor cell proliferation while sparing normal cells and inhibits angiogenesis, invasion, and metastasis considered as hallmarks of cancer. This chapter presents a succinct global overview of deregulated signaling pathways, antioxidant status and enzymes exacerbated in tumors and the role of polyphenols in modulating the end results in different site-specific cancers citing representative examples. We conclude highlighting chemosensitization of cancer with select polyphenol compounds – genistein, epigallocatechin-3-gallate, curcumin, quercetin, silibinin, and resveratrol – along with appraisal on ongoing clinical trials with primary objective of better clinical management of cancer patients with long-term survival benefits and designing novel analogs with improved bioavailability for future use.

Keywords
anticancer • cell signaling • chemoprevention • chemosensitization • polyphenols

1 Introduction

Polyphenols are a family of promising phytochemical compounds mainly derived from natural sources and considered beneficial if not critical to human health [1]. Their origin and persistence within edible plant parts and products is largely accountable as secondary metabolite ostensibly to protect against UV radiation and aggression to pathogens and predators [2, 3]. As functional nonnutritive food component, polyphenols are considered important because, in addition to their contribution to color, astringency, flavor, and oxidative stability, their antioxidant potential has been predicted to be higher than dietary antioxidants such as vitamin C and E. Major dietary sources contributing to total polyphenol intake include fruits, vegetables, cereals, chocolates, dry legumes, and beverages such as fruit juice, tea, coffee, and red wine. It has been estimated that approximately 100 g of fresh weight fruits such as grapes, apple, pear, cherries, and berries contains approximately up to 200–300 g of polyphenols, whereas a glass of red wine or a cup of freshly brewed tea or coffee typically contains approximately 100 mg of polyphenols. As a class, polyphenols are receiving increasing attention because emerging evidence strongly attests their role in prevention of cancer and other avenues related to health-promoting properties such as antimicrobial including antiviral effect, cardioprotective effect, antidiabetic effect, antiaging, neuroprotective effects, and protection against many other pathological events. This credence led to foundation in 2003 by Augustin Scalbert in Vichy, France, “1st International Conference on Polyphenols and Health.” This biennial conference includes sessions on range of relevant health-promoting effect topics including bioinformatics, metabolomics, biomarkers, cancer, and discussion on the issue of polyphenols in inflammation and oxidative stress.

Major insights and current knowledge on environmental factors associated with increased risk of developing cancer in humans include chemical and physical mutagens, e.g., cigarette smoke, exposure to heterocyclic amines, nitrosamines, polycyclic aromatic hydrocarbons (PAHs), tamoxifen, nickel, chromium, arsenic, chloromethyl ethers, asbestos, radon, and UV irradiation. Existing knowledge from appreciation of their mechanistic detail led to emergence of the field of “chemoprevention” which is defined as application of interventions (nutrients, drugs, and biologics) that inhibit or reverse the multistage carcinogenic process. This contributing chapter presents a global overview of our current knowledge relating to health-promoting effects of polyphenols focusing on pleiotropic and multitargeted mechanism of their action contributing to chemopreventive and anticancer effects (Fig. 71.1). We conclude with brief prologue on select polyphenol compounds underscoring clinical trials integrating these compounds either as mono- or adjunct therapy in cancer treatment strategy.

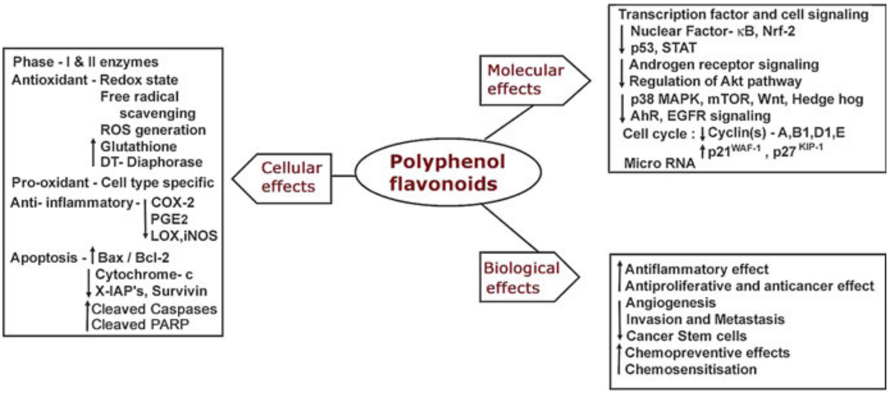
Phase - I & II enzymes
Antioxidant - Redox state
Free radical scavenging
ROS generation
↑ Glutathione
DT- Diaphorase
Pro-oxidant - Cell type specific
Anti- inflammatory - ↓ COX-2
PGE2
LOX,iNOS
Apoptosis - ↑ Bax / Bcl-2
↓ Cytochrome- c
X-IAP's, Survivin
↑ Cleaved Caspases
Cleaved PARP
Cellular effects
Polyphenol flavonoids
Molecular effects
Biological effects
Transcription factor and cell signaling
↓ Nuclear Factor- κB, Nrf-2
p53, STAT
↓ Androgen receptor signaling
Regulation of Akt pathway
↓ p38 MAPK, mTOR, Wnt, Hedge hog
AhR, EGFR signaling
Cell cycle : ↓ Cyclin(s) - A,B1,D1,E
↑ p21WAF-1 , p27 KIP-1
Micro RNA
↑ Antiflammatory effect
Antiproliferative and anticancer effect
↓ Angiogenesis
Invasion and Metastasis
Cancer Stem cells
↑ Chemopreventive effects
Chemosensitisation

Fig. 71.1 Multitargeted cellular, molecular, and biological effect of polyphenols relevant to cancer prevention and therapy

2 Chemistry and Classification of Polyphenols in Food

Polyphenols are derived from phenylalanine and characterized by the presence of several reactive hydroxyl groups on aromatic rings presenting great diversity in structures, ranging from rather simple molecules (monomers and oligomers) to polymers. As a chemical family, more than 8,000 distinct structures have been identified that can arbitrarily be classified into 10 different classes based conditionally on the existing number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another [1, 4] (Fig. 71.2). The most abundant and common polyphenols in foods are the low-MW phenolic compounds such as flavonoids and phenolic acids, along with less common stilbenes (e.g., resveratrol) and lignans (e.g., secoisolariciresinol). Flavonoids comprise the most studied group of polyphenols, and based on variation in the type of heterocycle group within this class, flavonoids have been further divided into six subclasses: flavones (e.g., apigenin), flavonols (e.g., quercetin, myricetin, and rutin), flavanones (e.g., naringenin), anthocyanins (e.g., cyanidin), flavan-3-ols (e.g., catechin, epicatechin, and epigallocatechin-3-gallate), and isoflavones (e.g., genistein) [1, 5]. An overview on pilot studies and trials from viewpoint of cancer prevention-related rationale, in which either healthy individuals or patients with premalignant conditions or cancer received polyphenol phytochemicals, has been reviewed and published by Thomasset et al. [6].

3 Polyphenols and Modulation of Phase I and II Biotransformation Enzymes

According to seminal scheme proposed by Lee Wattenberg, modulation of phase I drug-metabolizing enzymes and induction of phase II detoxifying enzymes have been inferred as an important strategy largely responsible for detoxification of

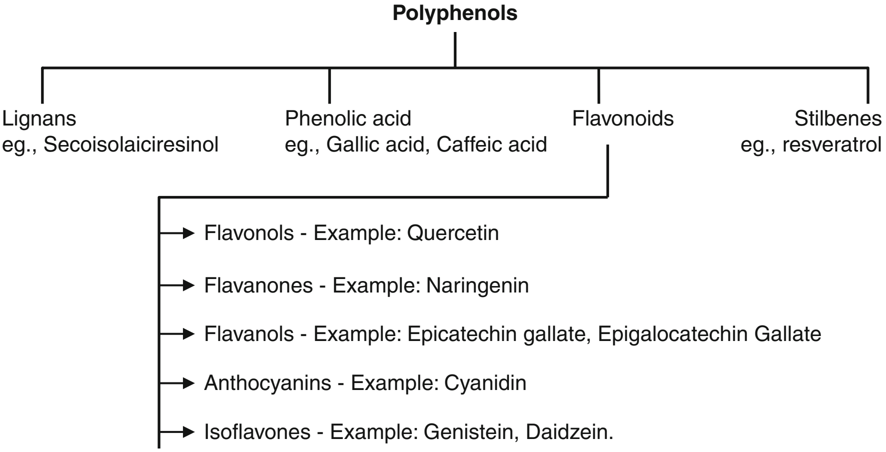
Polyphenols
Lignans
eg., Secoisolaiciresinol
Phenolic acid
eg., Gallic acid, Caffeic acid
Flavonoids
Stilbenes
eg., resveratrol
Flavonols - Example: Quercetin
Flavanones - Example: Naringenin
Flavanols - Example: Epicatechin gallate, Epigalocatechin Gallate
Anthocyanins - Example: Cyanidin
Isoflavones - Example: Genistein, Daidzein.

Fig. 71.2 Classification of major classes of dietary polyphenols

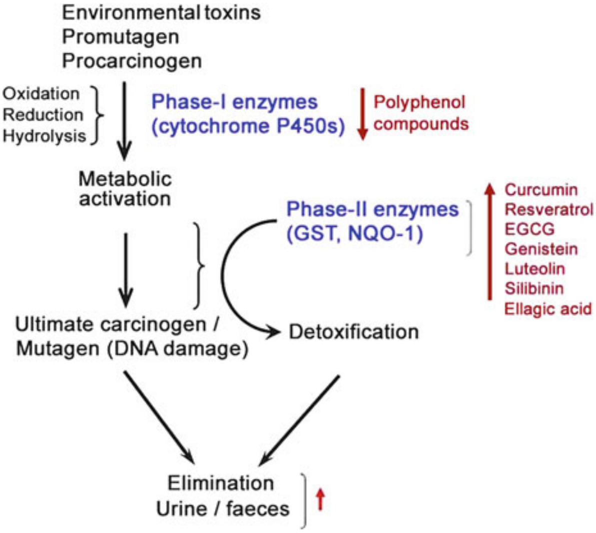
Environmental toxins
Promutagen
Procarcinogen
Oxidation
Reduction
Hydrolysis
Phase-I enzymes
(cytochrome P450s)
Polyphenol
compounds
Metabolic
activation
Phase-II enzymes
(GST, NQO-1)
Curcumin
Resveratrol
EGCG
Genistein
Luteolin
Silibinin
Ellagic acid
Ultimate carcinogen /
Mutagen (DNA damage)
Detoxification
Elimination
Urine / faeces

Fig. 71.3 Schematic representation of phase I and II reactions and its modulation by chemodietary polyphenol compounds

chemical carcinogens and by extension, cancer chemoprevention [7]. Essentially, phase I enzyme according to Wattenberg's perception catalyzes "functionalization reactions" mediated by the superfamily of heme-containing monooxygenase (cytochrome P450 (CYP) enzymes that metabolize inert chemical (pro)carcinogen to biologically inactive or chemically reactive electrophilic metabolites) (Fig. 71.3). The later undergoes additional metabolism by phase I or phase II enzyme systems (glutathione S-transferase (GST) isoenzymes, uridine diphosphate-glucuronosyltransferase, sulfotransferase, and catechol-O-methyltransferase) resulting in increased detoxification; these biochemical reactions generally increase water solubility, enhancing removal of harmful xenobiotic agents/carcinogens from the body, precluding them from attacking cellular DNA and initiating tumorigenesis [8, 9]. Further, within this conceptual framework, another observational paradigm classifying chemopreventive phytochemicals (including polyphenol compounds) primarily on the stage they effectively contribute to preventive effects has emerged and accordingly classified as: (a) inhibiting carcinogen formation, (b) blocking agents inhibiting tumor initiation, or (c) suppressing agents that specifically act as inhibitors of tumor promotion/progression.

Of interest to human health, it is now well established that some common dietary polyphenols – curcumin, resveratrol, genistein, epigallocatechin-3-gallate (EGCG), quercetin, silibinin, ellagic acid, etc. – show evidence of inhibiting carcinogen activation and being potent inducers of GST inhibit carcinogen-induced tumorigenesis in vivo in a number of animal models. Resveratrol, a polyphenol present in wine at concentration between 1 and 10 μM, restrains bioactivation of polycyclic aromatic hydrocarbons (PAHs), a class of ubiquitous environmental chemicals, through reduced expression of CYP1A1 and CYP1B1 genes in human bronchial epithelial cells [10]; increased CYP1A1 expression and activity are associated with a high risk of lung and colorectal cancer [11, 12]. Likewise, CYP1B1 activates procarcinogens and their elevated levels are associated with estrogen carcinogenesis [13]. Normal human breast and breast tumor tissues are known to express CYP1B1, leading to the generation of an carcinogenic entity – 4-hydroxyestrogen; hence, inhibition of CYP1B1 affects the production of this and other mutagenic estrogen 3,4-catechols [14]. Quercetin, found abundantly in fruits and vegetables, significantly inhibits benzo[a]pyrene (B[a]P)-induced CYP1A1 mRNA and protein expression within tumor cells [15]. Isoflavone – biochanin A (from red clover) and genistein (from soybean) have been found effective in inhibiting DMBA-induced DNA damage in cells by inhibiting CYP1A1 and CYP1B1 enzymes [16]. Furthermore, genistein and isoflavone analogs (daidzein and glycitein) show evidence of the potential to decrease the side effects of tamoxifen via inhibition of CYP1A2 that inhibits the formation of α-hydroxy tamoxifen and its sulfate conjugate believed to be responsible for DNA adduct formation [17, 18]. Baicalein, a flavone from the root of the *Scutellaria* species, reduces the CYP1A1/CYP1B1 mRNA expression induced by DMBA [19]. Other examples include flavones (chrysin, baicalein, and galangin), flavanones (naringenin), and isoflavones (genistein, biochanin A). All these compounds harbor the potential to inhibit the activity of aromatase (CYP19) enzyme diminishing estrogen biosynthesis and producing antiestrogenic effects vital in breast and prostate cancers [20]. Ellagic acid inhibits N-nitrosobenzylmethylamine (NBMA) metabolism in vitro and in vivo inhibiting NBMA-induced esophageal tumors [21, 22]. Further, the effect of dietary ellagic acid on hepatic and esophageal mucosal cytochromes P450 and phase II enzymes has been reported [23]. Another important plant polyphenol – curcumin – significantly affects CYP1A2 and CYP2A6 activity in humans [24]. Tea polyphenols exhibit an inhibitory effect on microsomal CYP enzyme system [25, 26]. Epigallocatechin-3-gallate (EGCG) has a strong inhibitory effect on CYP1A1 at the transcriptional level [27].

(−)-Epicatechin-3-gallate (ECG) – another catechin and major antioxidative phenolic constituent of green tea – inhibits inducible CYP450 isoforms and shown to abrogate up to 50 % benzo[a]pyrene-diol epoxide-DNA adduct formation in benzo [a]pyrene (B[a]P)-treated cells [28, 29]. Furthermore, in mouse model of transplacental induction of lymphoma, lung, and liver cancer by the polycyclic aromatic hydrocarbon – dibenzo[a,l]pyrene (DBP) – induction of cytochrome P450 (CYP1B1) in maternal liver by EGCG reduced the bioavailability of DBP to the fetus demonstrating that maternal ingestion of green tea during pregnancy and nursing may provide protection against transplacental carcinogenesis [30].

Glutathione S-transferase (GSTs) and NAD(P)H:quinine oxidoreductase 1 (NQO1)/quinone reductase (QR) have a considerably important role in the detoxification of carcinogens, and their induction above basal level by flavonoids and vegetable diets is associated with chemopreventive effects [31]. GST has been identified as one of the main detoxifiers of dihydrodiol and epoxide forms of benzo [a]pyrene (a known mutagen in tobacco), and GST-Pi plays a critical role in the development of lung carcinogenesis following exposure to tobacco-related carcinogens and urethane [32, 33]. EGCG enhances the activity of GST and NQO1 enzymes in mouse liver and small intestine and in breast and prostate cancer cells [34–37]. The effect of a biologically active flavonoid – silibinin derived from milk thistle (*Silybum marianum*) on phase II enzymes – reveals moderate to highly significant increase in both GST and QR activities in liver, lung, stomach, skin, and small bowel in a dose- and time-dependent manner demonstrating the bioavailability and phase II enzyme induction by systemically administered silibinin [38]. Genistein and daidzein have been testified to increase QR activities in murine hepatoma and human colon cancer cell line, and the inhibition of benzo[a]pyrene metabolite-DNA binding by genistein results from QR induction [39–41]. Topical applications of soy isoflavones (mixture of 33 mg of genistein and 67 mg of daidzein), 30 min prior to the application of tumor promoter – 12-O-tetradecanoylphorbol-13-acetate (TPA) on mouse skin – prevented decrease in GST activity [42]. Other studies reported that dietary genistein and daidzein are able to elevate the activities of phase II and antioxidant enzymes in rat liver and kidney and QR in the colon of rats in vivo [43, 44]. Thus, promotion of detoxification of chemical carcinogens by select polyphenol class of phytochemicals represents a promising rationale-based pharmacological approach for chemoprevention in susceptible individuals.

4 Anti-inflammatory Effect

In 1863 Rudolf Virchow envisaged a connection between inflammation and cancer that later supported the hypothesis that inflammatory process is a cofactor in carcinogenesis. Although inflammation under normal physiological conditions is beneficial for host’s defense, prolonged inflammation by chemical and physical agents and inflammatory reactions of uncertain etiology cause various chronic disorders including cancer. The complex circuitry of inflammation and cancer is less well understood. It is currently believed that at the site of inflammatory stimulation, the production of pro-inflammatory and immunosuppressive cytokines and diverse reactive oxygen species (ROS) and reactive nitrogen species (RNS) by locally recruited inflammatory cells induces genetic alterations which lead to malignant transformation and stimulate proliferation of initiated cells. Among the myriad of changes that occurs within the inflammatory environment, cyclooxygenase-2 (COX-2), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS) are important enzymes involved in provocating inflammatory processes; their improper upregulation has also been linked with the pathophysiology of certain types of human cancers as well as inflammatory disorders [45]. Thus, blocking inflammation signaling is usually recognized as potential mode for chemoprevention, and constellation of polyphenols targeting anti-inflammatory signaling pathways is described in the following sections. The natural occurring polyphenols – curcumin and resveratrol – are being recognized as one of the most promising nontoxic natural molecules to abate colitis and potentially colon cancer associated with colitis. Cui et al. reported that the polyphenol resveratrol significantly improves inflammation score and downregulates markers of inflammation and inflammatory stress (p53 and p53-phospho-Ser(15) reducing tumor incidence from 80 % to 20 % in mice [46].

4.1 Cyclooxygenase

The cyclooxygenase pathway converts arachidonic acid released by membrane phospholipids into eicosanoids such as prostaglandins, prostacyclins, and thromboxanes with cyclooxygenase-2 (COX-2) being the key regulatory enzyme for prostaglandin synthesis. Inhibition of COX-2 expression in malignant tumors is considered vital in clinical management of cancer since overexpression of COX-2 activity has been observed in practically every premalignant and malignant condition involving the colon, liver, pancreas, breast, lung, bladder, skin, stomach, head and neck, and esophagus [47]. Transcription factors – AP-1 and NF-κB – have been found to stimulate COX-2 transcription [48, 49]. Published studies indicate several classes of polyphenol compounds inhibit COX activity at transcriptional as well as enzyme level [50]. Polyphenol curcumin has an effect opposing TPA-induced tumor promotion by effectively inhibiting cyclooxygenase and lipoxygenase activities [51]. Theaflavin-2 (TF-2), a major component of black tea extract, suppresses inflammatory pathways in vitro and in vivo by modulating COX-2 gene expression at the transcriptional level alleviating inflammatory effect [50]. Mechanisms relating to anti-inflammatory effect by resveratrol targeting prevention and amelioration of carcinogenesis have been reported. Resveratrol treatment attenuates COX-2 induction stimulated by lipopolysaccharides (LPS), 12-O-tetradecanoylphorbol-13-acetate (TPA), or superoxide [52]. Resveratrol directly binds with COX-2 and this binding is absolutely required for resveratrol inhibition of the ability of human colon adenocarcinoma HT-29 cells to form colonies in soft agar [53]. Dietary resveratrol also antagonizes pro-inflammatory cytokines TNF-alpha and IL-1β and augments anti-inflammatory cytokine IL-10. Also, resveratrol reduces prostaglandin E synthase-1 (PGES-1), COX-2, and iNOS protein expression via downregulation of p38 mitogen-activated protein kinases (MAPK) signaling pathway [54]. Quercetin effectively suppresses COX-2 promoter activity in colon cancer cells [55]. Genistein inhibits COX-2 expression via LPS-mediated tyrosine kinase signaling pathway [56]. It has been reported that EGCG affects activity of COX-2 following interleukin-1β stimulation of human chondrocytes and also in LPS-induced macrophages [57, 58]. Green tea extract enriched with EGCG downregulated COX-2 expression induced by TPA in mouse skin and human mammary cells (MCF-10A) in culture [49]. It is currently being emphasized that chemopreventive compounds, capable of inhibiting COX-2 expression without affecting COX-1, needs prioritization for cancer prevention and therapy.

4.2 Lipoxygenase

Lipoxygenases (LOXs) are enzymes involved in biochemical events leading to generation of pro-inflammatory mediators such as leukotrienes and conjugated hydroxyeicosatetraenoic acids (HETEs) from arachidonic acid of membrane phospholipids. On basis of regiospecificity during interaction with substrates, lipoxygenases have been designated as 5-LOX, 8-LOX, 12-LOX, and 15-LOX [59]. Several lines of evidence comply that 5-LOX pathway interacts with multiple intracellular signaling pathways that control cancer cell proliferation [59]; thus, inhibiting 5-LOX pathway has been predicted useful in inhibiting ontogeny of tumor development in a number of animal model systems. A variety of polyphenol compounds have been evaluated for their ability to either inhibit the synthesis or action of leukotrienes in context of anticancer properties. Nordihydroguaiaretic acid (NDGA) is a well-characterized nonselective LOX inhibitor from resinous extracts of the creosote bush *Larrea divaricata*; it blocks cysteinyl leukotriene (CysLT) synthesis. It has been used for centuries by native North Americans as a remedy for diverse illnesses and inhibits the growth of various tumors both in vitro and in animals [60–64]. Curcumin inhibits COX and LOX activities as well as TPA-induced promotion in mouse skin [51]. Polyphenols in red wine and grape seed extracts inhibit colon adenocarcinoma cell proliferation concomitant with increased apoptosis accompanying inhibition of 5-LOX activity [65]. The beneficial effects of cocoa polyphenols – flavan-3-ols and procyanidins – have been ascribed to their ability to inhibit mammalian LOX enzyme leading to decline in the plasma concentrations of cysteinyl leukotrienes which are metabolites of the 5-LOX pathway of arachidonic acid metabolism [66]. Green and black tea polyphenols – (−)-epigallocatechin gallate, (−)-epicatechin-3-gallate, and theaflavins – reportedly inhibit LOX-dependent activity by 30–75 % in colon tumors [67].

4.3 Inducible Nitric Oxide Synthase (iNOS)

As the name suggests, iNOS is an inducible enzyme induced by various stimuli including pro-inflammatory cytokines, hypoxia, and redox transcription factor

NF-κB leading to production of high amounts of nitric oxide ($NO^{\bullet}$) upholding it for an extended period of time. The production of $NO^{\bullet}$ under inflammatory milieu induces oncogenic mutations in p53 genes invigorating pro-tumorigenic effects. Polyphenol compounds exhibit promise for their ability to inhibit this pathway. As an example, resveratrol suppresses iNOS expression and COX-2 in murine macrophage cell line (RAW 264.7) stimulated with LPS and interferon-γ (INFγ) [68, 69]. Quercetin obstructs the generation of LPS-induced NO through the suppression of iNOS expression and attenuates LPS-induced prostaglandin E(2) production both in vitro and in vivo [70]. In murine RAW 264.7 macrophages, genistein, apigenin, and kaempferol inhibited the production of nitric oxide and induction of iNOS by LPS probably via inhibition of the activity and expression of iNOS [71–73]. EGCG also blocks LPS- and IFNγ-activated iNOS expression and enzyme activity in vitro [74, 75]. iNOS is overexpressed in colon tumors, and selective iNOS-specific inhibitors are speculative of providing a selective and safe chemopreventive strategy for colon cancer. A comprehensive review article highlighting mechanisms underlying endotoxin-induced iNOS expression in macrophages and promising natural agents that may be useful for anti-inflammatory and anticarcinogenesis strategies has been published [76].

5 Antioxidant Potential of Polyphenols

The most well-known benefit of polyphenols on human health is ascribed to their antioxidant property. This is attributable to direct scavenging of various radicals due to their chemical substructure containing labile phenolic hydroxyl group as well as their ability to upregulate the expression of free radical scavenging and cytoprotective antioxidant enzymes – superoxide dismutase, catalase, and peroxidases. These enzymes under basal conditions do not operate at maximal capacities but are upregulated (induced) to much higher levels by polyphenol compounds, thereby markedly enhancing the capacity of cells and host to endure oxidative stress and related forms of toxicity. A great deal of research has evaluated the antioxidant effect of various classes of polyphenols.

Under normoxic conditions in an aerobic organism, ROS [mainly superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$)] and RNS (like nitric oxide (NO) and peroxynitrite anions ($ONOO^{-}$)) are generated as by-products of metabolism notwithstanding exposure to hazardous xenobiotics [77, 78]. These ubiquitously generated radicals not only potentially damage DNA and induce mutations but also partake in most carcinogenic processes by activating oncogene products and/or inactivating tumor suppressor proteins [79, 80]. The presence of a high level of H_2O_2 reported in several carcinomas including melanoma, neuroblastoma, colon, and ovarian carcinoma cell lines functions in triggering signaling leading to transcriptional activation of cell proliferating genes [81–83]. Thus, a surrogate paradigm – abrogating signaling events and the expression of responsive genes stimulating cell proliferation – resides in scavenging of *ROS* by phenolic antioxidants. Additionally, oxidative stress induced by ROS has also been linked to tumor promotion in many tissues and implicated as playing a significant role in the promotion of carcinogenesis [84–87]. Green tea polyphenols by virtue of its action as strong antioxidant function in trapping and preventing the formation of reactive species including superoxide radical, singlet oxygen, hydroxyl ROS, nitric oxide, nitric dioxide, and peroxynitrite and additionally are found to reduce hydrogen peroxide (H_2O_2)-induced cytotoxicity [88, 89]. Quercetin, present in many commonly consumed fruits and vegetables, is a powerful antioxidant and potent scavenger of ROS and RNS through increased Nrf2 expression in vitro [90–92]. Quercetin protects cells from ultraviolet A (UVA)-induced intracellular damage. Quercetin pretreatment strongly suppressed UVA-induced apoptosis in human keratinocyte HaCaT cells by markedly inducing the expression of antioxidative gene and reduced production of reactive oxygen species following UVA irradiation by increasing protein levels of the transcription factor Nrf2; these beneficial effects were greatly compromised by downregulating Nrf2 expression [93]. Genistein belonging to the isoflavone group of compounds also exhibits strong antioxidant activity, particularly through the increased expression of antioxidant and phase II detoxifying enzymes at physiological concentrations [94]. Mangiferin, a polyphenol found in mango, exerts cytoprotective effect on benzo[a]pyrene-induced lung carcinogenesis in mice by rescuing enzyme activities of catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase [95]. It has recently been documented that stilbene piceatannol induces cytoprotective heme oxygenase-1 expression in human mammary epithelial cells [96]. In addition to the aforementioned effects, the efficacy of other tea polyphenols, theaflavin-3,3′-digallate and (−)-EGCG, in inhibiting xanthine oxidase enzyme (a prooxidant) suppressing intracellular ROS and exerting antioxidative effect has been reported [97]. The effect of resveratrol to inhibit free radical formation has been validated in human promyelocytic leukemia cells treated with TPA [98]. Similarly, in mouse model of tumor induction, the epidermal hyperplastic response to TPA was inhibited by resveratrol through interference with pathways of reactive oxidants and, possibly, through the modulation of the expression of the proto-oncogene *c-fos* and TGF-β1 [99]. Furthermore, resveratrol (and other non-polyphenol antioxidants) tends to inhibit the development of adenomas and preneoplastic atypical hyperplasias in mice that are deficient in the 8-hydroxyguanine DNA glycosylase 1 (Ogg1) gene; this gene encodes an enzyme that repairs oxidative DNA injury by 8-oxoguanine (8-oxoG) [100]. Ogg1 gene deficiency enhances lung adenocarcinogenesis in mice by virtue of accelerated oxidative stress along with mutations of EGFR and K-Ras gene similar to that found in human lung cancer [100].

Intriguingly, in contrast to above mentioned scenario, emerging evidence suggests the accumulation of ROS at low to moderate concentrations could short-circuit signaling events that favor the induction of apoptosis by stimulating cytochrome c release (into cytosol) that triggers caspase activation in many biological systems [101]. In DU145 prostate cancer cells, ECG increases ROS formation and mitochondrial depolarization causing apoptosis induction [102]. Additionally, EGCG induces H_2O_2 formation in human lung adenocarcinoma (H661) and in Ha-ras gene-transformed human bronchial (21BES) cells to cause apoptosis; the effect seems to be abolished by addition of exogenously added catalase (CAT) confirming the involvement of H_2O_2 in provoking the induction of apoptosis upon treatment [103, 104]. Stilbene resveratrol has been shown to interact with mitochondria of cancer cells and induce an imbalance in cellular antioxidant activities, leading to a significant increase in the levels of both intracellular reactive oxygen species (ROS) and lipid peroxides. Resveratrol sensitizes colon cancer cells to 5-fluorouracil chemotherapy by inducing a state of increased oxidative stress mechanistically linked to the inhibition of Akt and STAT3 proteins, which are known to have oncogenic potential in colorectal carcinomas [105].

6 Polyphenols and Cell Cycle

The hyper-proliferation of tumor is a highly regulated process that progresses like normal cells through the four phases of cell cycle – G1, S, G2, and M – which are controlled by cell cycle regulatory proteins. These include cyclins (cyclin A, B, Ds, or E), cyclin-dependent kinase (CDK; CDK1, 2, 4, or 6), and associated CDK inhibitors (CKI) such as $p27^{KIP1}$, $p21^{CIP1/WAF1}$, p53, and phosphorylated retinoblastoma (pRb). In retrospect, cell cycle arrest occurs in response to cellular stress preventing the replication of damaged DNA and/or segregation of damaged chromosomes during mitosis through intricately balanced signaling ensuring completion of phase-specific events and maintaining genetic integrity [106]. Increasing experimental data therefore suggest that cell cycle control, particularly at the G1/S and G2/M transitions, represents a major task for the cell to ensure an accurate cell division. Accumulated evidence indicates the potential of polyphenol phytochemicals to induce growth inhibition of cancer cells by interfering with cell cycle checkpoints particularly at the G1/S and G2/M transitions through modulation of the expression and/or activities of key proteins.

The natural phytoalexin resveratrol was shown to result in inhibition in the expression of regulators of the G1/S transition of the cell cycle in particular cyclin D1, CDK4, and cyclin E in human breast cancer cells [107]. Benitez et al. found that treatment of androgen receptor-positive and androgen receptor-negative prostate cancer cells with resveratrol causes not only a significant reduction in the levels of expression of cyclins D1, E, and CDK4 but also a reduction in cyclin D1/CDK4 kinase activity compromising cells' capability to proliferate and causing an increase in apoptosis in time- and dose-dependent manner [108]. In HL-60 cells resveratrol induced apoptotic cell death in cells preferentially arrested in the G0/G1 phase concomitant with a gradual decrease in the anti-apoptotic Bcl-2 oncoprotein expression [109]. Quercetin exerts its antitumor effect through blocking cell cycle progression at the G0/G1 interface, parallel with CDK inhibition [110]. Many recent studies demonstrate that EGCG causes a predominant G1 cytostatic cell cycle arrest through regulation of cyclin D1, CDK4, CDK6, $p21^{CIP1/WAF1}$, and $p27^{KIP1}$ [111, 112].

We earlier reported that curcumin can completely downregulate cyclin D1 expression through both transcriptional and posttranscriptional mechanism(s) and showed a decrease in the formation of cyclinD1/CDK4 enzyme complex, resulting in suppression of proliferation and induction of apoptosis [113]. Corollary to our findings, several other independent studies reported that curcumin upregulates CDK inhibitors such as p21$^{CIP1/WAF1}$ and p27^{KIP1} and downregulated cyclin B1 and CDC2 [114]. CDK inhibitors (CDKIs) such as the p21$^{CIP1/WAF1}$ and p27^{KIP1} proteins abrogate formation of the enzyme complexes and block cell cycle progression. 3',4',7-Trihydroxyisoflavone, a metabolite of the soybean isoflavone daidzein, is a direct inhibitor of CDK2 and CDK4 [115].

In an in vivo model of intestinal tumorigenesis in Apc$^{Min/+}$ mice, feeding of grape seed extract reduced the total number of intestinal polyps compared to control mice [116]. The findings paralleled with decreased cyclin D1 and c-Myc protein levels in the small intestine along with downregulation in expression of other important molecules such as COX-2, iNOS, and β-catenin and increased expression of p21$^{CIP1/WAF1}$ with reduced cell proliferation and increased apoptosis [116]. Thus, the suppression of cell cycle components by natural polyphenol compounds could be an effective strategy in abating the proliferation of tumor cells in vivo.

7 Polyphenols, Transcription Factors, and Cell Signaling

Extensive research efforts to gain a better appreciation of molecular pathways have revealed the presence of a complex network of signaling pathways overtly active in cancer cells augmenting proliferation and suppressing apoptosis. The molecular basis of pleiotropic action of flavonoids and other class of polyphenols is emerging through examination of discreet regulatory pathways including Akt, NF-κB, mitogen-activated protein kinase (MAPK), p53, COX-2, Ras, and many other molecules that are known to regulate apoptosis without entailing unacceptable side effects (Fig. 71.4). Published studies reveal that insidious manipulation of multiple signaling cascades by dietary polyphenol compounds therapeutically interferes with signaling up- or downstream of target pathway contributing to the pleiotropic effects of these agents in chemoprevention strategy. We present a comprehensive indication of deregulated signaling mechanisms prevailing within tumor cells along with few select examples of polyphenol compounds so as to convey a broad overview relating to their biological and molecular basis of action in chemoprevention strategy. Furthermore, this highlights some noteworthy rationale of including cytoprotective polyphenol compounds in sensitizing tumors to improve the efficacy of chemotherapy in the clinics and warrants further examination to improve patient survival.

7.1 Polyphenols and Nuclear Factor-κB

The nuclear transcription factor-κB (NF-κB) signaling is one of the most vital components in the control of cell proliferation synchronizing survival of tumor cells, tumor invasion, metastasis, drug resistance, and stress response. Elevated levels of NF-κB have been detected in a number of human malignancies, and the subsequent activation

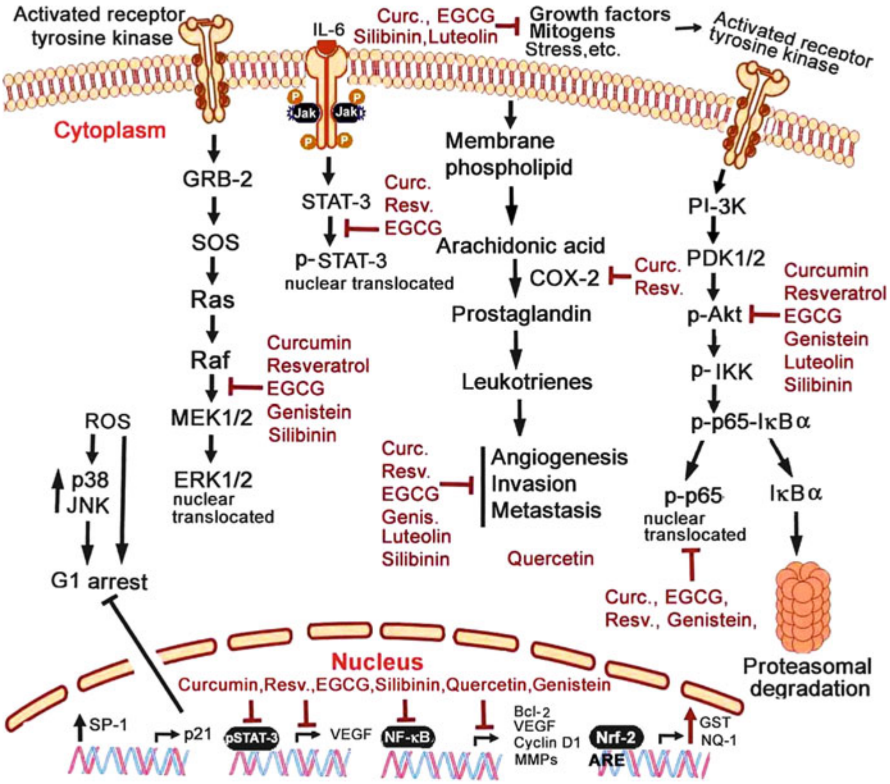
Activated receptor tyrosine kinase
IL-6
Curc., EGCG Silibinin,Luteolin
Growth factors
Mitogens
Stress,etc.
Activated receptor tyrosine kinase
Cytoplasm
Jak
Jak
P
GRB-2
SOS
Ras
Raf
MEK1/2
ERK1/2 nuclear translocated
Curcumin
Resveratrol
EGCG
Genistein
Silibinin
STAT-3
Curc.
Resv.
EGCG
p-STAT-3 nuclear translocated
Membrane phospholipid
Arachidonic acid
COX-2
Curc.
Resv.
Prostaglandin
Leukotrienes
Angiogenesis
Invasion
Metastasis
Curc.
Resv.
EGCG
Genis.
Luteolin
Silibinin
Quercetin
PI-3K
PDK1/2
p-Akt
p-IKK
p-p65-IκBα
Curcumin
Resveratrol
EGCG
Genistein
Luteolin
Silibinin
p-p65 nuclear translocated
IκBα
Curc., EGCG, Resv., Genistein,
Proteasomal degradation
ROS
p38
JNK
G1 arrest
Nucleus
Curcumin,Resv.,EGCG,Silibinin,Quercetin,Genistein
SP-1
p21
pSTAT-3
VEGF
NF-κB
Bcl-2
VEGF
Cyclin D1
MMPs
Nrf-2
ARE
GST
NQ-1

Fig. 71.4 A schematic summary of the molecular targets and cell signaling pathways altered by polyphenol compounds. Multiple growth factor receptors are activated at the cell surface during cancer. Polyphenol compounds promote transcriptional activation of a battery of detoxification and antioxidant proteins. The P13K–Akt, NF-κB, AhR, and STAT-3 pathways are of significance and targets of polyphenol compounds. By dephosphorylating these molecules, polyphenol compounds modulate downstream signaling pathways impinging on proliferation, angiogenesis, and apoptosis. NF-κB pathway is inactive as a result of the binding of p50 and p65 to IκBα. When IκBα is phosphorylated by IKKs and degraded, p50 and p65 are set free and are translocated into the nucleus to activate a specific set of genes. This pathway has been shown to be inhibited both in vitro and in vivo. Polyphenols inhibit COX-2 enzyme and reduce PGE2 levels

has been shown to suppress apoptosis and induce proliferation, invasion, metastasis, and chemoresistance signifying that activated NF-κB regulates downstream genes to promote cancer cell growth [117]. Therefore, NF-κB has emerged as a target for the prevention and/or treatment of cancer. Under non-stimulating conditions, NF-κB is sequestered in the cytoplasm through tight association with NF-κB inhibitory protein IκB. Following stimulation including cytokine binding to its receptor, activation of IκB kinase (IKK) complex occurs. This leads to phosphorylation and subsequent degradation of the inhibitory protein IκB, allowing NF-κB to translocate into the nucleus and bind to its target DNA to regulate the expression of NF-κB target genes (Fig. 71.4).

A number of studies reported in the literature reveal wide array of polyphenol compounds derived from common dietary sources and medicinal plants wield antitumor effects through the suppression of NF-κB DNA binding activity in myeloid, lymphoid, and several solid tumor cell lines derived from prostate, breast, head and neck, and pancreatic cancer cells resulting in the inhibition of downstream target genes that are critical for the establishment of aggressive cancers. This ultimately leads to the inhibition of cell growth and induction of apoptotic cell death [118–120].

Polyphenols such as curcumin, resveratrol, genistein, and tea components have also been found to potentiate the antitumor activity of chemotherapeutic agents through regulation of NF-κB. We and other investigators have reported that chemotherapeutic drugs such as cisplatin, Taxol, gemcitabine, oxaliplatin, and docetaxel could result in the activation of NF-κB in tumor cells, contributing to drug resistance in cancer cells [121–125]. Further, we have shown that pretreatment of cancer cells with isoflavone genistein followed by treatment with low concentrations of docetaxel, gemcitabine, cisplatin, or oxaliplatin elicited significantly greater inhibition of cell growth concomitant with induction of apoptosis compared to either agent alone [121–123, 126, 127]. The results could be recapitulated in established in vivo animal models. Collectively, our results and studies reported from other laboratories clearly suggest that polyphenol compounds, which inactivate NF-κB activity along with other cellular effects, may contribute to increased cell growth inhibition and apoptosis with suboptimal doses of chemotherapeutic agents.

7.2 Aryl Hydrocarbon Receptor (AhR)

The AhR is a ligand-dependent transcription factor belonging to the basic helix–loop–helix/Per–ARNT–Sim family of proteins responsible for the induction of drug-metabolizing enzymes. Induction of cytochrome P4501A1 (CYP1A1) expression has been studied extensively as a model of AhR action. A variety of structurally diverse xenobiotic compounds such as halogenated and polycyclic aromatic hydrocarbons (dioxins, benzo[a]pyrene, and 3-methylcholanthrene) bind to the AhR as ligands and cause toxicological effects including cancer by activating this transcription factor [128]. In AhR$^{-/-}$ mice, benzo[a]pyrene fails to induce skin carcinogenesis [129]. Substantial data reported in literature from cultured cells and animal models indicate that naturally occurring compounds such as flavonoids, curcumin, resveratrol, EGCG, and genistein can effectively suppress biological events related to AhR-mediated signaling [130–133]. An in-depth mechanism of flavonoids on aryl hydrocarbon receptor-mediated signal transduction has been reported recently [134]. Curcumin suppresses phosphorylation of the AhR and AhR nuclear translocator (ARNT) through the inhibition of PKC; the later can directly phosphorylate AhR [131]. EGCG inhibits AhR signal transduction by interacting with its partner protein heat shock protein 90 [135]. Resveratrol, being an AhR antagonist, decreases 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced expression of CYP1B1 expression and induces NAD(P)H:quinone oxidoreductase 1 (NQO1) expression [136, 137]. Such effects are important in breast cancer because estrogen genotoxicity may be initiated by cytochrome P450 (CYP1B1)-mediated oxidation of catechol estrogens to metabolites that react with DNA to form estrogen-DNA adducts [136]. Thus, by modulating AhR regulatory pathways, polyphenol compounds can influence breast cancer initiation and progression and other cancers as well.

7.3 Nuclear Factor E2 p45-Related Factor (Nrf2)

Nuclear factor E2 p45-related factor 2 (Nrf2) is a 1 (Bzip) transcription factor 2 sequestered in the cytoplasm by a cysteine-rich cytoskeleton-binding protein named Kelch-like ECH-associated protein-1 (Keap-1). After activation it translocates into the nucleus and binds to the “antioxidant response element” (ARE) in conjugation with small Maf proteins. Nrf2 plays a central role in the regulation (basal and/or inducible expression) of several phase 2 genes (e.g., *GST* and *NQO1*) by binding to the ARE in their promoters and portrayed as an important target for achieving chemoprevention (Fig. 71.4). ROS also activates the Nrf2–ARE pathway to activate antioxidant and detoxifying enzymes [138]. Most polyphenol compounds with chemopreventive status serve as transcriptional activators for the expression of glutathione S-transferase, NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1(HO-1), γ-glutamyl cysteine synthetase (γ-GCS), and antioxidant enzymes by coordinately regulating Nrf2-antioxidant/electrophile response element (ARE/EpRE) [139]. To cite a few cases in point, EGCG-rich green tea polyphenol treatment to human mammary epithelial cells results in the expression of glutamate-cysteine ligase, manganese superoxide dismutase, and heme oxygenase-1 antioxidant enzymes by Nrf2-mediated signaling upregulating antioxidant enzyme expression [140]. The antioxidant efficacy of quercetin is also reportedly mediated through increased Nrf2 expression [141]. Quercetin protects cells from ultraviolet A damage mainly by elevating intracellular antioxidative activity via the enhanced accumulation of this transcription factor for antioxidant genes [93]. Furthermore, it has been reported that quercetin upregulates the expression of Nrf2 mRNA and protein by inhibiting ubiquitination and proteasomal turnover [141]. EGCG upregulates the expression of γ-glutamyltransferase, glutamate-cysteine ligase, and heme oxygenase 1 in the liver and colon of mice [142]. Increased levels of glutathione S-transferase (GST)-P activity were detected in lymphocytes of volunteers with lower baseline GST-P activity when treated with 800 mg Polyphenon E per day for 4 weeks [143]. Flavonoids have been shown to induce the expression of NQO1 and GST following the release of Nrf2 from Keap-1 [144]. Curcumin supplementation resulted in increased expression of detoxification enzymes glutathione S-transferases, glutathione reductase, epoxide hydrolase, HO-1, catalase, and NQO1 in the liver, small intestine, and kidney tissues of mice [145]. These examples as “proof of concept” validate molecular basis for the action of polyphenols in cancer chemoprevention strategies.

7.4 Regulation of the Akt Pathway

Most human cancers display reduced expression of the Akt inhibitor *PTEN* (the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10). Hence, proteins regulating signaling through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway are frequently activated in tumors because of the loss of PTEN. Activated Akt phosphorylates downstream substrates involved in the regulation of cell survival, cell cycle progression, and cellular growth (Fig. 71.4). A wide array of polyphenolic compounds derived from dietary sources including genistein, resveratrol, EGCG, silibinin, curcumin, and quercetin and whole plant extracts have been reported to inhibit cancer cell growth and induce apoptosis through the inhibition of the Akt pathway in tumor cells [146–152]. Kaempferol found in tea, propolis, and grapefruit inhibits UVB-induced phosphorylation of Akt signaling attesting kaempferol as a putative antitumor promoting agent [153]. EGCG promotes apoptosis in T24 human bladder cancer cells by inhibiting PI3K/Akt activation that, in turn, results in modulation of Bcl-2 family proteins, leading to enhanced apoptosis [154]. In Apc$^{Min/+}$ mice, oral administration of the green tea component EGCG reveals marked chemopreventive effects in association with inhibition of Akt signaling [155]. Furthermore, it has been reported that EGCG and other green tea polyphenols (GTP) decrease Akt phosphorylation along with reduction in nuclear β-catenin level in human breast cancer cells, leading not only to induction of apoptosis but also compromised their invasive capacity [156, 157]. Recently Dave et al. showed that the isoflavone genistein prevents cancer by inducing apoptosis in target cell concurring with increased PTEN expression both in vivo and in vitro [158]. We and others have found that isoflavone genistein inhibits cancer cell growth and induces apoptosis through the downregulation of Akt in breast, lung, ovarian, prostate, and pancreatic cancer cells [126, 149, 159–161].

The existence of a potential crosstalk between Akt, NF-κB, and androgen receptor (AR) has been reported, and genistein could interrupt these crosstalks resulting in downregulation in the expression of AR and prostate-specific antigen (PSA) [159]. In pancreatic and ovarian cancer with constitutively active Akt, the ability of genistein to induce apoptosis and sensitization to cytotoxic chemotherapy (cisplatin/Taxotere) implies importance of the inhibition of phosphorylated Akt signaling as a clinically significant strategy [126, 162]. In spot of clinical drug development, Deguelin, a retinoid of flavonoid family with chemopreventive activities, has been found to decrease tumor incidence in animal models for lung, colon, mammary, and skin carcinogenesis through Akt inhibition [163–167]. Deguelin is also effective in reducing pAkt levels in the lung of Akt-inducible transgenic mice stimulating apoptosis and suppressing proliferation of premalignant and malignant human bronchial epithelial cells at doses in which only minimal effects were observed in normal bronchial cells [163, 168]. Collectively, these results reflect that Akt is an important target for action of chemopreventive agents not only in cancer prevention but also towards therapeutic approach.

Emerging evidence reveals that activated Akt is also critical for acquiring drug resistance in multiple cancers types [169–173]; therefore, one may anticipate downregulation of Akt by chemopreventive polyphenol agents would sensitize cancer cells to chemo- or radiotherapy. We and other investigators reported enhanced chemotherapeutic or radiation effects by isoflavone genistein being partially mediated by the inhibition of Akt signaling [121, 174, 175]. It has been found that genistein also enhanced necrotic-like cell death with the significant inhibition of Akt activity in breast cancer cells treated with genistein and adriamycin, suggesting that the enhanced growth inhibition by combination treatment is through the inactivation of the Akt pathway [176]. Phenoxodiol, one of the synthetic derivatives of genistein, inhibits Akt signaling pathway and subsequently activates the caspase system inhibiting X-linked inhibitor of apoptosis protein (XIAP) which in turn leads to increased chemosensitization [177]. Curcumin, the polyphenol from the plant *Curcuma longa*, downregulates Taxol-induced phosphorylation of Akt (and its interaction with NF-κB), revealing insight into antitumor effect of curcumin being mediated through the inactivation of the Akt and NF-κB pathways [178]. It is currently assumed that Akt inhibition is likely to emerge as a prognostic marker for patient risk stratification.

7.5 Polyphenols and MAP Kinase

Mitogen-activated protein kinases (MAPK) are a family of serine–threonine kinase proteins that essentially amplify and integrate signals from a variety of extracellular stimuli from the cell membrane to the nucleus. Accumulating evidence indicates constitutive and inappropriate activation of MAPK due to amplified or overexpressed growth factor receptors and oncogenic Ras as a critical component in a number of solid malignancies such as breast, prostate, and gastric cancers [179, 180]. Evidence from various studies has shown that modulation of this pathway by polyphenol compounds confers advantage for the prevention and/or treatment of human cancer [181]. In mammalian cells, three distinct but parallel MAPK cascades have been identified that lie downstream of the membrane-associated RTK: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), and the p38 MAPK. Although ERKs can be activated by mitogens and growth factors, JNK and p38 can be activated by many environmental stress stimuli such as UV and γ-irradiation as well as many inflammatory cytokines, which are frequently associated with the induction of apoptosis; therefore contextually, p38 and JNK have been generally linked to cell death and tumor suppression, whereas ERK pathway is thought to play a prominent role in conferring a survival advantage to cells and tumor promotion.

Modulation of MAPKs by flavonoid polyphenols such as kaempferol and genistein has been shown [182]. Huang et al. reported that genistein inhibited TGF-β-mediated p38 MAP kinase activation, matrix metalloproteinase type 2, and cell invasion in human prostate epithelial cells at concentrations associated with dietary consumption [183]. In other studies, genistein has been found effective in preventing cytokine-induced ERK1/2 activation and promoting apoptotic cell death [184]. Since genistein is a well-known inhibitor of tyrosine kinase, it is possible that genistein inhibit tyrosine kinase upstream of p38 MAPK and subsequently inhibit the phosphorylation of tyrosine on p38 MAPK, leading to the inactivation of MAPK pathway. EGCG has shown to inhibit 12-O-tetradecanoylphorbol-13-acetate and epidermal growth factor-induced transformation of mouse epidermal cell line JB6 which parallel with the inhibition of AP-1 transcription factor DNA binding and transcriptional activity [185, 186]. In Ha-ras-transformed human bronchial cells, treatment with EGCG inhibited c-Jun, ERK1/2 phosphorylation, and the phosphorylation of ELK1 and MEK1/2 [104, 187]. In prostate cancer, decrease in the MAPK signaling may contribute to the reduction in cell proliferation and induction of apoptosis and provide a cellular mechanistic basis for EGCG suppressing prostate cancer growth without toxicity [188]. In human gastric cancer cells, EGCG blocked tumor promoter-induced MMP-9 expression by suppressing MAPK signaling and AP-1 activation and in addition has shown to abrogate PMA-induced activation of extracellular-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) which is an upstream modulator of AP-1 [189]. In anaplastic thyroid carcinoma (ATC), one of the most lethal cancers because of its aggressiveness and the lack of efficacious therapy, EGCG showed potency towards growth inhibition and apoptosis by suppressing the phosphorylation of EGFR, ERK1/2, JNK, and p38 [190].

Paradoxically, contrary to above-mentioned findings, Bhattacharya and colleagues reported oxidation-triggered generation of intracellular reactive oxygen species leading to activation of JNK and p38 MAPK but not ERK in inducing apoptosis by theaflavins (TF) and thearubigins (TR) – the major polyphenols of black tea [191]. Phase II enzyme induction by green tea polyphenols involves the activation of MAPK [139, 192]. Curcumin inhibits the activation of MAPK and shown to inhibit JNK activation induced by various agonists, including PMA, ionomycin, anisomycin, UVC, gamma radiation, TNF, and sodium orthovanadate [193, 194]. Curcumin attenuates experimental colitis through a reduction in the activity of p38 MAPK and causes decrease drug resistance by its inhibitory effect on MAPK signaling [195–197]. Resveratrol significantly inhibits mitogen-activated protein kinase (MAPK) activities and immunoblot analyses revealed consistent reduction in the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), Jun N-terminal kinase (JNK-1), and p38 MAPK [198]. Resveratrol reduced prostaglandin E synthase-1 (PGES-1), COX-2, and iNOS protein expression via downregulation of p38 MAPK signaling pathway [54]. p38 MAPK pathway is involved in the mechanisms of the cell response to quercetin through the modulation of Nrf2 and glutathione-related enzymes [199]. Transcriptome and proteome profiling of colon mucosa of quercetin-fed rats point to tumor-preventive mechanism through downregulation of the potentially oncogenic MAPK in vivo [200]. Silibinin inhibits invasion of cancer cells by suppressing the MAPK pathway [201, 202]. Silibinin inhibits UVB-induced and regression of established skin tumors via modulation of MAPK and Akt signaling enhancing p53 in mouse skin, leading to decrease in UVB-caused proliferation and increased apoptosis, which might in part, be responsible for its overall efficacy against photocarcinogenesis and effectiveness both in prevention and intervention of human skin cancer [151, 203].

7.6 Wnt and Hedgehog Signaling and Polyphenol

In adult life Wnt signaling plays important homeostatic task, but inappropriate regulation and activation of this pathway is reportedly associated with several pathological disorders including cancer [204, 205]. In 60 % of the cases of colorectal cancer, either mutation or abnormalities in components of the Wnt/β-catenin signaling pathway are evident [206]. Other site-specific cancer with abnormal Wnt signaling has been reported for melanoma, hepatocellular carcinoma, gastric carcinoma, glioblastoma, leukemia, and pancreatic and breast cancer [207]. The antitumor effect of flavonoids in relation to their ability to modulate the Wnt/β-catenin signaling pathway has been reviewed by various authors [208–210].

Apigenin was the first described flavonoid as regulator of the Wnt pathway affecting the levels of β-catenin and Dsh proteins resulting in cell cycle arrest [207, 211]. EGCG inhibits Wnt signaling in breast, lung, and colon cancers by elevating transcriptional repressor protein levels (HBP1) which suppresses Wnt signaling [212]. Another mechanism reported in lung cancer cells includes promotion of demethylation of Wnt antagonist, WIF-1 (Wnt inhibitory factor 1), that inhibits Wnt signaling by direct binding to Wnt molecules [213]. The effect of green tea extract in reducing tumor multiplicity in the Apc$^{\mathrm{Min/+}}$ mouse has been linked to inhibition of translocation of Wnt mediator β-catenin to the nucleus [214, 215]. In rat mammary gland tumors, Su and colleagues demonstrated using Affymetrix rat 230A GeneChip arrays that soy isoflavone genistein inhibits Wnt expression through increased secretion of Wnt inhibitor (sFRP2) and decreased expression of Wnt5a attenuating Wnt signaling, without affecting β-catenin levels indicating noncanonical effect of genistein on mammary tumor [216]. In colon cancer, genistein inhibits β-catenin-mediated WNT signaling through increased expression of Wnt pathway antagonist sFRP2 gene expression by demethylating its silenced promoter [217]. Additionally, genistein has been reported to suppress GSK-3β and Akt phosphorylation which are upstream components of the β-catenin/Tcf pathway leading to suppression of β-catenin/Tcf transcriptional activity [218]. Other polyphenolic flavonoids – kaempferol, isorhamnetin, and baicalein – have been found and reported as negative regulators of β-catenin/Tcf signaling, and their inhibitory mechanism is related to the decreased binding of β-catenin/Tcf complexes to consensus DNA [218].

Flavonoid silibinin treatment decreased nuclear and cytoplasmic levels of β-catenin triggering growth inhibition and apoptosis in colon tumor cell lines such as SW480 where the Wnt pathway is altered [219]. The effect of resveratrol in colon cancer cells which do not have a basally activated Wnt pathway is due to significant decrease in the amount and proportion of β-catenin in the nucleus due to reduced expression of the two regulators (lgs and pygoI) of β-catenin localization [220]. The in vitro growth and apoptosis of several leukemia and lymphoma cell lines by quercetin are molecularly driven by inhibiting components of the Wnt signaling pathway [221]. Also, quercetin inhibits human SW480 colon cancer growth in association with inhibition of cyclin D1 and survivin expression through Wnt/beta-catenin signaling pathway [222].

The in vitro and in vivo effect of isoflavone genistein, EGCG, and resveratrol in the regulation of Hedgehog (Hh) signaling has also been reported [223]. Inhibition of Hh signaling correlates with delayed prostate tumor growth in vivo in TRAMP mice [223]. In transgenic LADY model of prostate cancer (alternative to TRAMP model), tumors rarely produce metastasis due to lack of increased Hh signaling during tumor development, evidence that strengthens the importance of Hh signaling in the process of metastasis [224].

7.7 Regulation of the STAT Pathway

Of the 7 STAT family members identified till date, aberrant constitutive activation of STAT3 family of cytoplasmic latent transcription factors has been identified in a number of human cancers including breast, lung, ovarian, pancreatic, skin, prostate, and in multiple myeloma, leukemia, and lymphomas. Active STAT3 dimers bind to consensus sequences in the promoters of genes regulating cell proliferation and anti-apoptotic behavior in cooperation with other transcription factors to regulate expression of genes such as Bcl-2 and Bcl-xL, Mcl-1, $p21^{CIP1/WAF1}$, and cyclin D1 [225–227]. Under normal conditions, multiple STAT3 endogenous negative regulators attenuate STAT3 signaling such as suppressors of cytokine signaling (SOCS) proteins, protein inhibitors of activated STATs (PIAS), and protein tyrosine phosphatases such as SHP-1 and SHP-2 that dephosphorylate active STAT3 complexes [228–230].

Natural chemopreventive agents such as green tea, resveratrol, and curcumin have shown to modulate STAT activation in tumor cells. In transgenic TRAMP mouse prostate cancer model, green tea polyphenol inhibited STAT3 expression inhibiting tumor growth and promoting apoptosis [231]. Polyphenon E (a standardized mixture of green tea polyphenols) suppresses STAT3 activation in breast cancer cells concurrent with inhibition of markers of angiogenesis [232]. Resveratrol inhibits Src tyrosine kinase activity blocking STAT3 activation [233, 234]. Bharti et al. demonstrated that curcumin inhibited IL-6-induced STAT3 phosphorylation abrogating nuclear translocation of activated STAT [235]. Administrating curcumin to athymic nude mice bearing ovarian tumors resulted in significant inhibition of STAT3 phosphorylation [236]. Luteolin, a flavonoid abundant in green vegetables, such as broccoli, cabbage, celery, green pepper and spinach, inhibits phosphorylation of STAT3 and targets it for proteasomal degradation, in this manner inhibits the expression of cyclin D1, survivin, Bcl-xL, and VEGF [237]. Thus, suppressing STAT signaling pathway by dietary polyphenol is expected to provide a window of opportunity for prevention and treatment of cancer.

7.8 Androgen Receptor (AR)

As a member of the steroid receptor superfamily of transcription factors, AR and its cognate natural ligand, androgen, are intimately associated with normal prostate development as well as in the growth and maintenance of hormone-resistant prostate cancer (HRPC) for which there is no curative therapy [238]. Most HRPC express AR and the androgen-inducible PSA. Studies have shown that polyphenol-rich food items such as soy products are associated with reduction of prostate cancer risk. The soy isoflavone genistein functions as a novel AR disrupting compound utilizing intracellular signaling pathways to alter AR nuclear accumulation and degradation by the ubiquitin proteasome pathway modulating androgen-dependent transcription program in prostate [239]. Furthermore, isoflavones have shown to modulate other regulatory molecules such as FOXO3a, GSK-3β, and β-catenin – these function as AR coregulators regulating the activity of AR. Experimentally, it has been demonstrated that isoflavone inhibits FOXO3a binding to the promoter of *AR*, but instead it promotes FOXO3a binding to the $p27^{KIP1}$ promoter, resulting in alteration of AR and $\mathrm{p27^{KIP1}}$ expression leading to the inhibition of cell proliferation and the induction of apoptosis in both androgen-sensitive and insensitive prostate cancer cells [240]. Using microarray techniques, it has been shown that resveratrol downregulates the expression of both AR and AR target genes by repressing AR transcriptional activity and not due to changes in nuclear translocation or DNA binding [241–243].

7.9 Mammalian Target of Rapamycin (mTOR) Signaling

The mTOR signaling is one of the major downstream signaling targets of PI3K/Akt and plays a critical role in promoting cellular proliferation and survival by phosphorylating two major targets – the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and the ribosomal protein S6 kinases (S6K1 and S6K2) that are required for cell cycle traverse from G1 to S phase [244]. Thus, inhibition of mTOR results in accumulation of cells in the G1 phase of the cell cycle and potential apoptosis. mTOR exists as two functionally distinct complexes, mTORC1 and mTORC2 differing in subunit compositions and biological functions. Dysregulated mTORC1 signaling is often observed in human tumors, and emerging evidence suggests that inhibition of mTOR signaling can be exploited as a potential tumor-selective therapeutic strategy. Initially, Rapamycin and its derivatives, CCI-779 and RAD001, showed much promise in clinical setting but later due to drug-mediated hyperactivation of Akt and ERK–MAPK pathways subsequently increased tumor cell viability accompanied by drug resistance [245]. Due to such negative clinical activity in a limited number of tumor types, it has now been rationalized to combine drugs which inhibit both (Akt and mTOR) signaling networks in therapeutic front. Recently, it was found and reported that the combined use of resveratrol and rapamycin resulted in modest additive inhibitory effects on the growth of breast cancer cells, mainly through suppression of rapamycin-induced Akt activation [246]. Similar findings have been reported in human glioma cells wherein rapamycin enhanced resveratrol-induced apoptosis [247]. Resveratrol inhibits insulin-stimulated Akt, S6 kinase, and 4E-BP1 phosphorylation and surges the association between mTOR and its inhibitor, DEPTOR, one mechanism by which resveratrol negatively regulates mTOR activity [248]. A combination of dietary grape polyphenols, resveratrol, quercetin, and catechin (RQC), at low concentrations induced apoptosis in gefitinib-resistant breast cancer cells and inhibited mammary tumor growth and metastasis in nude mice through mechanism involving inhibition of Akt and mTOR and activated AMP-dependent protein kinase (AMPK) even in the presence of gefitinib [249]. This emphasizes that anti-EGFR therapy can be potentiated by inhibition of Akt/mTOR signaling. Curcumin inhibits phosphorylation of the mTOR and its downstream effector molecules by dissociating raptor (a protein component of mTORC1 complex) from mTOR [250]. Silibinin from milk thistle plant inhibits translation initiation by inhibiting the mTOR signaling pathway [251]. Genistein modifies 4E-BP1 (Ser65) phosphorylation in tumor lines [252]. The antitumor activity of cisplatin by genistein is enhanced among others with reduced p-mTOR, p-p70S6K1, p-4E-BP1, and p-Akt [253]. EGCG, as key nodal ATP-competitive inhibitor of both phosphatidylinositol-3-kinase (PI3K) and mTOR (with K(i) values of 380 and 320nM), inhibits cell proliferation [254]. By targeting VEGF-R2-regulated Akt/mTOR/P70S6K signaling pathway, quercetin inhibits tumor growth and angiogenesis which in the future can be used as a potential drug candidate for cancer therapy [255].

7.10 Regulation of the Growth Factor Signaling Pathway

Over the past decade, extensive research has elucidated the functional relationship and molecular characterization between growth factor receptors (GFR) and their ligands that drive cell proliferation and tumor growth. Several chemopreventive phytochemicals including curcumin, genistein, resveratrol, and catechins have been shown to be potent inhibitors of several growth factor signaling pathways. Curcumin inhibits the ligand-stimulated activation of EGF (epidermal growth factor) receptor (EGFR) including an inhibitory effect on EGFR phosphorylation indicating that it has the potential to break the autocrine loops that are established in several advanced cancers and enhances the growth inhibitory effects of FU and oxaliplatin through EGFR and insulin-like growth factor receptor (IGFR) pathways [256–258]. Blocking EGF receptor directs the cancer cells to enter apoptosis, and this inhibition also abrogates the invasive potential of the cancer cells. The molecular effects and the mechanism by which EGCG and other catechins exert their protective effects towards dysregulated receptor tyrosine kinases (RTKs) in cancer cells have recently been summarized by Larsen et al. [259]. From ligand binding assays, it has been concluded that EGCG blocks the binding of EGF to its receptor to cause inhibition of EGFR phosphorylation [260]. In addition, it has been found that tea catechins inhibit receptor expression through a complex circuitry by inhibiting the activity of ERK, which regulates the transcription factor Egr-1, and that Egr-1 controls the expression of EGFR [260]. Inhibition of EGFR signaling has also been shown to decrease the production of vascular endothelial growth factor (VEGF) in cancer cells [261]. Adachi et al. demonstrated that EGCG disrupts lipid order and membrane organization; this causes internalization of EGFR such that EGF could no longer bind [262]. EGCG has also been shown to bind directly to EGF, VEGF, and platelet-derived growth factor (PDGF) ligands [263–265]. EGCG treatment potentiated the effects of the tyrosine kinase inhibitor erlotinib in head and neck tumors [112, 266]. In pancreatic cancer, growth inhibition and apoptosis was associated with inhibition of EGFR tyrosine kinase activity by flavonoid luteolin, present abundantly in several green vegetables [267]. Luteolin also inhibits VEGF-induced angiogenesis and tumor growth in a murine xenograft model [268]. Quercetin is reported to be a potent inhibitor of EGFR tyrosine kinase activity; it however does not directly inhibit EGFR but instead interferes with different signaling pathways downstream of EGFR that regulate cell proliferation and survival [269, 270]. In prostate cancer cells, growth inhibitory and apoptotic effects of silibinin could be achieved by targeting EGFR signaling. Silymarin and silibinin are also effective in inhibiting transforming growth factor-α (TGF-α) and EGF-mediated tyrosine phosphorylation of EGFR and its adapter protein Shc in androgen-independent human prostate cancer cells harboring constitutively active EGFR [271, 272]. IGFs are mitogenic ligands and their activity is firmly controlled by the presence of IGF-binding proteins (IGFBPs); similar to EGFR, IGF receptor (IGFR) signaling also promotes growth and survival of cancer cells. It has been shown that silibinin, apigenin, and EGCG downregulate IGF-IR signaling and significantly increase the levels of IGF-binding protein-3 (IGFBP-3) in prostate cancer inhibiting cell growth both in vitro and in vivo [273–275]. The anticancer properties of NDGA in breast cancer cells in vitro and in vivo relate to direct inhibition of the function of two receptor tyrosine kinases, the insulin-like growth factor receptor (IGF-1R), and the c-erbB2/HER-2/neu receptors resulting in inhibition of cellular anti-apoptotic signaling pathway [64].

8 Apoptosis

Apoptosis is an important defense mechanism against tumor development and major mechanism of anticancer action of chemopreventive and chemotherapeutic agents. At the molecular level, premalignant and tumor cells tend to skirt apoptosis by deregulating genes that bring about programmed cell death, and therefore, regulating apoptosis is considered as a relevant target in reducing the incidence of cancer. Contextually, overexpression of growth-promoting oncogenes and anti-apoptotic proteins such as Ras and Bcl-2 family members has been linked with promotion of cell proliferation which is fundamental to tumor growth. Additionally, in humans, the induction of apoptosis is closely related to normal p53 functioning since lack of its expression or function is associated with increased risk of tumor development. Two principal groups of Bcl-2 family proteins are anti-apoptotic proteins such as Bcl-2, Bcl-xL and Mcl-1 and proapoptotic proteins, which are further subdivided into multidomain proteins (Bax, Bak) as well as BH3-domain-only molecules such as Bim, Bid, Bik, Noxa, or Puma.

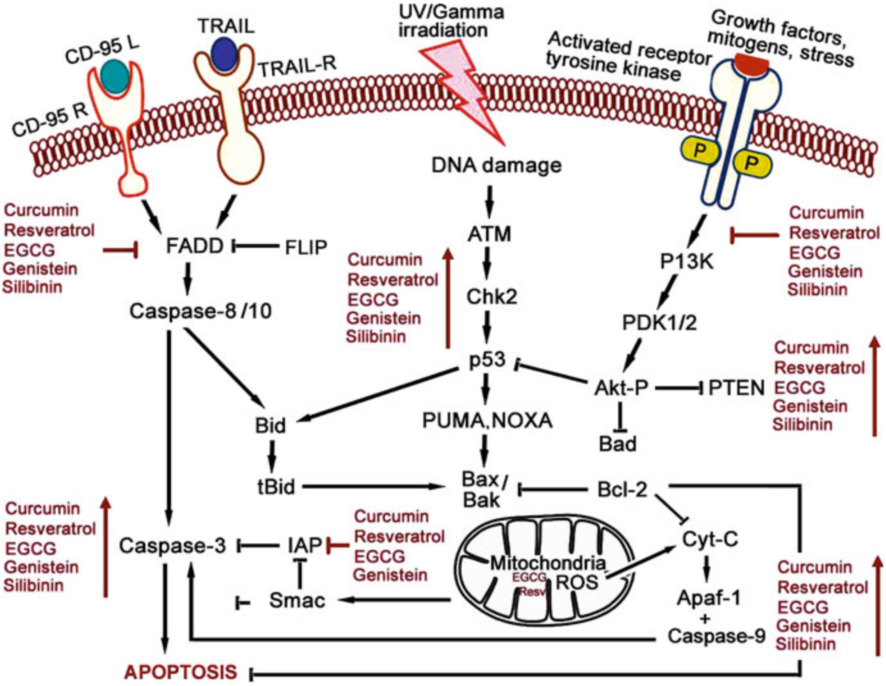
CD-95 L
TRAIL
UV/Gamma irradiation
Growth factors, mitogens, stress
Activated receptor tyrosine kinase
TRAIL-R
CD-95 R
P
P
DNA damage
Curcumin
Resveratrol
EGCG
Genistein
Silibinin
FADD
FLIP
Caspase-8 /10
ATM
Chk2
p53
P13K
PDK1/2
Akt-P
PTEN
Bad
Bid
tBid
PUMA,NOXA
Bax/ Bak
Bcl-2
Cyt-C
Apaf-1 + Caspase-9
Caspase-3
IAP
Smac
Mitochondria
EGCG
Resv
ROS
APOPTOSIS

Fig. 71.5 Potential pathways of apoptosis (extrinsic and intrinsic) and intracellular signaling elements modulated by polyphenols in chemoprevention and sensitizing cancer cells to cytotoxic chemotherapies

Most bioactive dietary polyphenols demonstrate potent apoptosis signaling in cancer cells as single agent augmenting the core apoptotic pathway that involves interference with mitochondrial integrity (intrinsic pathway) or synergize with death receptor of extrinsic pathway (Fig. 71.5). The extrinsic pathway is regulated by cytokines (mainly CD95L and TRAIL) secreted by cytotoxic T cells or natural killer (NK) cells and considered as important endogenous anticancer immunosurveillance agent inducing apoptosis selectively in tumor cells. Both these pathways converge through executioner caspases to cleave the death substrates (like poly (ADP-ribose) polymerase (PARP)) and eventually result in the classical morphological and biochemical hallmark of apoptosis such as chromatin condensation, translocation of phosphatidyl serine across plasma membrane, membrane blebbing, nuclear DNA fragmentation, and formation of apoptotic bodies [276]. As mentioned earlier, ROS contributes to mitochondrial depolarization and plays an important role in execution of intrinsic proapoptotic pathway.

Evidence for polyphenol-induced apoptosis has been observed in breast, colon, bone, leukemia, larynx, prostate, ovary, and pancreatic cancer cells. In most instances, the tested compounds have been documented to modulate apoptosis signaling through a number of targets in several cancer cell lines [277]. These targets include downregulation of the expression of apoptotic suppressor proteins such as Bcl-2 and Bcl-xL and upregulation of proapoptotic Bax expression, proteolytic activation of caspase-3, decrease in mitochondrial potential with subsequent release of apoptotic generating proteins cytochrome *c* and inhibitor of apoptosis (IAP) family.

Studies reported by us and several other laboratories have shown that isoflavone genistein induces apoptosis in breast, prostate, non-small cell lung cancer, head and neck squamous cell carcinoma, and pancreatic cancer cells [120, 121, 126, 278–281]. Phenoxodiol, the genistein derivative, under phase II clinical trials has been reported to bind to the tumor-associated NOX (tNOX) receptor and block its function and subsequently inhibits the anti-apoptotic proteins XIAP (X-linked inhibitor of apoptosis) and FADD-like ICE (FLICE) inhibitory protein, eventually inducing apoptotic cell death [177]. Bronikowska et al. tested and reported the cytotoxic and apoptotic activities of isoflavones – genistein, biochanin A, and neobavaisoflavone – in combination with TRAIL on HeLa cancer cells [282]. Isoflavone enhanced TRAIL-induced apoptosis indicating that isoflavones augment TRAIL cytotoxicity in cancer cells. In another study, the suppressive effect of genistein and resveratrol, alone and in combination in a transgenic model of spontaneously developing prostate cancer, revealed increasing evidence of apoptosis in the prostate along with reduced cell proliferation as core mechanism for the observed additive and synergistic effects in suppressing tumor growth. Furthermore, in this model genistein as a single agent induced apoptosis and found to decrease steroid receptor coactivator-3 (Src-3) in the ventral prostate [283].

Resveratrol has been shown to induce cell death in some tumor types expressing high CD95 (Fas, APO-1) by augmenting CD95 (FasL) expression, thereby manipulating CD95–CD95L system as “apoptotic trigger” to induce cell death [109]. The in vivo antitumor activity of grape-derived polyphenols against colon carcinoma cell xenograft in BALB/c mice and azoxymethane-induced aberrant crypt foci (preneoplastic lesions) has been viewed owing to increased apoptosis as indicated by TUNEL staining and active caspase-3 levels in tumor cells consistent with efficacy of these class of polyphenols in stimulating apoptosis under in vitro conditions [284]. Additionally, red wine polyphenols has been reported efficacious in reducing the number of azoxymethane-induced aberrant crypt foci (preneoplastic lesions) in colon by 49 % by mechanism involving inhibition of proliferation and promotion of apoptosis within tumor cells subsequent to upregulation of tumor suppressor genes. Resveratrol has been described to interfere with mitochondrial functions by inhibiting mitochondrial ATP synthesis through its binding to F1-ATPase [285]. The efficacy of treatment with polyphenolic extract of *Solanum nigrum* L. (SNPE) against hepatocellular carcinoma (HCC) was reportedly remarkable with almost complete inhibition of tumor weight as well as tumor volume at 2 μg/ mL. In vitro studies revealed sub-G(1) phase accumulation and caspase-3, 8, and 9 cleavages induced by SNPE attesting apoptosis induction as one of the underlying phenomenon in achieving cell growth inhibition [286].

In vitro, EGCG has been shown to cause growth inhibition and apoptosis in a number of human cancer cell lines including leukemia, melanoma, breast cancer, lung, bladder, and colon [47, 287–289]. Yamauchi (2009) reported EGCG in green tea polyphenols (GTP) as a potent apoptosis inducer that functions exclusively through a p53-dependent pathway in A549 cells [290]. Treatment with GTP and EGCG to nude mice bearing highly invasive estrogen receptor-negative human breast cancer cell line (MDA-MB-231) showed an effect in delaying the tumor incidence as well as reduction in tumor burden compared to control. Immunohistochemistry performed on tumors showed evidence of apoptosis induction that inhibited proliferation of cells strengthening proclamation that GTP and EGCG treatment inhibit proliferation and induce apoptosis cells in vitro and in vivo asserting antitumor properties [157].

In U-937 leukemia cells and mice harboring xenografts of this cell line, the proapoptotic effects of quercetin resulted in both the increased expression of the proapoptotic factor Bax and the inhibition of anti-apoptotic Mcl-1 [291]. Quercetin causes apoptosis in both transformed and primary leukemia cells but not in normal blood peripheral mononuclear cells at concentrations up to 50 mM [292]. Further, quercetin has been shown to be a potent enhancer of TRAIL-induced apoptosis in prostate and hepatocellular carcinoma cells [293, 294].

9 Invasion and Metastasis

A great deal of research has evaluated the effect of polyphenol compounds in inhibiting the invasive and metastatic behavior of cancer cells that represents the primary source of clinical morbidity and mortality in large majority of solid tumors. Currently, it is widely accepted that epithelial–mesenchymal transition (EMT) – a process that converts an epithelial cell to a mesenchymal cell by promoting the loss of cell–cell adhesion – leads to enhanced cancer cell migration and invasion. Gene expression analyses of human cancer specimens have identified cell migration genes, whose characteristic expression patterns can predict the risk of metastasis, and closely related to this phenomenon, high endogenous levels of uPA (urokinase-type plasminogen activator) and its receptor, uPAR, and matrix metalloproteases (MMPs) have been found in advanced metastatic cancers. Matrix metalloproteinases are proteolytic enzymes believed to provide cancer cells with their invasive potential by degrading the extracellular matrix. It is currently believed that inhibition of the activities of MMP-9 and uPA and levels of ERK and Akt might be one of the molecular mechanisms involved in the anti-invasive activity by polyphenols along with inhibition of AP-1 and NF-κB to block secretion of uPA. An in-depth molecular mechanism for anti-invasive and anti-metastatic activity of polyphenol and their related derivatives in various types of cancers has been recently reported by Weng and Yen [295]. In the following narrative, we summarize some key events associated with (select) polyphenol compounds such as EGCG, genistein, and resveratrol.

The pleiotropic anticancer effects of EGCG are linked with modulation of key elements in signaling pathways, which phenotypically retard invasion and metastasis of tumor cells from primary site including liver, lung, breast, prostate, colon, pancreatic, gastric, and oral site [296]. It has been reported that EGCG-mediated alterations in the expression of key regulators of EMT pathway including upregulation of E-cadherin, gamma-catenin, MTA3, ERα, and FOXO3 and downregulation of Snail reduce the invasive phenotype [297]. EGCG also increases the expression of the tissue inhibitor of MMPs (TIMP1 and TIMP2) at lower concentrations (1 μM) providing an additional mechanism and means to suppress the activity of MMPs [298]. Other investigators reported that EGCG significantly inhibits tumor cell invasion into basement membranes by reducing the expression of the MUC1, MMP-2, and MMP-9 proteins [299]. The invasion of lung carcinoma cells was attenuated by EGCG-mediated repression in expression of MMP-9 and nuclear localization of NF-κB [300]. The invasion and migration of human oral cancer cells, OC2, may partially be attributed to the decreased production of MMP-2/MMP-9 and uPA by EGCG [301]. Additionally, it has been shown that EGCG significantly suppresses the invasive ability of a variety of oral cancer cells by demethylating and enhancing RECK (an MMP inhibitor) and consequently inhibiting MMP-2/MMP-9 levels [302].

The effect of one of the most widely studied flavonoids – genistein on anti-metastatic properties on various types of cancer – has been reported. Genistein effectively inhibited the invasive potential of hepatocellular carcinoma (Bel7402 cells) by inhibiting focal adhesion kinase (FAK) signaling [303]. In breast cancer, the cell–matrix adhesion and migration of MDA-MB-231 cells are suppressed by genistein through inhibition of NF-κB and AP-1 and decreased titers of uPA secretion along with downregulation of MMP-2/MMP-9; MT1-, MT2-, and MT3-MMPs; and the chemokine receptors – CXCR4 and CXCL12 [304–307]. In melanoma, genistein suppressed adhesion-induced protein tyrosine phosphorylation to interrupt cell–ECM interactions contributing to inhibition of melanoma cell invasion [308]. The motility potential of melanoma cells is also decreased by genistein [309]. Genistein inhibits matrix metalloproteinase type 2 (MMP-2) activity in six out of seven prostate cell lines tested, blocks MMP-2 induction by TGF-β, and inhibited cell invasion. Efficacy was seen at low nanomolar concentrations, corresponding to blood concentrations of free genistein attainable after dietary consumption [183]. In orthotopic murine model of human prostate cancer metastasis, dietary genistein (before implantation) led to 96 % decrease in lung micrometastasis by 4 weeks along with nuclear morphometric changes in tumor cells indicative of increased adhesion (i.e., decreased detachment) and increased tumor levels of FAK, p38 MAPK, and HSP27 "promotility" proteins; however, the ratio of phosphorylated to total protein trended downward, indicating a failure to increase relative amounts of activated protein supporting the notion of cellular compensatory responses to antimotility effects induced by genistein [310].

In our laboratory, the effect of dietary genistein and purified soy extract [G2535, containing $\geq$97 % total unconjugated isoflavones and daidzein in a 2:1 ratio (G2535:daidzein)] on the growth of metastatic prostate cancer cells in an SCID-human experimental model of prostate cancer bone metastasis is being investigated. The findings demonstrate that genistein effectively inhibits prostate cancer cell growth in the bone environment and downregulates the transcription and translation of genes critically involved in the control of tumor cell invasion and metastasis in vitro and in vivo, suggesting possible therapeutic role of genistein and G2535 for metastatic prostate cancer [311, 312]. Other investigators have also demonstrated similar results showing that isoflavones inhibit bone metastasis of human breast cancer cells in a nude mouse model and metastasis of androgen-sensitive human prostate tumors in mice [313, 314]. Furthermore, studies reported from our laboratory have earlier documented that genistein intervenes in the regulation of the osteoprotegerin/receptor activator of NF-κB (RANK)/RANK ligand/MMP-9 signaling in prostate cancer, suggesting that isoflavone genistein could be a promising nontoxic agent that may synergize in augmenting the therapeutic outcome of metastatic prostate cancer with chemotherapeutic drugs [315]. Other reports indicate that genistein has the potential to therapeutically compensate endoglin deficiency – a key regulator of cell motility and induces metastatic suppressor kangai-1 (KA11) suggesting that genistein could be used for anti-metastatic therapies [316, 317].

Several studies have reported the anti-invasive and anti-metastatic effect of resveratrol as a potent arsenal in chemoprevention strategy. In hepatoma, Kozuki et al. and Miura et al. revealed that resveratrol can suppress the ROS-stimulated invasive capacity of the tumor cells by preventing rise of peroxide level and expression of HGF [318, 319]. Resveratrol inhibits MMP-9 expression in SMMC-7721 cells [320] and TNFα-mediated MMP-9 expression in HepG2 cells by downregulation of the NF-κB signaling pathway [320]. In lung cancer, anti-metastatic effect of resveratrol has been documented by a decrease both in the number and the weight of Lewis lung carcinoma (LLC) metastases in animal models via inhibition of LLC-induced angiogenesis [321, 322]. The migratory and invasive ability of A549 lung cancer cells is compromised by resveratrol treatment inhibiting the expression of heme oxygenase-1 (HO-1) and subsequently MMP-9 and MMP-2 expressions [323]. Resveratrol inhibits the migration of MDA-MB-231 breast cancer cells by inducing a rapid global array of filopodia and decreasing focal adhesions (also called cell–matrix adhesions) along with decrease in focal adhesion kinase (FAK) activity in these cells [324]. Quercetin, by downregulating uPA and its receptor uPAR and mRNA, culminates in inhibition of invasion, migration, and signaling molecules involved in survival and proliferation of cancer cells [325]. Apigenin substantially upregulated in colon cancer cells the anti-metastatic multifunctional cell-surface protein, CD26, which is downregulated in various cancers including colorectal carcinoma [326]. Thus, polyphenol compounds have the propensity to alter the metastatic behavior of tumor cells emphasizing their potential in chemoprevention armentorium.

10 Angiogenesis

Angiogenesis is the formation of new blood vessel from existing vasculature, an adaptive process to compensate tissue and cellular requirement for oxygen and nutrient import as much as the need to export metabolic wastes, and thus considered critical for growth and survival of tumors. Angiogenesis is also a prerequisite for metastatic tumor spread. As a result, intervention of the angiogenic process by polyphenol class of compounds has been viewed as a novel strategy for cancer control and therapy. Emerging evidence reveals tumor cells produce potent angiogenic factors such as vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) and fibroblast growth factor-2 (FGF-2) and concurrent downregulation of angiogenesis inhibitors (thrombospondin-1, angiostatin, endostatin) which sets in angiogenic phenotype within tumor [327, 328]. TGF-β signaling is known to upregulate angiogenesis. It has also been established that the α-subunit of potent tumorigenic factor, hypoxia-inducible factor-1 (HIF-1α), becomes elevated in tumors due to hypoxia and that HIF-1α transcriptional activity contributes to tumor angiogenesis, invasion, and progression [329]. The antiangiogenic properties of a number of naturally derived phytochemicals are essentially accomplished by inhibiting VEGF-mediated signaling and biological effects in endothelial cells and modulation of HIF-1α levels [330–332].

Great deal of evidence indicates the antiangiogenic effect of green tea extracts (GTE) and its main catechin EGCG. In in vitro model of cultured human umbilical vein endothelial cells (HUVECs), EGCG has been reported to disrupt VEGF-induced receptor dimerization inhibiting angiogenic signaling [333]. In human colorectal cancer cells, EGCG has also been shown to inhibit growth and activation of VEGF/VEGFR axis [334]. The effect of EGCG in heterotopic tumors induced by subcutaneous injection of gastric cells in nude mice recapitulates the in vitro data showing reduction in tumor microvessel density (MVD) by the treatment [335]. In breast cancer xenografts, green tea extract decreased the tumor blood vessel density, reducing the size of the tumor [336]. The antiangiogenic effect of other polyphenol compounds – genistein, resveratrol, quercetin, curcumin, and silibinin – has also been reported. Genistein has been shown to inhibit TGF-β signaling, impacting angiogenesis by suppressing TGF-β-induced expression of VEGF [337, 338]. Experiments conducted in nude mice xenograft and chick chorioallantoic membrane bioassay confirm genistein as a potent inhibitor of angiogenesis [339]. Mechanistically, genistein inhibits expression of angiogenesis-related factors, vascular endothelial growth factor [165], platelet-derived growth factor, tissue factor, and urokinase plasminogen activator (uPA), while, on the other hand, it upregulates angiogenesis inhibitors – plasminogen activator inhibitor-1, endostatin, angiostatin, and thrombospondin-1 [339]. Genistein significantly reduces nuclear accumulation of HIF-1α affecting angiogenesis by suppressing VEGF-mediated autocrine and paracrine signaling pathways between tumor cells and vascular endothelial cells [340]. Resveratrol suppresses tumor angiogenesis by blocking capillary-like tube formation induced by VEGF and also by intercepting the binding of VEGF to human umbilical vein endothelial cells [321]. Deguelin significantly reduces in vivo tumor angiogenesis and vascular tumor growth by interfering with several points in the angiogenic process, including inhibition of endothelial cell migration, invasion, and metalloprotease production and also inhibited HUVE cells' growth by inducing cell cycle arrest in the G0/G1 phase associated with induction of p21 and p53 and reduced survivin levels [341]. Deguelin anticancer activity in gastric, lung, and hepatocellular cancers also correlated with the inhibition of angiogenesis and induction of apoptosis [342–344]. Silibinin has been shown to strongly inhibit growth and survival of endothelial cells and inhibits VEGF- and IGF-1-induced cell proliferation and survival of endothelial cells [345]. Apigenin has been reported to inhibit hypoxia-induced or HIF-1α-mediated expression of VEGF in cancer cells [346]. Quercetin inhibits endothelial functions and in vivo angiogenesis induced by VEGF and inhibits tube formation by human microvascular dermal endothelial cells and HUVECs [347, 348]. Polyphenols of human interest need to be further analyzed in depth for their potential benefit in restricting neovascularization since blocking angiogenesis is a promising emerging therapeutic strategy to block or reverse tumor progression.

11 Polyphenols and MicroRNA

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNA molecules of 20–25 nucleotides in length cleaved from ~70 to 100 nucleotide hairpin pre-miRNA precursors [349]. Emerging evidence reveals pivotal role of miRNAs in a wide array of biological processes including their role as regulatory molecules targeting multiple signaling pathways found dysregulated in cancer and affecting approximately 30 % of human genes. The mechanism underlying miRNA dysregulation in cancer is not fully understood, although several lines of evidence suggest the involvement of an epigenetic mechanism. The miRBase Version 16.0 has 1,048 miRNA sequences annotated in the human genome with likelihood of further additional miRNA to be validated in the future [350, 351]. miRNAs represent two opposing roles – it can behave either as oncogenes or tumor suppressors depending on tissue type and presence of specific targets [352]. Increasing evidence attests that overexpressed miRNAs may function as oncogene and promote cancer development by negatively regulating tumor suppressor genes and/or genes that control cell differentiation or apoptosis [353, 354]. Such concept evolved from studies reporting upregulation or downregulation of specific miRNAs in various types of cancer and identifying their molecular targets. A good example is let-7, which negatively regulates expression of Ras oncogenes, and its downregulation in tumors contributes to activation of Ras signaling pathway [355]. Recently, Parasramka and colleagues and other investigators evaluated miRNAs as target for cancer chemopreventive agents along with information relating to miRNA biogenesis and mechanistic insight on epigenetic modifications in context of cancer [353, 356]. In the following sections, we present a broad overview with few representative examples of miRNAs as targets for action of polyphenols. Despite many of the published studies being descriptive, there exists much optimism in scientific community for future development of miRNA for use in therapy. Li et al. from our laboratory examined and reported genistein treatment upregulated miR-146a in pancreatic cancer cells subsequently, consequently impacting their invasive potential by downregulating EGFR, NF-κB, IRAK, and MTA-2 [357]. Further, in isogenic pancreatic cancer cells, isoflavone treatment led to upregulation of miRNAs belonging to miR-200 and let-7 families that were downregulated in gemcitabine-resistant cell lines relative to sensitive cell lines amending EMT-related transcription factors such vimentin, slug, and ZEB1 [354]. In human uveal melanoma cells (C918), genistein downregulated the expression of miR-27a in vitro and reduction in the size of xenografted tumors parallel with upregulation of its target tumor suppressor gene, *ZBTB10* [358]. The effect of EGCG treatment in altering the microRNA expression has been described in human hepatocellular carcinoma (HepG2), breast (MCF-7), and in lung cancer cells. In HepG2 cells, miR-16 which targets the anti-apoptotic Bcl-2 protein becomes upregulated by EGCG [359]. In MCF-7 cells, Polyphenon-60 treatment resulted in alterations of 23 miRNAs including downregulation of *miR*-21 and *miR*-27 which is generally overexpressed in these cells, with *miR*-21 being associated with downregulation of a tumor suppressor gene [360]. Resveratrol reportedly impairs several signature miRNAs in colon, lung, and prostate cancer cells with miR-21, miR-155, and miR-663 being recognized as the major miRNAs modulated by resveratrol with their targets linked to apoptosis induction and inhibition of cell proliferation and cell cycle regulation. MicroRNAs as promising molecular target for curcumin, and its synthetic analog with improved bioavailability (CDF, difluorinated curcumin), have been reported by us in pancreatic cancer cells and other investigators [361–363]. In a multidrug-resistant lung cancer cell line (A549/DDP), 342 miRNAs mainly targeting various oncogenic and tumor suppressors were altered (>2.5 fold) by curcumin treatment [363]. In pancreatic cancer, miRNA-199a, an oncogenic miRNA, was significantly decreased after curcumin treatment [361]. CDF in combination with gemcitabine attenuated the expression of miR-21 and miR-200 in tumor tissue and in parallel leads to the induction of tumor suppressor gene *phosphatase and tensin homologue* (*PTEN*) [362]. We recently reviewed the role of miRNAs in the regulation of cancer stem cell (CSC) phenotype and function during tumor development and progression and the potential role of naturally occurring agents believed to function by targeting CSC-related miRNAs [364].

12 Polyphenol and Cancer Chemoprevention

According to the American Cancer Society estimate, out of 1,638,910 numbers of new cancer cases diagnosed in the year 2012, a total of 577,190 deaths have been projected to occur in the United States in the current year [365]. Central to their mission and year 2015 goals, a 50 % reduction in age-adjusted cancer mortality rates and a 25 % reduction in age-adjusted cancer incidence rates along with a measurable improvement in the quality of life of all cancer survivors have been envisioned. Pertinent to the attainment of their goals in the foreseeable future, cancer prevention and early detection are deemed as promising strategy especially for people at high risk of cancer, and emerging evidence indicates polyphenol compounds strategically hold promise for cancer intervention. Based on accumulated information on their multitargeted mechanism of action, translational implications in preventing growth and metastasis of cancer are currently being actively investigated. Although the list is not complete, we report studies from literature highlighting the effect of few select polyphenol compounds – such as EGCG from tea, the flavonoids quercetin and genistein from onions and soya, curcumin in curry spice, flavanone silibinin from artichoke, and resveratrol from red grapes on some most common site-specific cancers in preclinical models of carcinogenesis.

12.1 Gastointestinal Cancers

The estimated new cases of gastrointestinal (GI) cancers in the USA in the current year are expected to be around 284,680, out of which 142,510 will succumb in the near future. Experimental studies reported over the past decade support the contention that bioactive polyphenol compounds show promise in inhibiting tumorigenesis in the digestive tract including esophagus, stomach, small intestine, and colon associated with increased apoptosis and reduced cell proliferation. The widely investigated model polyphenol compounds such as resveratrol, curcumin, silymarin, quercetin, and tea polyphenol(s) have been found effective in inhibiting colon tumorigenesis in Apc$^{Min/+}$ mouse model which are genetically predisposed to develop intestinal tumors as a result of mutation of the *APC* gene. As reported earlier, β-catenin plays an important role in the Wnt signaling that is most commonly dysregulated in colorectal cancer, and most polyphenol compounds exhibit efficacy in reducing the expression of β-catenin. EGCG added in drinking water (0.02–0.32 %, wt/vol) made accessible to Apc$^{Min/+}$ mice inhibited the development of small intestinal tumors along with inhibition of tumor multiplicity in a dose-dependent manner associated with increased expression of the signaling protein E-cadherin and decreased levels of nuclear *β*-catenin, c-Myc, phospho-Akt, and phospho extracellular signal-regulated kinase 1 and 2 (ERK1/2) [155]. Resveratrol administration through drinking water also inhibited intestinal tumorigenesis in Apc$^{Min/+}$ mouse model by about 70 % alongwith reduction in expression of genes that are directly involved in the progression or cell proliferation such as cyclin D1, D2 and DP-1 transcription factor and Y-box binding protein, and decrease in TGF-β levels [366]. Flavonone silibinin administered orally (750 mg silibinin/kg body weight) for 6 weeks decreased the total no of intestinal polyps by 55 % alongwith decrease in expression of signaling molecules such as beta-catenin, cyclin D1, c-Myc and phospho-glycogen synthase kinase-3β expression. Silibinin treatment was also effective in downregulating phospho-Akt, COX-2, iNOS, nitrotyrosine, and nitrite levels in polyps which are well-known precursors of intestinal/colon carcinogenesis [367]. Others have reported that silibinin inhibits DMH-induced colon carcinogenesis by modulating the Wnt/beta-catenin pathway and glutathione redox system [368]. In similar lines of development, the effect of Polyphenon E (PPE, a standardized green tea polyphenol preparation) made accessible through the diet (0.24 %) for 34 weeks on the development of azoxymethane (AOM)-induced adenocarcinoma in colon of rats revealed reduction in the incidence (57 % vs. 23 %) and multiplicity of adenocarcinoma, and adenoma was decreased by 80 % and 45 %, respectively [369]. Recently, Shimizu and colleagues demonstrated the inhibition of AOM-induced ACF formation in male C57BL/KsJ-db/db mice by EGCG (0.01 % and 0.1 % in drinking water) by suppressing the activities of the insulin-like growth factor (IGF)/IGF-1R axis [370]. Resveratrol also inhibits AOM and 1,2-dimethylhydrazine (DMH)-induced colon cancer in rats reducing the number of aberrant foci (ACF) by modulation in the expression pattern of p21 and Bax [371–373]. Other gastrointestinal-related site-specific effect of tea polyphenols includes diethylnitrosamine (DEN) and benzo[a]pyrene-induced forestomach cancer in A/J mice and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced gastric cancer in rats [374]. Resveratrol has been reported to be effective in inhibiting N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumor in rats [375], wherein upregulated expression of COX-2 and PGE-2 levels was all significantly lowered by resveratrol treatment [375]. Ellagic acid inhibited esophageal cancer incidence in rats (66.7 % vs. 100 % in controls) when administered following carcinogen–NMBA treatment [376].

12.2 Hepatocellular Cancer

Hepatocellular carcinoma (HCC) is the most common type of primary hepatic tumor, and the incidence of hepatocellular carcinoma is increasing in the United States and Western Europe [377]. Liver cirrhosis is acknowledged as a premalignant condition for developing HCC. Other risk factors include tobacco and alcohol consumption, aflatoxins, and sex hormones. Chemoprevention of liver cancer has been suggested as an alternative to current treatment modalities because of limited therapeutic advantage to present protocols of therapy. In line with this concept, the cancer-preventive effect of dietary polyphenols on liver cancer is supported by results from cell culture, animal, and clinical studies. In vitro cell culture studies show that polyphenols potently induce apoptotic cell death and cell cycle arrest in tumor cells but not in their normal cell counterparts. The major polyphenol of green tea – EGCG – was studied in C3H/HeNCrj mice spontaneously developing hepatoma. EGCG (0.1 % in drinking water) reduced the incidence of hepatoma-bearing mice from 83.3 (control) to 52.2 % and also reduced the average number of hepatomas per mouse from 1.83 (control) to 0.91 (0.1 % EGCG) at week 65. EGCG inhibited the growth and secretion of alpha-fetoprotein by human hepatoma-derived PLC/PRF/5 cells without decreasing their viability [378]. Preventive effects of EGCG on diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-db/db mice have been reported wherein EGCG prevented obesity-related liver tumorigenesis by inhibiting the IGF/IGF-1R axis leading to improvement in hyperinsulinemia and attenuating chronic inflammation [379]. According to author's speculation, EGCG may prove useful in the chemoprevention of liver tumorigenesis in obese individuals. In another study, the inhibitory effect of tea polyphenols in development of precancerous liver lesions in rats was deduced based on its inhibitory effect on GST-Pi overexpression, both at mRNA and protein levels [380]. It is believed that EGCG suppresses the growth of human hepatocellular carcinoma cells by inhibiting activation of the vascular endothelial growth factor–vascular endothelial growth factor receptor axis [381]. The efficacy of EGCG as monotherapy regimen and co-treatment with EGCG and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in HCC cells and xenograft tumors revealed synergistic apoptosis-inducing effect by downregulation of Bcl-2 and Bcl-xL caused by inactivation of NF-kappaB, supporting the notion that EGCG treatment may be useful for improving the prognosis of HCCs [382].

The effect of resveratrol in chemoprevention and treatment of hepatocellular carcinoma has been compiled and presented by Bishayee et al. [383]. Dietary resveratrol has been shown to prevent DEN-initiated and phenobarbitone-promoted hepatocarcinogenesis in rats possibly through anti-inflammatory effects by suppressing elevated levels of HSP70, COX-2, and NF-κB [384]. These beneficial effects combined with an excellent safety profile encourage the development of resveratrol for chemoprevention and intervention of human HCC that remains a devastating disease. Dietary resveratrol (50 ppm) has shown to inhibit proliferation and metastasis of tumor cells subcutaneously implanted with an ascites hepatoma cell line, AH109A in Donryu rats [385]. Treatment of H22 hepatoma cell-bearing mice with resveratrol (15mg/kg b wt) inhibited the growth of transplantable liver cancer by ~49 % along with decrease in the expression of cyclin B1 and CDC2 proteins [386]. The efficacy of other natural chemopreventive polyphenol compounds in inhibiting HCC such as ellagic acid administered concurrently with chemical carcinogen, N-2-fluorenylacetamide (FAA), in male ACI/N rats has been recorded. Ellagic acid treatment led to 30 % decline in tumor incidence relative to untreated control group [387]. Another report indicates significant decline in gamma-glutamyl transpeptidase-positive foci induced by aflatoxin B1 (AFB1) following treatment with ellagic acid (0.005 %) [388]. Silymarin also reduced the number of liver tumor nodules induced with carcinogen NDEA in rats along with elevated antioxidant enzymes in nodules [389]. Flavonoid quercetin exerts preventive effect on hepatic cancer development in rats via significant decrease of oxidative stress and antioxidant activity relative to untreated controls with several cancer-induced features being clearly observed in carcinogen-treated control group [390].

12.3 Prostate Cancer

Prostate cancer is the most common malignancy occurring in men and accounts for 29 % of all cancer diagnosed in the year 2012 in North America. Although polyphenol compounds have been found to inhibit growth and promote apoptosis of cultured prostate cancer cells, the conclusion thus far from preclinical models of prostate cancer complements polyphenols for primary prevention and preventing the progression of prostate cancer development. In transgenic mouse model of prostate cancer (TRAMP mice), resveratrol in the diet significantly reduced the incidence of poorly differentiated prostatic adenocarcinoma (by 7.7-fold) [391]. Mechanistically, this was found associated with decline in cell proliferation and growth factor, IGF-1 levels, along with downregulation of downstream effectors, phospho-ERKs 1 and 2, and increase in putative tumor suppressor estrogen receptor-beta [391]. Resveratrol has also been reported to be effective in inhibiting growth of advanced human prostate cancer xenograft in nude mice and those induced by chemical carcinogen 3,2'-dimethyl-4-aminobiphenyl in male rats.

In experimental models of prostate cancer, dietary genistein inhibited metastasis of human prostate cancer in mice [310]. In TRAMP mice, lifelong exposure to dietary genistein suppressed the development of cancerous lesions by 50 % and advanced prostate cancer by 35 % [392]. It is currently believed that genistein consumption is associated with reduced risk of prostate cancer by modulating androgen-dependent transcription program within prostate [239].

Mukhtar et al. showed that administration of a green tea polyphenol infusion (0.1 % in drinking fluid) to transgenic adenocarcinoma of the mouse prostate (TRAMP mice) for 24 weeks markedly inhibited prostate cancer development and distant site metastases [393, 394]. Conceivably, the inhibition of VEGF, MMPs, and IGF-1 signaling pathways contributed to the cancer prevention activity of green tea polyphenols although it is not clear whether tea polyphenols inhibit prostate carcinogenesis by direct action of tea polyphenols in the prostate or by an indirect action, such as by affecting circulating serum IGF-1 levels or by affecting androgen levels [369]. It has been speculated that green tea catechins inhibit prostate tumor formation in the TRAMP model through sustenance in the levels of clusterin (a protein involved in apoptosis and downregulated in the prostate during cancer progression) [395].

Oral silibinin has been reported to block prostate cancer growth and progression at PIN (prostatic intraepithelial neoplasia) stage in TRAMP mice via modulation within tumor IGF–IGFBP-3 axis and cell cycle regulation, and therefore, it has practical and translational potential in suppressing growth and neoplastic conversion of PIN to prostate cancer in humans [396]. Additionally, suppression of advanced human prostate tumor growth in athymic mice by silibinin feeding has been reported associated with reduced cell proliferation, increased apoptosis, and inhibition of angiogenesis [397].

12.4 Lung Cancer

Lung cancer has been rated as the second most site-specific cancer in males and females and is expected to account for 26 % of all female and 29 % of all male cancer deaths [365]. Mesothelioma represents 1.5 % of these cases, and small cell lung cancer (SCLC) accounts for approximately 13 %, with non-small cell lung cancer (NSCLC) being the rest. The potential of phytoalexin resveratrol treatment to mice bearing highly metastatic Lewis lung carcinoma cells (LLC) revealed significant reduction in the tumor volume (42 %), tumor weight (44 %), and metastasis to lung (56 %) which may be due to the inhibition of DNA synthesis in LLC cells and through the inhibition of tumor-induced neovascularization by resveratrol [321]. The anti-metastatic potential of resveratrol has been validated in another study reporting reduction both in the number and the weight of the lung metastases [322]. The inhibitory effect of resveratrol on enzyme-heme oxygenase-1 (HO-1) which is involved in angiogenesis and HO-1-mediated inhibition of MMP-9 and MMP-2 expression may account for the anti-invasive mechanism of resveratrol in lung cancer [323]. Matrix metalloproteinases (MMPs) are key enzymes in the degradation of extracellular matrix, and their expression is dysregulated in lung cancer metastasis. The chemopreventive potential of curcumin and resveratrol during promotional phase of benzo[a]pyrene (B[a]P)-induced lung carcinogenesis has been portrayed by Malhotra and colleagues. Although treatment with resveratrol and curcumin given separately to B[a]P-treated mice showed appreciable improvement in the histoarchitecture of the lung with improved antioxidant parameters, combined treatment resulted in a significant improvement in lipid peroxide and reduced glutathione levels, as well as in the activities of SOD along with noticeable improvement in the lung histoarchitecture [398].

Another commonly occurring polyphenol compound – ellagic acid – shows efficacy in the prevention of lung tumors induced by benzo[a]pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced pulmonary adenoma formation. In N-nitrosodimethylamine (NDMA)-induced model of lung cancer, ellagic acid treatment reduced the incidence of lung tumors to 20 % from the control value of 72 % concomitantly with increased level of antioxidant glutathione in the lungs of mice [399]. The effect of other classes of polyphenol compounds with reference to chemoprevention and therapy of lung cancer has been investigated. Quantification of micro-CT data in real time revealed that silibinin significantly decreases urethane-induced tumor number and size in B6/129 wild-type mice through inhibition of iNOS expression in lung [400]. Another study reported urethane-injected mice exposed to silibinin had statistically significantly low lung tumor multiplicity and reduced lung tumor expression of vascular endothelial growth factor (VEGF) and the two enzymes, inducible nitric oxide synthase and cyclooxygenase-2, that promote lung tumor growth and progression by inducing VEGF expression [401]. Oral silibinin repressed human non-small cell lung carcinoma A549 xenograft growth and enhanced the therapeutic response of doxorubicin in athymic BALB/c nu/nu mice with strong protective efficacy against doxorubicin-mediated adverse health effects.

A spectrum of chemoprotective effect of tea preparations (including green tea, black tea, EGCG, and theaflavins) in the inhibition of initiation, promotion, and progression stages of NKK-induced lung tumorigenesis in rat, mice, and hamsters has been reported [369]. Additionally, in metastasis model, oral administration of green tea infusion reduced the number of colonies of Lewis lung carcinoma cells in lung [402]. It has been recorded that administration of GTP prior to challenge with carcinogen B[a]P significantly reduces internal body tumor incidence in susceptible A/J mice [403]. The inhibitory effect of a standardized green tea polyphenol preparation containing 65 % (−)-epigallocatechin-3-gallate (Polyphenon E) administered through drinking fluid at 0.5 % concentration on NKK-induced lung tumor in A/J mice revealed an inhibition in the progression of lung adenomas to adenocarcinoma and reduced the lung tumor incidence by 52 % and tumor multiplicity by 63 % in tumor-bearing mice [404]. These favorable and protective changes at the cellular level were reflected by alterations such as increased apoptosis and alterations in the levels of c-Jun and extracellular signal-regulated kinase (ERK1/2) phosphorylation [404]. Additionally, black tea polyphenols have also been reported to be effective in inhibiting the pathogenesis of carcinogen (NKK and B[a]P)-induced lung cancer in mice despite the fact that bioavailability of GTP is higher than the high-MW polyphenols in black tea [405–407].

12.5 Breast Cancer

Studies reported in literature show inverse correlation between high intake of total dietary polyphenol and the incidence of breast cancer proclaiming cost-effective approach to control incidence of breast cancer. We earlier reported that resveratrol inhibits the tumor burden and multiplicity of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in Sprague Dawley rats [408]. Resveratrol also reduced N-methyl-N-nitrosourea-induced breast tumors in rats [409]. It has been reported that combination of polyphenols (resveratrol, quercetin, or catechin) at physiologically relevant concentrations is more effective than individual compounds in inhibiting ERα(−), ERβ(+) MDA-MB-231 breast cancer cell proliferation, cell cycle progression, and primary mammary tumor growth [410]. Additionally, it has been shown by the same research group that combined dietary grape polyphenols are effective in inhibiting not only mammary tumor growth but also site-specific metastasis especially to the liver and bone [411]. As a single agent in xenograft mouse model, resveratrol treatment culminated in a significantly lower tumor growth, decreased angiogenesis, and increased apoptotic index in ERα(−), ERβ(+) MDA-MB-231 tumors [412]. In HER-2/neu transgenic mice, resveratrol supplementation delayed the development of tumors and reduced the mean number, size, and metastasizing capacity of spontaneously developing tumors, an effect that was associated with downregulation of HER-2/neu expression and apoptosis within mammary tumors of these mice [413]. The flavonoid biochanin A (BCA) inhibits the growth of human breast cancer cells (MCF-7) in a murine xenograft animal model and is considered as a breast cancer-preventive agent [414]. The critical role of isoflavone genistein in inhibiting breast cancer has been thoroughly reviewed and presented [415–417]. The timing of exposure to genistein appears critical for its mammary protective effects. It has been reported that genistein early in life causes enhanced mammary gland differentiation, alterations in cell proliferation and apoptosis, and upregulation of tumor suppressor genes [418]. Neonatal administration of genistein had a protective effect against the subsequent development of mammary cancer induced by DMBA in Sprague Dawley rats [419]. Flavonolignan silibinin with chemopreventive activity in preclinical models of prostate and colorectal cancer failed to show similar effect on tumor development when tested in the C3(1) SV40 T,t antigen transgenic multiple mammary adenocarcinoma mouse model [420]. However, the effect of treatment with a complex of silibinin and phosphatidylcholine (IdB 1016) on the development of spontaneous mammary tumors was seen delayed in HER-2/neu transgenic mice along with reduction in the size and number of primary tumor masses and diminished lung metastases in these mice [421].

Ellagic acid inhibits 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced mammary cancer in female F344 rats [422, 423]. The effect of tea catechins on the progression or late promotion stage of mammary gland carcinogenesis is not robust due to low bioavailability of tea polyphenols in the mammary tissues. Intriguingly, black tea was found to reduce the tumor number and size in rats that were high on high-fat diets; it is thought that black tea affects tumorigenesis indirectly by affecting fat absorption and metabolism, which may subsequently influence estrogen metabolism and mammary tumorigenesis [369, 424].

12.6 Oral Cancer

Several experimental studies reported till date document the efficacy of polyphenol compounds in inhibiting chemical carcinogen-induced oral cancer. Studies reported in literature reveal naturally occurring plant phenolics such as caffeic, ellagic, chlorogenic, and ferulic acid(s) modestly influence the spectrum of pathological changes occurring within squamous epithelium concurrently with reduction in tumor incidence during in 4-nitroquinoline 1-oxide (4-NQO)-induced tongue carcinogenesis in rats [425]. Silymarin treatment during stages of tumor induction decreased the polyamine content and prostaglandin levels in tongue mucosa [426]. The modulatory efficacy of green tea polyphenols on 4-NQO-induced oral carcinogenesis showed a significant increase in the levels of expression of glycoconjugates (hexose, hexosamine, sialic acid, fucose) similar to that observed for immunological markers culminating in regression of oral cancer [427]. The antimutagenic effects of green tea catechins, (-)-epicatechin gallate (ECG), and EGCG on induction of 6-thioguanine (6TG)-resistant mutations induced by 4-NQO were observed only when these cells were post-treated with catechins during the mutation expression time after treatment with 4-NQO; this is suggestive that catechins may act intracellularly as antimutagenic blocking agent or suppressive agent. In preclinical model, EGCG efficiently inhibited phorbol-12-myristate-13-acetate (PMA)-induced cell invasion and MMP-9 expression, corroborating inhibition of growth of SCC-9 cell xenograft tumors in nude mice model [428]. According to the authors, the results hold promise that EGCG could reduce invasion and cell growth of tumor cells, and such a characteristic may be of great value in developing a potential cancer therapy. Resveratrol or a combination of resveratrol and quercetin, in concentrations equivalent to that present in red wines, is an effective inhibitor of oral squamous carcinoma cell (SCC-25) growth and proliferation [429]. Further, the effect of tea polyphenol constituents on growth of oral squamous carcinoma cells in vitro revealed a significant dose-dependent inhibition in cell growth including dose-dependent changes in cell morphology [430].

12.7 Bladder Cancer

Bladder cancer is another important health problem, and according to current estimate this ranks as the fourth most commonly diagnosed malignancy in males accounting 7 % of all cancer diagnosed at different sites [365]. Transitional cell carcinoma is the most common prevalent histological type of bladder cancer in Western population. From literature preview, one concludes that some classes of investigated polyphenols show promise in inhibiting bladder cancer. Studies reported thus far show that silymarin inhibits bladder cancer induced by tobacco smoke carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine in ICR mice [431]. In another study, green tea polyphenol (Polyphenon E) was evaluated for chemoprotective efficacy against 4-hydroxy butyl(butyl)nitrosamine-induced bladder cancer. Administration of Polyphenon E to rats caused dose-dependent decrease in palpable urinary bladder cancer relative to control group of animals [432]. The effect of several soy components including genistein and isoflavone-rich soy phytochemical concentrate (SPC) on growth and metastasis in clinically relevant model of orthotopic bladder cancer and subcutaneously growing tumors in mice resulted in the induction of tumor cell to undergo apoptosis along with inhibition of tumor angiogenesis [433]. Furthermore, SPC, but not genistein, significantly inhibited lung metastasis in this model, in part, by its effect on modulating the expression of NF-κB and IGF-1 [433].

Overall, these information support interest of dietary natural polyphenol in chemoprevention of important site-specific cancers which can be exploited further in sensitizing therapy to anticancer agents as discussed in the following sections.

13 Polyphenols and Chemosensitization of Cancer Therapy

Overwhelming evidence from numerous preclinical studies and clinical trials provides increasing evidence that some polyphenol has more benefit in clinical therapy of cancer than chemotherapy alone, rendering this class of phytocompounds highly attractive for therapeutic interventions. The majority of patients diagnosed with cancers receive chemotherapy and or radiotherapy under multimodal treatment protocol. Unfortunately, findings from many clinical studies reveal the emergence of drug-resistant phenotypes. Reversal of such resistance, whether intrinsic or drug induced, is critical for the success of cancer treatment. Even though association has been found between diet and cancer risk, polyphenols have not been established as a direct cause or cure for cancer. From foregoing myriad of biological responses due to pleiotropic effect of polyphenol compounds on cellular and molecular events, a rationale for combining polyphenol compounds with conventional therapeutics can be expected to have higher efficacy because of inactivation of survival signaling and simultaneous activation of multiple death pathways. Conceptually, this may sensitize tumor cells to therapeutics. Furthermore, clinical studies suggest that a common form of multidrug resistance (MDR) in human cancers results from the expression of the MDR1 gene that encodes P-glycoprotein (P-gp), a member of

ABC transporter family that has been described for drug resistance and low bioavailability of drugs by pumping drugs out of the cells at the cost of ATP hydrolysis [434]. Various P-gp inhibitors have been synthesized and studied extensively to reverse MDR. Of interest, a few select polyphenol compounds have shown to intensify action of chemotherapeutic drugs by suppressing the MDR phenomenon [435–439]. Studies reported thus far combining polyphenol compounds with cytotoxic anticancer therapies augmenting chemosensitization in a variety of preclinical murine model of human cancers reinforce biological basis towards underlying potential therapeutic application of polyphenols. This concept has been summarized in the following description exemplifying polyphenol phytochemicals such as EGCG, genistein, curcumin, resveratrol, quercetin, and silibinin. These compounds beside dietary component exhibit notable efficacy in preclinical models of carcinogenesis.

We reported that isoflavone genistein potentiated growth inhibition and apoptotic cell death caused by cisplatin, erlotinib, docetaxel, doxorubicin, gemcitabine, and CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) in cancers of prostate, breast, pancreas, lung, and lymphoma [122, 123, 127, 440–442]. We have also found that dietary genistein in vivo could enhance the antitumor activities of gemcitabine, cisplatin, oxaliplatin, and docetaxel in a tumor model, resulting in apoptotic cell death and the inhibition of tumor growth [121, 124, 126, 312]. Similar observations have been reported by other investigators showing that the antitumor effects of chemotherapeutics, including 5-fluorouracil (5-FU), adriamycin, cytosine arabinoside, tamoxifen, and perifosine, could be potentiated by genistein [176, 443–447]. Genistein enhanced the antitumor effect of bleomycin in HL-60 cells, but not in normal lymphocytes in an in vitro study [448]. The synergistic action of genistein and cisplatin or carmustine (BCNU) on the growth inhibition of glioblastoma and medulloblastoma cells has also been observed [449]. In ovarian cancer, genistein potentiated the antiproliferative and proapoptotic effect of antibodies directed against the cell adhesion molecule L1-CAM [450]. Furthermore, despite limitations in the cytotoxic effect of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) in gastric and pancreatic adenocarcinoma cell lines, subtoxic concentrations of genistein sensitized these TRAIL-resistant cells to TRAIL/Apo2L-mediated apoptosis [451, 452]. In radiotherapy, experimental studies from Dr. Hillman's laboratory have demonstrated that the combination of genistein and radiation exerts enhanced inhibitory effects on tumor growth and progression of renal cell carcinoma and prostate tumor in orthotopic models [453, 454]. Genistein also enhanced radiosensitivity in human esophageal and cervical cancer cells, suggesting the beneficial effects of genistein in cancer radiotherapy [174, 175].

Studies have viewed resveratrol could be potentially useful in chemosensitization when used in combination with a variety of anticancer drug treatment mainly due to its effect on apoptosis augmenting therapeutic efficacy. Combination of resveratrol with platinum drugs, cisplatin and oxaliplatin, has been reported to sensitize the ovarian cancer cells to platinum-induced apoptosis providing a mode of overcoming drug resistance [455]. In another study resveratrol has been found to augment the growth inhibitory effects of cisplatin and doxorubicin on human ovarian (OVCAR-3) and uterine (Ishikawa) cancer cells diminishing cardiac toxicity of doxorubicin [456]. Significant antitumor synergistic cytotoxicity by combination of resveratrol with dexamethasone, fludarabine, and bortezomib in Waldenström macroglobulinemia (a rare, slow-growing non-Hodgkin lymphoma) has been reported [457]. Furthermore, in drug-sensitive promyelocytic leukemia HL-60 cell line and its multidrug-resistant variant HL-60/VCR (P-gp positive), resveratrol exhibited proapoptotic activity when combined with multiple anticancer drugs – doxorubicin, cycloheximide, busulfan, gemcitabine, and paclitaxel – resulting in enhancement of the combination therapy [458]. The antitumor effect of combination of resveratrol with 5-fluorouracil (5-FU) on transplantable murine hepatoma 22 model has been reported with inhibition up to 77 % in combination treatment group compared to 53 % in 5-FU alone antagonizing its toxicity markedly [459]. Furthermore, in pancreatic cancer, the potential of resveratrol regarding therapeutic efficacy was recapitulated in a relevant preclinical orthotopic model wherein the response outcome between only gemcitabine treatment versus resveratrol plus gemcitabine was significantly greater compared to monotherapy [460]. Resveratrol sensitizes human melanoma cells that were sensitive or resistant to temozolomide and to paclitaxel in lung cancer cells [461, 462]. Additionally, resveratrol sensitizes leukemic cells to proteasome inhibitors via regulation of FOXO1 transcriptional activity and accumulation of $p27^{KIP1}$ and protects against cisplatin-induced cardiotoxicity by alleviating oxidative damage [463, 464]. In ER positive breast cancer explants, the efficacy of combination of cyclophosphamide and resveratrol revealed enhanced antiproliferative actions along with differential expression of cell cycle, apoptosis, and stress factors encouraging clinical trial of the regimen in the future [465].

The chemosensitizing effect of green tea and its constituents has been reported both in vivo and in vitro. EGCG, the most potent constituent among catechins present in green tea, downregulates P-gp and BCRP in a tamoxifen-resistant MCF-7 cell line and induced apoptosis and growth inhibition when combined with tamoxifen in human breast cancer MDA-MB-231cells [466, 467]. Furthermore, in a murine model of breast cancer, EGCG synergistically sensitized the tumor cells to paclitaxel by diminishing the expression of endoplasmic reticulum chaperone GRP78 (*g*lucose-*r*egulated *p*rotein *78*) augmenting apoptosis [468]. EGCG reversed doxorubicin resistance in solid human carcinoma xenograft model [469]. In orthotopic mouse glioblastoma models, EGCG significantly enhanced therapeutic efficacy of temozolomide by lessening expression of GRP78 known to be responsible for chemoresistance to temozolomide [470]. Additionally, sensitization of glioma cells to cisplatin and tamoxifen by tea catechin has been reported to be associated with decreased expression of telomerase improving drug sensitivity [471, 472]. In human prostate cancer, EGCG sensitizes carcinoma cells to TRAIL-mediated apoptosis and synergistically inhibits the biomarkers associated with angiogenesis and metastasis such as VEGF, uPA, angiopontin, and MMPs [231, 472]. Stearns et al. reported that combination therapy of EGCG and doxorubicin inhibits prostate tumor metastasis and tumor growth in SCID mice [473]. In an in vivo mouse model for chemoresistant liver cancer, green tea catechin, EGCG, augmented the antitumor activity of doxorubicin by markedly enhancing the intracellular accumulation of doxorubicin along with downregulation of MDR1 and HIF-1α expression [439]. In a mouse model of human melanoma, EGCG sensitizes melanoma cells to interferon-induced growth inhibition with increase in Fas protein levels and decrease in NF-κB [474]. A synergistic inhibitory action of a combination of Polyphenon E with the cholesterol-lowering agent, atorvastatin, against NNK-induced lung cancer in A/J mice resulting from downregulation of anti-apoptotic protein (Mcl-1 and Bcl-xL) has been reported [475]. The synergistic growth inhibitory effect of EGCG and erlotinib in cell culture and nude mouse xenograft model of squamous cell carcinoma of head and neck suggests that erlotinib treatment activates the tumor suppressor protein p53, which dictates synergistic growth inhibition outcome by inhibiting NF-κB signaling pathway [476]. A comparative study evaluating the efficacy of mitomycin C and polyphenolic catechins (Polyphenon E®) in rat bladder tumor model revealed similar efficacy of both substances in preventing intravesical tumor growth [477].

A comprehensive review by Raina et al. on the efficacy and mechanisms of combinatorial strategies for cancer eradication by silibinin and cytotoxic agents has been published [478]. Silibinin restored paclitaxel sensitivity to paclitaxel-resistant human ovarian carcinoma cells [479]. Quercetin greatly improved therapeutic index of doxorubicin against breast cancer by its opposing effects on HIF-1alpha in tumor and normal cell, and combining intratumoral doxorubicin injection and quercetin synergistically induced rejection of established breast cancer in mice [480, 481]. In human leukemia, colon, prostate, hepatoma, and non-small cell lung cancer cell lines, quercetin enhanced TRAIL-mediated apoptosis [293, 294, 482–484]. Curcumin has been shown to inhibit the growth of cancer cells in vitro with strong anticancer outcome in animal models of carcinogenesis. The reported benefit of curcumin in combination with taxane chemotherapy for hormone refractory prostate cancer (HRPC) treatment suggests that curcumin in combination with taxane could be useful in HRPC patients. Curcumin also sensitizes TRAIL-resistant xenografts of human prostate tumor cells, suggesting that combination of curcumin and TRAIL could be useful for the prevention and treatment of human prostate cancer [485]. In lung cancer, the synergistic effect of curcumin and cisplatin via downregulation of thymidine phosphorylase and excision repair cross-complementary 1 (ERCC1) protein levels has been reported [195]. Sreekanth et al. reported that liposomal curcumin augments the anticancer therapy of paclitaxel in experimental mouse model of cervical cancer by downregulating anti-apoptotic factors and survival signaling molecules such as NF-κB, Akt, and MAPK that play key roles in cell proliferation, survival, angiogenesis, and metastasis [196]. The effect of curcumin on multidrug resistance-associated protein 5 (MRP5) in pancreatic cancer sensitized these tumor cells to the cytotoxic effects of 5-fluorouracil and gemcitabine [486]. Combination of curcumin and chemotherapeutics as a novel strategy for the treatment of breast and colon cancer has been reported by us and others [125, 487]. Several clinical trials using curcumin in combination treatment are being conducted to test the effects and toxicity of the combination in patients with multiple myeloma, rectal, colon, and pancreatic cancers.

From preceding account, it thus becomes clear that chemopreventive polyphenol compounds as efficient multitargeted agents could be considered for the design of anticancer therapy. Preclinical studies reported till date supports their inclusion to achieve favorable increase in the efficacy of chemotherapeutic index.

14 Clinical Trials for Cancer Therapies by Polyphenols

Based on information available at http//www.clinicaltrials.gov several clinical trials are under way which mirror the clinical usefulness of individual class of polyphenols in cancer patients (Table 71.1). Much of these ongoing trials or yet to open for participant recruitment relating to polyphenols are focused on investigating the effects of genistein, isoflavone, EGCG, resveratrol, curcumin, and silibinin. We believe that in the future these compounds will prove valuable in combination with conventional therapeutics or together with radiation therapy for the treatment of human malignancies. Thomasset et al. reviewed and published results of pilot studies and trials, in which either healthy individuals or patients with premalignant conditions or cancer received polyphenol compounds from the viewpoint of chemoprevention-related rationale. Their conclusion based on collected evidences surmises that tea polyphenols should tentatively move forward into phase III clinical intervention trials, while in the case of curcumin and soya isoflavones more studies in premalignant conditions are warranted [6]. Later a prospective study ($n = 383$) found that plasma genistein levels inversely correlate with subsequent incidence of breast cancer in both pre- and postmenopausal Dutch women supporting the hypothesis that high isoflavone and, in particular, genistein intake may be associated with reduced breast cancer risk [488]. Additional, meta-analysis of 18 published epidemiological studies supports the hypothesis that soy isoflavones may reduce breast cancer risk.

The beneficial effects of isoflavone on early stage prostate cancer draw closer from a clinical trial demonstrating that supplementing early stage prostate cancer patients with soy isoflavones alters surrogate markers of proliferation such as serum prostate-specific antigen (PSA) and free testosterone in large number of subjects than the group receiving placebo [489]. Currently, a clinical trial is being conducted using AXP107-11 (a crystalline form of genistein) in combination with gemcitabine in patients with locally advanced or metastatic, unresectable adenocarcinoma of the pancreas (stage III–IV) (NCT01182246; Table 71.1). Phenoxodiol™ one of the isoflavone analogs (derived from equol) has shown a broad-spectrum anticancer effect [472, 490]. In phase I trial, phenoxodiol delivered by intravenous infusion continuously for 7 days every 2 weeks in cohort of patients with solid tumors was well tolerated up to a dose of 27mg/kg/day [491]. Another phase I and pharmacokinetic study involving either repeated weekly infusions of phenoxodiol administered over 1–2 h or as single bolus intravenous dose in patients with advanced cancer with no treatment-related death has been reported [472, 492]. Phenoxodiol is

Table 71.1 Current ongoing clinical trials

Genistein/isoflavone	
NCT01028001	Phase II trial of preoperative soy isoflavone supplementation and molecular markers in the prevention of head and neck squamous carcinoma
NCT01036321	Phase II clinical trial of purified isoflavones in prostate cancer: comparing safety, effectiveness, and mechanism of action between African, American and Caucasian men
NCT01174953	High-risk prostate cancer prevention study
NCT01126879	Phase 2 trial of genistein in men with circulating prostate cancer cells
NCT00499408	Phase II trial of vitamin D and soy supplementation for biochemically recurrent prostate cancer following definitive local therapy
NCT01489813	Phase II randomized placebo-controlled clinical trial of genistein in reducing the toxicity and improving the efficacy of intravesical therapy
NCT01538316	Clinical trial on the effectiveness of the flavonoids genistein and quercetin in men with rising prostate-specific antigen
NCT01325311	Phase IIa, randomized placebo-controlled trial of single high-dose cholecalciferol and daily genistein (G2535) versus placebo in men with early stage prostate cancer undergoing prostatectomy
NCT01182246	Safety, pharmacokinetics, and efficacy of AXP107-11 in combination with standard gemcitabine (Gemzar®) treatment in patients with locally advanced or metastatic, unresectable adenocarcinoma of the pancreas, stage III–IV: a prospective, open-label, multicenter, sequential phase Ib/IIa study (the drug substance, AXP107-11, is a crystalline form of genistein)
Green tea/EGCG	
NCT01317953	Phase study of oral green tea extract as maintenance therapy for extensive-stage small cell lung cancer
[a]NCT01589887	Clinical and biologic evaluation of Polyphenon E, an extract of green tea containing EGCG, in plasma cell dyscrasias – pilot study
[a]NCT00942422	The clinical and biologic evaluation of Polyphenon E, an extract of green tea containing EGCG, in plasma cell dyscrasias – pilot study
[a]NCT00262743	A phase I/II study of daily oral Polyphenon E in asymptomatic, Rai stage 0–II patients with chronic lymphocytic leukemia
NCT00917735	Phase II, randomized, double-blind, placebo-controlled study of the efficacy of green tea extract on biomarkers of breast cancer risk in high-risk women with differing catechol-O-methyltransferase (COMT) genotypes
NCT01060345	A pilot study of chemoprevention of green tea in women with ductal carcinoma in situ (DCIS)
[a]NCT00676793	Phase II clinical trial to determine if Polyphenon E inhibits c-Met signaling and activation of pathways contributing to breast cancer progression
[a]NCT00516243	Phase IB randomized, double-blinded, placebo-controlled, dose escalation study of Polyphenon E in women with a history of hormone receptor-negative breast cancer
NCT01360320	Minimizing the risk of metachronous adenomas of the colorectum with green tea extract MIRACLE
NCT00596011	Phase II, randomized, double-blind, multicentered study of Polyphenon E in men with high-grade prostatic intraepithelial neoplasia (HGPIN) or atypical small acinar proliferation (ASAP)
[a]NCT00233935	Phase IB randomized, double-blinded, placebo-controlled, dose escalation study of Polyphenon E in patients with Barrett's esophagus

(*continued*)

Table 71.1 (continued)

[a]NCT01606124	Randomized phase II trial of Polyphenon E versus placebo in patients at high risk of recurrent colonic neoplasia
NCT01116336	Phase I chemoprevention study with green tea and erlotinib in patients with premalignant lesions of the head and neck
NCT01032031	The effect of dietary bioactive compounds on skin health in humans in vivo
Curcumin	
NCT01160302	An exploratory biomarker trial of the food substances curcumin C3 Complex® in subjects with newly diagnosed head and neck squamous cell carcinoma
NCT01294072	Phase I clinical trial investigating the ability of plant exosomes to deliver curcumin to normal and malignant colon tissue
NCT00927485	Use of curcumin for treatment of intestinal adenomas in familia adenomatous polyposis (FAP)
NCT00641147	Curcumin for treatment of intestinal adenomas in familial adenomatous polyposis (FAP)
NCT00689195	Evaluation of curcumin formulation and Ashwagandha root powder extract in the management of advanced high-grade osteosarcoma
[a]NCT00969085	Phase II trial of curcumin in cutaneous T-cell lymphoma patients
[a]NCT01490996	A phase I/IIa study combining curcumin (Curcumin C3-Complex, Sabinsa) with standard care FOLFOX chemotherapy in patients with inoperable colorectal cancer
[a]NCT01238198	Oral curcumin for radiation dermatitis in breast cancer patients
[a]NCT01608139	Pilot study of curcumin, vorinostat, and sorafenib in patients with advanced solid tumors
[a]NCT01219673	A study of reducing the symptom burden produced by chemoradiation treatment for head and neck cancer
[a]NCT01269203	A phase II randomized study of the efficacy of curcumin for reducing symptoms during maintenance therapy in multiple myeloma patients
Resveratrol	
NCT01476592	A biological study of resveratrol's effects on Notch-1 signaling in subjects with low-grade gastrointestinal tumors
NCT01489319	Evaluation of the ovarian dynamic response and the inflammatory response to oral lipid challenge in relation to body composition in polycystic ovary syndrome
Silibinin	
[a]NCT01129570	Phase I trial of Siliphos in patients with advanced hepatocellular carcinoma

[a] Study is not yet open for participant recruitment

currently undergoing in phase II/III clinical trials to investigate its effect combined with docetaxel in patients diagnosed with ovarian, fallopian tube, or primary peritoneal cavity tumors (NCT003038880). Phase II results on safety and efficacy of intravenous phenoxodiol in combination with cisplatin or paclitaxel in women with platinum/taxane-refractory/resistant epithelial ovarian, fallopian tube, or primary peritoneal cancers have recently being published [493].

Pilot clinical studies relating to administering EGCG at dose of 800 mg/day to healthy individuals were reportedly well tolerated with minimal toxicity ($\leq$ grade I) [494]. Subsequent studies revealed that EGCG delivered in the form of capsule

(200 mg p.o. for 12 weeks) was effective in patients with human papillomavirus-infected cervical lesions [495]. A 1-year "proof-of-principle" study by Bettuzzi et al. in volunteers with high-grade PIN suggested that green tea polyphenol may reverse or delay prostate carcinogenesis [496]. Later, a follow-up update by Brausi et al. confirmed the benefit of green tea intake is limited only in precancerous stage of the disease and those with high-grade prostatic intraepithelial neoplasia (HGPIN) on biopsy, but in studies involving men with more advanced prostate cancer, green tea consumption did not show benefits, confirming the conclusion from mouse prostate cancer model that the effect of green tea polyphenol decreases with advancing age [497].

Polyphenon E, a highly characterized green tea extract standardized to EGCG and other green tea polyphenols received IND (*i*nvestigational *n*ew *d*rug) status and is currently undergoing pilot trials for plasma cell dyscrasias (NCT01589997; NCT00942422) and in men with HGPIN or atypical small acinar proliferation (ASAP) (NCT00596011). The results of a randomized, double-blind, placebo-controlled trial of Polyphenon E in prostate cancer patients before prostatectomy have recently been reported [498]. The primary aim of this study was to determine the bioavailability of green tea polyphenols in prostatic tissue with secondary end points being measurement of modulation of systemic and tissue biomarkers related to prostate carcinogenesis. Phase I and II clinical trial of Polyphenon E in patients with asymptomatic Rai stage 0 to II chronic lymphocytic leukemia revealed that chronic daily oral administration of the preparation is well tolerated with signs of clinical activity accompanied by reduction in absolute lymphocyte count (ALC) and/or lymphadenopathy in majority of the patients [499, 500]. Recently, a phase III clinical trial is being conducted using combination of EGCG and erlotinib to chemoprevent head and neck cancer with premalignant lesion (NCT 01116336).

Clinical trial using formulated resveratrol SRT501 combined with bortezomib in patients with multiple myeloma has to be terminated because of the failure of kidney function. Nonetheless, one currently ongoing clinical trial relates to biological study of resveratrol effect on Notch-1 signaling in subjects with low-grade gastrointestinal tumors (NCT01476592). Polyphenol curcumin has a wide margin of safety. Table 71.1 summarizes the ongoing clinical trials of curcumin in cancer of different sites and origin. However, pharmacokinetics and bioavailability studies of curcumin revealed its poor absorption and rapid elimination from the body. A study that investigated the safety and feasibility of combination therapy using curcumin and gemcitabine treatment in pancreatic cancer patients revealed no dose-limiting toxicities in the phase I study and recommended a dose for oral curcumin as 8 g/day for phase II study [501]. Further investigation into efficacy of treatment outcome using this recommended dose in pancreatic cancer patients is awaited. Siliphos (main component being silibinin) is being tested for intervention in advanced hepatocellular carcinoma (NCT01129570).

From above discussion, it is obvious that including polyphenol compound or its derivates either as monotherapy or in combination may synergize the efficacy of current therapy supporting further therapeutic interest of using polyphenol or its derivatives in clinical application.

15 Prologue on Select Polyphenol Compounds from Natural Dietary Sources

15.1 Genistein

Genistein (4,5,7-trihydroxyisoflavone) is the predominant isoflavone in soybean-enriched foods and related products such as tofu and soy drinks. Its consumption in Asian countries varies between 20 and 80 mg/day, whereas it is only 1–3 mg/day in the USA [502]. The soy isoflavone genistein has been well studied and is found to inhibit cancer cell growth in vivo and in vitro. It has multiple molecular targets including various receptor, enzymes, and pathway interactions affecting cell growth by inducing apoptosis and reduces invasiveness by downregulation of matrix metalloproteinase expression [94, 239, 415]. Genistein is also a tyrosine kinase inhibitor, but inhibition of receptor tyrosine kinase requires higher circulating genistein levels in humans; a dose of 10/mg/kg soy-rich diet yields circulating genistein concentration of about 1μM, which is far below that required for significant inhibition of tyrosine kinase [503]. Isoflavone genistein was originally identified as having a close similarity in structure to estrogens and harboring weak estrogenic activity and, as such, was labeled as a phytoestrogen; nonetheless, review of data from literature and clinical trials suggests an inverse correlation between genistein intake and risk of breast and other hormonally related reproductive organ cancers. A study among women in Shanghai, China, found that plasma isoflavone concentration was inversely associated with the risk of nonproliferative and proliferative benign fibrocystic conditions as well as breast cancer [504]. Clinical studies in osteopenic and osteoporotic postmenopausal women support breast and uterine safety of purified naturally derived genistein administered for up to 3 years accompanied by attenuation of postmenopausal problems [415, 505]. Genistein has close structural similarity to 17β-estradiol and shown to compete with 17β-estradiol in interaction with estrogen receptor (ER) and preferentially binds and activates ER-β isoform. The latter has been shown by cluster analysis to upregulate the expression of genes that are important in the negative regulation of cellular proliferation and affect the expression of genes associated with signaling events that repress ER activities including cellular proliferation, thus exerting a potential favorable role in the prevention of hormone-related cancers [415].

In parallel with its effects on estrogen-sensitive cancer, relatively high levels of soy isoflavones have been found in the serum, urine, and prostatic fluid of Asian men who consume a soy-rich diet presumably contributing in lowering the risk and incidence of prostate cancer in several cohorts and case-controlled studies [506–509]. Genistein affects androgen receptor-mediated gene expression in vivo and is classified as an androgen antagonist in prostate tissue at a concentration relevant to human exposure on a soy-rich diet. However, other different tissue- and tumor-specific concentration of genistein in human population has not been determined.

15.2 Resveratrol

The phytoalexin resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a non-flavonoid polyphenol compound found enriched in dietary items such as grapes, peanuts, plums, pines, blueberries, and cranberries. Red wine constitutes a major source of human consumption of resveratrol. Jang et al. first reported cancer chemopreventive potential of this bioactive compound effectively intervening at all three discrete and overlapping major stages of carcinogenesis [510]. This led other researchers to evaluate its potential to suppress tumor growth in context of cancer chemoprevention and as therapeutic adjunct; their assessment revealed that resveratrol suppresses the growth of a variety of cancers including lymphoma; multiple myeloma; breast, prostate, colon, and pancreatic cancer; melanoma; head and neck squamous cell carcinoma; ovarian carcinoma; and cervical carcinoma [511, 512]. Further, the diverse bioactivity associated with resveratrol stimulated its investigation in several human clinical trials. A myriad of molecular events that decrease tumor size and volume in preclinical models associated with resveratrol intake have been compiled and presented [5, 513]. Their anticancer properties can be summarized as (a) growth inhibitory and cell proliferation activity; (b) cell cycle arrest in the S, G1/S, or G2/M phase of cell cycle depending on cell and tissue specificity through a mechanism involving the downregulation of activator molecules and upregulating their inhibitors and tumor suppressor p53 transcription factor and their responsive genes; (c) induction of apoptosis by suppressing expression levels of anti-apoptotic proteins; and (d) antiangiogenic, anti-invasion, and anti-metastatic characteristics.

15.3 (−)-Epigallocatechin Gallate

The cultivation of green tea (*Camellia sinensis*) dating more than 5,000 years ago was initially associated with medicinal usage, until its current status as one of the most popular consumed beverage worldwide along with purported recognition of its beneficial health effects including anticancer properties. Relative to other tea, the chemistry of green tea has been well characterized, and the chemopreventive effect of green tea is mainly attributed to its polyphenol content known as catechins. (−)-Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea and represents 50–80 % of the catechins by weight in a typical 200–300 mg per brewed cup of green tea [289, 514]. Besides EGCG, other major catechins in green tea are (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epicatechin. Recently, the molecular targets that could account for the chemopreventive effect of EGCG and other tea compounds have been reviewed and succinctly presented [289, 369, 515, 516]. Overall, its broad-spectrum pleiotropic effects on cellular proteins and signaling pathways attribute in curtailing tumor growth and constitute underlying mechanism by which EGCG and other tea polyphenol constituents protects against cancer reflecting its chemopreventive efficacy. In addition the ability of green tea polyphenols to inhibit matrix metalloproteinases, cyclin-dependent kinases, ubiquitin/proteasome degradation pathways, DNA methyltransferase, and receptor tyrosine kinase (RTK) pathways has been proposed as promising avenues for enhancing therapeutic efficacy [259]. Additionally, the antioxidant activity of green tea polyphenols and, more recently, prooxidant effects of these compounds have been suggested as cellular basis for cancer prevention [88, 517]. Several evidences point to the effect of tea polyphenol in inhibiting tumor incidence and multiplicity in different organ sites such as the skin (UV radiation and chemically induced), lung, liver, breast, prostate, stomach, pancreas, bladder, and colon based on preclinical, observational, and clinical trial data [289, 516]. Besides cancer, EGCG has demonstrated beneficial effects in other diseases such as diabetes, Parkinson's disease, Alzheimer's disease, stroke, and obesity.

15.4 Silibinin

The flavonolignan silibinin is a purified polyphenolic compound derived from the seeds of herb milk thistle (*Silybum marianum*). It was originally used as a remedial hepatoprotective agent being capable of scavenging both free radicals and reactive oxygen species by enhancing the antioxidant defense machinery of cells. Additionally, silibinin has effects on multiple targets including abrogation of mitogenic and survival signaling and cell cycle regulation. Silibinin has been reported to target the receptor tyrosine kinase (EGFR, IGF-1), MAPK, and NF-κB pathways in various cancers [518]. It has been vigorously evaluated in different animal species and models with no signs of adverse effects, and thus, its consumption as a drug or dietary supplement appears to be safe with no systemic toxicity. Silibinin has been examined against different cancers (lung, oral, colon, prostate, and pancreas) for over a decade with outcome suggesting its further evaluation in large cohort of patient population harboring different site-specific cancer.

15.5 Quercetin

The flavonoid quercetin derived from fruits and vegetables (apple, onion, tomatoes) has been considered as prototype chemopreventive agent targeting key molecules due to its antioxidant, anti-inflammatory, antiproliferative, proapoptotic, and antiangiogenic effects interfering against the "initiation" to invasion and metastasis of tumor progression. As reported, multitargeted cancer prevention by quercetin includes modulation of several signal transduction pathways including those associated with the processes of inflammation and carcinogenesis [519]. Further, a number of investigators have examined and reported that quercetin is effective in inducing cell death and cell cycle arrest in tumor cells through downregulation of selective oncogenes. Recently, Russo et al. reviewed the status of quercetin in disease prevention and therapy concluding the necessity of further research on possible use of quercetin in adjunct cancer therapy [92].

15.6 Curcumin

As diferuloylmethane, is a polyphenolic compound extracted from the dried ground rhizome of the perennial herb *Curcuma longa*. Curcumin has been extensively researched and reviewed with regard to its mechanism of action that mediates its chemopreventive and therapeutic effects. Several known pleiotropic effects of curcumin action have emerged including inhibition of signaling pathways at multiple levels such as transcription factors NF-κB, STAT (constitutively and drug induced), enzymes (COX-2, MMPs), cell cycle arrest with downregulation of cyclin D1 and cyclin E and upregulation of p21$^{\text{CIP1/WAF1}}$ and p27$^{\text{KIP1}}$ cyclin-dependent kinase inhibitors, inhibitory effect on oncogenic proliferation (EGFR and Akt), anti-invasive pathway-related molecules (β-catenin and adhesion molecules), and anti-metastatic effect. Curcumin upregulates caspase family proteins and downregulates anti-apoptotic genes (Bcl-2, Mcl-1, and Bcl-xL). The effect of curcumin is not only limited to cancer but also reported to display therapeutic effect against various human disorders such as metabolic and infectious diseases, diabetes, psoriasis, rheumatoid arthritis, atherosclerosis, and Parkinson's and Alzheimer's diseases. In animal models, the anticarcinogenic effects of curcumin include a spectrum of cancers such as leukemias, lymphomas, multiple myeloma, brain cancer, and melanoma as well as skin, cervix, lung, prostate, breast, ovarian, bladder, liver, gastrointestinal tract, pancreatic, and colorectal cancers. However, on the downside, therapeutic applications of curcumin in humans are limited by its high metabolic instability as well as poor absorption and bioavailability. To overcome this shortcoming, many research laboratories including ours, are focussing on developing novel, synthetic analogs and formulations of curcumin including its complexation with polymeric micelles or nanoparticle-based encapsulation towards greater chemical stability, systemic bioavailability, and antitumoral activities than naturally occurring curcumin [520, 521].

16 Conclusion and Perspective

Emerging scenario from multifaceted preclinical and clinical studies reveal that polyphenol class of compounds have the potential to intervene and modulate many strategic targets predicted in ontogeny of tumor initiation and progression. Many of these events are currently believed to result from the high frequency of mutations prevailing in cancer cells along with multiple deregulated signaling mechanisms. This knowledge is paving way to explore novel approaches for "preventive intervention" at a time point where it can be most effective to therapeutic modules. Drugs that are currently in clinical practice have shown response in clinics, but still most patients diagnosed with cancer succumb to recurrence or advanced disease that lacks effective therapy. With farfetched knowledge gained with respect to cancer prevention research and chemopreventive phytochemicals, superimposing conventional dogma of cancer treatment with new testament of "chemosensitisation" by multitargeting phytochemicals into continum of cancer care and therapy holds promise for the future. Dietary intake of fruits, vegetables, beverages, and edible plant parts rich in polyphenol contents is proposed to help in preventing and lowering the risk of cancer in susceptible population. This chapter summarized a prognostic overview of the potential of polyphenol compounds covering multitargeted mechanism including modulation of inflammation, antioxidant status, regulation of key intracellular signaling events pertaining to cancer cell proliferation and other biological events such as apoptosis, cell cycle arrest, angiogenesis, and metastasis. Collectively, all these evidences reinforce our conviction that polyphenol compounds could be ideal candidates for further exploration and incorporation in "prevention intervention." Much of what we know presently, the realization of bioavailability of bioactive polyphenol in context of therapeutic response remains a challenge that need to be vigorously addressed. Another important issue relates to recommended dose range associated with optimal biological activity and maximal therapeutic indices. Further, using computer modeling and other modules such as systems biology approach, development of novel stable semisynthetic derivatives and formulation of natural polyphenol compounds needs to be prioritized and further evaluated in genetically modified transgenic and knockout models in setting of prevention and sensitization to therapy. This can later be extended into clinic either as monotherapy or in combination with current cancer therapies in patients leading to improvement in health care.

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Tannins and Anthocyanins of Wine: Phytochemistry and Organoleptic Properties

72

Pierre-Louis Teissedre and Michael Jourdes

Contents

1 Introduction 2256
2 Tannins and Anthocyanins in Grape Berries 2257
2.1 Structure of Tannins 2257
2.2 Structure and Impact of Anthocyanins 2260
3 Tannins and Anthocyanins in Wines 2262
4 Organoleptic Properties of Tannins 2263
5 Organoleptic Properties of Anthocyanins 2265
6 Tannin Evolution and Organoleptic Perception 2266
7 Mechanisms of Polymerization and Condensation of Tannins and Anthocyanins During Aging 2269
8 Conclusions 2270
References 2271

Abstract

The occurrence of tannins and anthocyanins from grapes and wines is well documented. Various chemical reactions involving anthocyanins and/or flavanols have been demonstrated to occur during red wine aging. Current knowledge regarding the reaction mechanisms involved in some of these processes and the structures of the resulting products is reviewed. Their effects on wine organoleptic quality are also discussed.

Keywords
Anthocyanins • Astringency • Bitterness • Color • Grape wine • Phenolics • Tannins • Quality

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1 Introduction

Phenolic compounds are products of secondary metabolism of plants; they are particularly important in grape wine because of their effects on wine color and it's organoleptic properties [1]. Polyphenols are defined as a wide range of chemicals including at least one aromatic nucleus and one or more hydroxyl groups, in addition to other constituents [2]. Tannin has been defined by as follows: "water-soluble phenolic compounds, molecular weight of between 500 and 3,000 Da, which, in addition to the properties of usual phenols, have the ability to precipitate alkaloids, gelatine and other proteins." Anthocyanins are defined as water-soluble vacuolar pigments that may appear red, purple, or blue according to pH. They belong to a parent class of molecules called flavonoids. Anthocyanins are glucoside of anthocyanidins. Anthocyanins have been shown to act as a "sunscreen," protecting cells from high-light damage by absorbing blue-green and UV light, thereby protecting the tissues from photoinhibition or high-light stress.

Tannins belong to the flavan-3-ols and can be considered as the most diverse category of flavonoids. These compounds range from simple monomers, (+)-catechin and its isomer (−)-epicatechin, to oligomers and polymers called proanthocyanidins. The proanthocyanidins are formed of catechin and epicatechin by oxidative coupling between the positions C4 and C6 or C8 of the adjacent monomer. The oligomers of procyanidins are formed by 2–5 units of (+)-catechin or (−)-epicatechins, the polymers being formed by 6 or more units [3, 4]. In addition, flavan-3-ols may be esterified with gallic acid (i.e., epicatechin gallate) or hydroxylated to form prodelphinidin (i.e., gallocatechins, epigallocatechin). The flavan-3-ols, present in grapes, are mostly in the form of polymers. The seed tannins are made up of procyanidins (polymers of catechin and epicatechin), partially galloylated, while those of skins also contain prodelphinidins (polymers of gallocatechin and epigallocatechin) [5]. The average number of monomeric units, defined as the mean degree of polymerization (mDP), may go up to 18 in tannin fraction from seeds and around 30 units in skin tannin extract. The anthocyanidins are widely present in the plant kingdom, mainly in the form of glycosides, especially in black/red grape skins, where they are responsible for the colors red, blue, and purple depending on the pH of the cell compartment [6]. The most common anthocyanidins are the pelargonidin, cyanidin, delphinidin, peonidin, and malvidin, but these compounds are only present under their glycosylated forms, called anthocyanins. The anthocyanidins are also capable of conjugation with hydroxycinnamic acids (e.g., *p*-coumaric acid, caffeic acid) and other organic acids (e.g., malic and acetic acids). Unlike other species, North American *Vitis* species have significant levels of diglycosylated anthocyanins in positions C3 and C5; *Vitis vinifera* contains only traces of these and is characterized by the presence of a majority of anthocyanin monoglucosides, particularly the malvidin-3-*O*-glucoside and its acylated derivatives. The anthocyanins are present in the red grapes at about 500–3,000 mg/kg, but can reach higher values in cultivars called "dyers" as Alicante Bouschet (5,000 mg/kg) in which the anthocyanin concentration in pulp is also high.

Table 72.1 Anthocyanin and tannin content in the different parts of the grape berry

	Pulp	Skins	Seeds
Tannins (mg/kg)	Traces	100–500	1,000–6,000
Anthocyanins (mg/kg)	–	500–3,000	–

Source: Teissedre and Chervin [7]

Table 72.2 Catechin content by cultivars

Cultivars	Catechins (mg/kg)	% In stalk	% In seeds	% In skins	% In pulp
Alicante bouschet	551	15	64	10	11
Cabernet sauvignon	344	10	83	7	T
Carignan	94	27	54	19	T
Cinsault	154	47	37	9	T
Grenache blanc	144	17	51	32	T
Grenache noir	173	25	64	11	T
Merlot	601	9	81	11	T
Mourvedre	171	25	58	17	T
Pinot noir	1,165	4	94	2	T
Colobel (hybrid)	862	8	79	7	6
Mean	377	20	65	14	1
					T = traces

Source: Bourzeix et al. [8]

The grape berries also contain large amounts of tannins mainly concentrated in the seeds and skins and large amount of anthocyanins concentrated in skins (Tables 72.1 and 72.2).

2 Tannins and Anthocyanins in Grape Berries

The major phenolic compounds are the anthocyanins and tannins (proanthocyanidins or condensed tannins) [9–12]. The identification of these compounds and their distribution in the grape berry have been investigated.

2.1 Structure of Tannins

Tannins are abundant in grapes and wine, and in the grape, they are found in both the seed and skin. These are often specifically called the flavan-3-ols to identify the location of the alcohol group on the C ring. The flavan-3-ols are the most reduced form of the flavonoids, since both positions 2 and 3 on the C ring are saturated, stereoisomers exist, and two are found in grapes (Fig. 72.1). The *trans* from is (2R,3S) (+)-catechin, and the *cis* form is (2R,3R) (−)-epicatechin. Both catechin and epicatechin have the 3′,4′ catechol substitution on their B ring. The only other

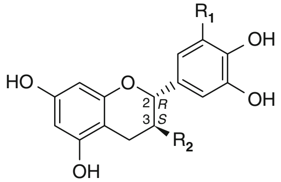
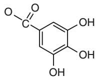
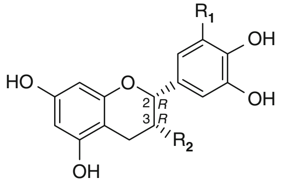
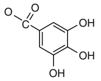
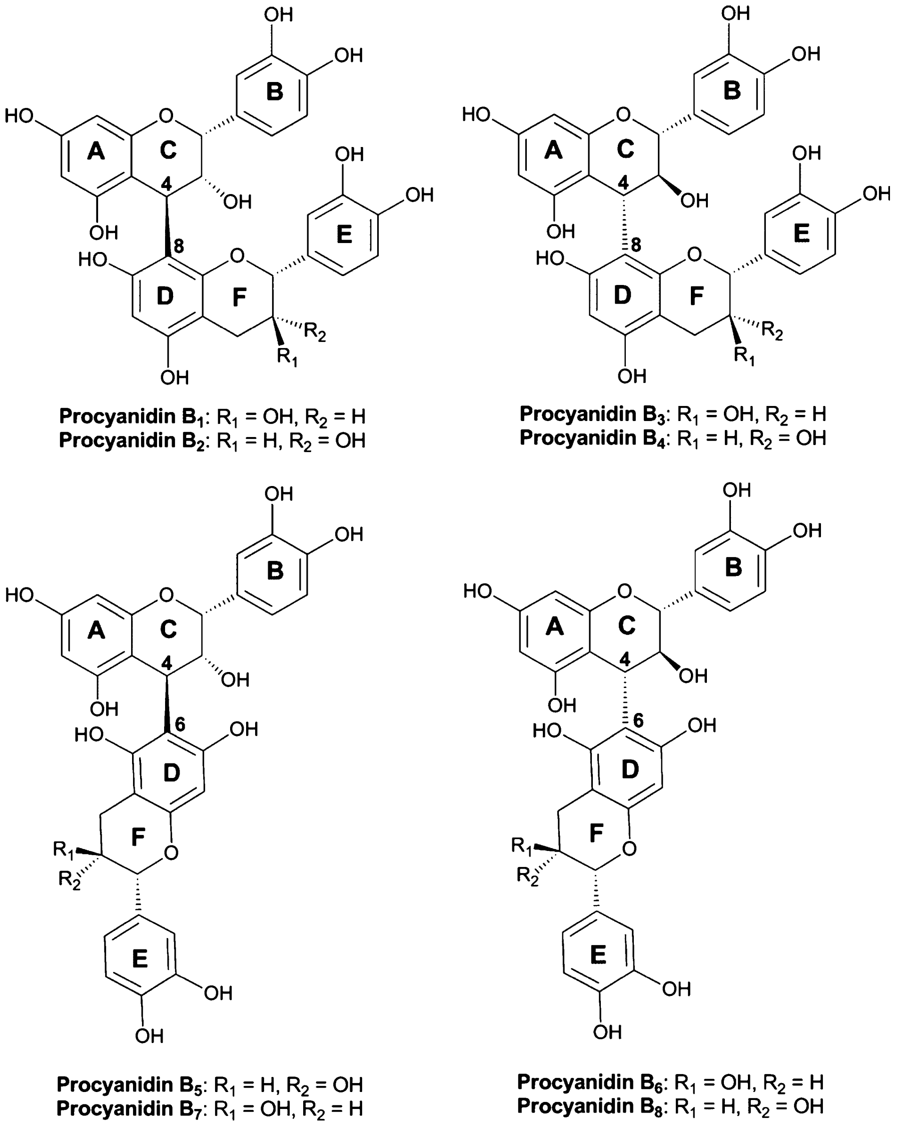
	Flavan-3-ols	R_1	R_2	C-2	C-3
	(+)-Catechin	H	H	*R*	*S*
	(+)-Gallocatechin	OH	H	*R*	*S*
	(+)-Gallocatechin gallate	OH		*R*	*S*
	(–)-Epicatechin	H	H	*R*	*R*
	(–)-Epigallocatechine	OH	H	*R*	*R*
	(–)-Epicatechine gallate	H		*R*	*R*

Fig. 72.1 Chemical structure of principal flavanol monomers of grapes and wine

B ring substitution pattern found in wine flavan-3-ols is the 3′,4′,5′-trihydroxy form, appropriately called the gallocatechins. Epigallocatechin is found in grape skin, but gallocatechin is not found in significant amounts. Gallate esters are also found, and the gallic acid is esterified at position 3 of the epi-series only. Epicatechin gallate represents a small but significant proportion of the flavan-3-ol pool in grapes in the seeds. Thus, for the simple series of monomeric flavanols, there are four different ones found in wine. These monomeric flavan-3-ols are sometimes referred as the "catechins." The levels of total monomeric flavan-3-ols in typical red wine ranged from 40 to 120 mg/L with the majority usually being catechin [13]. The levels are strongly affected by seed extraction techniques and are higher when extended maceration techniques are used. The majority of phenolic compounds in red wine are from the condensation of flavan-3-ol units to yield the oligomers (proanthocyanidins) and polymers (condensed tannins). The condensation occurs to form covalent bonds between flavan-3-ol units, the most common linkages being 4 → 8 and 4 → 6 positions (Fig. 72.2). On average, epicatechin is the predominant unit in condensed tannins from grapes and wine; catechin is the next most abundant (often found at the end or terminal units – those with no bonds at position 4). In typical red wines, the amount of polymer plus oligomer is a sizable fraction of the total phenolic level, being in the range of 20–50% in new wines and a higher proportion in older wines. Levels are between 0.5 g/L and 2 g/L or higher for some red wines in function of the grape variety, while in white wine, levels are lower ranging from 10 to 50 mg/L and highly dependent on pressing techniques [11]. The monomeric catechins are bitter and astringent, while in polymer, the bitterness is minimal, but the astringency remains [14]. Over several years of aging, a disproportionation reaction can occur, perhaps with some oxidation, so the polymers continue to increase in size until they are no longer soluble in wine and form the precipitate commonly observed in older red wines. During aging, the amount of phenolics in a wine decreases.



Procyanidin B$_1$: R_1 = OH, R_2 = H
Procyanidin B$_2$: R_1 = H, R_2 = OH

Procyanidin B$_3$: R_1 = OH, R_2 = H
Procyanidin B$_4$: R_1 = H, R_2 = OH

Procyanidin B$_5$: R_1 = H, R_2 = OH
Procyanidin B$_7$: R_1 = OH, R_2 = H

Procyanidin B$_6$: R_1 = OH, R_2 = H
Procyanidin B$_8$: R_1 = H, R_2 = OH

Fig. 72.2 Procyanidin dimers of flavanols from grapes and wines

The proanthocyanidins form a considerable portion of the tannins found in wine and in particular contribute heavily to the color and flavor of red wines. Proanthocyanidins are high-molecular-weight polymers formed from flavan-3-ol monomeric unit (i.e., (+)-catechin and (−)-epicatechin). Oxidative condensation occurs between carbon C-4 of the heterocycle C ring and carbons C-6 or C-8 of the attached aromatic A rings (Fig. 72.3) [15]. The procyanidins B1–B4 are the most

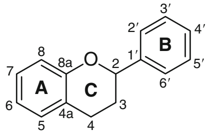


Fig. 72.3 Basic structure and numbering system of flavonoids

common dimers and are characterized by the C4–C8 linkage; they are occasionally accompanied by corresponding C4–C6-linked isomers (B5–B8) (Fig. 72.2).

It was reported that esterification of (−)-epicatechin and procyanidin B2 by gallic acid can occur and that these gallate esters are only found in the grape seed extract. Grape seed extract contains oligomer procyanidins made up of dimers or trimers of (+)-catechin and (−)-epicatechin.4-6 The procyanidin dimers are comprised of procyanidins B1, B2, B3, B4, B5, B6, B7, and B8. There are six procyanidin trimers which include procyanidins C1 and C2.

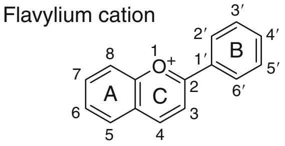
Furthermore, several galloylated procyanidins, which are most commonly the gallate esters of the dimeric procyanidins, and some free gallic acid are present [16, 17]. Tetramers or greater of these flavonols would be known as polymeric proanthocyanidins, and the astringency of the molecule would increase accordingly. Therefore, oligomeric proanthocyanidins are less astringent, bind less strongly to proteins, and are more soluble [17].

2.2 Structure and Impact of Anthocyanins

Anthocyanins belong to the flavonoid group of polyphenols. They have a C6C3C6 skeleton typical of flavonoids. Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium cation, i.e., the flavylium cation [18]. The main part of anthocyanins is its aglycone, the flavylium cation (Table 72.3), which contains conjugated double bonds responsible for absorption of light around 500 nm causing the pigments to appear red to human eye. The aglycones are called anthocyanidins, which are usually 3,5,7,3′,4′-penta- or 3,5,7,3′,4′,5′-hexasubstituted. Different anthocyanidins are known today (Tables 72.1 and 72.2), but only six of them are significant and most common from food point of view [19]. The most important anthocyanidins are pelargonidin, cyanidin, peonidin, delphinidin, malvidin, and petunidin (Fig. 72.4). These aglycones differ in the number of hydroxyl and methoxyl groups in the B ring of the flavylium cation.

Winemakers observed sensory differences in Cabernet Sauvignon wines made from three pruning treatments in a single vineyard, particularly in mouthfeel characteristics. The relationships between wine composition and wine sensory characteristics were examined and then compared to berry weight and composition and wine quality scores. Cabernet Sauvignon from three pruning treatments – machine, cane, and spur – was harvested at commercial harvest date, and replicate wines were made from each for three vintages [20]. The composition of the wines from all three pruning systems was generally similar. Differences in individual descriptive attributes did not separate the wines from the three treatments, or across vintages, despite

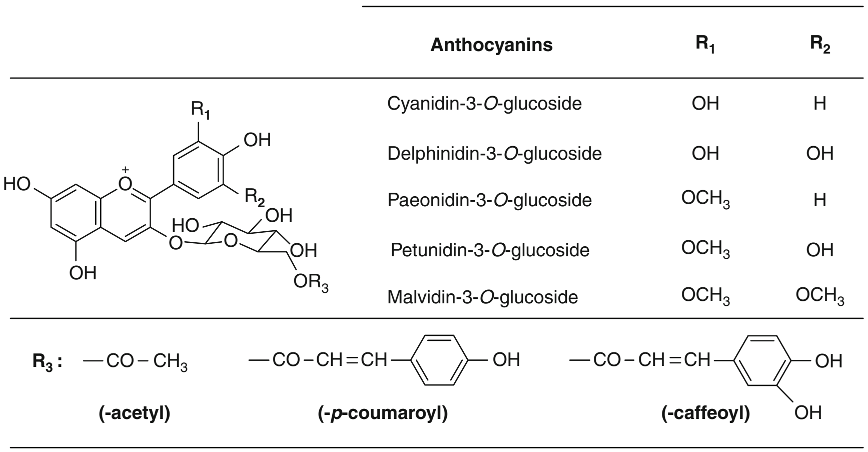
Table 72.3 The substitution pattern of flavylium cation forming the naturally occurring anthocyanidins known today



Anthocyanidin	3	5	6	7	3′	4′	5′	Color
Carajurin	H	H	OH	OH	H	OCH_3	OCH_3	–
Arrabidin	H	H	OH	OH	H	OH	OCH_3	–
3′-Hydroxyarrabidin	H	H	OH	OH	OH	OH	OCH_3	–
Apigenin	H	OH	H	OH	H	OH	H	Orange
Luteolin	H	OH	H	OH	OH	OH	H	Orange
Tricetinidin	H	OH	H	OH	OH	OH	OH	Red
Pelargonidin	OH	OH	H	OH	H	OH	H	Orange
Aurantinidin	OH	OH	OH	OH	H	OH	H	Orange
Cyanidin	OH	OH	H	OH	OH	OH	H	Orange red
5-Methylcyanidin	OH	OCH_3	H	OH	OH	OH	H	Orange red
Peonidin	OH	OH	H	OH	OCH_3	OH	H	Red
Rosinidin	OH	OH	H	OCH_3	OCH_3	OH	H	Red
6-Hydroxycyanidin	OH	OH	OH	OH	OH	OH	H	Red
6-Hydroxydelphinidin	OH	OH	OH	OH	OH	OH	OH	Bluish red
Delphinidin	OH	OH	H	OH	OH	OH	OH	Bluish red
Petunidin	OH	OH	H	OH	OCH_3	OH	OH	Bluish red
Malvidin	OH	OH	H	OH	OCH_3	OH	OCH_3	Bluish red
Pulchellidin	OH	OCH_3	H	OH	OH	OH	OH	Bluish red
Eupinidin	OH	OCH_3	H	OH	OCH_3	OH	OH	Bluish red
Capensinidin	OH	OCH_3	H	OH	OCH_3	OH	OCH_3	Bluish red
Hirsutidin	OH	OH	H	OCH_3	OCH_3	OH	OCH_3	Bluish red
Ricciniodin A[a]	OH	H	OH	OH	H	OH	H	–

[a] Ring closure on the basis of either linkage between the 3- and 6′-positions + an additional OH-group at the 2′-position (Adapted from F.J. Francis [19], O. Andersen [79], and Devia et al. [80]. The anthocyanidins in bold are the most important ones regarding foods)

differences in overall quality scores. Principal component analysis (PCA) could separate the wines by pruning and by vintage using wine composition or sensory parameters. Higher concentrations of anthocyanins, tannins, and phenolics in berries did not always result in higher concentrations in wines. In this study, higher wine tannin or wine phenolic concentrations did not result in higher wine astringency, and wine color measures and phenolic composition were not good indicators of individual wine sensory properties or wine quality. Wine composition was not necessarily directly influenced by berry composition. Few studies focus on the berry to wine sensory continuum, particularly over more than one vintage or in a commercial



Anthocyanins	R_1	R_2
Cyanidin-3-*O*-glucoside	OH	H
Delphinidin-3-*O*-glucoside	OH	OH
Paeonidin-3-*O*-glucoside	OCH_3	H
Petunidin-3-*O*-glucoside	OCH_3	OH
Malvidin-3-*O*-glucoside	OCH_3	OCH_3

Fig. 72.4 Structures of principal wine anthocyanins

context. This study highlighted how complex the relationships among berry qualities, wine sensory properties, and wine quality can be, particularly within a single vineyard. In another work, the authors [21] try to determine how changes in grape composition brought about by artificial shading (sunlight exclusion) influence wine properties including color, flavonoid composition, and sensory attributes. Prior to flowering, bunches of Shiraz grapes were enclosed in boxes designed to eliminate light without altering bunch temperature and humidity. This artificial bunch shading had little effect on berry ripening and accumulation of sugar, but at harvest, the shaded bunches had smaller berries and higher seed weight, juice pH, and titratable acidity. The amount of anthocyanins in the fruit was not significantly changed, but anthocyanin composition in the shaded berries was shifted toward dioxygenated anthocyanins (i.e., glucosides of cyanidin and peonidin). Shaded fruit shows an increased in seed tannin level and a decreased in skin tannin concentration, but the largest relative change in flavonoid composition was a marked decrease in flavonols in the shaded fruit, similar to previous studies. Wines made from shaded fruit had lower wine color density, total phenolics, anthocyanins, and tannins when the wine was bottled and after aging for up to 3 years. Sensory analysis of the wines indicated no significant difference in aroma attributes, but the wines made from shaded fruit were rated lower for astringency, fruit flavor, and flavor persistence in-mouth sensory attributes. The results indicate that extreme shading of Shiraz fruit can decrease wine color, anthocyanins, and tannins as well as altering sensory attributes.

3 Tannins and Anthocyanins in Wines

Anthocyanin pigments and tannins are particularly important for red wine quality. Their extraction depends on their location in the berry and their solubility.

All phenolic compounds are unstable and undergo numerous enzymatic and chemical reactions. Color and taste changes during red wine aging have been described as anthocyanin–tannin reactions. The structures and properties of tannins and pigmented tannins from these reactions are often misunderstood. Wine phenolic composition is subject to evolve in wine during aging and storage [22]. Five key points have to be taken under consideration: (1) reactions of tannins yield both larger polymers and smaller species; (2) anthocyanin reactions can generate colorless species as well as polymeric and small various pigments; (3) some polymeric pigments undergo sulfite bleaching, while some low-molecular-weight pigments do not; (4) polymers are both soluble and astringent, so the astringency loss during aging may involve cleavage rather than polymerization; and (5) sensory properties of anthocyanins and tannins are modulated by interactions with other wine components.

The effect of the use of SO_2 and pectolitic enzymes in the prefermentative phase, maceration time, and oak aging on color, anthocyanins, tannins, (+)-catechin, (−)-epicatechin content of Aglianico wine has been realized [23]. The addition of SO_2 and pectolitic enzymes before fermentation caused an increase in color intensity, color stability, total phenolics level, anthocyanins, (+)-catechin, and (−)-epicatechin content in Aglianico wine. Longer maceration times made wines richer in total phenolics and gave them better chromatic characteristics. Storage in oak caused a decrease in anthocyanins, (+)-catechin, (−)-epicatechin content, but an increase in total phenolic content, and a stabilizing effect on color also occurred.

4 Organoleptic Properties of Tannins

Grape phenolics are nonvolatile, and therefore, they cannot be smelled. Anthocyanins provide the visual component in wines. Flavan-3-ol monomers are bitter and thus have taste. That is because some hydroxyl groups of polyphenols bind to salivary proteins that some of them as tannins are defined as astringent giving a sensation of dryness in the mouth. The tannins or proanthocyanidins are astringent, but the perception is complex. Tannins are compounds to be deterrents to herbivores and fungi. Tannins can accomplish this property because of their ability to bind strongly to proteins [24]. Considering wine, it is generally considered that we observe this as a loss of lubrication due to the tannins binding and precipitating salivary proteins. To put it simply, tannins are astringent, terribly astringent [25, 26]. Astringency is a tactile sensation, and therefore, we feel it. This gives rise to the common term used to describe tannins in wine: mouthfeel. Beyond astringency, tannins can also possess bitterness, which is a taste sensation and is brought about by the lowest-molecular-weight tannins [14, 27–29]. It is generally considered that too much bitterness in wine is not desirable, and based upon the reduction in the lowest-molecular-weight tannins observed during berry maturation, this may provide a structural explanation for why tannin quality improves with fruit maturity. Considering tannin perception in total, the astringency of tannins has a distinct temporal aspect to their perception [30]. When wines have an excess quantity of tannins, the astringency of the wine can linger beyond that of other components. This persistence is generally thought of as being undesirable. Although bitterness and astringency are found in red wines, it is not a descriptor that is often used in a production setting. Instead, winemakers tend to describe tannins in terms that provide subquality information. Sensory scientists and chemists have tried to understand these more subtle aspects of tannin perception [29, 31–40]. From these investigations, it is clear that the perception of astringency in wine could be influenced by many components in wine including ethanol [29, 41], acidity [29, 41, 42], viscosity [43], simple sugars[44], polysaccharides [45, 46], and anthocyanins [45]. The major difficulty to conduct this type of study results in the influence of the variation in human response to astringency and bitterness [41]. Some complex interaction between tannins and other macromolecules found in wine occurs and modifies the mouthfeel perception [29, 46–49].

How can different grape components influence tannin perception? Tannins and acid are balanced with ethanol sugar and polysaccharides. The goal to get an optimized red wine quality is to balance these components. When the grape berry is mature, the composition should be balanced. The enologist has the ability to modulate wine perception by adjusting the balance particularly with the decision of the harvest date and with adapted winemaking and aging process. To simultaneously explore the primary and interactive effects of proanthocyanidin (“tannin”), ethanol, anthocyanin, and wine polysaccharide concentrations on the mouthfeel perception of wine-like media, a sensory study based on an incomplete factorial design was conducted [50]. Two grape polyphenol fractions, i.e., grape seed tannins and anthocyanins, and two fractions of wine polysaccharides (mannoproteins + arabinogalactan proteins and rhamnogalacturonan II) were prepared and analyzed. A panel of 15 trained judges generated a series of mouthfeel descriptors and rated their intensities while samples containing various levels and combinations of the components were held in mouth and after expectoration. The sensory perception was primarily determined by tannin concentration. However, the attribute ratings were also strongly influenced by all other factors both directly and through interactions. The intensities of all astringency descriptors increased with tannin concentration and were reduced when rhamnogalacturonan II was added. Bitterness increased with ethanol level and decreased in the presence of proteoglycans. Secondary effects observed included both masking and enhancement of the primary effects but also specific interaction effects. The latter are probably related to differences in the structural organization and properties of molecular assemblies involving polyphenols, polysaccharides, and ethanol.

The taste and mouthfeel properties of three different types of tannin-like polyphenolic compounds, representative of some of the tannin-like polyphenolic compounds found in red wines, were determined using descriptive sensory analysis [50]. Ethyl-bridged flavan-3-ols were produced by reaction of (+)-catechin with acetaldehyde under acidic conditions. Red-colored tannin-like polyphenolic compounds from wine and from wine pomace were isolated by multilayer coil countercurrent chromatography (MLCCC). Mouthfeel attributes and bitterness of the fractions dissolved in a model wine (MW) medium were rated while the fractions were held in the mouth and after expectoration. The sensory properties of the fractions described above were compared to those of apple procyanidins (tannins) with degree of polymerization (dp) 3 and 9. Both wine and pomace-derived colored tannin-like polyphenolic compounds were rated as significantly less astringent when compared to both the apple tannin fractions but were similar to the MW. The ethyl-bridged flavanols (with mean dp of 5) were rated significantly more bitter than both the apple procyanidin fractions. Astringency of the ethyl-bridged flavanols was lesser than the apple procyanidin fraction with dp9 and more than that with dp3. Highly purified grape anthocyanidin monoglucosides and coumaroylated monoglucoside, prepared either by solid-phase extraction or by multilayer coil countercurrent chromatography, were rated similarly to the MW indicating that free anthocyanins, like the colored tannin-like polyphenolic compounds from wine and pomace, do not contribute astringency or bitterness to wine.

Storage conditions that may influence the chemical and sensory properties of young bottled Cabernet Sauvignon and Merlot wines have been also studied [51]. Low- and high-tannin wines ($\leq$400 mg/L and $\geq$ 800 mg/L catechin equivalents, respectively) stored at 23 °C for 0 day (baseline) and at either 27 °C or 32 °C for 40, 55, and 70 days were used for chemical and sensory analyses. In both low- and high-tannin wines, storage at 32 °C resulted in significant increase in small polymeric pigment ($p \leq 0.05$) with a corresponding decrease in anthocyanin concentrations over time, which was more pronounced in Cabernet Sauvignon. In both varieties, high-tannin-content wines contained larger polymeric pigment than the low-tannin-content wines ($p \leq 0.05$). A trained sensory panel ($n = 21$) gave higher astringency ratings to high-tannin-content wines than low-tannin-content wines for both varieties, which remained constant throughout the study. An increased perception of bitterness was associated with storage at 32 °C storage for 70 days, while alcohol burn intensity was comparable in Cabernet Sauvignon. No significant differences in bitterness and alcohol burn intensity were found in Merlot. These results indicate that storage temperature and storage time contributed to change the chemical composition of typically aging red wines but did not impact perceived astringency. Tannin concentration was positively correlated with perceived astringency ($r = 0.882$) in Cabernet Sauvignon, while small polymeric pigment and low polymeric pigment had lower correlation with perceived astringency for both varieties.

5 Organoleptic Properties of Anthocyanins

The relationships between the levels of polyphenols, acidity, and red pigments in Shiraz wines and their perceived textural profiles as quantified by a trained sensory descriptive analysis panel were explored [52]. A “chamois-like” feeling when the wine was held in the mouth appeared to be related to an absence of polyphenols. The in-mouth “chalklike” texture was strongly associated with anthocyanin concentration and was negatively associated with alcohol level and acidity. The astringent subqualities of “velvetlike” and “emery-like” roughing were mostly related to polyphenol levels, but these attributes could not be adequately differentiated by the compositional variables under study. Wines that elicited a “puckery” sensation were characterized by relatively low anthocyanin levels, high acidity, and high pigmented polymer and tannin concentrations. The results of the study suggest that the in-mouth textural properties of Shiraz red wine are associated not only with their tannin composition and concentration but also with their acidity and anthocyanin and alcohol concentrations.

Two fractions containing the major polysaccharides present in wine have been isolated, one comprising a mixture of neutral polysaccharides, mannoproteins, and arabinogalactan proteins and the other containing the acidic polysaccharide rhamnogalacturonan II. A grape anthocyanin fraction was also prepared (Vidal et al. [36]). A trained sensory panel, using formal sensory descriptive analysis methods to rate the intensity of mouthfeel attributes while the samples were held in the mouth and after expectoration, individually assessed the fractions dissolved in a model wine at levels commonly encountered in red wines. Both polysaccharide fractions significantly increased the "fullness" sensation above that of the base wine. The rhamnogalacturonan II fraction significantly decreased the attribute ratings associated with the astringency of the model wine, whereas the neutral wine polysaccharide fraction had less affect on reducing the ratings for these attributes. The anthocyanin fraction tended to increase "fullness" although the effect was not great enough to be statistically significant. Unlike the polysaccharides, this fraction also increased perceived astringency, but this effect could be due to the presence of some derived tannins in the sample.

The contribution of anthocyanins or tannin–anthocyanin reaction products to the mouthfeel properties of wines prepared from both red and white berries with and without pomace contact and anthocyanin addition was also investigated [53]. A trained sensory panel rated mouthfeel and taste attributes in wines after 6 months of bottle storage, and phenolic measures were obtained. A white wine made in the same manner to how a red wine is made did not exhibit the same mouthfeel sensory attributes of a red wine: it was lower in viscosity, less particulate in nature, and lower in intensity for the astringency descriptors fine emery, dry, and grippy. It was found that differences in ratings of mouthfeel attributes could not be related closely to phenolic composition or structure. This study has shown that the presence of anthocyanins during fermentation increases the intensity of astringency attributes. Using the mouthfeel wheel, the differences between a white wine made like a red wine and a red wine could be quantified. It was also shown that the presence of anthocyanins in red wine can explain the differences perceived between the mouthfeel properties of a white and a red wine.

6 Tannin Evolution and Organoleptic Perception

Many wines are consumed after a period of aging. Aging modifies various organoleptic wine properties and implies a significant financial cost in the wine final price. The present-day wine consumers expect wine from a particular vintage and age to possess unique qualities that differentiate it from other wines of the other vintages.

Flavan-3-ols are the most abundant phenolic compounds, with oligomeric and polymeric procyanidins (condensed tannins) in red wine often representing 25–50% of the total phenolic constituents [54]. As these attributes contribute so much to overall red wine quality, an understanding of the relationship between proanthocyanidin composition, sensory perception, and age of red wine is critically important. It was possible to correlate a chemical parameter describing wine structure such as proanthocyanidin mean degree of polymerization (mDP) and a sensory parameter describing proanthocyanidin mouthfeel characteristics such as astringency with the age of vintage of Bordeaux Cabernet Sauvignon wines for 23 wine samples of known aging periods from Chateau Mouton Rothschild (*premier grand cru classé Pauillac*) kept under similar conditions during and after the winemaking process. An extended aging period was considered, covering a time frame of 27 years. The dates of production of these wines range from 1978 to 2005. The oligomeric and polymeric proanthocyanidins were depolymerized in the presence of a nucleophilic agent (phloroglucinol) in acid medium [55]. Reversed-phase HPLC analysis of the products formed allows determination of the structural composition of proanthocyanidins, which are characterized by the nature of their constitutive extension units (released as flavan-3-ols phloroglucinol adducts) and terminal units (released as flavan-3-ols). To calculate the apparent mDP, the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, in moles) was divided by the sum of all flavan-3-ol monomers (in moles). Regarding sensory analysis, fifteen judges were trained to evaluate astringency intensity using a 0–7-point scale (0 = amorphous, 1 = hollow, 2 = soft, 3 = mellow, 4 = slight astringency, 5 = tannic, 6 = hard, 7 = rough). A solution of 1.0 g/L aluminum sulfate was presented to judges as an astringency marker. We found that the mDP decreases with aging [55], with values of R^2 above 0.8 (Fig. 72.5a). This regression model behaves fairly well in the range of years explored; the lowest values of mDP were measured for 1978 vintage, whereas the highest were found for the 2001 followed by the 2005 vintage. If the wine is included between vintages 1978 and 1998, its DPm will vary from 1.81 to 3.68. The wine DPm fluctuates between 3.94 and 7.13 for the vintages 1999–2005. During aging, rearrangement reactions between phenolic compounds may explain decreases in the mDP. In particular, the presence of oxygen in wine will lead to the formation of acetaldehyde via the coupled oxidation of oxygen and phenolic compounds [56]. In young wines, free sulfur dioxide is present; formed acetaldehyde is considered as the bisulfite adduct and therefore would not be available for reaction with phenolic compounds [57]. Concerning astringency intensity, Fig. 72.5b suggests that astringency decreases during aging ($R^2 = 0.598$, $p = 0.000$). Wine aging is a process in which the organoleptic properties are modified as the result of a series of reactions whose effect is to strengthen certain desired characteristics while reducing or eliminating other unwanted ones. This change of perception could be attributed to mDP, since the higher the mDP is, the higher the overall astringency is perceived [50]. The correlation between astringency intensity and mDP ($R^2 = 0.509$, $p = 0.049$) permits the qualitative assessment of tannins. Scale patterns between wine mDP and tannin perception are proposed (Fig. 72.6). The judges perceived wines having an mDP inferior to 2 to be soft and mellow, while wines with an mDP between 2 and 4

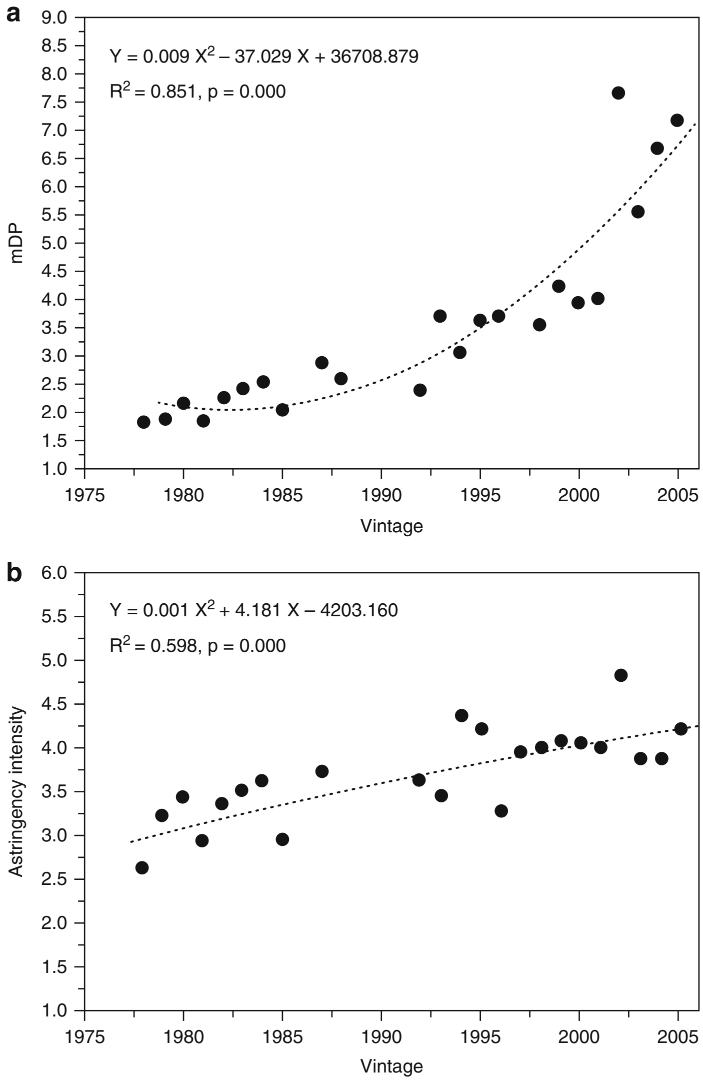


Fig. 72.5 Correlation between mDP, astringency intensity, and vintage. (**a**) Mean degree polymerization (mDP) and vintage; (**b**) astringency intensity and vintage

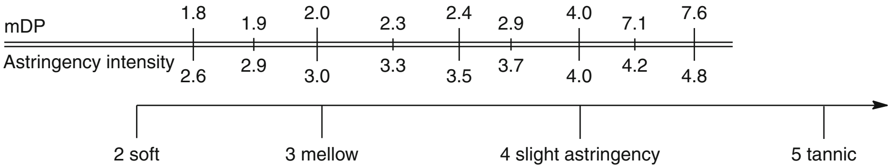

mDP
1.8
1.9
2.0
2.3
2.4
2.9
4.0
7.1
7.6
Astringency intensity
2.6
2.9
3.0
3.3
3.5
3.7
4.0
4.2
4.8
2 soft
3 mellow
4 slight astringency
5 tannic

Fig. 72.6 Astringency variation according to mDP

were characterized as mellow and slightly astringent, and wines with an mDP bigger than 4 were perceived rather tannic. The function of this scale pattern is not the identification of anonymous wines, but the exercise of astringency quality evaluation, to give sensory information taking into consideration a specific wine chemical analysis. This might classify its value for consumption and to see whether it has the optimized astringency required prior to consumption. mDP and astringency intensity parameters are critically required for the wine industry in order to judge wine quality.

7 Mechanisms of Polymerization and Condensation of Tannins and Anthocyanins During Aging

Oxidative reactions in wine have been subject to thorough review with the oxidation of ethanol to acetaldehyde (via hydrogen peroxide) widely accepted as a major factor [58, 59]. This reaction followed by subsequent reactions of the acetaldehyde with tannins and anthocyanins to form ethyl-linked structures is very complex [60–62]. It is affected by a range of wine parameters including phenolic content, temperature, pH, sulfur dioxide concentration, and light penetration. In addition to the oxygen mediated reactions, other reactions involving phenolics but not oxygen can also take place in wine [63]. These reactions include polymerization and condensation of tannins, breakdown and condensation of anthocyanins, and precipitation of larger polymers. In addition to reactions that affect color and tannin structure, the presence or absence of oxygen also influences wine aroma evolution and improvement [64–66]. The acetaldehyde formed during wine aging reacts with anthocyanins. It can react with anthocyanins either alone to form ethylidene-bridged anthocyanin dimers [67–70] (Fig. 72.7, reaction 5) and vitisin B [71, 72] (Fig. 72.7, reaction 6) or in combination with Fl (flavan-3-ols) to form ethylidene-bridged copolymers (Fig. 72.7, reaction 7). Previous studies have shown that acetaldehyde addition increased the rate of anthocyanin-flavan-3-ol copolymerization [57, 69, 73]. Ethylidene-bridged copolymers up to tetramers were synthesized in model wine containing malvidin-3-*O*-glucoside and (−)-epicatechin. The obtained oligomers contained at most two anthocyanins units, which were always in the terminal position [61, 74]. The copolymers formed are transient and may evolve into substances with higher degrees of condensation, which may finally precipitate [61, 74]. The depolymerization of ethylidene-bridged Fl or the dehydration of ethyl alcohol-flavan-3-ol adduct formed during acetaldehyde-flavan-3-ol condensation may lead to the formation of *p*-vinylphenols (Fig. 72.7, reaction 4). These compounds may react with anthocyanins and pyranoanthocyanins to form more complex compounds such as *p*-vinylpyranoanthocyanin-flavan-3-ol [61, 74–78] in which the ethylidene bridges are rearranged and are no longer quantifiable as EDP ethylidene-diphloroglucinol with the EDP phloroglucinolysis (Fig. 72.7, reaction 8). Clearly, more research on microoxygenation as well as on the effect of oxygen on red wine composition in general needs to be carried out in order to get a better understanding of all the mechanisms and species formed in the complex media.

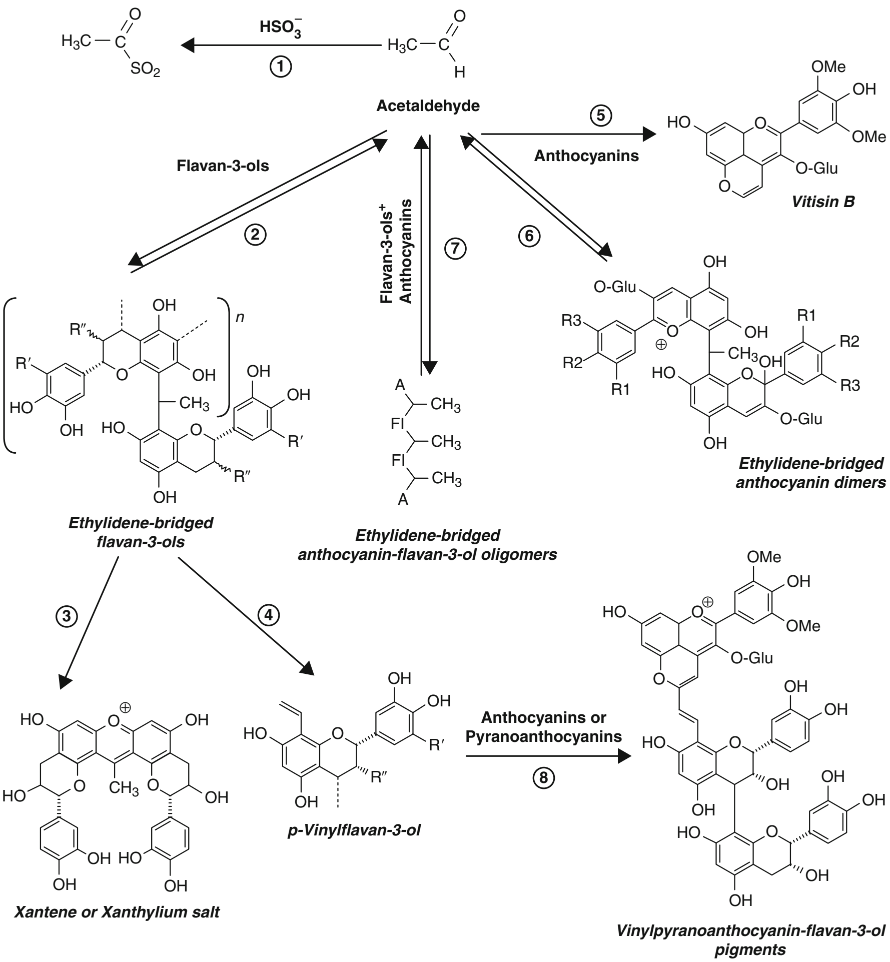

HSO$_3^-$
①
Acetaldehyde
⑤
Anthocyanins
Vitisin B
Flavan-3-ols
②
Flavan-3-ols+ Anthocyanins
⑦
⑥
Ethylidene-bridged flavan-3-ols
Ethylidene-bridged anthocyanin-flavan-3-ol oligomers
Ethylidene-bridged anthocyanin dimers
③
④
Xantene or Xanthylium salt
p-Vinylflavan-3-ol
Anthocyanins or Pyranoanthocyanins
⑧
Vinylpyranoanthocyanin-flavan-3-ol pigments

Fig. 72.7 Reaction pathways of acetaldehyde with Fl (flavan-3-ols) and anthocyanins. Fl, R′ = H or OH, R″ = OH or O-gallate; *O*-glucose (O-Glu), R1 = OH or OCH3 and R = H, OH, or OCH3

8 Conclusions

Tannins and anthocyanins are key wine component groups. Since the skins and seeds contain the most of the phenolics, red wine is a whole berry extract. In addition, because of the significant sensory effects of these substances on bitterness and astringency, they are controlled by winemakers. The methods of control involve the manipulation of extraction as well as fining with protein to precipitate and remove tannin from finished wine; technological enological process as nano- or ultrafiltration is now seeing some use. While many studies have examined astringency, the lack of a clear, accepted definition that delineates the oral sensations it encompasses makes it difficult to effectively compare results. The potential interaction of astringency with basic tastes in many complex foods and beverages suggests that the physiological and psychological mechanisms underlying the perception of astringency should be further studied. However, it is essential to ensure that consumer expectations created by the vintage information on the wine label are not mismatched by the sensory experience of consuming the wine. Scale pattern between wine aging and both mDP and astringency intensity is a first answer to these expectations.

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Grapevine Stilbenoids: Bioavailability and Neuroprotection 73

Pierre Waffo-Téguo, Stéphanie Krisa, Alison D. Pawlus, Tristan Richard, Jean-Pierre Monti, and Jean-Michel Mérillon

Contents

1 Introduction 2277
2 Chemistry of Stilbenoids 2278
2.1 Distribution of Stilbenoids 2279
2.2 Determination of Stilbenoids in Wine 2282
3 Pharmacological Applications and Mechanisms of Action 2283
3.1 Neurodegenerative Diseases 2283
3.2 Wine and Neurodegenerative Diseases 2284
3.3 Stilbenoids and Aβ peptides 2285
3.4 Resveratrol and Tauopathies 2289
3.5 Stilbenoids and Neuroinflammation 2289
4 Toxicity of Resveratrol 2291
5 Bioavailability and Metabolism 2292
5.1 In Vivo and In Situ Studies in Rodents 2292
5.2 Studies in Human 2293
5.3 Levels of Resveratrol and Its Metabolites in Target Tissues 2295
6 Other Biological Activities 2295
6.1 Cancer Chemoprevention 2296
6.2 Cardiovascular Protection 2298
6.3 Aging and Longevity 2300
7 Clinical Trials 2301
8 Conclusion 2302
References 2303

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Abstract

Vine and wine are an abundant source of stilbenoids that constitute a large class of compounds, including a series of monomers and oligomers (dimers, trimers, tetramers). Stilbenoids can reach a level of over 100 mg L^{-1} in red wine. Several epidemiological studies have correlated the consumption of grapes and wines with significant health benefits. The most studied stilbenoid, resveratrol, exhibits multiple biological activities across multiple diseases including cancer and cardiovascular and neurodegenerative diseases. This compound shows promise in both the prevention and the treatment of these diseases. Moreover, resveratrol mimics calorie restriction and extends the lifespan in species ranging from yeast to multicellular animals (not in normal mammals) by a sirtuin-dependent mechanism. Resveratrol is well absorbed yet rapidly metabolized; however, the tissue concentrations found in some studies in animals and humans are compatible with its biological activities. Resveratrol is well tolerated at doses of up to 1 g day^{-1} in human and does not cause serious adverse events up to 5 g day^{-1}. Interestingly, other wine and grape stilbenoids such as piceatannol, pterostilbene, viniferins, and vitisins also have beneficial activities. In this chapter, we have discussed chemistry and protective role of stilbenoids in general, and resveratrol in particular, during various pathological conditions such as neurodegeneration, neuroinflammation, cancer, cardiovascular diseases, and antiaging process. Works related to bioavailability and clinical trials of resveratrol are also presented.

Keywords

Biological activities • polyphenols • resveratrol • toxicity • *Vitis vinifera*

Abbreviations

AA	Arachidonic acid
AD	Alzheimer’s disease
ADI	Acceptable daily intake
AP-1	Activator protein 1
APP	Amyloid precursor protein
Aβ	β-Amyloid peptide
COX	Cyclooxygenase
HDL	High-density lipoprotein
IGF	Insulin-like growth factor
IL	Interleukin
iNOS	Inductible nitric oxide synthase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NOAEL	No observed adverse effect levels
PGE_2	Prostaglandin E_2

ROS	Reactive oxygen species
SIRT	Sirtuin
TNF	Tumor necrosis factor
TxA2	Thromboxane A2

1 Introduction

Vitis vinifera L. is a perennial woody vine belonging to the Vitaceae family. It is a productive plant, considered the world's premier fruit, occupying 7.6 million hectares of viticultural land in 2010. It is used mainly for wine (6.8 million hectares) but also for juice, fresh consumption (table grapes), dried fruit, and distilled liquor.

Polyphenolics are important constituents of grapes in determining the color, taste, and body of wines. Unlike other alcoholic beverages, red wine, which is obtained after about 10 days of maceration, contains phenolic compounds in substantial concentrations of up to 4 g L^{-1}. Due to the lower maceration time, white and rosé wines contain about a tenth of the polyphenol levels of red wines [1]. Among these compounds, stilbenoids constitute an important subclass, whose overall levels can reach over 100 mg L^{-1} in red wine. Resveratrol is one of the major stilbenoids of red wine with numerous promising biological activities.

Several epidemiological studies have shown a correlation with red wine consumption and significant health benefits. According to the statistics of the World Health Organization (1995), the reduction in the mortality rate from coronary heart disease in France as compared to the USA was 61% for men and 69% for women. As compared to the UK, the reduction was 68% and 71%, respectively [2, 3]. This finding constitutes the "French paradox" since the saturated fat intakes and serum cholesterol levels are similar in these three countries. This paradox has been attributed, in part, to moderate wine consumption [2]. A prospective cohort study by Renaud et al. was performed on the health effects of wine and beer drinking in 36,250 middle-aged men from eastern France [4]. Compared to abstainers, a moderate daily intake of wine, defined as 22–32 g of alcohol or two to three glasses, was associated with a lower risk of death due to cardiovascular diseases (40%), cancer (22%), other causes (42%), and all causes (33%). Similar results were found in a large Danish cohort [5].

Many epidemiological studies in different countries have examined the relationship between alcohol consumption and the risk of other diseases, including neurodegeneration. In prospective studies in the United States, Mukamal et al. observed a pronounced reduction in the risk of incident dementia among older adults (5,900 subjects) with moderate alcohol consumption (one to six drinks per week) [6], and Stampfer et al. found that in women (12,000 participants; 70–81 years old), up to one drink per day decreases the risk of cognitive decline of about 20% [7]. In these two studies, no significant difference was found according to the type of beverage. Additional cross-sectional (French men and women and

African and Japanese Americans) and prospective studies examined cognitive performance among moderate drinkers and abstainers, with the majority of these studies showing a beneficial effect [7, 8].

In regard to wine consumption, a number of studies have looked at its impact on neurodegenerative disease risks by examining cognitive performance and rates of dementia. The most frequent age-related degenerative disease is Alzheimer's disease, which accounts for approximately 70% of the cases of dementia. In the Tromsø study from Norway, light-to-moderate wine consumption was associated with better performance on cognitive tests after a 7-year follow-up of 5,000 subjects [9]. Moreover, a protective effect of wine against the occurrence of dementia was found in the Aquitaine region of France (the French Paquid cohort) in a prospective study that involved approximately 4,000 subjects aged 65 and over [10]. The risk of developing a dementia was decreased by about a factor of 2 in moderate drinkers (250–500 mL per day) compared to nondrinkers and light drinkers. In the Copenhagen City Heart study and the Chongqing study in China, authors found also that light-to-moderate drinking of wine is associated with a lower risk of dementia in elderly people [11, 12]. Similar results were obtained in two studies performed in Canada and New York, where a lower risk for dementia was correlated with wine consumption but not with spirits and beer consumption [13, 14]. In these last four studies, the consumption of wine was from one serving a week to three servings a day.

While epidemiology studies show wine to be protective against the development of many diseases, this correlation is complicated by other factors. For instance, some self-reporting studies have found that wine drinkers have a healthier diet than people who drink beer or spirits. In Danish supermarkets, wine buyers made more purchases of healthy food items such as olives and other fruits and vegetables than people who buy beer [15]. Other recent studies in Scotland and in China suggest that prior intelligence (defined by childhood IQ scores), socioeconomic status and general lifestyle moderation may partly explain the association between moderate alcohol intake and better cognitive function [16, 17].

It is prudent to note that only the moderate consumption of wine has shown a potential decrease in the risk of certain diseases, whereas excessive consumption is harmful and associated with increased mortality. Recently, supplementation with Concord grape juice for 12 weeks has been found to improve memory function in older adults (sample of 12 persons) in pre-dementia conditions [18].

2 Chemistry of Stilbenoids

Over the last 100 years, the grapevine *Vitis vinifera* L. (Vitaceae), the primary source of wine, has been the subject of numerous chemical studies. While stilbenoids are found in a number of plant species [19], grapes and related products are the most significant dietary source of these substances [20]. Stilbenoids are non-flavonoid polyphenols with an essential structural skeleton of two aromatic rings joined by an ethylene bridge (C_6–C_2–C_6). Over 1,000 stilbenoids have been structurally characterized in the plant kingdom, which primarily vary in the number and position of hydroxyl groups and various substitutions with sugars, methyl, and methoxy groups in addition to the structural conformations of the molecules and oligomerization patterns.

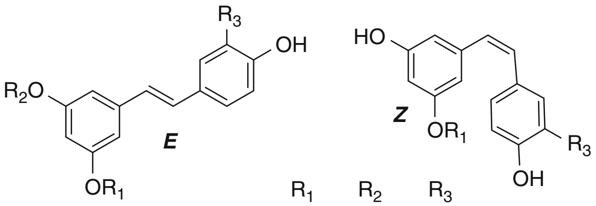
Within the grapevine, *V. vinifera*, including all parts of the plant and cell cultures, there are a little over 60 known stilbenoids. However, the majority of these have not been identified as wine constituents. Of the total known *V. vinifera* stilbenoids, 17 are monomers, all resveratrol and piceatannol derivatives, with eight having been identified in wine. The majority of the stilbenoids are dimers, with 24 dimers known in the plant, but just 9 of these have been reported in wine. In addition to these dimers, there are six trimers in *V. vinifera*; none have been identified in wine. While 16 tetramers are known *V. vinifera* plant part constituents, only one, hopeaphenol, has been found in wine [21].

2.1 Distribution of Stilbenoids

In *V. vinifera*, stilbenoids can either be constitutively expressed or induced through an abiotic or biotic stress. While there are exceptions, the stilbenoids are primarily constitutively expressed in the lignified organs (roots and stems) and are mainly induced substances, serving as phytoalexins, in leaves and berry skins [22, 23]. Within the roots and stems, stilbenoids are found as major constituents. In leaves, stilbenoids have been shown to be highly inducible, and a number of stilbenoids have been identified in infected and treated leaves. The stilbenoids in roots, stems, and leaves have been widely studied due to their importance in disease resistance. While these plant parts are not commonly utilized in the diet, they may be sources of novel stilbenoids with potent biological activities.

2.1.1 Grape Berries

In the berry, stilbenoids are produced primarily in the skin and to a lesser extent, the seeds. The stilbenoid monomers, *E*-resveratrol and *E*- and *Z*-piceid (Fig. 73.1) are



	R_1	R_2	R_3
E/Z-resveratrol	H	H	H
E/Z-piceid	Glc	H	H
E/Z-piceatannol	H	H	OH
E/Z-astringin	Glc	H	OH
E-pterostilbene	CH_3	CH_3	H

Fig. 73.1 Stilbenoids monomers

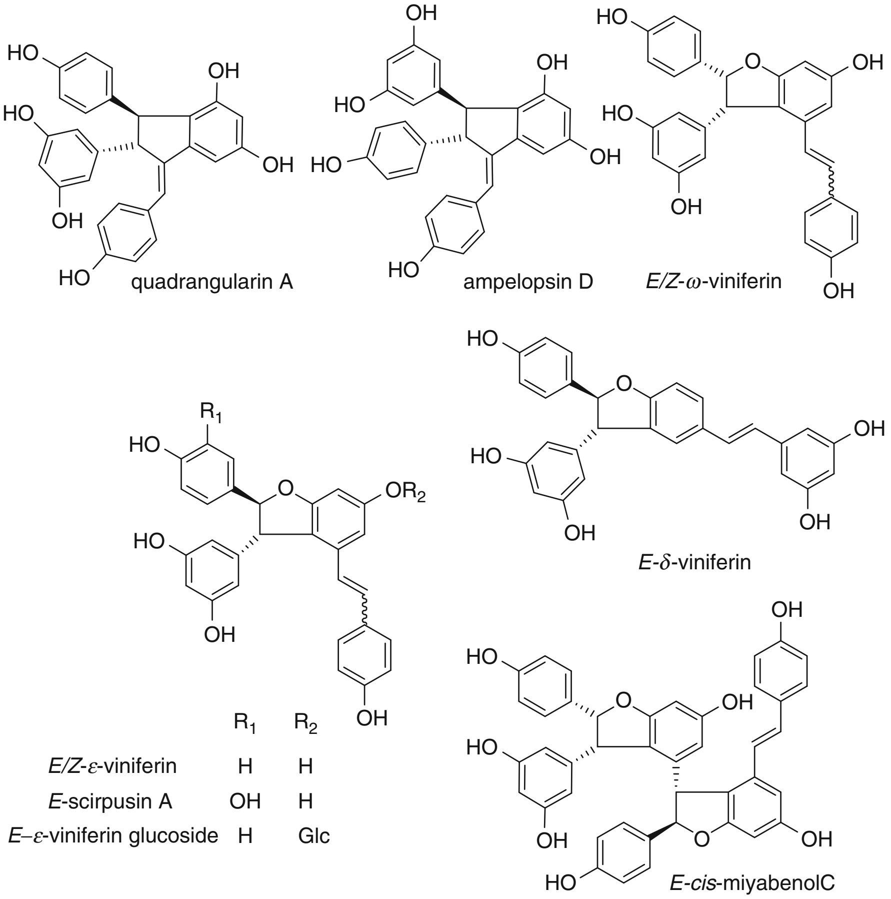

quadrangularin A
ampelopsin D
E/Z-ω-viniferin
E-δ-viniferin
R1 R2
E/Z-ε-viniferin H H
E-scirpusin A OH H
E–ε-viniferin glucoside H Glc
E-cis-miyabenolC

Fig. 73.2 Stilbenoids dimers and trimer

found in grapes, with the concentration of the glucosides usually being significantly higher than the aglycone [1]. An additional monomer, pterostilbene, has also been detected in healthy and immature grape berries, but in low levels [24].

2.1.2 Leaves

Stilbenoids are highly inducible in leaves using a number of chemicals, UV-C treatment, and infectious agents [25]. To date, the majority of stilbenoids from leaves have been identified from stressed leaves. In some of the preliminary studies by Langcake and Pryce using leaves infected by *Botrytis cinerea*, the main stilbenoids detected were *E*-resveratrol, *E*-ε-viniferin (Fig. 73.2), and α-viniferin [26]. The resveratrol dehydrodimer, *δ*-viniferin, and piceids were detected in leaves induced by UV irradiation [27, 28]. More recently, Mattivi et al. isolated and identified 14 stilbenoids from the leaves of a hybrid *V. vinifera* infected with *Plasmopara viticola* [23]. These compounds included two new dimers and two new to *V. vinifera*: *E*-ω-viniferin,

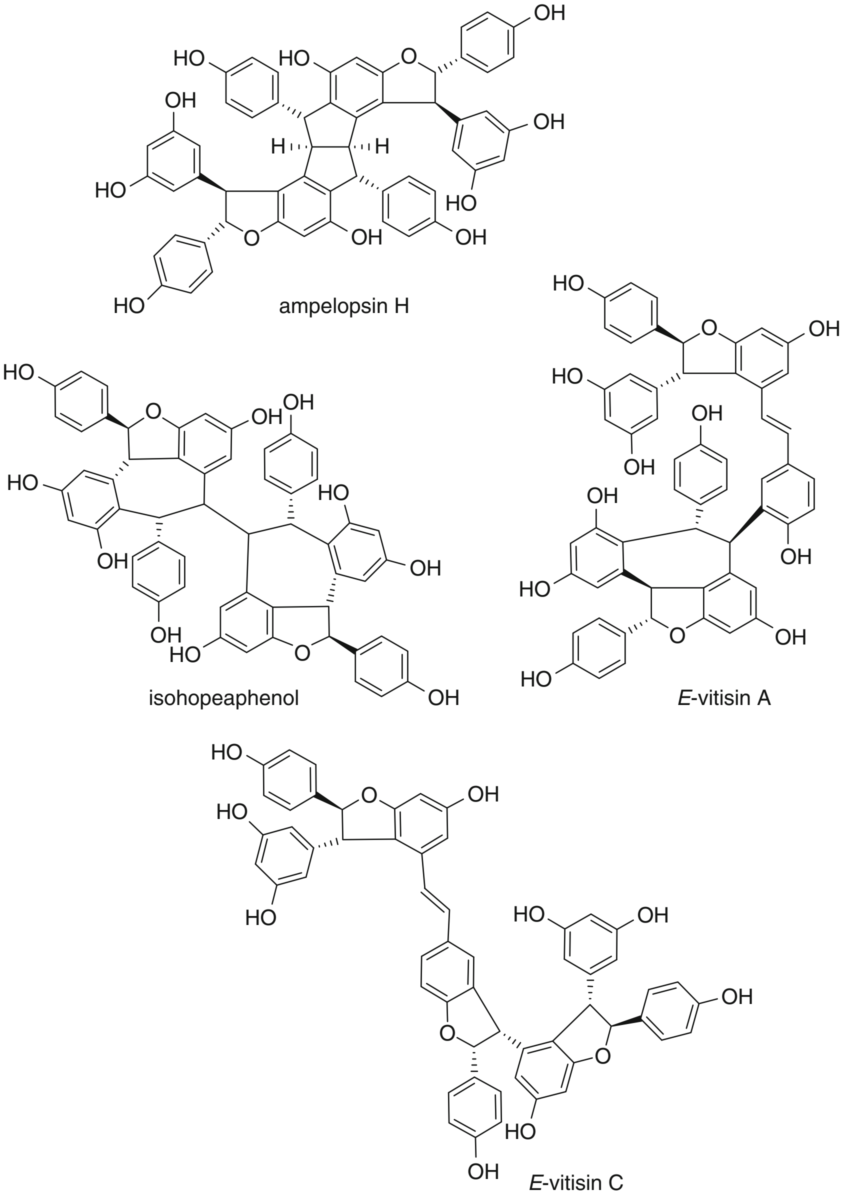


Fig. 73.3 Stilbenoids tetramers

Z-ω-viniferin, ampelopsin D, quadrangularin A, respectively, together with the known dimers: *E*- and *Z*-ε-viniferin, and pallidol. In addition, four trimers were found, *Z*-miyabenol C and *E*-*cis*-miyabenol C new in *V. vinifera*, along with α-viniferin and *E*-miyabenol C. Three tetramers new to grapevine, isohopeaphenol, ampelopsin H, and a vaticanol C-like isomer, were also characterized (Fig. 73.3, [23]).

2.1.3 Stems

In the stems, a greater number of stilbenoids have been isolated and identified than in other plant parts. These include the monomers and dimers *E*- and *Z*- resveratrol, piceatannol, *E*-piceid, (+) − ampelopsin A [29], pallidol [30], scirpusin A [31], ε-viniferin [29], ε-viniferin glucoside [32], viniferifuran [33], and (−)-malibatol A [34], along with the trimers *E*-miyabenol C [35] and (+)-viniferol D [36] and tetramers viniferol A, hopeaphenol, isohopeaphenol [34], viniferol B and C [37], *E*-vitisin A, B, and C [38], and vitisifuran A and B (Fig. 73.3, [33]).

2.1.4 Roots

From the roots, five stilbenoids have been isolated, including resveratrol, ε-viniferin (dimer), gnetin H (trimer), hopeaphenol, and r-viniferin (tetramers) [39].

2.2 Determination of Stilbenoids in Wine

Of the 18 stilbenoids identified in various wines, 13 have been quantified. These molecules include *E*- and *Z*-resveratrol, *E*- and *Z*-piceid, *E*- and *Z*-piceatannol, and *E*- and *Z*-astringin, hopeaphenol, pallidol, parthenocissin A, *E*-ε-viniferin, and *E*-*δ*-viniferin.

Since the first report of the presence of *E*-resveratrol in wine by Siemann and Creasy in 1992, the quantification of these stilbenoids, especially resveratrol, has been carried out by a variety of analytical methods, primarily with HPLC-UV [40] and GC/MS [41–43]. These methods often require prior sample treatment before injection. Nevertheless, HPLC with UV detection has been the most frequently used technique due to its widespread availability. Other quantification techniques include HPLC with a fluorescence detector and LC-MS often with little or no sample preparation required [44].

Resveratrol occurs in wine in free (*E* and *Z*) and glycosidically bound forms. *E*-resveratrol is present in concentrations ranging from trace amounts to 36 mg L^{-1}, averaging 2 mg L^{-1}, in red wines and has been found up to 8 mg L^{-1} in white wines [45]. For the bound forms of resveratrol (piceids), concentrations are reported to be in a range of 0.3–9 mg L^{-1} in red and 0.1–2.2 mg L^{-1} in white wines [41, 46]. Ribeiro de Lima et al. determined piceids and *E*-astringin in Portuguese red wines in concentrations up to 68 mg and 36 mg L^{-1}, respectively [47].

Besides the stilbene monomers, some resveratrol dimers have been characterized from wines. From German commercial Riesling white wines, ε-viniferin diglucosides and pallidol mono- and diglucosides have been identified at very low levels (<0.05 mg/L) [48]. From French commercial red wines, low levels (from 0.5 to 4.8 mg L^{-1}) of *E*-ε-viniferin, parthenocissin A, and pallidol have been isolated [44, 49]. From Brazilian red wines, *trans*-*δ*-viniferin was found, but only in one of the vintages tested (2002), with an average level of 11.7 mg L^{-1} [50].

The only known tetramer to be unambiguously identified in wine, hopeaphenol, has been quantified in Pinot Noir wines from Burgundy in addition to a range of North African wines, where their concentrations have ranged from undetectable levels to 2.1 mg L^{-1} [51, 52].

HPLC-MS has now become the analytical method of choice for the quantitation of stilbenoids in the grapevine and wine. Buiarelli et al. developed a method for the quantitation of several stilbenoid monomers (*E* and *Z* forms of resveratrol, piceid, piceatannol, and astringin) in 19 red and 3 white Italian wines by HPLC-MS-MS using a triple quadrupole mass spectrometer without the need for sample preparation [53]. The detection limits were about 50 ng mL^{-1} for all these monomers.

Red wines contain larger amounts of stilbenoids than white wines, regardless of the enological technology applied. The extent of maceration with skins during fermentation is the main factor determining the concentration of stilbenoids in wines. They generally require long maceration times to be extracted efficiently [42, 54]. The increase of alcohol, due to fermentation during maceration, also promotes the extraction of stilbenoids from the grape skins.

3 Pharmacological Applications and Mechanisms of Action

3.1 Neurodegenerative Diseases

For demographic reason, the percentage of neurodegenerative diseases is increasing. These disorders, which result from deterioration of neurons, are classified into two categories: (1) movement disorder pathologies such as Parkinson's disease and (2) cognitive deterioration pathologies and dementia such as Alzheimer's disease (AD). The majority of neurodegenerative studies have focused on AD, which is the most common type of neurodegenerative disorder, accounting for 65% of all dementias. The prevalence of AD is estimated to be between 1% and 5% among people aged 65 years and doubles approximately every 4 years, where it reaches about 30% at 80 years [55]. The number of people with AD could rise from about 26 million today to more than 100 million worldwide by 2050 [56].

Histopathology studies reveal that one of the major characteristics of AD is the excessive accumulation of two types of proteins, tau proteins and β-amyloid peptide (Aβ) (Fig. 73.4, [57]). β-amyloid originates from proteolytic cleavages of the transmembrane amyloid precursor protein (APP) [58]. APP can be cleaved by different proteases, called α-, β-, and γ-secretases. The α-secretase cleaves APP into a nontoxic amyloid form, whereas β-secretase, followed by γ-secretase, cleaves the APP to form Aβ peptide. Normally, this Aβ peptide is quickly removed from the brain through clearance mechanisms. However, buildup of the Aβ peptide can occur by either overproduction or defective clearance. This buildup can lead to extracellular self-aggregation into assemblies, ranging from soluble high-molecular-weight Aβ oligomers to fibrils and amyloid plaques. These different assemblies promote inflammation and activate neurotoxic pathways, leading to dysfunction and subsequent death of brain cells [56, 59]. Recent studies suggest that the small Aβ oligomers cause more damage to synaptic and cognitive functions than larger amyloid plaques [56]. Moreover, these Aβ oligomers can interact with cell surface receptors which stimulate NF-κB signaling in microglia (resident immune cells) and astrocytes, triggering the release of inflammatory and neurotoxic mediators [60].

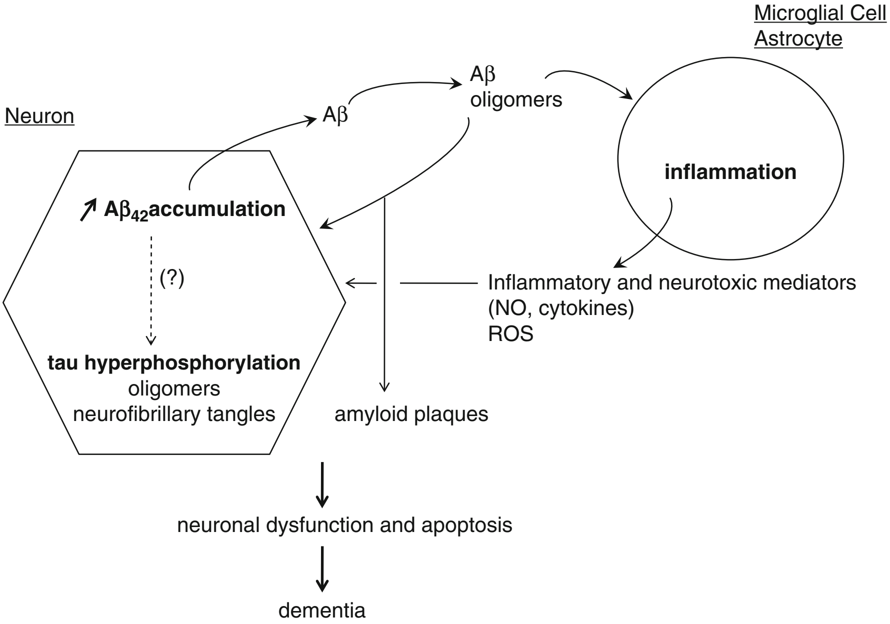

Microglial Cell
Astrocyte
Aβ
oligomers
Neuron
Aβ
inflammation
↗ Aβ42accumulation
(?)
Inflammatory and neurotoxic mediators
(NO, cytokines)
ROS
tau hyperphosphorylation
oligomers
neurofibrillary tangles
amyloid plaques
neuronal dysfunction and apoptosis
dementia

Fig. 73.4 Main key players involved in AD

Proinflammatory cytokines can interact with the processing and production of Aβ peptide in neurons, suggesting that chronic, unresolved inflammation may be the initial steps in AD in certain individuals [61]. Indeed, the inflammatory activity of immune cells (macrophages and microglia) and of astrocytes increases with age, which is the most important risk factor for AD [56]. The accumulation of soluble Aβ oligomers is also accompanied by markedly increased intracellular calcium, due to the upregulation of calcium channels expression, leading to neuronal cell dysfunction and death [62]. The second major protein implicated in Alzheimer's pathology, tau protein, is a microtubule binding protein, which can become detached from microtubules after abnormal hyperphosphorylation and also self-aggregate into oligomers and inclusions, known as neurofibrillary tangles, within neurons. The presence of inclusions gradually filling the intracellular space, combined with destabilization of the microtubules, can seriously impair neuronal function and kill neurons [63]. Studies support that Aβ peptide can facilitate tau pathology [64], but Small and Duff hypothesize an alternative model that links Aβ elevations and tau hyperphosphorylation by separate mechanisms driven by a common upstream molecular defect [65].

3.2 Wine and Neurodegenerative Diseases

To test the ability of red wine consumption on the development of an Alzheimer's disease, two different wines were administered to mice in two separate studies.

For both studies, the transgenic mice (Tg2576), a model of Alzheimer's disease was used. These mice manifest Aβ peptide accumulation in the brain after 7 months of age. In the first study, Wang et al. used a Cabernet Sauvignon in drinking water with a final ethanol concentration of 6%, from 4 to 11 months [66]. Red wine consumption was compatible with general good health of the mice and attenuated spatial memory decline and Aβ neuropathology. The concentration of Aβ peptides and amyloid plaque burden in the neocortex and hippocampus of treated mice was notably reduced in comparison with ethanol controls. Red wine promoted the non-amyloidogenic pathway, through an increase in α-secretase activity, which may prevent the synthesis of toxic Aβ peptides. The quantity of red wine consumed by mice was equivalent to a human daily intake of two to three glasses, which is a moderate consumption. The role of resveratrol in this study was not well established because the wine used contains only a very low level of resveratrol (0.2 mg/L) and the analysis of additional stilbenoids was not performed. In a second study, these authors tested a wine from another species, Muscadine wine made from *V. rotundifolia*. Muscadine wine has been found to contain resveratrol, but very little additional chemical studies have been performed on this plant. Using HPLC-UV and comparison with known standards, the authors demonstrated a distinctive chemical profile of major compounds from the previously used Cabernet Sauvignon wine [67]. Muscadine treatment also attenuated AD phenotype in AD mouse model but through a different mechanism. Both treatments inhibited the aggregation of Aβ peptides, but the α-secretase pathway was not promoted by Muscadine. These studies suggest that specific polyphenols from the different red wines may beneficially modulate AD phenotypes through multiple Aβ-related mechanisms. Consuming a combination of multiple polyphenols with complementary activities, present in foods and/or drinks, may be optimal for AD prevention. These authors also tested a grape seed polyphenol extract comprised of catechins in monomeric (8%), oligomeric (75%), and polymeric (17%) forms, using the same transgenic mouse model of AD [68]. When orally administered to the mice at a dose equivalent to 1 g day^{-1} in humans, this extract significantly reduced the level of Aβ peptides in the brain, mainly the soluble extracellular high-molecular-weight oligomeric Aβ species, considered a major risk factor for the onset and the progression of AD dementia. The extract might exert this effect through the prevention of Aβ oligomerization, as shown in vitro [68]. A reduction in amyloid plaque burden was also found. These beneficial effects were correlated with the attenuation of cognitive impairments.

3.3 Stilbenoids and Aβ peptides

Several studies have investigated the effects of resveratrol and of several other stilbenoids on AD for their ability to modulate multiple mechanisms of AD pathology [60]. In vitro studies have shown that resveratrol may protect against the Aβ peptide-induced toxicity in PC12 neuronal cells or primary neurons by influencing apoptotic signaling pathways, reducing changes in mitochondrial membrane potential, and inhibiting the accumulation of reactive oxygen species [69–72]. The stilbenoid tetramer, vitisin A, and piceatannol exert much stronger protective effects than resveratrol on Aβ-induced PC12 cell death [71, 73]. Marambaud et al. also showed that resveratrol markedly lowers the levels of secreted and intracellular Aβ peptide produced in different APP-transfected cell lines, but resveratrol did not reduce Aβ synthesis due to its lack of effect on the β- and γ-secretases [74]. The treatment of these cells with selective proteasome inhibitors significantly blocked the resveratrol-induced decrease of Aβ, which demonstrated a potential proteasome-dependent anti-amyloidogenic activity of resveratrol. In another study, however, Jeon et al. reported that resveratrol and scirpusin A have a relatively specific inhibitory activity on β-secretase [75]. These results all together suggest that resveratrol and other stilbenoids may protect neurons against Aβ injuries.

Recently, a therapeutic approach that interferes directly with the neurodegenerative process in AD, particularly the accumulation and the aggregation of Aβ, is considered one of the most promising targets to alter the progression of the disease, rather than merely treating the symptoms [76]. Several studies suggest that polyphenols could prevent AD or delay its onset by directly inhibiting the Aβ aggregation and the formation of Aβ fibril deposits in the brain [77, 78]. This would be particularly advantageous since monomeric Aβ could act as natural antioxidant that prevents neuronal cell death caused by oxidative stress, whereas Aβ oligomers and fibrils are neurotoxic [79]. Using in vitro assays, we recently reported that resveratrol derivatives inhibit the aggregation of the peptide (25–35 fragment) and destabilize the preformed oligomers and fibrils [80–82]. Initial screening of 4 monomers, 4 dimers, and 6 stilbenoid oligomers from grapevine for inhibition studies was performed at a concentration of 10 μM, with curcumin as the positive control. Five stilbenoids exhibited peptide aggregation inhibition activity better than that of curcumin. These were further tested to determine their IC_{50} values. The IC_{50} values of all compounds are summarized in Table 73.1.

Among all the compounds tested, resveratrol, piceid, and the dimers, scirpusin A and ε-viniferin glucoside, exhibited the most significant inhibition of Aβ aggregation. Of the monomers tested, resveratrol and its glucoside (piceid) showed the strongest inhibition of the peptide aggregation. Examination of the inhibitory data for the stilbene monomers suggests some potential structure-activity relationships (Table 73.1) [82]. The presence of a single hydroxyl group in the *para* position on ring B and at least one hydroxyl group on ring A is essential for the protective activity against Aβ aggregation, suggesting that hydrogen bonds might play an important role in the binding process. Of the three stilbene dimers, two had considerable inhibitory activity: scirpusin A (0.7 ± 0.3 μM) and ε-viniferin glucoside (0.2 ± 0.3 μM). These two compounds differed only by two substituents. Scirpusin A had an additional hydroxyl group, whereas ε-viniferin glucoside has a glucose unit. It is very difficult to draw any conclusions on the structure-activity relationship using this limited data set. Nevertheless, their strong inhibitory activity in vitro warrants further investigation into their

Table 73.1 Inhibitory activity of stilbenoids on βA fibril formation

Compound	Inhibition %	EC_{50} (μM)
Curcumin	**45** ± **9**	**10** ± **2**
Monomers		
Resveratrol	**63** ± **6**	**6** ± **2**
Piceid	**62** ± **6**	**6** ± **2**
Piceatannol	25 ± 9	–
Pterostilbene	35 ± 7	–
Dimers		
Ampelopsin A	**46** ± **6**	**10** ± **2**
Scirpusin A	**80** ± **9**	**0.7** ± **0.3**
E-ε-viniferin	25 ± 9	–
E-ε-viniferin glucoside	**93** ± **3**	**0.2** ± **0.3**
Oligomers		
Miyabenol C	15 ± 5	–
Nepalensinol B	17 ± 7	–
Hopeaphenol	13 ± 6	–
Isohopeaphenol	21 ± 9	–
Vitisin C	32 ± 9	–
Viniferol E	17 ± 10	–

Bold values indicate the molecules exhibiting inhibitory activity at least equal to that of curcumin reference

potential as therapeutic agents in AD treatment and prevention. Unlike the stilbene dimers, the stilbene oligomers, both the trimers and tetramers, were weak inhibitors. These results suggest that spatial constraints are critical in the binding process. However, other oligomers need to be tested to confirm that bulkier compounds lack activity since results indicate that the inhibitory effect depends not only on the specific ring substituents but may also depend on the overall 3D structure [81].

Since the full-length Aβ (1–40) and Aβ (1–42) peptides are more biologically relevant than the fragment Aβ (25-35) peptide, we then investigated the effects of ε-viniferin glucoside on their aggregation and cytotoxicity [83]. ε-Viniferin glucoside inhibited the fibrillization and the toxicity in PC12 cells of both full-length peptides with the same level as the fragment peptide. Using mass spectrometry (Fig. 73.5), we observed non-covalent complex between ε-viniferin glucoside and Aβ (1–40) peptide at 2/1 stoichiometry. Therefore, ε-viniferin glucoside could act on Aβ polymerization at an early stage in the process. The binding may lock the peptide conformation and thus prevent nucleation. NMR experiments will be conducted to extend the molecular knowledge of this interaction.

When orally administered to a transgenic mouse model (Tg19959) of Alzheimer's disease at a dosage of 300 mg/kg for 45 days, resveratrol reduces plaque formation mainly in medial cortex (−48%), striatum (−89%), and hypothalamus (−90%) without alterations in APP processing or SIRT-1 activation [84].

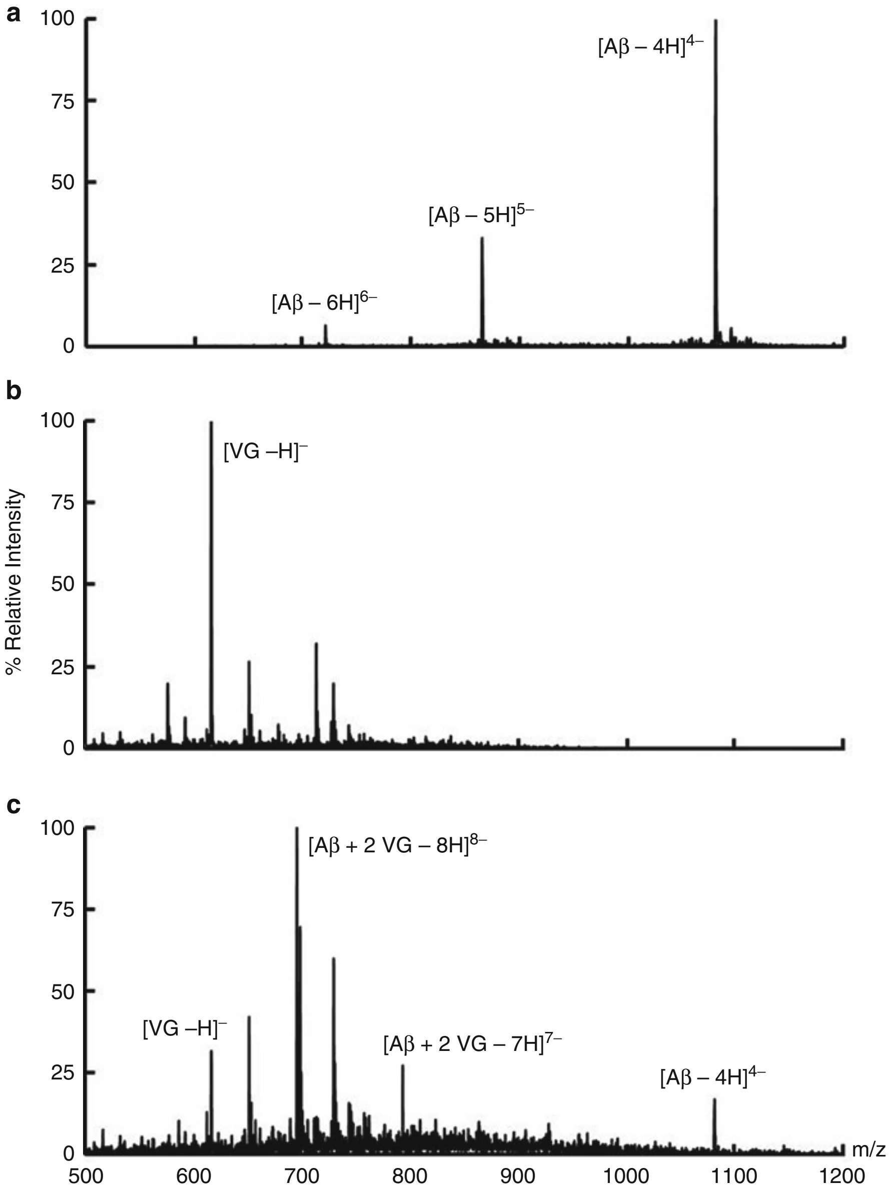


Fig. 73.5 Electrospray ionization mass spectra of (**a**) Aβ (1–40) peptide at 10 μM; (**b**) ε-viniferin glucoside (VG) and (**c**) the mixture of Aβ (1–40) and VG (1:1 molar ratio, 10 μM)

However, resveratrol was not detectable in the brain, but the precise mechanisms by which it reduces Aβ pathology was not elucidated; however, it did alter brain glutathione levels. Using a scaling factor of 0.08, the authors find a human equivalent of 1.68 g per day for a 70-kg person.

3.4 Resveratrol and Tauopathies

To test the effect of resveratrol on Alzheimer's disease and tauopathies, the inducible p25 transgenic mouse model [85, 86] was used. For this study, resveratrol was dosed via intra-cerebroventricular injection for 3 weeks, which prevented neurodegeneration in the hippocampus and cognitive decline [87]. Moreover, resveratrol reduced the extent of cell death caused by p25 in transfected primary neurons. SIRT-1 deacetylase was found to be involved in these resveratrol's neuroprotective effects in both the in vitro and in vivo studies.

3.5 Stilbenoids and Neuroinflammation

Inflammatory processes within the nervous system are driven by mononuclear microglial cells and are thought to play an important role in the pathogenesis of neurological disorders, including meningitis, encephalitis, and Alzheimer's and Parkinson's diseases [88]. Microglia are normally quiescent but can be activated by exposure to bacterial lipopolysaccharide (LPS) or Aβ [89, 90]. Thus, activated microglia are capable of producing a variety of proinflammatory mediators including proinflammatory cytokines such as interleukins (IL-1β, IL-6) and tumor necrosis factor (TNF)-α, reactive oxygen species (ROS), nitric oxide (NO), and prostaglandin E_2 (PGE_2) [91, 92]. Although NO and ROS contribute to the killing of microbial pathogens, they also interact to form peroxynitrite, which is toxic to the neurons when cocultured with LPS- or Aβ-stimulated microglia [93]. In addition, they can exacerbate proinflammatory cytokine production in response to these stimuli [94]. Since proinflammatory cytokines can, in turn, contribute to the synthesis of NO, this may lead to a self-propelling circle of NO, ROS, and proinflammatory cytokine production and uncontrolled inflammation [91].

A number of stilbenoids have been tested using an activated microglia model. In vitro studies show that resveratrol inhibits LPS-induced TNF-α, NO, iNOS expression, and ROS production in microglia [72, 95–97] by inhibiting IκB degradation, which prevents the nuclear translocation of the transcription factor NF-κB [96]. This activity was also found with piceatannol and pterostilbene [92, 97]. Resveratrol also potently inhibited prostaglandin E_2 production in LPS-activated primary rat microglia without affecting COX-2 expression in this model [98]. In our lab, we have evaluated the effect of several stilbenoids from grapevine and wine at 10 μM concentration on NO, TNF-α, and ROS production by LPS-activated BV2 microglia (Table 73.2). Hopeaphenol, and *E*-vitisin A were found to be toxic to BV-2 cells, whereas *Z*-piceid, ampelopsin A, quadrangularin A, and *E*-miyabenol C were not active. Ten stilbenoids were effective in reducing LPS-induced nitrite production at 10 μM, with the percentage of inhibition ranging from 10.9% to 89.1%. The IC_{50} values of *E*-vitisin B, *E*-piceatannol, *E*-ε viniferin, *E*-δ-viniferin, and *E*-resveratrol were all less than 15 μM, at 6.4, 7.8, 8.3, 12.4, and 13.9 μM, respectively. Among these compounds, only *E*-vitisin

Table 73.2 Inhibitory activity of stilbenoids on NO production induced by LPS in microglial cells

	Compound	Inhibition (%)[a]	IC_{50} (μM)
Monomers	*Z*-piceid	NA	
	E-resveratrol	42.2 ± 6.3***	13.9
	E-piceid	13.7 ± 8.4	
	E-resveratrol-2-*O*-*β*-glucoside	NA	
	E-piceatannol	63.2 ± 9.2***	7.8
	E-astringin	11.2 ± 8.2	
	E-oxyresveratrol	10.9 ± 1.0	
Dimers	*E*-*δ*-viniferin	37.3 ± 5.3*	12.4
	E-ε-viniferin	49.7 ± 9.3***	8.3
	E-scirpusin A	25.3 ± 6.7**	
	Pallidol	29.5 ± 8.8*	
	Ampelopsin A	NA	
	Quadrangularin A	NA	
	Leachianol **F**	NA	
	Leachianol **G**	NA	
Trimers	*E*-miyabenol C	NA	
Tetramers	Hopeaphenol	T	
	E-vitisin A	T	
	E-vitisin B	89.1 ± 0.8***	6.4

BV-2 cells were treated with LPS at 1 μg/mL and stilbenes at 10 μM concentration

[a] Means the inhibition (%) of NO production relative to the LPS control. Concentration of production of NO in cells alone was (1.0 ± 0.5 μM) and in cells activated with 1 μg/mL of LPS was (9.3 ± 2.1 μM)

T, means cytotoxic effect was observed; NA, means not active

***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ are significantly different from the value in BV-2 treated with 1 μg/mL of LPS

All compounds were examined in a set of experiments repeated at least four times

B significantly reduced LPS-induced ROS (29.0%) and TNF-α (41.9%) production. Our findings indicate that glycosylation decreases the activity of *E*-resveratrol monomers. Similarly, the presence of an *ortho*-hydroxy structure on ring B is relevant for monomer activity, if we compare those of *E*-resveratrol and *E*-piceatannol. Interestingly, the stilbenoid tetramer, *E*-vitisin B, was more effective than the other stilbenoids tested. However, as stated previously, *E*-vitisin A, which only slightly differs from *E*-vitisin B, was found to be cytotoxic at 10 μM to BV-2 microglia. We therefore investigated the effects of a lower concentration of *E*-vitisin A and found that 5 μM reduced NO production by LPS-activated BV-2 cells (71%) without being cytotoxic. These findings are in accordance with those obtained in the RAW 264.7 macrophage cell line [99]. Since *E*-vitisin B and *E*-vitisin A are both tetramers derived from *E*-resveratrol, they may exert anti-inflammatory effects similar to resveratrol through decreased activation of the NF-κB signaling pathway, thus reducing the expression of TNF-α and iNOS.

Vitisin B is a stilbenoid with promising antioxidant and anti-inflammatory effects that may prove effective in slowing down microglia-driven neuroinflammatory processes.

In vivo studies show that dietary supplementation of resveratrol reduced the LPS-induced deficits in spatial working memory in aged mice and also the production of interleukin-1β (a proinflammatory cytokine) in the periphery and the brain. In addition, resveratrol was shown to inhibit interleukin-1β production by LPS-stimulated microglia in vitro [100]. In healthy volunteers, the activation of NF-κB in circulating peripheral mononuclear cells was prevented by the moderate consumption of alcohol drinks containing polyphenols such as red wine during a fat-enriched diet [101]. In a gerbil ischemia model, administration of resveratrol during the early stage of cerebral ischemia protects against neuronal cell death in the hippocampal CA1 area and concomitantly inhibits glial cell activation [102]. Again, in a colchicine-induced Alzheimer's disease model, chronic treatment with orally administered resveratrol (10–20 mg kg^{-1}) greatly reduced the cognitive impairment and oxidative stress markers in the rat brain [103].

Stilbenoids, and resveratrol in particular, appear to interact with various molecular mechanisms involved in AD and thus exert neuroprotective properties.

4 Toxicity of Resveratrol

Safety studies have been conducted on *E*-resveratrol synthesized by DSM Nutritional Products Ltd. in multiple in vitro and in vivo animal studies (>99% purity) [104]. It was shown in this work that resveratrol is nonirritating to the skin and eyes of rabbits and non-sensitizing. It was non-mutagenic in the Ames test with *Salmonella typhimurium* and *Escherichia coli*. A positive clastogenic activity was found in a chromosome aberration test with human lymphocytes, but in an in vivo micronucleus test in rats, with a dose up to 2 g kg^{-1}, resveratrol was not genotoxic. To evaluate the possible oncogenicity of resveratrol, p53 knockout mice were treated daily by gavage for 28 consecutive days [105]. These mice deficient in the tumor suppressor gene p53 showed no increase in the incidence of malignant or benign tumors at the maximum tolerated dose, 1 g kg^{-1} day^{-1}. No evidence of oncogenicity was found with this model. Higher doses (2 or 4 g kg^{-1} day^{-1}) induced toxicity and mortality. The target organ was mainly the kidney [105, 106]. Histopathology showed hydronephrosis and also urothelial hyperplasia [105].

Some studies have found that resveratrol exhibited a weak estrogenic activity in vitro [107]. Indeed, resveratrol was shown to have a low affinity for the estrogen receptor, about 5 orders of magnitude lower than diethylstilbestrol and estradiol [104]. However, a recent in vivo uterotrophic assay with immature female rats was negative [108]. Moreover, the administration of resveratrol at a dose of 0.75 g kg^{-1} day^{-1} to pregnant rats did not induce any adverse effects on embryo-fetal survival and development [104]. The no observed adverse effect levels (NOAEL) for resveratrol obtained in different general toxicity studies in rats, rabbits, and dogs, from subacute to chronic in duration, 1–6 months, are always dosed between 0.25 and 0.75 g kg^{-1} day^{-1} [104, 108, 109]. These data show a good interspecies concordance, and no increase in the toxicity of resveratrol has been observed with the increase of the treatment duration. Due to these studies, the 0.75 g kg^{-1} day^{-1} dose was defined as the NOAEL for resveratrol [108]. To calculate the acceptable daily intake (ADI) in human, a standard default safety factor of 100 is often applied to data from toxicity studies, which gives an ADI of 0.45 g^{-1} day^{-1} for a 60-kg individual. However, the adverse effects in humans have been investigated in some studies after ingestion of higher doses of resveratrol, up to 5 g^{-1} day^{-1} for a single dose and for chronic administration [110–112]. The consumption of resveratrol by humans at these doses did not cause serious adverse events in these studies. For example, after 29 daily doses of 0.5, 1, 2.5, or 5 g resveratrol, healthy volunteers exhibited mild adverse effects only for the doses in excess of 1 g per day [112]. The majority of the participants on the two highest dose levels presented at least one adverse effect. The most common toxicity possibly due to resveratrol was gastrointestinal including nausea, flatulence, abdominal discomfort, and diarrhea. Typically, the onset of gastrointestinal symptoms was 1 h after ingestion with improvement throughout the day. It was also found that resveratrol at a dose of 1 g day^{-1} might manifest adverse metabolic drug interactions in inhibiting certain cytochrome P450 isoenzymes, which could potentially lead to increased adverse drug reactions or altered drug efficacy in people on medication [113].

5 Bioavailability and Metabolism

5.1 In Vivo and In Situ Studies in Rodents

Since 1995, several studies have contributed to the clarification of *E*-resveratrol bioavailability. The first experiments on absorption and distribution of resveratrol in vivo were performed by Bertelli and coworkers in rats [114]. They evaluated the concentration of resveratrol in the plasma, urine, heart, liver, and kidney after oral administration of 4 mL of red wine containing 6.5 mg L^{-1} of resveratrol. The result shows that resveratrol is quickly absorbed, reaching its peak concentration (20 ng mL^{-1}) approximately 60 min after wine ingestion, with initial resveratrol concentration observed after 30 min. In the liver and kidneys, the highest concentrations were reached after 1 h. A second group of rats was given a daily dose of 2 mL of red wine containing 6.5 mg L^{-1} of resveratrol for 15 days. The values obtained indicate a higher concentration in the different organs, particularly in the liver. However, the authors suggest that the amount of resveratrol found in these different tissues was lower than that required for pharmacological activity.

Numerous studies have shown that dietary polyphenols are subjected to metabolic conversion in the liver and also during their absorption in the intestine before reaching the systemic circulation. Andlauer et al. investigated the absorption of resveratrol using an isolated preparation of luminally and vascularly perfused rat small intestine [115]. They showed that 46% of the luminally administered resveratrol was extracted by the small intestine and 21% appeared on the vascular side. The majority of the absorbed resveratrol was two glucuronide derivatives (80%). However, they did not identify the glucuronidation sites of these stilbenoids. In our laboratory, we identified two monoglucuronides of resveratrol using human liver microsomes, which corresponded to glucuronidation at positions 3 and 4′ [116].

Several studies describe the bioavailability of labeled resveratrol in vivo. Soleas et al. used tritiated resveratrol and showed that 77–80% of this stilbenoid may be absorbed in the rat intestine [117]. However, only trace amounts of radioactivity were detectable in the liver, kidney, heart, or spleen. In our laboratory, Vitrac et al. investigated the absorption and tissue distribution of ^{14}C-resveratrol following oral administration to mice [118]. The concentrations of drug-related radioactivity in the various organs are measured. Three hours after administration, radioactivity was found in the brain, lung, heart, liver, kidney, spleen, duodenum, colon, and testis. The highest concentration of radioactivity per organ was found in the duodenum. The kidneys contained the second highest amount of labeled resveratrol, followed by the lungs and liver. Substantial amounts were present in the colon and spleen, while moderate radioactivity was present in the heart, testis, and brain. The low concentration of radioactivity in the colon suggested that there are low levels of fecal elimination. On the contrary, decreasing concentrations of radioactivity in kidney over time indicates that renal excretion might be one of the major modes of elimination of the ^{14}C labeled resveratrol, which was also supported by the high concentrations found in urine. We also found that the concentration of radioactivity in whole blood was relatively low and constant during the experiment period (6 h). The observations were consistent with the recent results found by Juan et al. [119]. Ninety minutes after intravenous administration of 15 mg kg^{-1}, resveratrol and its conjugates were widely distributed in all the tissues studied (liver, kidney, lungs, testis, and brain). The highest concentrations were found in kidney and the lowest in brain.

5.2 Studies in Human

After these in vivo and in situ experiments in animals, the absorption of resveratrol by humans was performed. Soleas et al. [120] was the first to measure free and conjugated resveratrol in plasma and urine. Volunteers were given 25 mg of resveratrol orally in 120 mL of white wine. The plasma concentrations of free resveratrol and glucuronide and sulfate conjugates peaked at 30 min. At this time, the concentration of resveratrol conjugates was 48-fold higher than the free resveratrol (338 μg L^{-1} vs. 7.1 μg L^{-1}). Over the 24-h collection period, 24.6% of the resveratrol was recovered in the urine, predominantly in conjugated form. Walle et al. evaluated the absorption after oral administration of 25 mg of ^{14}C-labeled resveratrol [121]. They determined a peak plasma resveratrol equivalent concentration, based on total radioactivity, of 491 μg L^{-1} at 1 h after the dose.

In these two studies, the plasma concentration of the free resveratrol aglycone was less than 2% of the total resveratrol concentration. Walle et al. showed that most of the radioactivity after oral dosing was recovered in urine (53–85%) but also in feces (0.3–38%) [121]. For the structure identification of resveratrol metabolites in urine, a larger unlabeled dose (100 mg) was given to one subject. Five major metabolites were identified, including two resveratrol monoglucuronides, a dihydroresveratrol monoglucuronide, a resveratrol sulfate, and a dihydroresveratrol sulfate. The sulfate conjugates excreted in urine were the major metabolites. Intestinal microflora was suggested as the potential source of hydrogenated resveratrol metabolites formed by saturation of the aliphatic double bond. Boocock et al. determined six major conjugated metabolites in the plasma and urine of human volunteers after administration of an oral dose of 1 g: two monosulfate conjugates, one disulfate, two monoglucuronides, and one glucuronide sulfate [122]. Two more conjugates (one disulfate and one diglucuronide) were identified in plasma and urine from nine volunteers after oral administration of 85.5 mg of piceid, the major resveratrol glucoside found in wine and grapes, by Burkon and Somoza [123].

Some studies aimed to characterize the effect of matrix and food on the absorption of resveratrol. Golberg et al. reported no major differences among the three matrices used, grape juice, white wine, and V-8 homogenized vegetable cocktail, containing 25 mg of resveratrol [124]. It appears that the biggest impact on resveratrol pharmacokinetics occurs when administered with a high-fat source. La Porte et al. who investigated the pharmacokinetics of 2 g of resveratrol administered twice daily with food, showed that a high-fat breakfast decreased the area under the plasma concentration–time curve from 0 to 12 h and maximum plasma concentration by 45% and 46%, respectively, when compared with the standard breakfast [125]. These data suggest that food has an important effect on the systemic exposure of resveratrol.

In a dose escalation study, Boocock et al. evaluated the safety and pharmacokinetics of resveratrol administered as a single dose (0.5, 1, 2.5, or 5 g) [111]. The peak plasma concentration of the parent molecule across the four dose levels ranged from 73 to 539 ng mL^{-1} (0.3–2.4 μM). The authors suggest that these concentrations are markedly below the resveratrol concentration required in in vitro experiments to elicit pharmacological effects associated with cancer chemoprevention (>5 μM). The most abundant metabolites were resveratrol 3-sulfate and two resveratrol monoglucuronides, with area under the plasma concentration curve values up to 23 times greater than those of resveratrol.

Since the first investigations on the bioavailability of resveratrol, the improvement of analytical techniques has allowed increasingly lower detection limits of resveratrol, in addition to allowing the identification and measurement of its metabolites. While there is great heterogeneity in the experimental conditions used in research on resveratrol in humans, a few conclusions can be made. Upon oral administration, resveratrol is rapidly absorbed, yielding peak plasma concentration between 0.5 and 2 h post dose. Resveratrol has a low bioavailability even with high doses. The low concentration of the free form in plasma is due to a rapid and extensive metabolism in the intestine and liver before distribution to various tissues. The main metabolites described in plasma and urine are the conjugated metabolites of resveratrol (glucuronic acid and/or sulfate), but some authors also identified conjugates of dihydroresveratrol.

5.3 Levels of Resveratrol and Its Metabolites in Target Tissues

Currently, some questions remain unanswered. For example, in vivo studies in rodents report the parent compound and metabolites in target tissues. Abd El Moshen et al. described the tissue distribution of ^{3}H-resveratrol in rat following oral administration of 50 mg kg^{-1} [126]. They detected resveratrol and its glucuronide conjugate in plasma and tissues (kidney, liver, heart, lung, testis, and brain). More recently, Juan et al. reported that resveratrol glucuronide was identified as the major metabolite after an intravenous administration of resveratrol (15 mg kg^{-1}), but the sulfated form was also widely distributed (in lower levels than resveratrol or the glucuronide) [119]. In humans, only one study has quantified resveratrol and its metabolites in target tissue, where it was examined in tumor and normal colon tissues [127]. In this study, 20 patients with histologically confirmed colorectal cancer consumed eight daily doses of resveratrol at 0.5 or 1 g before surgical resection. Parent compounds plus two monoglucuronides, two sulfates, one disulfate, and one resveratrol sulfate glucuronide were identified. The highest mean concentrations of resveratrol and metabolites are found in normal tissue localized proximal to the tumor. These concentrations reached 674, 86, 67 nmol g^{-1} for resveratrol, resveratrol-3-glucuronide, and resveratrol-3-sulfate, respectively. These authors suggested that daily oral doses of resveratrol produce levels in the human gastrointestinal tract at a level sufficient to elicit anticarcinogenic effects. Even if some quantitative differences exist between human and rodent studies, it seems that resveratrol and its glucuronide and sulfate conjugates are present in organs. Recent data suggests that the 3- and 4′-sulfates engage several mechanisms consistent with anticancer activity [128, 129]. However, more investigations are needed in the evaluation of the potential biological activity of resveratrol metabolites. On the other hand, some authors have suggested that sulfatases and ß-glucuronidases could convert the metabolites back to resveratrol in human target tissues and that other stilbenoids, such as piceid and oligomers of resveratrol, may represent alternative forms of resveratrol administration [123, 130].

6 Other Biological Activities

Stilbenoids have a wide range of pharmacological and biological actions, primarily in regard to cancer and cardiovascular diseases. Moreover, resveratrol appears to mimic calorie restriction and extend lifespan in certain species.

6.1 Cancer Chemoprevention

Clifford et al. reported that a diet with red wine solids delayed the onset of tumors in transgenic mice, which spontaneously develop externally visible tumors without carcinogen pretreatment [131]. Systemic administration of resveratrol has been shown to inhibit the initiation and the development of tumors in about 30 rodent cancer models, but there are a few exceptions in which no benefit has been found [132]. Moreover, the study of tumorigenesis in a mouse skin cancer model showed that topical application of resveratrol reduced the number of skin tumors per mouse by up 98% and lowered drastically the percentage of mice with tumors [133]. Resveratrol also exerts protective effects against ultraviolet radiation, mediating oxidative stress and cutaneous skin damage, including skin cancer [134].

Additionally, several reports indicate that *E*-resveratrol inhibits the proliferation of a wide variety of human cancer cells including breast, prostate, colon, gastric, lung, pancreatic, liver, thyroid, and ovarian cancers, leukemia, lymphoma, osteosarcoma, squamous cell carcinoma, multiple myeloma, and medulloblastoma [135]. Among the other stilbenoids, piceatannol, α- and ε-viniferin, hopeaphenol, pallidol, ampelopsin A, vaticanol B and C, and pterostilbene also showed cytotoxicity and/or antiproliferative effect on different tumor cell lines [136–145].

The cancer chemopreventive activity of *E*-resveratrol was established in various assays reflecting the three major stages of carcinogenesis [133, 146]. Indeed, resveratrol may inhibit carcinogenesis by affecting the initiation, promotion, and progression of cancer through several different mechanisms, via the modulation of signal transduction pathways that control cell division, apoptosis, inflammation, angiogenesis, and metastasis [135, 147–149]:

- The anti-initiation activity was demonstrated by its antioxidant and antimutagenic effects, inhibition of carcinogen bioactivation, induction of phase II drug-metabolizing enzymes, and the stimulation of DNA repair.
- The anti-promotion activity was shown by its blocking action of the stimuli-mediated MAPK pathway activation, inhibition of polyamine synthesis and increase of polyamine catabolism, and inhibition of the production of proinflammatory mediators via cyclooxygenase-2 and lipoxygenase pathways. Resveratrol may also inhibit NF-kB and AP-1 activation.

Resveratrol can modulate the major cell cycle mediators, including cyclins, cyclin-dependent kinases, and inhibitor proteins which lead to arrest the cell cycle through mechanisms varying with the cancer cell type, in G1, S, or G2/M phase; and can induce apoptosis through the p53-dependent mechanism in several cancer cell lines. Previously, we described a cell surface resveratrol receptor on the extracellular domain of integrin αVβ3 in breast cancer cells. Binding of resveratrol to integrin, principally to the β3 monomer, was essential for transduction of the stilbene signal into p53-dependent apoptosis of these cells [150]. Resveratrol may also act through p53-independent mechanism in some cell types.

- The inhibition of progression and invasion mechanisms has also been reported. Resveratrol can affect the expression of the inducible nitric oxide synthase gene, which is partly controlled by NF-kB and thus reduces the abnormal level of NO, which contributes to inflammation and angiogenesis. Moreover, resveratrol can inhibit angiogenesis through the inhibition of polyamine synthesis, the vascular endothelial growth factor, and of the expression of adhesion molecules and matrix metalloproteinases, which is also involved in tumor invasion and metastasis. In vivo studies show the inhibition of tumor-induced neovascularization and metastasis by resveratrol. *E*-Resveratrol markedly reduced proliferation and migration of liver myofibroblasts, major actors in the development of liver fibrosis and cancer progression. It can also deactivate human liver myofibroblasts [151]. From numerous studies using various liver cancer cell lines, chemically induced tumors, as well as implanted cancer in animal models, it now appears that resveratrol is one of the most promising molecules for the prevention and also the therapy of metastatic diseases of the liver [152].

Resveratrol could also affect cancer stem cells based on cancer stem cell theory [153]. This theory asserts that several human cancers are initiated from and maintained by a very small population of tumorigenic cells that are capable of continuous self-renewal which produces the tumor mass. Indeed, resveratrol modulates two major pathways playing a pivotal role in cancer stem cell self-renewal [153].

Resveratrol can be converted to piceatannol by cytochrome P450 enzymes in the liver [154] or overexpressed in a wide variety of human tumors [155]. Piceatannol has a known antileukemic activity and is also a tyrosine kinase inhibitor [156]. Piceatannol can also induce apoptosis in human tumor cell lines [144], inhibit the lipopolysaccharide-induced production of critical mediators of the inflammatory response in different models [157, 158], and suppress NF-kB activation induced by various inflammatory agents [159]. Moreover, piceatannol has antimetastatic activities which might be due to the inhibition of angiogenesis [137]. Recently, Kwon et al. studied the mechanisms underlying these effects [160]. They demonstrated that piceatannol inhibits migration and invasion of prostate cancer cells by the inhibition of interleukin-6/STAT3 signaling. STAT3 is a transcription factor controlling several oncogenic processes.

Several other stilbenoids also have promising cancer chemopreventive activities. The dimer ε-viniferin displayed a more potent inhibitory effect than resveratrol on human cytochrome P450 enzymes involved in bioactivation of numerous carcinogens [161]. ε-Viniferin also possesses anti-inflammatory properties [162], as well as vaticanol B, α-viniferin, vitisin A, vitisifuran A, and hopeaphenol [163, 164]. An orally administered extract containing bergenin, hopeaphenol, vaticanol B and C, and ε-viniferin, exhibited an antitumoral effect against subcutaneously allografted sarcoma in mice [140]. Analysis of structure-activity relationships showed that the substitution of hydroxyl groups of resveratrol to methoxy groups potentiated cytotoxic activity [165, 166]. Pterostilbene exerts various effects including antiproliferative, proapoptotic, antioxidant, anti-inflammatory, anti-invasive, and antimetastatic activities.

6.2 Cardiovascular Protection

Resveratrol and other stilbenoids could be beneficial to cardiovascular health by acting on multiple targets, such as lipoproteins and oxidative stress, platelet aggregation, vasodilation, and myocardial infarction [132, 167, 168].

6.2.1 Antioxidant Activity

The oxidation of low-density lipoproteins (LDL) plays a critical role in the initiation of atherogenesis. Oxidized LDLs are a key component in endothelial injury. They may directly injure the endothelium and play an initial role in the increased adherence and migration of monocytes and lymphocytes into the subendothelial space [169]. Oxidized LDL may bypass the normal tight control exercised by the classical LDL receptor in the macrophages and be endocytosed via nonregulated scavenger receptors. This leads to a rapid accumulation of cholesterol and cholesteryl esters and subsequent foam cell formation. Hence, it is conceivable that oxidative stress accelerates atherogenesis by enhancing LDL oxidation and increasing its accumulation into foam cells. The presence of antioxidants can interfere with the peroxidation process by removing the alkoxyl or peroxyl radicals.

In our laboratory, we have studied the antioxidant activities of pure stilbenoids found in wine [170]. On Cu^{2+}-induced lipid peroxidation on LDL, we showed that three stilbenoids (resveratrol, astringin, and piceatannol) were more effective than Trolox, the water soluble vitamin E analogue. No significant difference was found between *E* and *Z* structures of each molecule, except for resveratrol, with a better activity for *trans*-resveratrol. The glycosylation of *trans*-stilbenoids reduces their activity when compared to the corresponding aglycones, whereas a catechol structure in the B ring increases it. Among these molecules, the most potent antioxidant is piceatannol (aglycone, catechol structure) which was two times more efficient than Trolox. These results are in agreement with other reported data concerning structure-activity relationship studies of resveratrol and its analogues [171]. These compounds can also act by another mechanism, i.e., their complexation with metal ions (iron, copper), which are involved in the generation of free radicals and lipid peroxidation [172]. Moreover, α-tocopherol (the principal form of vitamin E) which functions as a major antioxidant in human LDL can be recycled from its free radical form (α-tocopheryl) by a phenolic compound.

Resveratrol can also prevent the initial events of atherosclerosis in endothelial cells by inhibition of the enzymatic systems producing reactive oxygen species, such as NADPH oxidase and hypoxanthine/xanthine oxidase, and by the inhibition of both the expression of adhesion molecules and the monocyte adhesion to endothelial cells [167]. Moreover, red wine polyphenols, in particular *E*-resveratrol, have been shown to inhibit the proliferation and the migration of vascular smooth muscle cells in intima, notably involved in the formation of atherosclerotic plaques [173]. In fact, resveratrol specifically blocks the mTOR pathway, which is activated by oxidized LDL, and regulates the proliferation of smooth muscle cells [174]. Other stilbenoids have been shown to exert an activity on this target as well. For example, pterostilbene inhibits vascular smooth muscle cell proliferation with an IC_{50} value less than 1 μM [175], whereas vitisin B, a resveratrol tetramer, enhances their proliferation but exhibits an anti-migratory effect [176].

Using a hamster model of atherosclerosis, Auger et al. showed aortic fatty streak area was significantly reduced (76%) in the group receiving resveratrol at a level corresponding to a moderate consumption of red wine [177]. Orally administrated resveratrol in rats maintained on a hypercholesterolemic diet improves the plasma lipid levels altered such as total cholesterol, triglycerides, LDL cholesterol, and HDL cholesterol [178].

6.2.2 Antithrombotic and Vasoprotective Effects

Studies suggested that *E*-resveratrol is particularly active on human platelet aggregation by inhibiting the synthesis of certain eicosanoids [179]. We screened various stilbenoids for their antiplatelet properties on human platelet-rich plasma in which aggregation was induced either with arachidonic acid (AA), ADP, collagen, or the TxA2 mimetics U-46619 [180]. Significant inhibition of AA-induced aggregation was only observed for the aglycones, *E*- and *Z*-resveratrol and piceatannol, with IC_{50} values similar to that of acetylsalicylic acid. These aglycones also showed an antiplatelet activity when collagen or ADP was used as stimulating agent, but only at much higher concentrations. In addition, all derivatives were found to be inactive when U-46619 was tested as aggregation inducer. Based on these findings, the anti-aggregating profile observed for stilbenoids points to an interaction of the compounds with the platelet arachidonic acid pathway through a mechanism consistent with an inhibition of cyclooxygenase. Significant inhibition of AA-induced aggregation was also observed for the tetramer, vitisin A, with an IC_{50} value inferior to that of acetylsalicylic acid, but ε-viniferin (a dimer) had no inhibitory effect [181]. Moreover, *E*-resveratrol was found to modulate intracellular free calcium in stimulated human platelets, which is an essential component of the aggregation process [167]. Interestingly, orally administrated resveratrol prevents in vivo platelet aggregation and thrombus formation in hypercholesterolemic rabbit and mice, respectively [132]. Clinical study showed that resveratrol can inhibit aggregation of platelets from high-risk cardiac patients who are aspirin resistant [182].

Several studies have shown that the extent of coronary artery stenosis due to atherosclerotic plaque formation and expansion into the arterial lumen is not sufficient to explain the incidence of clinical events associated with atherosclerosis [183]. It appears that the generation of clinical events involves plaque rupture, resulting in thrombus formation and arterial occlusion. This rupture is induced by vasomotor disturbances in which oxidized low-density lipoproteins may be involved. Resveratrol is able to regulate vasomotion, which is impaired in atherosclerosis. The key regulators of the vasomotor function are the vasodilator NO and the vasoconstrictor endothelin-1 [167]. A number of in vitro and in vivo studies have shown improved vascular function in response to resveratrol [184, 185]. Resveratrol enhances expression and activity of endothelial nitric oxide synthase [186] and inhibits endothelin-1 secretion and endothelin-1 gene expression in human umbilical vein endothelial cells [187]. Intragastric administration of resveratrol for 12 weeks to hypercholesterolemic rabbits improved the endothelial function, reduced plasma endothelin-1 levels, and induced a significant elevation in NO levels [188, 189]. It was also reported that coronary flow velocity reserve and flow-mediated dilatation of the brachial artery increased specifically after the intake of a red grape polyphenol extract by patients with coronary heart disease [190]. This extract contained mainly flavanols and phenolic acids in addition to *E*-resveratrol and ε-viniferin. These effects may be due to the improvement of endothelial function and the vasorelaxing effects of polyphenols.

6.2.3 Myocardial Infarction

Numerous data in animals strongly suggest that resveratrol might protect against ischemic damage during myocardial infarction [132, 178]. Perfusion of rat hearts with resveratrol before ischemia leads to a better recovery of developed pressure and aortic flow, reduction of malondialdehyde concentrations and of infarct size, and increase of functional recovery of the coronary flow. Hung et al. found a positive correlation between the antioxidant activity of stilbenoids and their cardioprotective efficacy, with piceatannol being notably more active than resveratrol [191]. While the antioxidant properties of stilbenoids are at least partially involved in this myocardial protection, the implication of NO-dependent mechanisms has also been shown [192]. These authors showed that rats receiving drinking water containing a low concentration of resveratrol for 15 days have the heart protected from ischemic reperfusion injury. Resveratrol can also enhance neovascularization in infarcted rat myocardium [193].

Some studies in animals suggest that resveratrol might be useful to protect against brain damage following cerebral ischemia [132].

6.3 Aging and Longevity

Calorie restriction is a very effective nutritional intervention inducing slow metabolism that can extend the lifespan in species ranging from yeast to primates and also protect against obesity, diabetes, and cardiovascular disease [194, 195]. Calorie restriction is believed to extend lifespans, in part, by increasing the activity of sirtuins, NAD^+-dependent deacetylases, although their physiological role may be more complex than expected [195]. Under calorie restriction, the altered oxygen consumption modifies the NAD^+/NAD ratio and leads to an NAD^+-dependent activation of sirtuin, an evolutionary conserved enzyme family which acts by deacetylating histones and nonhistone proteins such as transcription factors, NF-kB, and the p53 tumor suppressor involved in longevity [167, 196]. In a famous paper by Howitz et al. [197], it was shown that resveratrol mimics calorie restriction by stimulating a sirtuin and extending *Saccharomyces cerevisiae* lifespan by 70%, piceatannol lacking any activity. Then, this activity of resveratrol was also found in multicellular animals (short-lived invertebrates), the worm *Caenorhabditis elegans* and the fly *Drosophila melanogaster*, with an increase of longevity of 14% and 29%, respectively, through sirtuin activation [198]. Likewise, this effect was also observed in a short-lived vertebrate, the fish *Nothobranchius furzeri*; resveratrol added to the food caused an extension of the maximum lifespan by up to 59% [199]. In addition, resveratrol delays the age-dependent decay of locomotor activity and cognitive performance in the fish and prevents age-dependent neurodegeneration by decreasing aggregated proteins in the brain [199]. Some controversial results have been found as no increase in lifespan of different yeast strains and *C. elegans* in the presence of resveratrol and no direct activation of a mammalian sirtuin (SIRT1) was observed [195, 200–202]. However, resveratrol has proven to be a valuable tool for mammalian cells in vitro, in which it has been shown to produce SIRT1-dependent effects, but this may occur through an indirect mechanism [203]. No extension of the normal lifespan by resveratrol has been found in mammals, as in mice on a standard diet. However, Baur et al. showed that resveratrol improves health and survival of mice on a high-calorie diet, preventing the early mortality associated with obesity [203]. In fact, resveratrol shifts the physiology of mice consuming excess calories toward that of mice on a standard diet affecting favorably a variety of parameters such as motor function, insulin sensitivity, organ (liver) pathology, mitochondrial number, and 144 of the 153 pathways significantly altered in high-caloric mice (whole-genome microarrays on liver) [203]. Sirtuin activation may constitute a potential strategic target in aging associated diseases such as neurodegenerative diseases and cardiovascular and metabolic diseases [196].

7 Clinical Trials

There is little published evidence of pharmacodynamic effects of resveratrol in humans demonstrating protective or therapeutic effects. The database "clinicaltrials.gov" of the US National Institutes of Health shows a total of 32 clinical studies with resveratrol. Of these studies, seven are examining the effects of this compound on brain functions, five are either active or recruiting, and two are completed. The results from one of these clinical trials are currently available [204]. These authors assessed the effects of resveratrol on cerebral blood variables and cognitive performance in 22 healthy adults by a double-blind, placebo-controlled, crossover study. Cerebral blood flow was evaluated by measure of total and deoxygenated hemoglobin levels in the prefrontal cortex using near-infrared spectroscopy. Single doses of orally administered resveratrol, 250 or 500 mg, led to a significant increase of the cerebral flow in dose-dependent manner during task performance, but cognitive function was not affected. This beneficial action can be due to the vasorelaxatory properties of resveratrol, likely due to an induction of the NO synthesis. A reduced cerebral blood flow is observed in primary neurodegenerative diseases, and in natural aging, which is associated with impaired NO-mediated vasodilatation [204, 205]. In this study, the bioavailability data show the presence of low levels of resveratrol and its metabolites in the plasma, mainly the sulfated conjugate. The second completed study, currently not published, carried out by the same authors was similar to the first one, except the co-supplementation with piperine. The aim was to investigate whether this alkaloid from black pepper may be able to enhance the bioavailability of resveratrol, as previously demonstrated for other phenolic compounds through inhibition of their metabolism [206, 207].

In other pharmacological domains such as cancer, a few studies have been carried out in humans. In the UK study described by Patel et al. [127], twenty patients with confirmed colorectal cancer consumed eight daily doses of resveratrol (0.5 or 1 g) before surgical resection. A small, 5%, reduction in cell proliferation in colorectal tissue after resveratrol consumption was observed, which suggests that these doses are sufficient in the human gastrointestinal tract to favorably alter cell proliferation. Resveratrol (parent) was recovered from tumor tissue and proximal to tumor at maximal levels of 94 and 674 nmol/g, respectively, where it represents the major form, although its conjugates, sulfates, and glucuronides were the dominant forms present in the plasma. These tissue concentrations are compatible with cancer chemopreventive activity of resveratrol because resveratrol has been shown to exhibit growth inhibition and apoptosis induction in human-derived colon cells in vitro with IC_{50} values of about 20 nmol mL^{-1} [127, 208]. The same team also showed that ingestion of resveratrol at 2.5 g day^{-1} for four weeks by healthy volunteers caused a decrease in a circulating insulin-like growth factor (IGF) and an IGF binding protein, which might contribute to chemopreventive activity. Indeed, the IGF signaling system can significantly influence the development of malignancies. In this study, the repeated ingestion of high doses of resveratrol leads to micromolar concentrations of parent and much higher levels of conjugates in the plasma [112].

8 Conclusion

Vine and wine are abundant sources of polyphenolic compounds, including flavonoids and stilbenoids. The latter appear to constitute a large class of compounds, and a number of stilbenoids have exhibited potent biological activities on multiple targets in rodent models and cells in vitro. Within wine, there are almost 20 known stilbenoids, and over 60 in grapevine, a number of which have demonstrated promising biological activities, yet many others have undergone little or no biological evaluations. The sum of these stilbenoids in wine may help explain the health benefits of wine reported in epidemiological studies.

Among the stilbenoids in grapevine, *E*-resveratrol is the most studied due to its promising biological activities in regard to the prevention and treatment of leading diseases, including cancer, cardiovascular diseases, and neurological diseases. Currently, resveratrol is the subject of over 30 clinical trials in the USA alone, including studies on its bioavailability and pharmacokinetics.

In regard to neurological diseases, resveratrol and several other stilbenoids can modulate multiple pathways in neurodegenerative disease pathologies. In Alzheimer's disease, several stilbenoids, including resveratrol, have shown several different activities against the fibrillization of Aβ peptide and prevention of toxicity-induced damage in neuronal cells, including the microglia-driven neuroinflammatory processes. Some data are now available on its bioavailability, biological effects, and toxicity in humans, which have shown the absorption levels based on multiple dosing and acute and chronic side effects of high dosages. Additionally, further investigations are still necessary as to the identification of the endogenous compounds responsible for the biological activity within target tissues and the precise mechanism of its indirect interaction with sirtuins.

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Proanthocyanidins of Cocoa: Bioavailability and Biological Activities

74

Manuel Rusconi, Maria Teresa Pinorini, and Ario Conti

Contents

1 Introduction: Health Benefits of Cocoa 2312
2 Proanthocyanidins in Cocoa and Cocoa-Derived Products 2313
2.1 Factors Affecting the Quantity and Quality of Proanthocyanidins 2316
3 Properties of Proanthocyanidins 2318
3.1 The Promising Role of Proanthocyanidins 2320
3.2 Bioavailability and Metabolism of Proanthocyanidins 2321
3.3 Human Studies 2323
4 Bioactivity of Proanthocyanidins 2324
4.1 Redox Activity 2325
4.2 Nonredox Activity 2325
4.3 Atherosclerosis, Cytotoxicity, Carcinogenicity, Platelet Aggregation, Vasodilatation, Vasoconstriction, Reperfusion, and Cellular Adhesion 2326
4.4 Water-Insoluble Polyphenols 2327
5 Conclusion 2328
References 2328

Abstract

Cocoa and dark chocolate contain many active compounds, which seem to play some pharmacological roles both in vitro and in human. Many studies have focused their attention on polyphenol content, which is elevated in cocoa beans and in some kind of dark chocolate, depending on harvest and postharvest handling. Procyanidins monomers and oligomers modulate a large quantity of biological mechanisms. Indeed, scientists might be more interested to better understand pharmacological aspects of such compounds in vivo and their

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mechanisms of action and interactions with other molecules as well as new medicines. Moreover, some potential adverse effects have to be taken into prompt consideration. This chapter, which is not the result of a specific experimental work but only a short study of recent bibliography on the specific subject, might help in understanding some aspects connected with the chemistry of cocoa and chocolate.

Keywords
Chocolate • cocoa • proanthocyanidins • human health • adverse effects

Abbreviations

APP	Amyloid precursor protein
Aß	Amyloid beta
AUC	Area under the curve
BBB	Blood-brain barrier
COX-2	Cyclooxygenase 2
EDR	Endothelium-dependent relaxation
EGCG	Epigallocatechin gallate
HDL-c	High-density lipoprotein cholesterol
iNOS	Nitric oxide synthases
LDL-c	Low-density lipoprotein cholesterol
mDP	Mean degree of polymerization
NFCS	Nonfat cocoa solids
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOAEL	No observed adverse effect level
ORAC	Oxygen radical absorbance capacity
pKa	Acidity constant
UGT	Uridine 5′-diphospho-glucuronosyltransferase

1 Introduction: Health Benefits of Cocoa

Over the last two decades, the scientific community has become aware of the potential health-related benefits of antioxidants and the properties of polyphenols-rich dark chocolate and cocoa. More than 200 studies were reported on bioactive compounds, chemical compositions, and health benefits of cocoa and cocoa products. Many of the proposed health-protective activities associated with the consumption of cocoa and chocolate have been attributed to flavan-3-ols, including monomers. Reported pharmacological activities of procyanidins include antioxidative and anticancer effects, protection against cardiovascular disease, risk reduction of blood clotting, protection against urinary tract infections, decrease of LDL-c, decrease of blood pressure, and improvement of endothelium vasodilatation. Moreover, there is some scientific evidence about an increase in blood flow and perfusion of the brain by

Fig. 74.1 Cocoa beans in a cacao pod

polyphenols, and experimental data suggests that flavanols may delay the onset of neurodegenerative diseases such as Alzheimer's disease through a number of different mechanisms. Preliminary in vitro and ex vivo investigations suggested that cocoa flavanols and procyanidins might possess immunoregulatory effects [1–3]. The potential mechanisms through which flavanols and cocoa might exert their beneficial activity on cardiovascular health include: activation on NO, antioxidant, anti-inflammatory, and antiplatelet effects, which in turn might improve endothelial function, lipid levels, blood pressure, and insulin resistance [4–6].

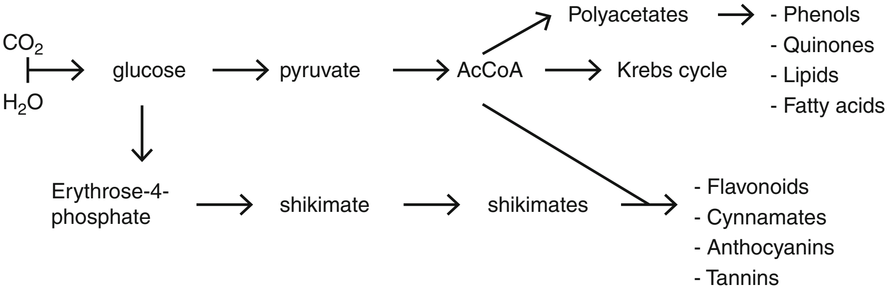
More recently, studies begin to emphasize the bioactivities of flavonoids through modulating several cellular signaling pathways involved in cell apoptosis, proliferation, survival, and inflammatory responses.

Procyanidins seem to represent the second most abundant natural phenolic after lignin and, with other substances, they might be responsible for the oral sensation of astringency in ripening fruits (apples, peaches, grapes, berries), beverages (tea, wine), cocoa and chocolate [7, 8].

It has been showed that chocolate is one of the most polyphenol-rich foods. According to Lee et al. [9], cocoa contains a higher content of flavonoids per serving than teas or red wine. Moreover cocoa powder is one of the richest dietary sources of flavanols (on a weight basis) identified so far, exceeded only by a few food ingredients such as buckwheat hulls, sorghum, cinnamon, and some "superfruit" [10]. Cocoa is also one of the richest dietary sources of polyphenols listed in the Phenol-Explorer database [11] and one of the richest sources of procyanidins in the USDA Database for the Proanthocyanidin Content of Selected Foods [12] (Fig. 74.1).

2 Proanthocyanidins in Cocoa and Cocoa-Derived Products

Polyphenols are common antioxidants present in a wide range of foods and beverages of plant origin. Although still debated and with many differences depending from the studied population, a mean intake of about 1 g per day has been proposed [13, 14].



Scheme 74.1 Secondary metabolism of plants. Glucose metabolism: the shikimate and the acetate pathway

Phenolic compounds or polyphenols constitute one of the most abundant and widely distributed groups of substances in the plant kingdom with more than 8,000 phenolic structures currently known. They are products of the secondary metabolism of plants and arise biogenetically from two main primary synthetic pathways: the shikimate pathway and the acetate pathway. Both acetic acid and shikimic acid are derived from glucose metabolism [15] (Scheme 74.1).

Moreover, according to chemical structure, polyphenols can be divided in classes as simple phenols, benzoquinones, phenolic acids, acetophenones, phenylacetic acids, hrodroxycinnamic acids, phenylpropenes, coumarins, isocoumarins, chromones, naphthoquinones, xanthones, stilbenes, anthraquinones, lignans, neolignans, lignins, and flavonoids. The latter allow to consider 13 subclasses with more than 5,000 compounds (Fig. 74.2).

The absolute structure of flavonoids can vary dramatically; however all plant flavonoids share a common 15-carbon structural backbone designated as C_6–C_3–C_6. Flavonoids share a common structure consisting in two aromatic rings (A and B); differences in the structure of the heterocyclic C ring result in distinct classes of flavonoids, including flavanols, flavanones, flavones, isoflavonols and anthocyanidins (Figs. 74.3, 74.4).

In nature, many flavonoids exist as glycosides, making characterization of compounds even more complex.

Cocoa is a source of many polyphenolic compounds, including hydroxybenzoic acid (gallic, syringic, protocatechuic, vanillic acids), hydroxycinnamic acids and analogues (caffeic, ferulic, p-coumaric, phloretic acids, clovamide, dideoxyclovamide), flavonols (quercetin), flavones (luteolin, apigenin), flavanones (naringenin), and flavan-3-ols monomers ((+)-catechin, (−)-catechin), oligomers and polymers (proanthocyanidins) [18, 19].

Epicatechin and catechin (Fig. 74.4) represent the basis on which to build more complex molecules such as procyanidin polymeric forms. Proanthocyanidins are polymer chains of flavonoids such as flavan-3-ols. Mainly, two primary forms of procyanidins occur in plants: A-type and B-type, which differ by the linkage between individual compounds. A-type procyanidins form 4–8 and 2–7 cross-links and has

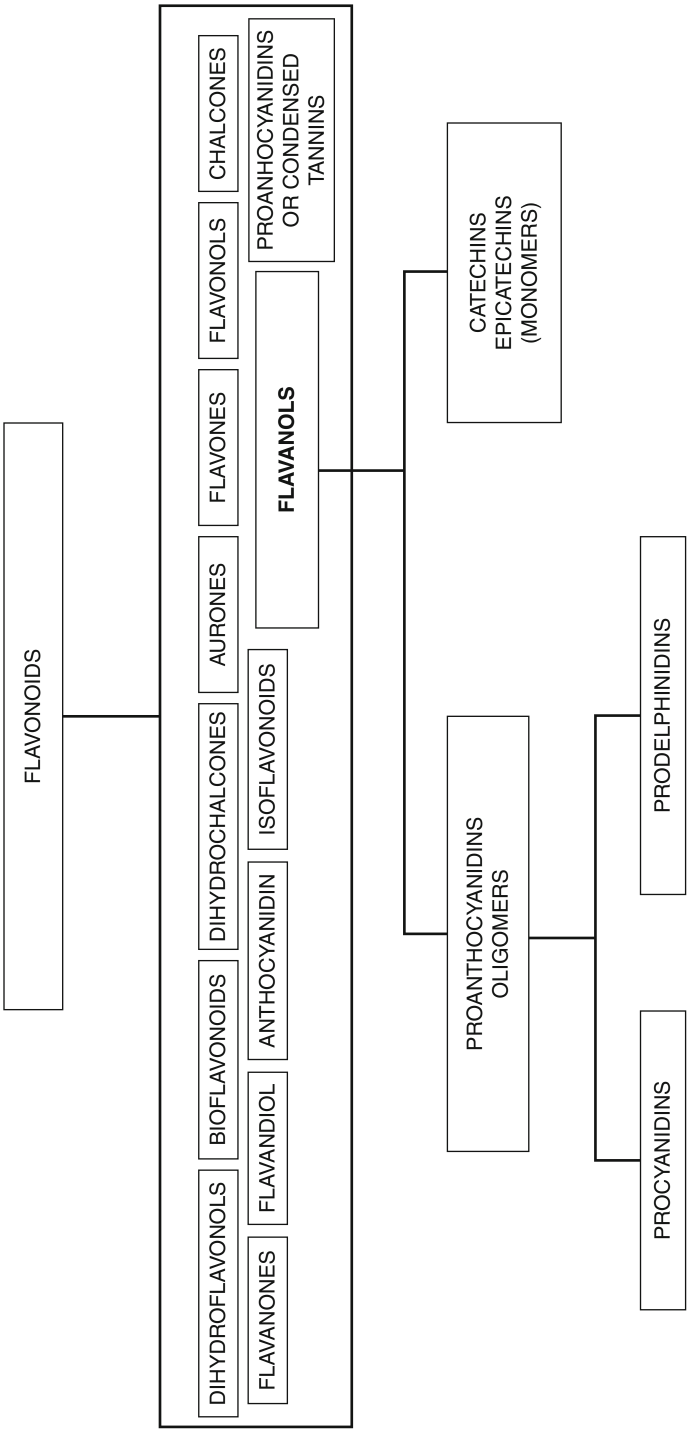

FLAVONOIDS
DIHYDROFLAVONOLS
BIOFLAVONOIDS
DIHYDROCHALCONES
AURONES
FLAVONES
FLAVONOLS
CHALCONES
FLAVANONES
FLAVANDIOL
ANTHOCYANIDIN
ISOFLAVONOIDS
FLAVANOLS
PROANHOCYANIDINS OR CONDENSED TANNINS
CATECHINS EPICATECHINS (MONOMERS)
PROANTHOCYANIDINS OLIGOMERS
PROCYANIDINS
PRODELPHINIDINS

Fig. 74.2 Classification scheme for flavonoids (Adapted from Robbins et al. [16])

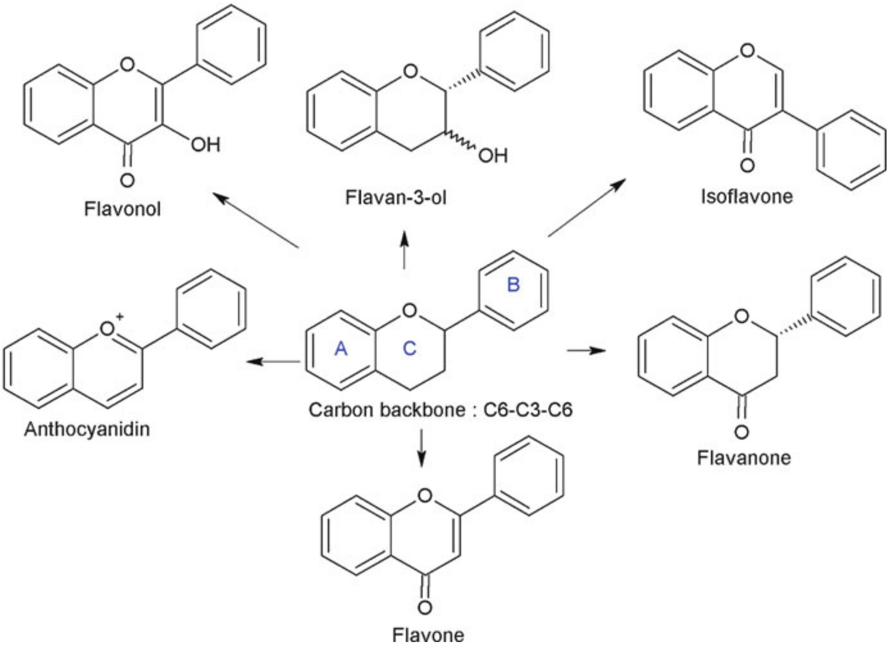


Fig. 74.3 The generic three ring (A, B, and C) carbon backbone structures (C6–C3–C6) of the major flavonoid subclasses found in food [17]

been described in cranberries. The B-type procyanidins form 4–8 cross-links. Moreover, B-1-B-4-types differ only in the arrangement of catechin and epicatechin units, with predominant forms being procyanidin B-1 found in grape, sorghum, cocoa, and cranberry, type B-2 found in apple, cocoa, and cherry, type B-3 found in strawberry and hops, and finally type B-4 found in raspberry and blackberry [20]. Kelm et al. [21] indicated that unfermented cocoa beans contain monomers up to 14 subunits (tetradecamer).

2.1 Factors Affecting the Quantity and Quality of Proanthocyanidins

The amount of flavonoids and flavanols in cocoa and chocolate might be highly variable and this variability is multifactorial. For example, plant's genetic predisposition dictates the biosynthesis of the primary (sugars, amino acids, etc.) and secondary metabolites (proanthocyanidins, saponins, alkaloids, etc.) while genetics can cause as much as a fourfold difference in flavan-3-ol content of fresh cocoa beans [22, 23]. Variability with respect to secondary metabolites exists between varieties (or cultivars) of the same species. However, various environmental factors determine the extent to which genetic potentialities are

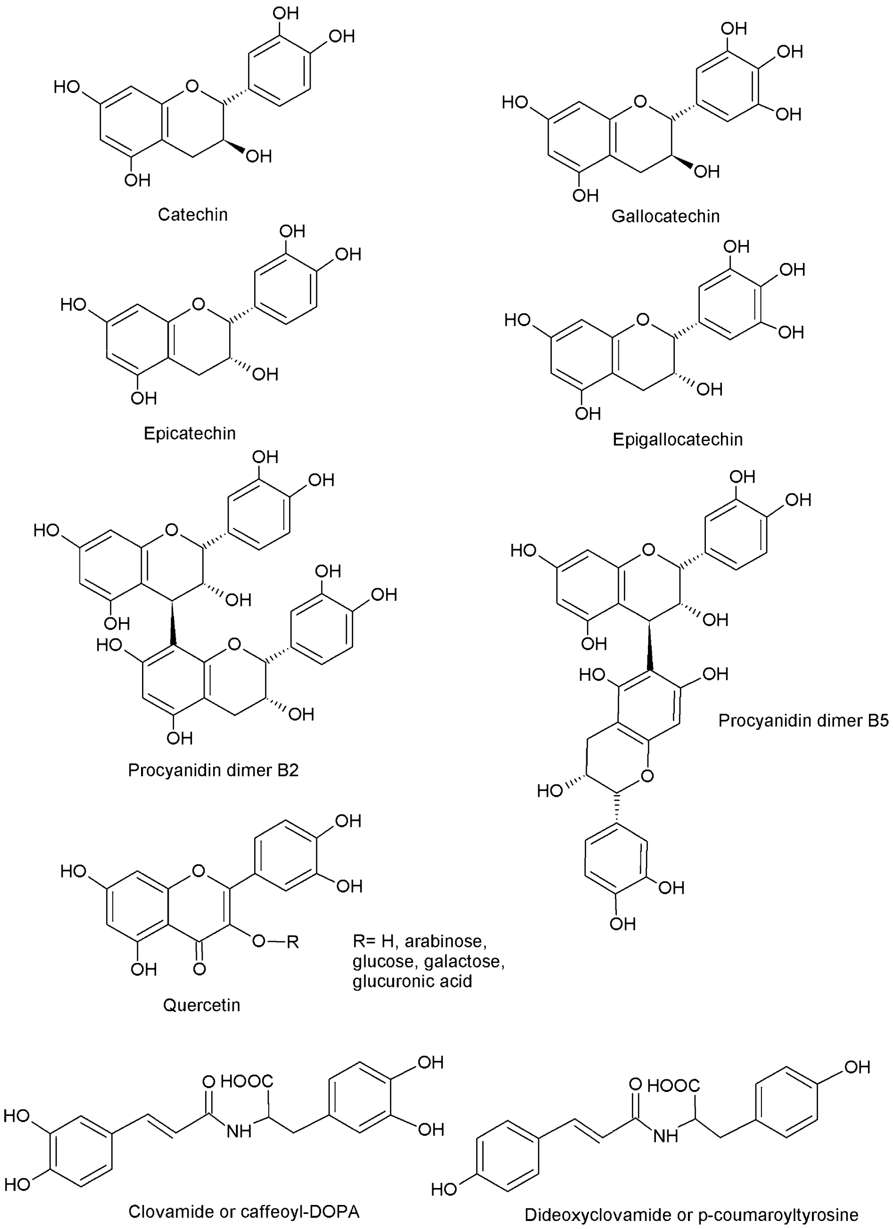

Catechin
Gallocatechin
Epicatechin
Epigallocatechin
Procyanidin dimer B2
Procyanidin dimer B5
Quercetin
R= H, arabinose, glucose, galactose, glucuronic acid
Clovamide or caffeoyl-DOPA
Dideoxyclovamide or p-coumaroyltyrosine

Fig. 74.4 Chemical structure of selected cocoa polyphenols [17]

achieved. Ecology, drought, soil type/structure, disease, herbivore damage, and farming practices (i.e., pruning, application of pesticides, etc.) do have an influence on secondary plant metabolism. The postharvest handling (storage time, temperature, modified atmospheres, etc.) of fruits and vegetables can also impact metabolite levels. Processing effects, such as heating, fermentation, roasting, alkaline treatment (Dutch processing), shearing, and baking, have been shown to reduce both the level of total procyanidins and the level of low molecular weight flavanols. Finally, variability in food component values may be attributable to the differences in analytical methods used to determine the values [12].

Various type of dark chocolate with high flavonoid contents are today available in the market. These chocolates are produced by controlling postharvest processes: fermentation, heating, and alkalization treatments. By controlling the process involved in preparing the chocolates, high-flavonoid chocolate can be produced that preserves up to 70 % of the flavonoids present in the final product. Current regulations do not require that antioxidant capacity and/or polyphenol content be provided on food labels. Inclusion of this information has been suggested [24, 25].

3 Properties of Proanthocyanidins

The discovery of flavan-3-ols and their procyanidin polymeric forms in cocoa can be traced back to the beginning of the last century and are now identified as catechins [26–28]. In 1939, leucoanthocyanin phenolic compounds were identified [29] and, in 1955, fractionation and characterization of these compounds were reported [30]. Procyanidins in cocoa have more recently been fractionated into monomers through decamers with even higher forms existing [31].

Proanthocyanidins have been the subject of more than 2,700 bibliographic entries in the last 65 years [8].

The chemical structures of flavonols and procyanidins might be important for antioxidant activity because of free radical trapping and chelation of redox-active metals properties. Adamson et al. [32] indicated that polyphenol content positively correlated with antioxidant properties as measured by ORAC. All polyphenols possess antioxidant properties in vitro but this activity is not well characterized in human.

Procyanidin-rich chocolate increased the antioxidant capacity, decreased the oxidative stress, and decreased the leukotriene-prostacyclin ratio in humans and human aortic endothelial cells [33, 34]. Procyanidins extracted from cocoa exhibited EDR through activation of nitric oxide synthase activity in rabbit aortic rings in vitro [35]. The results were reported to be due to the tetramers and higher polymers of epicatechin, and monomers, dimers, and trimers were not capable of contributing to EDR. In vivo studies, both in human and animals, indicated that epicatechin from cocoa could enhance the antioxidative activity in plasma. In theory, these antioxidant actions can result in a reduction of the steady-state concentration of free radicals and other oxidants, diminishing the following oxidation of target molecules such as lipids, proteins, and nucleic acids. However, plasma concentrations of flavanol and procyanidin observed after consumption of foods rich in such compounds were relatively low. The actual concentrations that can be

Table 74.1 Polyphenols and procyanidins content of different cocoa products. Data are expressed as mean per gram of product

Type	Polyphenols (mg/g of GAE[a])	Procyanidins (mg/g)
CS-1	4.05	0.91
CS-2	4.79	0.89
CS-3	3.66	0.37
MC-1	4.5	0.71
MC-2	5.38	0.9
MC-3	3.25	0.43
DC-1	12.3	2.78
DC-2	11.73	4.1
DC-3	14.88	4.06
BC-1	29.7	16.33
CP-1	60.2	23.71
CP-2	45.3	19.28
CP-3	51.7	22.44

CS chocolate syrup, *MC* milk chocolate, *DC* dark chocolate, *CP* cocoa powder (Adapted from [20])

[a]Expressed as gallic acid equivalents (GAE)

reached in plasma of humans subjected to realistic polyphenol consumption are believed to be in the nanomolar range [36, 37]. This low bioavailability leads to a kinetically unfavorable condition with respect to other compounds with similar free radical scavenger capabilities present in blood in significantly higher micromolar concentrations, that is, tocopherols and ascorbate. According to Galleano et al. [38], a function of flavanols as direct free radical scavengers is unlikely to be relevant, and could be limited to the blood and other tissues directly exposed after consumption, that is, gastrointestinal tract. Moreover, it has been suggested that other mechanisms, compatible with the concentration at target site reached by flavanols, may explain the observed changes in cell or tissue oxidation levels after flavanol consumption. These mechanisms are beyond the ability of flavanols and other flavonoids to directly prevent free radical-mediated tissue damage [37].

Procyanidins have been reported at concentrations from 370 to 23,710 mg/kg in various cocoa products [19, 20, 39] (Table 74.1). Besides other proanthocyanidins, (−)-epicatechin has been reported as the major monomeric flavanol in cocoa, representing ca. 35 % of the total phenolic content [40].

Catechin and epicatechin have been found at concentrations of 150–1,580 mg/kg in chocolate and 2,530–3,170 mg/kg in cocoa liquor [39, 41–44].

Many phenols are found as glycosides in cocoa, mainly glucoside, galactoside, and arabinose. Miller et al. [45] showed that there is a strong degree of correlation between flavanols, with the possible exception of catechin, and % NFCS in chocolate and cocoa-containing products, with cocoa powder being highest and chocolate syrup being lowest in these compounds.

Supplementation of cocoa for 4 weeks significantly improves platelet function among healthy subjects [1]. However, there was no correlation between cocoa intake and plasma antioxidant status. Similarly, cocoa intake decreased LDL oxidation without changes in antioxidant potentials and oxidative stress level in plasma [46]. Dark chocolate supplementation for 3 weeks in healthy subjects significantly increased HDL-c compared to their unsupplemented counterparts [47]. However, there were no changes in total antioxidant capacity and oxidative stress biomarker (8-isoprostane). Similarly, Wan et al. [48] demonstrated that cocoa powder and dark chocolate supplementation improved HDL levels by 4 % compared to control diet, but there were no changes in oxidative stress biomarkers. Milk chocolate bar consumption increased HDL levels compared to high carbohydrate snacks among young men [49]. These studies clearly indicated that cocoa administration did not exert their antioxidative properties in plasma of healthy subjects, although there were significant health outcomes. This could be due to the status of subjects recruited in the study. Cooper et al. [44] reported that healthy subjects may already have optimum dietary status and supplementation will not produce meaningful outcomes.

Cocoa powder exerted anticancer properties in in vivo studies. Amin et al. [50] indicated that cocoa liquor extract lowered the activity of tumor marker enzymes (alkaline phosphatase, gamma-glutamyl transpeptidase, glutathione-S-transferase, and glutathione reductase activities) in plasma and/or liver of hepatocarcinogenic male Sprague-Dawley rats, which were induced with diethylnitrosamine and 2-acetylaminofluorene.

Cocoa powder supplementation significantly reduces the incidence of prostate carcinogenesis compared to positive controls using the *N*-methylnitrosourea and testosterone propionate prostate tumor model on 60 male Wistar-Unilever rats [51]. The supplementation also increased the life span of the tumor-bearing rats. Bisson et al. [52] reported that cocoa powder dose-dependently decreased prostate hyperplasia through reducing dihydrotestosterone level and prostate size ratio. To a greater extent, long-term supplementation of cocoa powder improved cognitive performance in aged rats compared to unsupplemented rats [53]. Daily cocoa extract administration prevented the overproduction of free radicals after heat exposure and thus protect from cognitive impairments [54, 55].

3.1 The Promising Role of Proanthocyanidins

In a mice model of Parkinson's disease, catechins are thought to chelate metal ions such as copper (II) and iron (II) and therefore prevent the generation of potentially damaging free radicals, to suppress the translation of APP mRNA, to reduce holo-APP, and to decrease Aβ levels. After the oxidation of catechins by free radicals, a dimerized product is formed with an increased iron-chelating potential and ability to scavenge superoxide anions [56–58].

In vivo studies on mice have shown that 0.33 % of an EGCG administration can reach the brain and that frequent consumption enables the body to maintain a high level of catechins [59].

Other properties of catechins monomers and oligomers can be summarized as follows:

- Increase antioxidant enzymes such glutathione peroxidase and reductase, superoxide dismutase, and catalase.
- Increase the total plasma antioxidants, whereas decrease the plasma peroxide level.
- Decrease oxidative stress by inhibiting the activity of xanthine oxidase, a ROS-generating system.
- Protect lipids from oxidation in the liver, serum, and brain. For instance, it has been demonstrated that catechins could protect against lipid peroxidation induced by 6-hydroxydopamine, hydrogen peroxide, and iron.

These antioxidant effects are observed in vitro with concentrations ranging from 1 to 50 μM. Interestingly, higher concentrations (100–500 μM) and in the presence of copper (II) or iron (III), EGCG exacerbated oxidative stress, cytotoxicity, and DNA damage induced by hydrogen peroxide. Moreover, EGCG promotes cell survival by restoring the protein kinase C activity, a critical regulator of cell proliferation and survival [57, 60–68].

3.2 Bioavailability and Metabolism of Proanthocyanidins

When discussing the biological activity of flavonoids in general, and flavanols in particular, there are some major factors to be considered: bioavailability from food, matrix effects, absorption and metabolism in the gastrointestinal tract, tissue and cellular distribution after absorption, possible interactions and/or accumulation, which are the chemical form(s) biologically available to the cell/tissue and their potential metabolism at cellular level [3, 42, 69, 70].

Recently, there have been two major approaches commonly used to determine the availability of phenolic compounds either by measuring their concentration in plasma and urine after ingestion of known amount of foodstuffs or ingestion of the pure compounds [71].

Factors that influence the extent and rate of absorption of ingested compounds by the small intestine include molecular size, lipophilicity, solubility, pKa, gastric and intestinal transit time, membrane permeability, and first-pass metabolism [72, 73].

The procyanidins that cross the intestinal barrier are conducted to the liver via the portal vein, where they further degrade into metabolites by methylation, glucorination, and sulfation, which result in the potential antioxidants capacity of flavanols. These metabolites can possibly reach all tissues within hours following consumption as described in radiolabeled experiments with rats. Further modification in the colon gives rise to other bioactivities that are attributed to flavanols. Urine and feces analysis confirmed the presence of low molecular weight metabolites, which indicates that polymeric procyanidins are absorbed through the intestinal barrier after degradation in low molecular weight metabolites, most probably by gut microflora [74, 75].

Although it has been suggested that the metabolism of procyanidin polymers (specifically dimer B-3 and trimer C-2) by intestinal microflora is limited [76], human fecal microflora, grown under anaerobic conditions in vitro, have the ability to degrade procyanidins (mDP = 6) to low molecular weight metabolites within 48 h.

In vitro studies showed that procyanidin oligomers (dimers and trimers) passed through the human epithelial Caco-2 cell monolayer, whereas polymers did not [77].

Epigallocatechin gallate enter the brain after a gastric administration and methylated flavonoids cross the BBB more readily, whereas glucuronide or glycoside conjugates have greater difficulty [59, 78, 79].

It is well recognized that orally administered flavonoids would undergo extensive presystemic first-pass metabolism. Substantial intestinal and hepatic glucuronidation have been found in a number of structurally diverse flavonoids.

Glucuronides of phenolic compounds have generally been assumed to be rapidly excreted in vivo and to be pharmacologically inactive, but several studies are demonstrating that some drug glucuronides may be pharmacologically active [80, 81].

Studies on the stability of procyanidins oligomers isolated from *Theobroma cacao* L. has shown that these compounds are unstable under acidic conditions and decompose to epicatechin monomeric and dimeric units, but also to other oligomeric units [81].

During digestion and transfer across the small intestine, and in the liver, flavanols are rapidly metabolized in phase I and phase II biotransformations to various *O*-sulfated, *O*-glucuronidated and *O*-methylated forms. Various UGT isozymes, expressed in the intestine and in the liver, have been identified to catalyze the glucuronidation of flavonoids. In humans consuming cocoa, plasma levels of non-methylated epicatechins such as epicatechin-7-sulfate and methylated metabolites such as 3′-*O*-methylepicatechin have been reported to occur in micromolar concentrations within 1 h after intake. Metabolic studies have confirmed the presence of these conjugates in the plasma and urine of rodents and humans, as well as in the bile and brain of rats. It has been reported that colonic microflora can break flavonoids flavan structure to form simple phenolics and rig-fission metabolites that may be physiologically relevant.

Studies incubating EGCG with intestinal fluid (pH 6.5) for only 5 min resulted in a significant decrease (81.6 %) in the amount of EGCG, whereas a similar incubation in plasma (pH 7.4) resulted in only a 29.3 % decrease [82].

In summary, non-metabolized flavanols or metabolites of flavanols can exert biological effects depending essentially on flavanol metabolism and presence at target tissue. Certain monomers may be better absorbed than others. For example, in humans consuming a cocoa beverage containing equal amount of epicatechin and catechin, epicatechin was identified to be the predominant plasma flavanol absorbed, with plasma catechin levels reaching less than 10 % of epicatechin concentrations. Part of these differences in plasma flavanol concentrations, could be due to procyanidin degradation: dimers have been shown to form epicatechin and methylated epicatechin under certain conditions, although the physiological relevance of such degradation remains to be confirmed. Although it is uncertain whether the trimers and higher polymers of the flavanols are readily absorbed, it has been suggested that over prolonged exposure, bacterial breakdown and colonic absorption of smaller molecules may occur. Both absorption and metabolism of cocoa-derived catechins appear to be influenced by chocolate matrix [17, 38].

In fact, in a complex food matrix, the pH changes are likely to be buffered on the inside for long periods of time, thus limiting flavanol oxidation during intestinal transit.

Several studies have been performed regarding the influence of milk protein on the bioavailability of epicatechin from cocoa beverages and chocolate. Serafini et al. [83] reported that milk resulted in a reduced AUC for epicatechin relative to control in chocolate confections, while others reported no statistical difference between the AUC of epicatechin from cocoa beverages consumed with water or milk. Recently Mullen et al. [84] reported that milk decreases urinary excretion but not plasma pharmacokinetics of cocoa flavan-3-ol metabolites. Ortega et al. [85] observed that the fat content of certain cocoa samples enhances the digestibility of some phenolic compounds, especially procyanidins during duodenal digestion. Recently, Neilson et al. [86] suggested that chocolate confections containing high levels of sucrose might enhance plasma level of the predominant catechin and epicatechin metabolites as compared to milk chocolate confections, while confections containing moderate levels of sucrose and no milk deliver intermediate plasma levels of these compounds. However, the physical state of the product may significantly modulate this effect.

3.3 Human Studies

According to Donovan et al. [87], the concentration of (–)-catechin was higher to that of (+)-catechin in chocolates. However, bioavailability of (–)-catechin was less than the (+) form of catechin, resulting in low plasma concentration of (–)-catechin. On the other hand, Fraga et al. [88] reported that (+)-catechin was 100 times more efficient than quercetin in an in vivo oxidative stress model [55].

In a human clinical trial, the administration of 148 mg of procyanidins had increased plasma epicatechin at 2 h compared to baseline (0 h) [33]. In addition, plasma epicatechin concentration increased to 21.2 nmol/L after consumption of the procyanidins and then returned to normal levels [33]. Similarly, plasma procyanidin dimer, (–)-epicatechin and (+)-catechin can be detected as early as 0.5 h and reach maximal concentrations by 2 h after acute consumption of cocoa [36]. Murphy et al. [1] indicated that administration of procyanidins increased plasma (–)-epicatechin and (+)-catechin by 81 % and 28 %, respectively. Moreover, (–)-epicatechin was detected as early as 0.5–1 h after chocolate or cocoa consumption and they are present mainly as sulfate conjugates, glucuronides, or methylated forms [89]. A study related to plasma kinetics of epicatechin in men after consumption of 40 and 80 g of dark chocolates, detected that epicatechin increased markedly after chocolates consumption, reaching a maximum between 2 and 3 h [90]. The maximal concentration and AUC of plasma kinetics correlate well with the dose of chocolates. This indicates that epicatechin is absorbed from chocolates and is rapidly eliminated from plasma. Attainable plasma levels were 0.7 μmol/L (free epicatechin and epicatechin conjugates) from 80 g of black chocolates, which contain 164 mg of epicatechin. To a greater extend, Baba et al. [91] indicated that administration of procyanidins B2 [epicatechin-(4β-8)-epicatechin] (Fig. 74.4) extracted from cocoa powder was absorbed in the plasma and excreted in the urine. The compounds appeared maximally in the plasma at 30 min and decreased gradually from 30 to 300 min [55].

Baba et al. [89] reported that (–)-epicatechin from chocolate or cocoa is present in plasma of human volunteers as sulfate, glucuronide, and sulfate-glucuronide (mixture of sulfate and glucuronide) conjugates, rather than methylated forms. It was reported that plasma concentration of glucuronide conjugates of non-methylated and methylated (–)-epicatechin were higher compared to other forms. Using a pure (–)-epicatechin compound, Da Silva showed that (–)-epicatechin is present in plasma in the forms of glucuronide and sulfate-glucuronide conjugates (free and *O*-methylated) [92]. The glucuronidation of (–)-epicatechin occurs at the position 3′ of the B ring in humans [93]. Natsume et al. [93] found that (–)-epicatechin metabolites present in human plasma are different than the forms present in rats. The glucuronidation of (–)-epicatechin occurs at the 7 position of the A ring and the 3′ position of the B ring in rats and humans, respectively. This glucuronidation compound showed low antioxidant activity compared to intact compounds as it has lost the catechol structure of the B ring responsible for the antioxidative effects. (–)-Epicatechin metabolites are present in urine within 24 h in the range of 25–30 %. There is a significant reduction in total (–)-epicatechin metabolites after 6 h and the remaining conjugates are mostly present as the *O*-methylated form. Excretion of (–)-epicatechin metabolites in urine was observed to be dose dependent [94]. Moreover, it was observed that the level of (–)-epicatechin metabolites excreted in urine was in the close range after equivalent ingestion of pure (–)-epicatechin and (–)-epicatechin from cocoa powder. Hence, the bioavailability of (–)- epicatechin was not influenced with the presence of other compounds present in cocoa powder [55].

4 Bioactivity of Proanthocyanidins

Proanthocyanidins are the principal vasoactive polyphenols in red wine that are linked to a reduced risk of coronary heart disease and to lower overall mortality [95]. The French Paradox introduced the idea that flavonoids found in red wine and fresh fruit may be responsible for the lower rates of cardiovascular diseases in France. In fact, red wine was the first polyphenol-rich food (year 1979) to be inversely associated with ischemic heart disease deaths in industrialized countries [96] and with deaths from coronary heart disease in segments of the French population [97]. Hertog et al. [98] linked a lower mortality from cardiovascular disease in an elderly population of Dutch males with a high flavonol intake, principally with the consumption of onions, apples, and tea.

Today, there is much epidemiological evidence that diets rich in fruits and vegetables can reduce the incidence of different diseases such as cardiovascular diseases, diabetes, cancer, and stroke. These protective effects are attributed, at least in part, to phenolic secondary metabolites. Initially, the protective effect of dietary phenolics was thought to be due to their antioxidant properties, which resulted in a lowering of the levels of free radicals within the body. However, there is now more evidence that the metabolites of dietary phenolics, which appear in the circulatory system in nmol/L to low mmol/L concentrations, exert modulatory effects in cells through selective actions on different components of the intracellular signaling cascades vital for cellular functions such as growth, proliferation, and apoptosis. In addition, the intracellular concentrations required to affect cell signaling pathways are considerably lower than those required to cause an antioxidant effect [99].

4.1 Redox Activity

Several studies indicate that catechins and procyanidins are powerful scavengers of ROS. Some findings regarding the antioxidant activity of proanthocyanidins are listed in Ref. [100]. Other antioxidant mechanisms are the chelation of transition metals, as well as the mediation and inhibition of enzymes. The metal-chelating activity of proanthocyanidins is thought to be due to their capacity to reduce the concentration, and thus the oxidative activity, of hydroxyl radicals formed by Fenton reaction catalyzed by iron or copper. Flavanols also influence oxidative stress via enzyme modification and modulation of cell signaling pathways; the extent of the effect relies greatly on flavanol structure-related protein reactivity [101].

4.2 Nonredox Activity

Nonredox mechanism of actions of flavonoids might also be relevant in vivo. It has been suggested that flavonoids metabolites could interact with cell signaling cascades, influence the cell at a transcriptional level, or downregulate pathways leading to apoptosis [102].

Epigallocatechin gallate have been shown to inhibit COX-2 and iNOS expression by preventing NF-kB activation [103]. Oligomers of 5–12 subunits, with the most powerful pentamers, are active on different human cancer cell lines [104].

Moreover, monomers and dimers were shown to repress NO production, TNF alpha secretion, and NF-kB-dependent gene expression induced by interferon γ, whereas the trimeric procyanidin C2 and Pycnogenol enhanced these parameters. In addition, in unstimulated RAW 264.7 macrophages, both procyanidin C2 and Pycnogenol increased TNF gamma secretion in a concentration and time-dependent manner. These results demonstrate that procyanidins act as modulators of the immune response in macrophages [105].

Results from Vestraeten et al. [106] indicate that dimers and trimers isolated from peanut and cocoa interact with lipid membranes and thus can modulate membrane fluidity. The alteration of membrane fluidity can per se affect numerous cellular processes influencing, among others, the functionality of membrane-associated enzymes and certain intracellular transport mechanisms and membrane receptors. In addition, procyanidins may reduce the incorporation into the bilayer of hydrophobic compounds that can affect, either directly or indirectly, the integrity of the membrane.

With respect to the physiological relevance of dimers and trimers, it has been reported that dietary procyanidins are relatively stable during gastric transit [40]; thus, high micromolar concentrations of both dimers and trimers can be predicted to occur throughout the gastrointestinal tract following the consumption of procyanidin-rich foods. While absorption of trimers from the gut is thought to be very limited, monomers and dimers can be readily detected in the plasma pool within 2 h after the consumption of flavonoid-rich foods or beverages. Thus, while the potential protective effects of dimers and trimers are probably most relevant for the gastrointestinal track, the dimers may also provide some protection to cells in circulation, as well as vascular endothelial cells [106].

4.3 Atherosclerosis, Cytotoxicity, Carcinogenicity, Platelet Aggregation, Vasodilatation, Vasoconstriction, Reperfusion, and Cellular Adhesion

Other nonredox mechanisms of actions of proanthocyanidins are involved in amelioration of several steps in the process of atherosclerosis [107].

Procyanidin B2 (dimer), isolated from loquat leaves, was found to inhibit the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced activation of Epstein-Barr virus early antigen in Raji cells [108]. Animal studies and cell models suggest that flavonoids act as anticarcinogens through influencing molecular events in the initiation, promotion, and progression stages of cancer [109].

Regarding cytotoxic activities, the 3,3′-di-*O*-gallate derivative of procyanidin B2 was found to be active against human leukemic cells (HL-60) and a melanoma cell line but was inactive toward several other tumor cell lines. Several other galloylated procyanidin dimers were also shown to inhibit the growth of human lung and colon carcinoma cell lines [7, 108].

In vitro experiments with whole blood showed that cocoa procyanidin trimers and pentamers increased expression of platelet activation markers (fibrogen binding conformation of GPIIb-IIIa and P-selectin) in unstimulated platelets but suppressed platelet activation response to epinephrine [110]. Both short-term (2–6 h) studies and a long-term (28 day) study with human subjects demonstrated that consumption of proanthocyanidin-rich cocoa beverage lowered P-selectin expression and platelet aggregation (ADP-, collagen-, epinephrine induced) in ex vivo experiments [110, 111].

The inhibition of Ca^{2+} influx through Ca^{2+} channels thereby activating NO release is a proposed mechanism in nitric oxide–dependent vasodilation by proanthocyanidins [112].

Angiotensin II is a vasoconstrictor that is produced in the pulmonary capillaries by angiotensin-converting enzyme and can be involved in the development of hypertension and atherosclerosis [113]. Several proanthocyanidins and preparations containing them inhibited angiotensin-converting enzyme activity in both in vitro and in vivo experiments. These included pycnogenol, proanthocyanidins isolated from red grapes, and extracts of *Erythroxylum laurifolium* (endemic species on Reunion Island in the Indian Ocean) and of fruits of *Cupressus sempervirens* L. (Italian cypress) [107].

Hearts from red wine proanthocyanidin-fed rats were more resistant to ischemia-reperfusion injury than hearts from control animals [114, 115]. Blood flow parameters were improved, whereas infarct size, formation of hydroxyl radicals, and malondialdehyde levels of heart perfusate were all modulated as a result of feeding animals proanthocyanidins or proanthocyanidin-containing ingredients. These same dietary treatments also reduced the levels of proapoptotic factors JNK and c-Jun, as well as the proportion of apoptotic cardiomyocytes.

Proanthocyanidins and/or their metabolites [116] containing high proportion of A-type linkages are beneficial in the case of urinary tract infection. The primary effect is inhibition of cellular adherence of P-type (mannose-resistant) uropathogenic strains of *Escherichia coli* [117, 118].

4.4 Water-Insoluble Polyphenols

Among the dietary constituent, cocoa highly contributes to the intake of water-insoluble polyphenols. Recent studies [119] have demonstrated that procyanidin content in food is largely underestimated. Authors suggested that the measurement of non-extractable polyphenols may be crucial in assessing reliable dietary intake of polyphenols and this is of particular importance when the benefits exerted by polyphenols bound to dietary fiber throughout the gastrointestinal tract are investigated. Fogliano et al. [120] have shown that insoluble polyphenols are able to exert antioxidant activity through the whole gastrointestinal tract; despite being still bound to other macromolecules and that the human digestive process solubilizes a significant part of the bound polyphenols and it increases their bioaccessibility.

4.4.1 Potential Adverse Effects

Procyanidins have been considered antinutritional compounds because they can interact with proteins, starch, essential amino acids, and carbohydrates and inhibit certain enzymes [121–123]. This binding depends on the degree of polymerization: the larger molecules tend to bind more efficiently [7]. However, at the dose present in cocoa no adverse effect has been observed [124, 125]. In addition, the level of flavonoids required to induce mutations and cytotoxicity may not be physiologically achievable through dietary sources; however the use of flavonoid supplements could result in exposure to potential toxic levels [126]. Reported flavanol-related health detrimental effects include activation of pro-carcinogens, pro-oxidant activity, hemorrhage formation, initiation of hepatotoxicity, genotoxic effects, interference with thyroid hormone biosynthesis, alteration of pharmacokinetics of therapeutic drugs, increased estrogenic tumor formation, mutagenicity, modification of plasma biochemistry, instigation of gastroenteritis, antinutritive activity, and weight loss [100, 127–129].

Proanthocyanidin-rich products have been reported to possess the ability to cause cell toxicity to normal, healthy tissue [129] and although generally considered safe, ingestion of higher concentration of proanthocyanidins instigates destruction of mucosal lining of the digestive tract, gastroenteritis, and congestion of the intestinal wall in rats, hemorrhagic gastroenteritis in rabbits [130], and striking lesions in the digestive tract of sheep [131]. Structurally similar to endogenous steroid hormones, flavonoids may also promote estrogenic activity by initiating increased expression of aromatase (CYP19), which correlates with tumor initiation, promotion, and progression [132, 133]. Different studies on rats have found approximately the same NOAEL for procyanidins, which correspond to an average of 12 g daily by an adult for 3 months. Moreover, a grape seed proanthocyanidins extract is under study in a Phase I pilot chemopreventive study in healthy women at high risk of developing breast cancer [8, 134, 135].

5 Conclusion

Regarding the general healthy population, the intake of moderate quantities of chocolate might be considered as safe. Some kind of chocolate, in particular dark chocolate, contains high amounts of polyphenols and proanthocyanidins, which are actually believed to possess different pharmacological activities. The most studied activity is undoubtedly the antioxidant, but some nonredox mechanism of actions could be also relevant in vivo: some flavonoids and flavonoids metabolites could interact with cell signaling cascades, influence the cell at a transcriptional level, or downregulate pathways leading to apoptosis.

Although the scientific evidence on the antioxidant properties of polyphenols and proanthocyanidins is relevant, this is not yet the case for the pharmacokinetic and pharmacodynamic aspects of such compounds and their metabolites, in particular when considering mechanisms other than the antioxidant. Interactions with other compound and food matrix could alter the effects. Moreover the health status of the subject could explain the differences in clinical outcomes.

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Polyphenols and Beer Quality

75

Sonia Collin, Vesna Jerkovic, M. Bröhan, and D. Callemien

Contents

1 Introduction 2334
2 Catechins and Proanthocyanidins 2334
  2.1 Colloidal Instability 2335
  2.2 Astringency 2342
  2.3 Color 2343
  2.4 Health Properties 2343
3 Prenylchalcones and Derived Flavanones 2343
  3.1 Health Properties 2345
4 Flavonols 2346
  4.1 Bitterness 2346
  4.2 Health Properties 2346
5 Hydroxybenzoic Acids, Hydroxycinnamic Acids, and Derived Compounds 2346
  5.1 Flavor 2347
  5.2 Color 2350
6 Stilbenes 2350
  6.1 Health Properties 2351
7 Conclusion 2352
References 2353

Abstract

Beer phenols issued from malt and hop can contribute directly to several characteristics of beer, mainly flavor, astringency, haze, body, and fullness. Some phenolic structures can also impart very interesting health properties. Yet phenolic structures also evolve through storage. Low-molecular-weight phenols like 4-vinylsyringol can impart off-flavors in aged beer, while oxidized

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flavonoids strongly influence astringency, haze, and color. The instability of stilbenes, prenylchalcones, and derived flavanones could also modify their health potential.

Keywords
Aging • beer • brewing process • colloidal stability • flavor • polyphenols

Abbreviations

AEDA	Aroma extract dilution analysis
APCI	Atmospheric-pressure chemical ionization
C	Catechin
E	Epicatechin
EGC and GC	Epigallocatechin and gallocatechin
ESI	Electrospray ionization
FD	Dilution factor
HPLC or LC	High-performance liquid chromatography
MS	Mass spectroscopy
MS/MS	Tandem mass spectroscopy
NP	Normal phase
P1 to P10	Procyanidins from monomers to decamers
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
RP	Reversed phase
UV	Ultraviolet

1 Introduction

Beer phenols issued from malt and hop can contribute directly to several characteristics of beer, mainly color, flavor, astringency, and haze. As antioxidants, they can also protect raw materials from oxidative degradation throughout the process, minimizing therefore off-flavors such as *trans*-2-nonenal.

In this chapter, all phenolic structures that have been found in beer will be described.

2 Catechins and Proanthocyanidins

Even compared to grapes, hop emerges as an exceptional source of catechins and proanthocyanidins. Therefore, although added in 100 times lesser quantity than malt, it can account for 30% of total beer polyphenols. Among hop cultivars, the lower the bitterness, the higher the flavonoid level (up to 1% “total flavanoids” in Saaz pellets, as expressed in terminal unit weight [1]). During mashing, malt flavonoids are progressively dissolved in the wort (monomers dissolve much faster than oligomers). From mash filtration to boiling, a great proportion of them will be lost through oxidation, adsorption to spent grains, linkage to coagulated proteins, etc. According to the type of hop conditioning used (CO_2 extracts are much poorer in polyphenols than pellets and cones) and the stage of addition, more or less flavonoids will be brought into the wort in the boiling kettle [2].

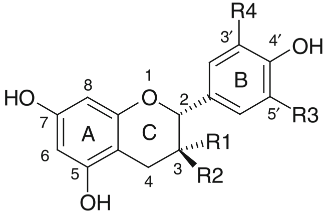
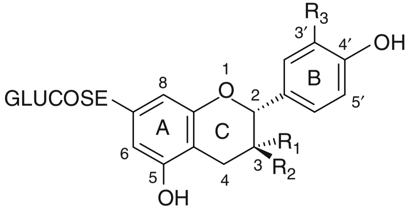
In dried hop cones or pellets, (+)-catechin and (−)-epicatechin monomers can reach up to 2,821 and 1,483 ppm, respectively [3–7]. Malt contains only 10–100 ppm (+)-catechin (and no epicatechin at all) [3, 5, 8–10]. The main monomeric unit identified in beer is (+)-catechin (0.5 to 6.9 mg.L^{-1}), but (−)-epicatechin (0.8–1.9 mg.L^{-1}), (−)-catechin gallate, (−)-epicatechin gallate, and two glycosides have also been detected (Table 75.1) [8, 11–21].

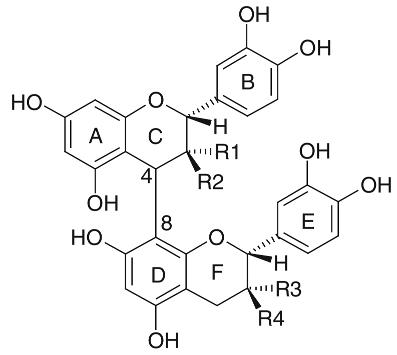
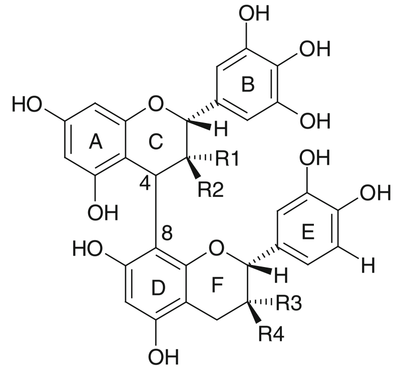
Hop is also an excellent source of flavonoid oligomers (proanthocyanidins, known as anthocyanogens in the brewing field). For instance, B3 and B4 procyanidin dimers have been detected at levels up to 0.1% [3, 5–7]. Malt contains two B3 dimers (prodelphinidin and procyanidin) at lower levels than in hop, but with higher amounts of gallocatechin units [3, 5, 10, 22]. Many trimers have also been detected in malt (catechin and gallocatechin units) and hop (catechin, epicatechin, and gallocatechin units, but always a catechin unit at the terminal position). Thiolysis hyphenated to RP-HPLC-ESI(−)-MS/MS was recently optimized by our group to investigate beer polyphenolic oligomers [17]. Thiolysis indicated that most beer dimers are procyanidins B3 (two catechin units), while most trimers are prodelphinidins (catechin in terminal units and gallocatechins or catechins in extension units). Despite the absence of chromatographic peaks corresponding to oligomers above trimers, an apparent degree of polymerization (mDP) close to 6 was calculated in a total LH20 extract. Detailed structures were determined by RP-HPLC-ESI(−)-MS/MS [16]. Four dimers were identified: three procyanidins (B1, B3, and B4) and one prodelphinidin (B3) (Table 75.1). Previously detected in hop or malt, three trimers (the procyanidin C-4∝-8-C-4∝-8-C and two prodelphinidins, GC-4∝-8-C-4∝-8-C and GC-4∝-8-GC-4∝-8-C), were distinguished for the first time in beer. As expected, according to previous thioacidolysis data, most beer proanthocyanidins carry a catechin as terminal unit.

2.1 Colloidal Instability

Colloidal instability due to interactions between polyphenols and proteins limits the shelf life of beer. A lag phase is usually observed in lager beers before chill-haze development [23–25]. The time needed to form critical amounts of tanning polyphenols leading to visible chill-haze particles corresponds to the lag phase. As described by Leemans et al. [25] for different batches, the longer the lag phase, the better the colloidal stability.

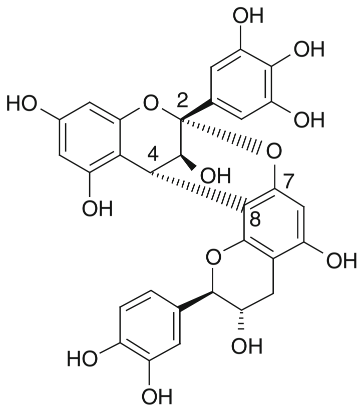
Table 75.1 Flavan-3-ol monomers, polymers, and range of concentrations in beer [8, 11, 13, 14, 16, 19–21, 31, 62, 70, 124–126]

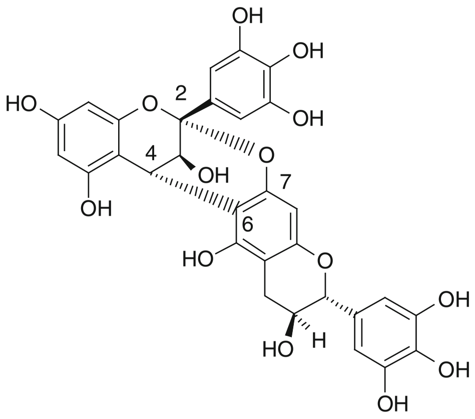
Structures	Compounds	R1	R2	R3	R4	Concentrations in beer (mg.L^{-1})
Monomers						
 R4 3′ 4′ OH B R3 5′ HO 7 8 1 O 2 A C 6 5 4 3 R1 R2 OH	(+)-Catechin	H	OH	H	OH	0.28–6.9
	(−)-Epicatechin	OH	H	H	OH	<0.10–1.9
	(−)-Catechin gallate	H	Gallate	H	OH	5–20
	(−)-Epicatechin gallate	Gallate	H	H	OH	5–20
	3-O-methylcatechin	H	OCH$_3$	H	OH	Detected
 R$_3$ 3′ 4′ OH B 5′ GLUCOSE 8 1 O 2 A C 6 5 4 3 R$_1$ R$_2$ OH	Catechin-7-O-β-D-glucopyranoside	H	OH	OH		Detected
	Catechin-7-O-β (6”-O-nicotinoyl)-β-D-glucopyranoside	H	OH	OH		Detected

Dimers						
Procyanidins B						
 OH OH HO O B A C H R1 4 R2 OH OH OH 8 HO O E D F H R3 R4 OH	**B1** (−)-Epicatechin-(4*β*-8)-(+)-catechin	OH	H	H	OH	Detected
	B3 (+)-Catechin-(4α-8)-(+)-catechin	H	OH	H	OH	Traces–3.1
	B4 (+)-Catechin-(4α-8)-(−)-epicatechin	H	OH	OH	H	Detected
Prodelphinidins B						
 OH OH HO O B OH A C H R1 4 R2 OH OH OH 8 HO O E H D F H R3 R4 OH	**B3** (−)-Gallocatechin-(4α-8)-(+)-catechin	H	OH	H	OH	Traces–3.3
	B9 (−)-Epigallocatechin-(4*β*-8)-(+)-catechin	OH	H	H	OH	Detected

(*continued*)

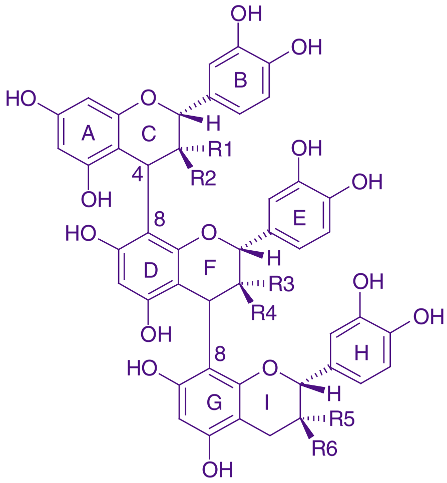
Table 75.1 (continued)

Structures	Compounds	R1	R2	R3	R4	Concentrations in beer (mg.L^{-1})
Prodelphinidins A						
 OH OH HO O 2 OH O 4 OH 7 OH 8 O OH HO OH OH	*ent*- (−)-Epigallocatechin -(4α-8, 2α-O-7)-(+)-catechin					Detected

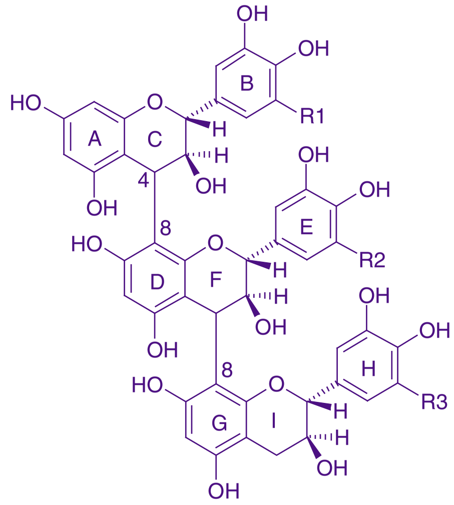
 OH OH OH HO O 2 4 OH OH O 7 6 HO O OH HO H OH OH	*ent*- (−)-Epigallocatechin -(4α-6, 2α-O-7)-(+)-catechin	Detected

(*continued*)

Table 75.1 (continued)

Structures	Compounds	R1	R2	R3	R4	Concentrations in beer (mg.L^{-1})
Trimers						
Procyanidins C						
	**C2** (+)-Catechin-(4α-8)-(+)-catechin -(4α-8)-(+)-catechin	H	OH	and R5 = H	and R6 = OH	Detected

OH
OH
HO
O
B
A
C
H
R1
4
R2
OH
OH
OH
8
HO
O
E
D
F
H
R3
R4
OH
OH
OH
8
HO
O
H
G
I
H
R5
R6
OH

Prodelphinidins C						
	(−)-Gallocatechin-(4α-8)-(−)-gallocatechin-(4α-8)-(+)-catechin	OH	OH	H	/	Detected
	(−)-Gallocatechin-(4α-8)-(+)-catechin-(4α-8)-(+)-catechin	OH	H	H	/	Detected

OH
OH
B
HO
O
A
C
H
R1
H
4
OH
OH
OH
OH
8
HO
O
E
D
F
H
R2
H
OH
OH
OH
OH
8
HO
O
H
G
I
H
R3
H
OH
OH

Chill haze (or reversible haze), defined by non-covalent bonds between polyphenols and active proteins, can eventually turn into permanent haze that no longer dissolves as the beer warms.

Catechin does not rapidly induce strong haze. Upon storage, however, it does. Likewise, colloidal instability caused by dimers and trimers is enhanced after oxidation (not true for tetramers and pentamers) [26–28]. Free radicals are known to enhance haze [29]. Tannoids have been defined by Chapon [30] as intermediates in the oxidation of simple flavanoids to tannins, forming complexes with proteins. On the other hand, according to O'Rourke et al. [28], oxidized flavanols cause chill haze, but only subsequent polymerization leads to tannoids and permanent haze [28, 31].

Leemans et al. [25] have proposed a model in which aldehydes and oxygen play key roles in tanning polyphenol formation [25, 31]. Not only dissolved oxygen but also shaking, higher temperature, polyphenol-rich raw materials, light, and heavy metals will significantly increase colloidal instability [25, 31].

Beer contains less haze-active polyphenols than haze-active proteins. Derived from barley hordeins, haze-active proteins (10–30 kDa) are acidic hydrophilic polypeptides, rich in both proline and glutamic acid [26] and glycosylated [32]. Much more haze is produced near pH 4.0 than at pH 3.0 or above pH 4.2. At the beer pH, ethanol at low concentration causes a modest decline of haze, while strong haze is observed at higher concentrations [33].

To preserve beer colloidal stability, brewers usually remove haze-active materials [34]. To get rid of haze-active proteins, precipitation with tannic acid, hydrolysis with papain and adsorption to bentonite [35] or silica gel [36, 37] are very effective, but unfortunately in some cases, such procedures also remove foam proteins. To remove haze-active polyphenols, the most usual way is adsorption to polyvinylpolypyrrolidone-PVPP. Because of the structural analogy between these compounds and proline [38], pyrrolidone rings bind polymerized flavanoids through hydrogen and ionic bonds.

New combined absorbents are now proposed to brewers, such as PVPP mixed with silica xerogel, PVP bound onto silica, and tannin linked to silica [23, 39]. Another innovative way is the use of flavan-3-ol and proanthocyanidin-free malt which allows affording an excellent colloidal stability [40].

2.2 Astringency

In beer, flavanoids could be also responsible for astringency [41, 42]. Catechin and epicatechin thresholds lie between 1 and 20 ppm [41, 42], with higher values for the beer matrix (20 ppm). Astringency is intensified at low pH, especially near 4.0–4.2 [43], but a higher astringency has been measured by François et al. [44] in beers with a pH close to 5. In this case, it was suspected that the pH of the samples fell in the mouth before polyphenol/protein interactions occurred. Sensory analyses applied to top-fermented beers have shown that storage (20°C or 40°C with air in the headspace) decreases bitterness and post-bitterness but intensifies astringency [45]. On the other hand, no significant astringency-related deterioration was measured in lager beers aged for 5 days at 40°C (with or without oxygen) [44]. In both cases, an increase in DP (global assay) and a decrease in total flavanoids were mentioned, especially at higher temperature or pH, in the presence of air [44, 45].

2.3 Color

Beer color increases through storage, especially in the presence of oxygen and at higher temperature. At pH 3, colorless catechin-derived products are formed after enzymatic oxidation, whereas at pH 6 after chemical degradation, yellow products including dehydrodicatechin A dimers differing by their interflavan linkages can be detected in model media (Fig. 75.1) [46, 47]. Recently, Callemien and Collin [15] detected dehydrodicatechin A in a beer spiked with catechin after storage.

2.4 Health Properties

Flavan-3-ols induce cardioprotective effects, including antioxidant effects (protection against LDL oxidation) and inhibition of platelet activity and vasodilatation [48, 49]. Flavonoids might reduce the risk of cancer, although some procarcinogenic activities have also been reported [48, 50]. Flavonoids alter the synthesis of eicosanoids (mediators of inflammation). They decrease the leukotriene/prostacyclin ratio by modifying lipoxygenase activity [51, 52]. Immune regulation has also been observed [53]. Hop proanthocyanidins can help prevent nitric oxide-related disorders such as Alzheimer's and Parkinson's diseases [7].

3 Prenylchalcones and Derived Flavanones

More than twenty prenylchalcones and derived flavanones, studied mainly for their biological effects, have been identified in hop [54]. Concentrations higher than 0.6%, with a predominance of xanthohumol and desmethylxanthohumol, are usually found (Fig. 75.2) [55]. Levels of 80 and 90 ppm have been reported for the corresponding flavanones, isoxanthohumol and hopein. The higher the α-acid content (bitter acids in hop), the higher the xanthohumol level [56].

Since hop is the only source of these compounds in beer, a relation can be established between their concentration and the rate of hopping. Xanthohumol isomerizes easily during the brewing process into isoxanthohumol [57]. Only 15–50% hop xanthohumol remains in the final beer [58, 59], leading to concentrations often below 1 mg.L^{-1} [18, 55, 60, 61]. Stout- and Porter-style beers are characterized by slightly higher levels because dark malts contain compounds inhibiting xanthohumol isomerization [57]. The use of xanthohumol-enriched hop products (obtained by ethanol-CO_2 extraction) combined with late hopping makes

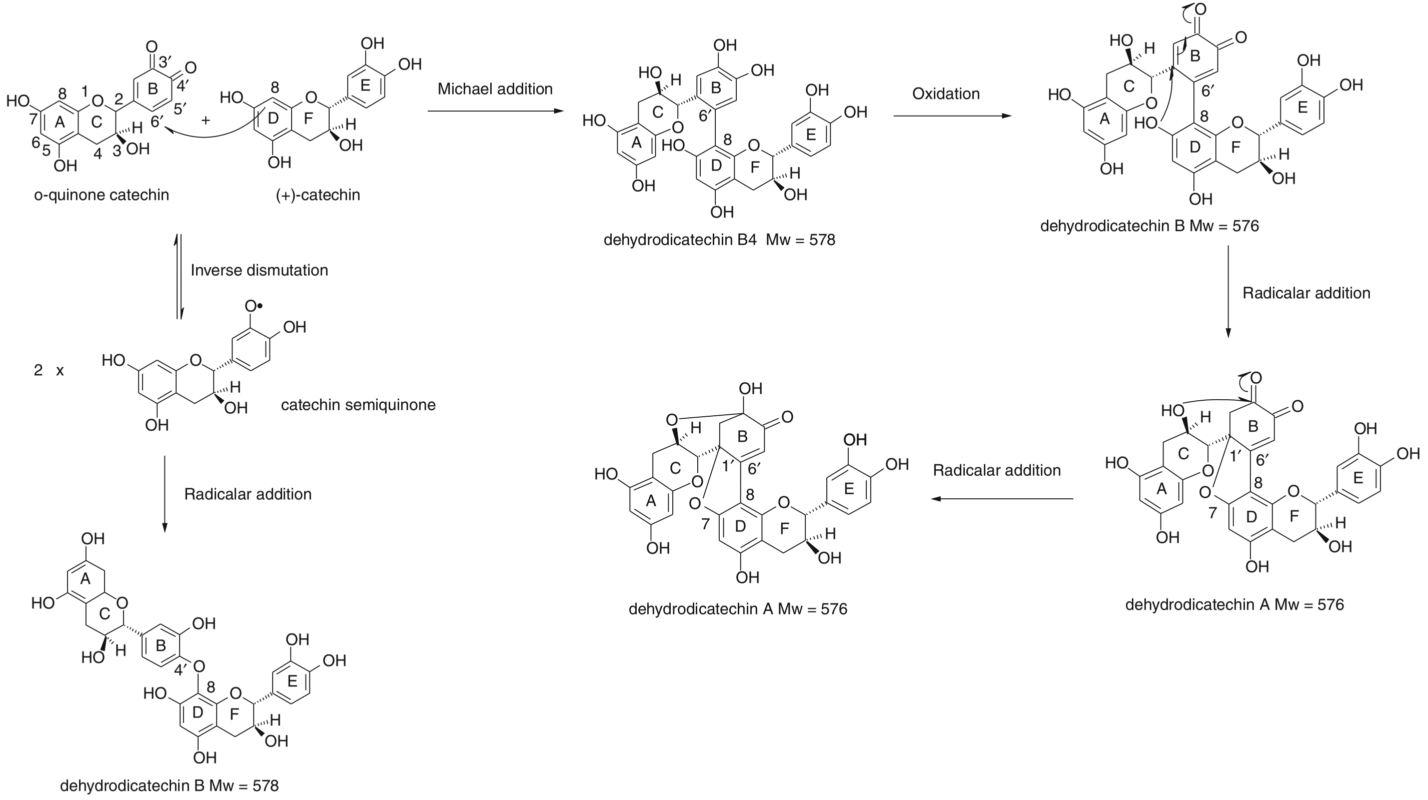

o-quinone catechin
(+)-catechin
Michael addition
dehydrodicatechin B4 Mw = 578
Oxidation
dehydrodicatechin B Mw = 576
Radicalar addition
dehydrodicatechin A Mw = 576
Radicalar addition
dehydrodicatechin A Mw = 576
Inverse dismutation
2 x
catechin semiquinone
Radicalar addition
dehydrodicatechin B Mw = 578

Fig. 75.1 Proposed degradation schemes of (+)-catechin to form colorless compounds with Mw = 578 and yellow compounds with Mw = 576 [15, 46]

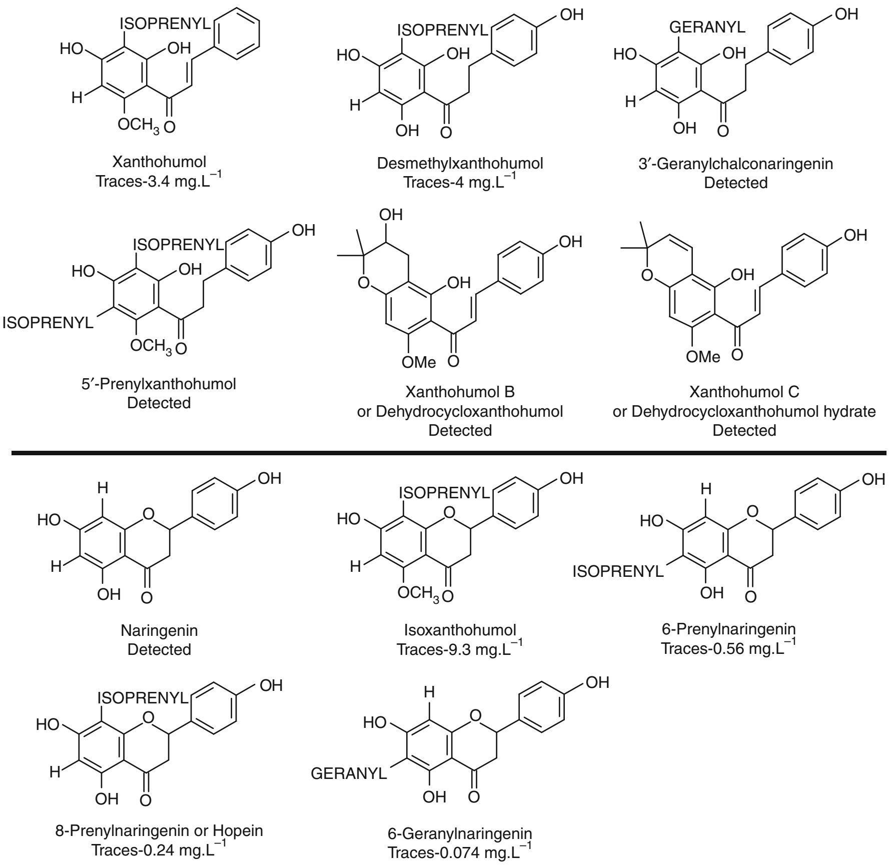

ISOPRENYL
HO
OH
H
OCH_3 O
Xanthohumol
Traces-3.4 mg.L^{-1}
ISOPRENYL
OH
HO
OH
H
OH O
Desmethylxanthohumol
Traces-4 mg.L^{-1}
GERANYL
OH
HO
OH
H
OH O
3′-Geranylchalconaringenin
Detected
ISOPRENYL
OH
HO
OH
ISOPRENYL
OCH_3 O
5′-Prenylxanthohumol
Detected
OH
OH
O
OH
OMe O
Xanthohumol B
or Dehydrocycloxanthohumol
Detected
OH
O
OH
OMe O
Xanthohumol C
or Dehydrocycloxanthohumol hydrate
Detected
H
OH
HO
O
H
OH O
Naringenin
Detected
ISOPRENYL
OH
HO
O
H
OCH_3 O
Isoxanthohumol
Traces-9.3 mg.L^{-1}
H
OH
HO
O
ISOPRENYL
OH O
6-Prenylnaringenin
Traces-0.56 mg.L^{-1}
ISOPRENYL
OH
HO
O
H
OH O
8-Prenylnaringenin or Hopein
Traces-0.24 mg.L^{-1}
H
OH
HO
O
GERANYL
OH O
6-Geranylnaringenin
Traces-0.074 mg.L^{-1}

Fig. 75.2 Prenylchalcones, derived flavanones, and range of concentrations in beer (Adapted from [31])

it possible to increase significantly the xanthohumol and isoxanthohumol potential of beer (close to 10 mg.L^{-1}).

3.1 Health Properties

Xanthohumol is a "broad-spectrum" cancer chemopreventive agent acting on all three stages of carcinogenesis. Xanthohumol and isoxanthohumol are both active ROS scavengers, while only the former is active in superoxide scavenging assays. Isoxanthohumol, 8-prenylnaringenin, and xanthogalenol may also exert chemopreventive effects [18, 61–64].

Prenylflavanones have mainly been studied for their estrogenic activity. Hopein is a very potent phytoestrogen. The authors recommend its application in prevention or treatment of (post)menopausal symptoms and osteoporosis [61, 63, 65]. Weak estrogenic activity has been observed for close analogs like 6-prenylnaringenin, 8-geranylnaringenin, 6,8-diprenylnaringenin, and isoxanthohumol. Prenylchalcones like xanthohumol and xanthogalenol also show low activity [66].

4 Flavonols

Sixteen flavonol glycosides (mainly mono-, di-, and triglycosides of quercetin and kaempferol) have been detected in hop [31]. Although boiling can extract 91% of the kaempferol and 88% of the quercetin glycosides, only a few ppms of flavonols are found in the final beer (Fig. 75.3) [8, 11, 13, 67, 68].

4.1 Bitterness

In beer, flavonols could be responsible for bitterness but do not participate in beer haze formation [31].

4.2 Health Properties

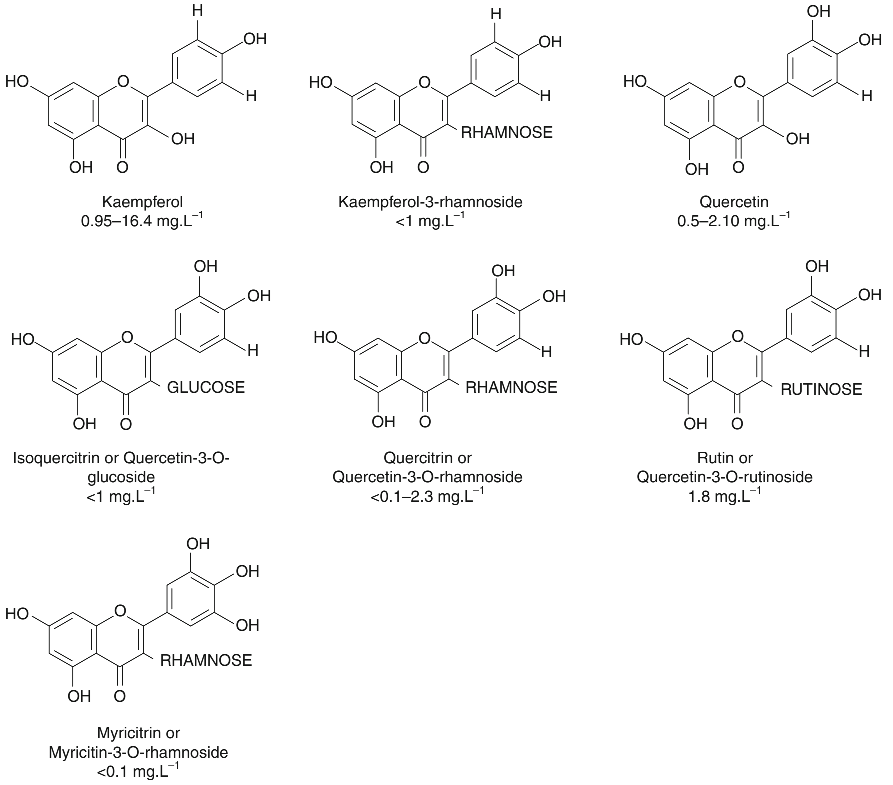
Flavonols induce cardioprotective effects, including antioxidant effects (protection against LDL oxidation) and inhibition of platelet activity and vasodilatation [48, 69], while very little information is available on their potential anticancer effects.

5 Hydroxybenzoic Acids, Hydroxycinnamic Acids, and Derived Compounds

Malt and hop contain various hydroxybenzoic acids, which are retained at least partially up to the final beer (Fig. 75.4). Total hydroxybenzoic acids – mainly *p*-hydroxybenzoic, vanillic, and gallic acids – usually reach a few ppms in beer. They have also been found as glycosides or other bound forms [8, 11–14, 70–74].

Hydroxycinnamic acids are partially recovered in beer (Fig. 75.4). Most of them are in combined forms in the raw materials, either with quinic acid, glucose, or cell-wall constituents [8, 11–14, 70–78]. In malt, *p*-coumaric and ferulic acids are esterified with arabinoxylans [79]. They can be both water extracted and enzymatically solubilized by cinnamoyl esterases [80]. After mashing, an additional release of ferulic acid may occur during fermentation due to yeast cinnamoyl esterases [81].

Three coumarins issued from orthohydroxycinnamic acid cyclization have also been found in beer (Fig. 75.5) [8, 73].



Kaempferol
0.95–16.4 mg.L^{-1}

Kaempferol-3-rhamnoside
<1 mg.L^{-1}

Quercetin
0.5–2.10 mg.L^{-1}

Isoquercitrin or Quercetin-3-O-glucoside
<1 mg.L^{-1}

Quercitrin or
Quercetin-3-O-rhamnoside
<0.1–2.3 mg.L^{-1}

Rutin or
Quercetin-3-O-rutinoside
1.8 mg.L^{-1}

Myricitrin or
Myricitin-3-O-rhamnoside
<0.1 mg.L^{-1}

Fig. 75.3 Flavonols and range of concentrations in beer (Adapted from [31])

5.1 Flavor

Hydroxybenzoic and hydroxycinnamic acids are characterized by relatively high flavor thresholds (> ppm, mainly bitter taste and astringency) [82]. On the other hand, their decarboxylated derivatives (Fig. 75.5) can impart very strong phenolic/clove/smoked flavors to beer because of their low threshold values (ppb order).

Decarboxylation can occur either by thermal degradation [83] during malt kilning and in the boiling kettle [77, 84], or during fermentation. In this last case, decarboxylation is catalyzed by the phenylacrylic acid decarboxylase found in *Saccharomyces cerevisiae* strains displaying the Pof^{+} phenotype (phenolic off-flavor) [85, 86] and in some contaminating microorganisms like *Brettanomyces*/*Dekkera* spp. [87] or *Enterobacteriaceae* [88]. In this way, 4-vinylguaiacol is issued from ferulic acid, while 4-vinylphenol derives from *p*-coumaric acid. 4-Vinylguaiacol has been also found in hop [89].

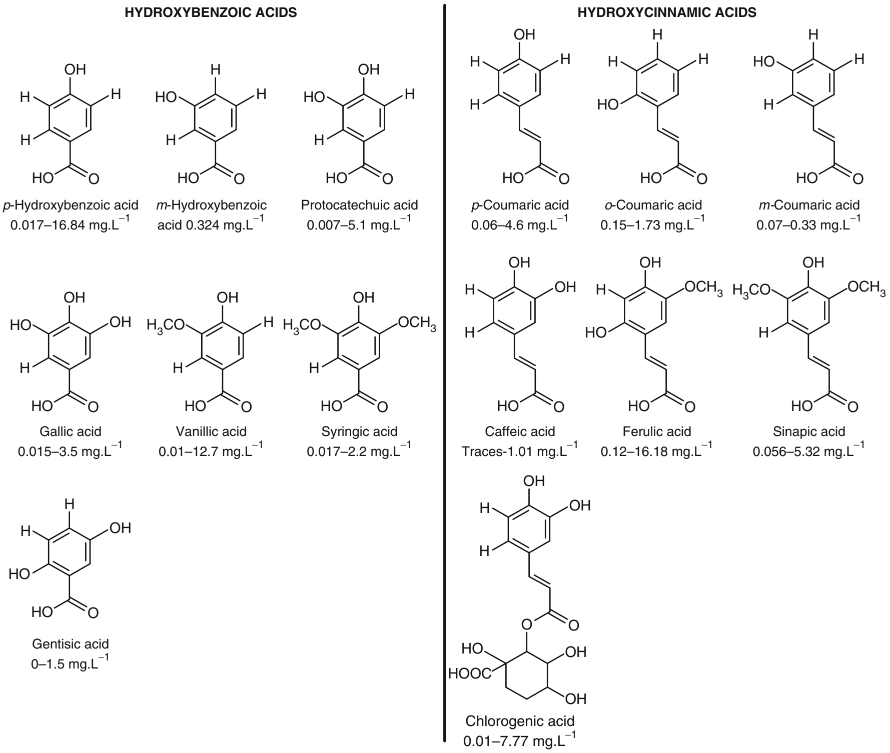

HYDROXYBENZOIC ACIDS
HYDROXYCINNAMIC ACIDS
p-Hydroxybenzoic acid 0.017–16.84 mg.L^{-1}
m-Hydroxybenzoic acid 0.324 mg.L^{-1}
Protocatechuic acid 0.007–5.1 mg.L^{-1}
Gallic acid 0.015–3.5 mg.L^{-1}
Vanillic acid 0.01–12.7 mg.L^{-1}
Syringic acid 0.017–2.2 mg.L^{-1}
Gentisic acid 0–1.5 mg.L^{-1}
p-Coumaric acid 0.06–4.6 mg.L^{-1}
o-Coumaric acid 0.15–1.73 mg.L^{-1}
m-Coumaric acid 0.07–0.33 mg.L^{-1}
Caffeic acid Traces-1.01 mg.L^{-1}
Ferulic acid 0.12–16.18 mg.L^{-1}
Sinapic acid 0.056–5.32 mg.L^{-1}
Chlorogenic acid 0.01–7.77 mg.L^{-1}

Fig. 75.4 Hydroxybenzoic acids and hydroxycinnamic acids and range of concentrations in beer (Adapted from [31])

In Belgian white beer production, enzymatic decarboxylation of ferulic acid occurs linearly through fermentation at a rate close to 140 ppb/day (Fig. 75.6). The rate decreases strongly during secondary fermentation, down to 20 ppb/day. Compared to *p*-coumaric acid, ferulic acid is preferentially degraded by yeast (*p*-coumaric acid remains unmodified until the ferulic acid concentration reaches 2 ppm) [31]. Concentrations up to 6.2 ppm in 4-vinylguaiacol and up to 3.2 ppm in 4-vinylphenol have been reported in wheat beers [8, 77–79, 81, 90–97]. For instance, 4-vinylguaiacol contributes to the specificity of Belgian white beers (made with unmalted wheat) and German rauch and weizen beers (made with malted wheat) [92, 94, 97, 98]. According to its concentration, 4-vinylguaiacol can lead either to strong pharmaceutical off-flavor defects [91] or to pleasant clove flavors [79], while 4-vinylphenol is always considered to be an off-flavor [79]. These vinyl compounds can be further oxidized or reduced into smaller molecules like vanillin, 4-ethylguaiacol, guaiacol, and 4-ethylphenol through chemical reactions [99] or through the activity of wild yeasts like *Brettanomyces*/*Dekkera* spp. [87].

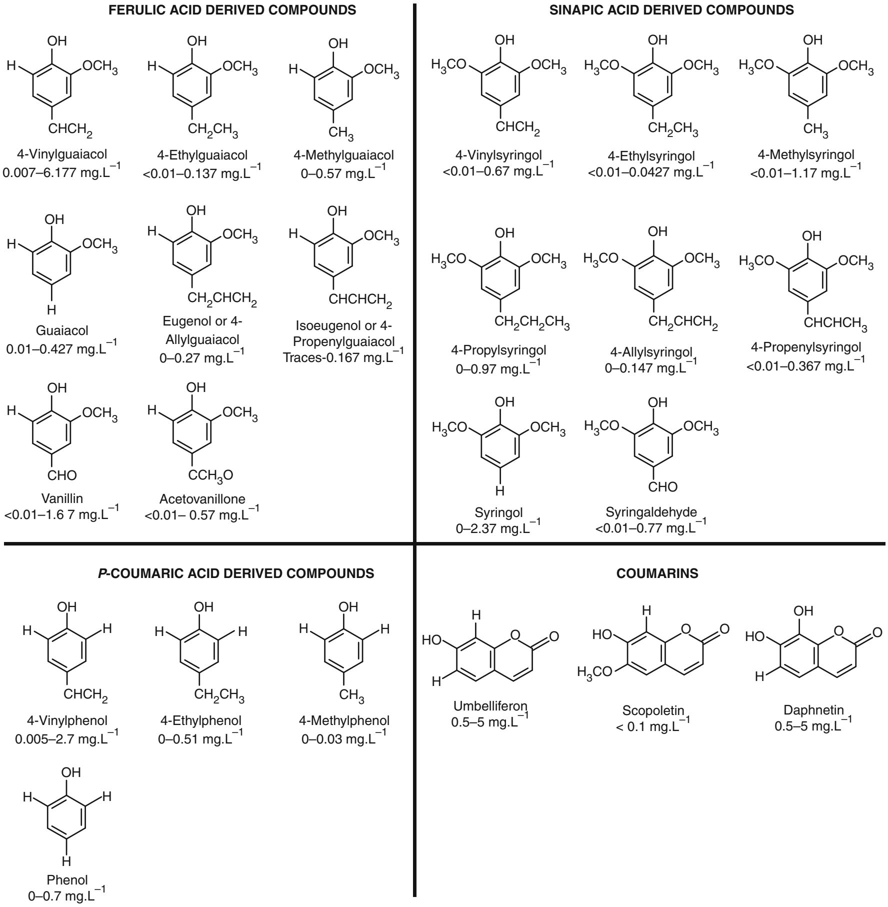

FERULIC ACID DERIVED COMPOUNDS
4-Vinylguaiacol
0.007–6.177 mg.L^{-1}
4-Ethylguaiacol
<0.01–0.137 mg.L^{-1}
4-Methylguaiacol
0–0.57 mg.L^{-1}
Guaiacol
0.01–0.427 mg.L^{-1}
Eugenol or 4-Allylguaiacol
0–0.27 mg.L^{-1}
Isoeugenol or 4-Propenylguaiacol
Traces-0.167 mg.L^{-1}
Vanillin
<0.01–1.6 7 mg.L^{-1}
Acetovanillone
<0.01– 0.57 mg.L^{-1}
SINAPIC ACID DERIVED COMPOUNDS
4-Vinylsyringol
<0.01–0.67 mg.L^{-1}
4-Ethylsyringol
<0.01–0.0427 mg.L^{-1}
4-Methylsyringol
<0.01–1.17 mg.L^{-1}
4-Propylsyringol
0–0.97 mg.L^{-1}
4-Allylsyringol
0–0.147 mg.L^{-1}
4-Propenylsyringol
<0.01–0.367 mg.L^{-1}
Syringol
0–2.37 mg.L^{-1}
Syringaldehyde
<0.01–0.77 mg.L^{-1}
P-COUMARIC ACID DERIVED COMPOUNDS
4-Vinylphenol
0.005–2.7 mg.L^{-1}
4-Ethylphenol
0–0.51 mg.L^{-1}
4-Methylphenol
0–0.03 mg.L^{-1}
Phenol
0–0.7 mg.L^{-1}
COUMARINS
Umbelliferon
0.5–5 mg.L^{-1}
Scopoletin
< 0.1 mg.L^{-1}
Daphnetin
0.5–5 mg.L^{-1}

Fig. 75.5 Hydroxybenzoic- and hydroxycinnamic acid-derived compounds and range of concentrations in beer (Adapted from [31])

Degradation of 4-vinylguaiacol through natural aging (25% after 20 days) or at 40°C (50% after 20 days) has been reported [77, 94, 100]. This compound could be partially transformed to 4-ethylguaiacol, vanillin, and guaiacol [79, 101].

By using the AEDA methodology on aged lager beers, 4-vinylsyringol was identified as a strong old-beer-like phenolic odorant (FD value as high as that of *trans*-2-nonenal, responsible for the cardboard off-flavor in aged beer) [102, 103]. Its release through aging should be due to acidic hydrolysis of a glycoside, since sinapic acid decarboxylation occurs much earlier in the process, either in the boiling kettle or during fermentation.

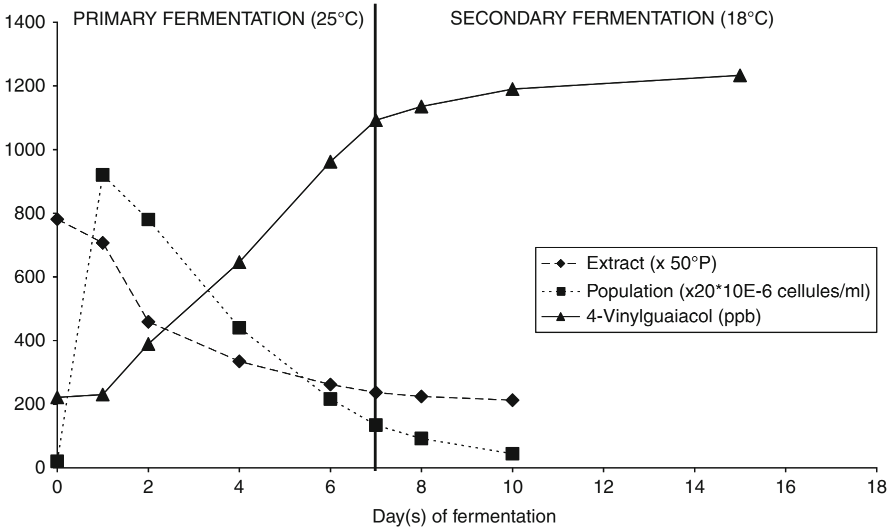


Fig. 75.6 Evolution of extract, cell population, and 4-vinylguaiacol concentration through fermentation of a Belgian white beer

5.2 Color

Phenolic acids do not participate in beer haze formation [27, 104], but vinylphenol and cinnamic acid have been described as potential pigments [105–108].

6 Stilbenes

Our group recently discovered three stilbenes in hop: *trans*-resveratrol, *trans*-piceid, and *cis*-piceid [4, 109]. Concentrations ranging from 0.7 to 11 ppm *trans*-piceid and from 0.03 to 2.3 ppm *trans*-resveratrol have been reported in hop cones [110]. A strong influence of geographic origin and harvest year has been shown [110], but American aromatic cultivars like Willamette and Cascade emerge in all cases as the best sources of stilbenes. Resveratrol is very sensitive to heat and light [110]. Even during hop storage, a significant loss occurs, especially in highly oxygen-sensitive varieties, leading to new analogs like *cis*-resveratrol and dimers [111]. Likewise, hop pelletization induces strong degradation [111, 112]. *trans*-Resveratrol and glycosides are absent from malt [113], so one should not be surprised to find only traces of stilbenes in beer (Fig. 75.7). Up to 5 μg.L^{-1} *trans*-resveratrol was detected by our group in Belgian commercial beers [114].

Taking into account a concentration of 1–10 ppm stilbenes in hop and hopping close to 200 g.hL^{-1} in wort, a maximum of 2–20 μg.L^{-1} stilbenes could be

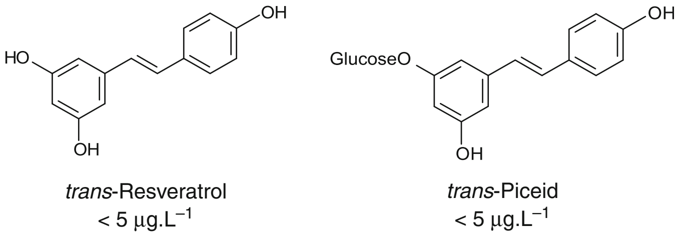


Fig. 75.7 Stilbenes and range of concentration in beer [114]

expected in beer. Moreover, massive degradation of *trans*-resveratrol is known to occur in the boiling kettle (60% degradation after 7 min of boiling – Fig. 75.8a). *trans*-Piceid is much more stable during heat treatment, and it can be converted to free resveratrol through wort fermentation (only 60% recovered from a beer prepared by spiking an industrial wort at 10 mg.L^{-1} before fermentation – Fig. 75.8b). When the wort was not pitched, *trans*-piceid remained stable, suggesting that yeast enzymes catalyze this hydrolysis to free resveratrol, as previously described for wine [114]. On the other hand, the *trans*-resveratrol concentration decreased even in the absence of yeast (40% degradation), most likely because of reactions with wort components (apparent degradation of 17% in the presence of yeast due to the equilibrium with piceid).

In order to increase the stilbene level, stilbene-enriched hop products and brewery process modifications are needed (e.g., adding a stilbene-enriched ethanolic hop extract after fermentation significantly increases the beer stilbene potential).

6.1 Health Properties

trans-Resveratrol shows an impact on platelet aggregation and vasodilatation, and through its effect on the antioxidant status, regulates gene expression and decreases the total lipid concentration (cholesterol and triglycerides) [115]. Although less potent, *cis*-resveratrol, *trans*-, and *cis*-piceid also improve the antioxidant activity [116, 117].

Piceid absorption is enhanced by the presence of its sugar [118].

trans-Resveratrol inhibits the initiation and growth of tumors. It inhibits cyclo-oxygenase, ornithine decarboxylase, and angiogenesis [119, 120]. *trans*-Piceid is a weaker inhibitor of ROS production [121]. As flavonoids, *trans*-resveratrol alters the synthesis of eicosanoids (mediators of inflammation) and decreases the leukotriene/prostacyclin ratio by modifying lipoxygenase activity [120–122]. Estrogenic activity has recently been reported for some stilbenes, especially *trans*-resveratrol. *cis*-Resveratrol appears less potent [120, 123].

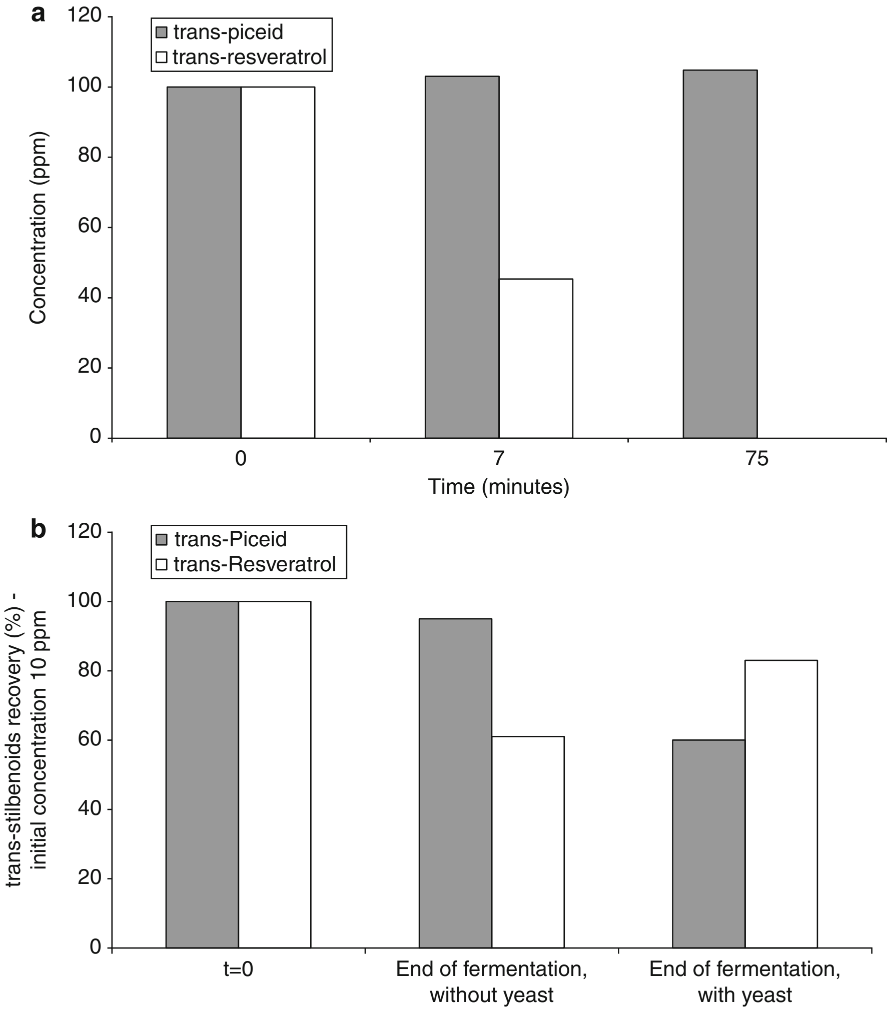


Fig. 75.8 Follow-up of the degradation of *trans*-resveratrol and *trans*-piceid (**a**) in an aqueous model medium previously flushed by nitrogen-mimicking wort ebullition (**b**) through wort fermentation [112, 114]

7 Conclusion

The aim of the present chapter was to review all phenolic structures that have been found in beer. Each family was discussed according to its properties and stability through storage. However, at the end of this chapter, it is very difficult to advice brewers as to which phenols should be kept in the final beer and at what levels.

Beer phenols issued from malt and hop can contribute directly to several characteristics of beer, mainly flavor, astringency, haze, body, and fullness. Some phenolic structures also impart very interesting health properties. However, degradation of such compounds will inevitably lead to alteration of fresh beer. On the other hand, as antioxidants, these compounds can considerably protect raw materials from oxidative degradation throughout the process.

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Wine Polyphenols and Vascular Protective Effects

76

Cyril Auger and Valérie B. Schini-Kerth

Contents

1 Introduction 2362
2 Mechanism of Endothelium-Dependent Vascular Protection 2362
2.1 The Endothelium-Derived Nitric Oxide 2363
2.2 The Endothelium-Derived Hyperpolarizing Factor (EDHF) 2365
2.3 The Prostacyclin (PGI_2) 2365
3 Vascular Protection by Wine Polyphenols: In Vitro and Ex Vivo Studies 2366
3.1 Red Wine Polyphenols Induce the Activation of eNOS In Vitro 2366
3.2 Red Wine Polyphenols Induce Endothelium-Dependent Relaxations Ex Vivo ... 2369
3.3 Red Wine Polyphenols Have Beneficial Effects on the Endothelial Function In Vivo 2371
4 Clinical Studies on the Beneficial Effects of Wine Polyphenols 2372
5 Conclusion 2373
References 2373

Abstract

Numerous studies indicate that regular intake of polyphenol-rich food and beverages such as red wine is associated with a protective effect on the cardiovascular system. In addition to the antioxidant property, polyphenols may also induce a beneficial effect on the cardiovascular system by several other mechanisms including the improvement of the vascular function. Indeed, experimental and clinical studies indicate that polyphenols are potent inducers of two major endothelial vasoprotective mechanisms, the formation of nitric oxide (NO) and the induction of endothelium-derived hyperpolarization (EDH).

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1 Introduction

Diet is one of the major risk factors of mortality by cardiovascular diseases associated with lifestyle [1–3]. For example, the Mediterranean diet has been reported to have a favorable effect on lipid profile, endothelium-dependent vasodilatation, insulin resistance, metabolic syndrome, antioxidant capacity, and myocardial and cardiovascular mortality [4, 5]. Moreover, a recent meta-analysis of epidemiological studies showed that greater adherence to a Mediterranean diet is associated with a significant reduction (9 %) in mortality from cardiovascular diseases [6]. The Mediterranean diet is defined in part by its high content in polyphenols coming from fruits, vegetables, and the regular and moderate consumption of red wine. Numerous epidemiological studies have shown a protective effect against cardiovascular diseases of various rich sources of polyphenols such as red wine [7–9], vegetables and fruits [10–13], green tea [14, 15], as well as chocolate and cocoa [16, 17].

Endothelial function is a key regulator of the vascular health, and its alteration has been shown to be an independent predictor of cardiovascular outcome in subjects with cardiovascular risks [18]. Numerous polyphenols and polyphenol-rich sources have been shown to improve the endothelial function; thus, polyphenol-rich sources such as red wine might prevent the development of cardiovascular diseases, at least in part, by improving the endothelial dysfunction.

This chapter summarizes experimental and clinical evidence suggesting that red wine and other sources rich in polyphenols and flavonoids improve the endothelial function in health and diseases.

2 Mechanism of Endothelium-Dependent Vascular Protection

The endothelium is a monolayer of cells covering the lumen of all blood vessels. While the endothelium has been considered for a long time as an inert barrier between the vessel wall and the blood, studies in the late 1970s revealed that endothelial cells play a major role in the regulation of vascular tone [19, 20]. Indeed, Furchgott and Zawadzki first reported that acetylcholine induced relaxations of rabbit aortic rings suspended in organ chambers only in the presence of an intact endothelium [19]. Further investigations indicated that the endothelium plays a key role not only in the control of vascular tone but also in the adhesion and aggregation of platelets and leucocytes to the blood vessel, in endothelial permeability, smooth muscle cell proliferation, and blood coagulation [21–26].

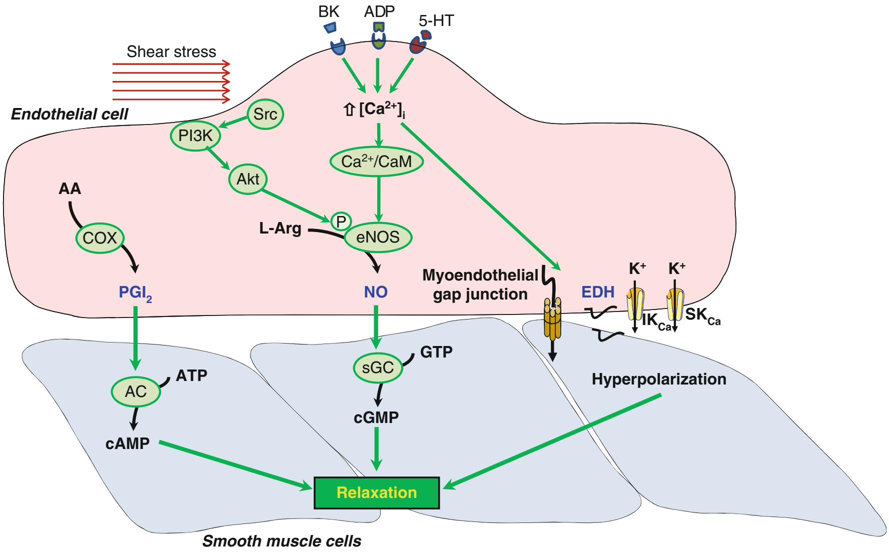

Fig. 76.1 *Endothelium-derived relaxing factors*. Endothelial nitric oxide synthase (eNOS) generates nitric oxide (NO) from L-arginine (L-Arg) after its activation by shear stress via the Src/PI3-kinase/Akt pathway or by a variety of agonists (*5-HT* serotonin, *BK* bradykinin, *ADP* adenosine diphosphate) via the calcium/calmodulin pathway (Ca^{2+}/CaM). NO induces relaxation of the vascular smooth muscle by the activation of soluble guanylyl cyclase (sGC). Endothelium-derived hyperpolarization (EDH) often involves the activation of endothelial SK$_{Ca}$ and IK$_{Ca}$ channels (small and intermediate conductance Ca^{2+}-activated K$^+$ channels, respectively) inducing hyperpolarization (⁓) of the endothelium, which is, thereafter, transmitted, in part, to the underlying vascular smooth cells via myo-endothelial gap junctions with subsequent relaxation. Prostacyclin (PGI$_2$) is also a potent vasodilator, which has potent platelet inhibitory effects. Abbreviations: *Src* Src family kinase, *PI3K* phosphatidylinositol 3-kinase, *ATP* adenosine triphosphate, *cAMP* cyclic adenosine monophosphate, *GTP* guanosine triphosphate, *cGMP* cyclic guanosine monophosphate, *AC* adenylyl cyclase, *AA* arachidonic acid

The regulation of the vascular tone by the endothelium is due predominantly to the endothelial formation of several potent vasorelaxing factors involving nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin (PGI$_2$) (Fig. 76.1).

2.1 The Endothelium-Derived Nitric Oxide

The main relaxing factor produced by the endothelium is nitric oxide or NO. After the first report by Furchgott and Zawadzki in 1980 that described an endothelium-derived relaxing factor (EDRF), it took 7 years to identify the EDRF as NO [19, 27, 28]. NO is a labile gas produced by the conversion of L-arginine to L-citrulline by NO synthases, a group of heme-containing dimeric enzymes. Among the three isoforms, one is induced by several pro-inflammatory cytokines such as interleukin and is named inducible NO synthase (iNOS or type II NOS), while the other two are constitutively activated and observed mainly in neural tissue (neuronal NOS, nNOS, or type I NOS) and in endothelial cells (endothelial NOS, eNOS, or type III NOS). The eNOS is a 135 kDa protein that is primarily found in the Golgi apparatus as well as in caveolae, the membrane association requiring myristoylation and palmitoylation [29]. Several physiological stimuli including shear stress, bradykinin, serotonin, and ADP have been reported to induce the activation of eNOS by both calcium/calmodulin-dependent and -independent events. Indeed, shear stress induces a sustained NO formation in native endothelial cells that is dependent on an initial increase of the intracellular concentration of free calcium, while the sustained phase is not sensitive to chelation of calcium or inhibition of calmodulin [30, 31]. Under basal conditions, eNOS is mostly in an inactivated state due to its association to caveolin 1, the caveolae scaffold protein, and to HSP90 (heat-shock protein). The calcium-dependent activation of eNOS is mainly due to the increase of the intracellular concentration of free calcium followed by the association of calcium with calmodulin. The calcium-calmodulin complex then interacts with HSP90, which directly activates eNOS by allowing its phosphorylation by kinases and the dissociation of eNOS from caveolin 1 [32]. The activated eNOS will then relocate to the Golgi apparatus where it will generate NO [33].

In addition to the calcium-dependent activation, the activity of eNOS can also be regulated positively or negatively by the phosphorylation of the enzyme. Indeed, it has been reported that phosphorylation of the residues serine 1177 or 615 activates eNOS while the phosphorylation of the residue threonine 495, tyrosine 657, or serine 114 causes an inhibitory posttranslational modification [34]. Activation of eNOS subsequent to its phosphorylation on serine 1177 in endothelial cells has been observed in response to shear stress, bradykinin, and VEGF [34]. Several kinases are involved in the phosphorylation of serine 1177 including the protein kinase A (PKA), protein kinase B (Akt), or Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII) (for review, see [34]). On the other hand, eNOS is constitutively phosphorylated on threonine 495. This phosphorylation is associated in endothelial cells with a reduced binding ability of the calcium-calmodulin complex to eNOS [35].

While eNOS can be activated by both the calcium signal and phosphorylation, the availability of the substrate and the presence of endogenous inhibitors might also contribute to regulate the activity of eNOS and the endothelial formation of NO. Indeed, several studies indicate that cardiovascular diseases are often associated with an increased expression and/or activity of arginase, an enzyme which competes with eNOS for its substrate L-arginine, and also with a reduced L-arginine transport in endothelial cells leading to a reduced availability of the substrate and, thus, to a reduced formation of NO [34, 36].

After its activation, eNOS converts its substrate L-arginine into NO and L-citrulline. NO can diffuse freely in the vascular wall and reach the smooth muscle cells where it activates soluble guanylyl cyclase, an enzyme-transforming guanosine-5′-triphosphate (GTP) into guanosine 3′,5′-cyclic monophosphate (cGMP).

The increase in cGMP activates the cyclic-dependent protein kinase (PKG), which reduces the intracellular concentration of calcium and activates the myosin light chain phosphatase (MLCP), leading to relaxation of the vascular smooth muscle. In addition, NO can also exert a large number of effects such as inhibition of platelet adhesion and aggregation, smooth muscle proliferation, monocyte adhesion, as well as activation of glucose uptake, glycolysis, and fatty acid oxidation (for reviews, see [23, 37]).

2.2 The Endothelium-Derived Hyperpolarizing Factor (EDHF)

The EDHF is an endothelium-derived relaxing factor which importance increases with the decrease of the size of the artery. Indeed, EDHF-mediated relaxations are not observed in the aorta, the largest conductance artery, while it is the main endothelium-derived relaxing factor in small resistance arteries [38]. Initially, EDHF responses were defined as the relaxations that persisted in the presence of inhibitors of eNOS and cyclooxygenases and were associated with the hyperpolarization of the vascular smooth muscle (for review, see [39]). The classical EDHF pathway has been described as an increase in the intracellular free calcium concentration in endothelial cells inducing the opening of two calcium-dependent potassium channels, the small and intermediate conductance calcium-dependent potassium channels (SK_{Ca} and IK_{Ca}, respectively). Activation of these channels induces the hyperpolarization of endothelial cells, which will be transmitted to the underlying smooth muscle cells either by myo-endothelial gap junctions or activation of inwardly rectifying potassium channel (K_{IR}) and ouabain-sensitive Na^+/K^+-ATPase. In addition, an EDHF pathway independent of the endothelial hyperpolarization has also been described. This EDHF pathway seems to depend on the hyperpolarization of smooth muscle cells by opening of various potassium channels such as ATP-sensitive and large-conductance potassium channels (K_{ATP} and BK_{Ca}, respectively$_{)}$, most likely subsequent to the release of several factors by endothelial cells. These endothelial factors have not been totally elucidated and seem to vary depending on the species and the vascular bed studied, but include so far NO, hydrogen peroxide, prostacyclin, epoxyeicosatrienoic acids (EETs), vasoactive peptides, and carbon monoxide (for review, see [39]). Due to this complex and multifaceted origin, it has been recently suggested that the term EDHF was no longer adequate and should be replaced by EDH for endothelium-derived hyperpolarizations.

2.3 The Prostacyclin (PGI_2)

In 1976, Moncada et al. reported that microsomes isolated from rabbit and pig aortas were able to convert endoperoxides into a factor which has strong vasorelaxant and anti-aggregant properties [40]. The product was later identified as the prostacyclin PGI_2, which is the major prostanoid produced by endothelial cells. Its synthesis starts by the conversion of arachidonic acid into endoperoxides by the action of the two isoforms of cyclooxygenases, before the subsequent conversion of endoperoxides into prostacyclin by the PGI_2 synthase. After diffusion toward the underlying smooth muscle, PGI_2 activates the adenylyl cyclase, which converts adenosine-5′-triphosphate (ATP) into 3′,5′-cyclic adenosine monophosphate (cAMP). cAMP, in turn, activates protein kinase A leading to a decrease in the free calcium concentration and inhibition of the myosin light chain kinase, an enzyme responsible for phosphorylating smooth muscle myosin, thus inducing relaxation of the vascular smooth muscle.

3 Vascular Protection by Wine Polyphenols: In Vitro and Ex Vivo Studies

The first study on the potential of red wine and grape-derived polyphenols to inhibit the vascular tone was reported in 1993 by Fitzpatrick et al. using rat aortic rings suspended in organ chambers [41]. Following this first study, numerous other groups have studied the polyphenol-induced activation of the eNOS leading to an increased formation of the vasoprotective NO using in vitro, ex vivo, and in vivo approaches. In addition, several studies have reported that polyphenols are able to induce vasorelaxations through EDH in isolated arteries, suggesting that polyphenols can activate both pathways in the endothelium (Fig. 76.2).

3.1 Red Wine Polyphenols Induce the Activation of eNOS In Vitro

The in vitro characterization of the vascular effects of polyphenols from red wine has focused mainly on the potency of red wines to activate the NO pathway in endothelial cells. In 2002, Leikert et al. reported that exposure of cultured endothelial cells (HUVECs and EaHy926 cells) to a red wine extract for several hours resulted in the induction of eNOS expression and subsequent formation of NO [42]. This effect was later confirmed using French red wine by Wallerath et al. [43, 44]. The characterization of the pathways involved indicated that red wine polyphenols activate a redox-sensitive mechanism. Indeed, using the redox-sensitive probe dihydroethidine, it has been shown that red wine polyphenols cause the intracellular formation of superoxide anions in cultured coronary artery endothelial cells [45]. Moreover, the activation of eNOS in endothelial cells induced by a red wine polyphenolic extract is significantly inhibited by intracellular antioxidants like *N*-acetylcysteine, membrane-permeant analogs of superoxide dismutase (SOD), such as Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) and polyethylene glycol-SOD (PEG-SOD). The formation of superoxide anions in endothelial cells, in turn, triggers a Src/PI3-kinase/Akt pathway, which ultimately causes eNOS activation by increasing the phosphorylation level of Ser 1177, a positive regulatory site, and the dephosphorylation of Thr 495, a negative regulatory site [46, 47]. Moreover, it has been reported that the formation of NO in cultured endothelial

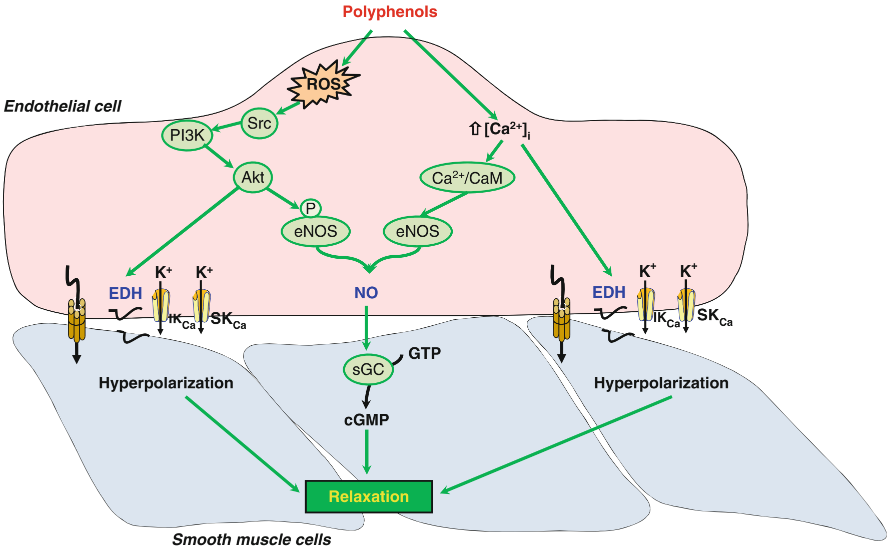

Fig. 76.2 *Polyphenols and polyphenol-rich sources induce endothelial-dependent NO- and EDH-mediated relaxations.* Polyphenols are potent inducers of the endothelial formation of nitric oxide (NO) and endothelium-derived hyperpolarization (EDH) via a redox-sensitive mechanism. *SK_{Ca}* small conductance calcium-activated potassium channels, *IK_{Ca}* intermediate conductance calcium-activated potassium channels, *Src* Src family kinase, *PI3K* phosphatidylinositol 3-kinase, *eNOS* endothelial NO synthase, *L-Arg* L-arginine, *sGC* soluble guanylyl cyclase, *GTP* guanosine triphosphate, *cGMP* cyclic guanosine monophosphate, *AA* arachidonic acid, *COX* cyclooxygenase, *ATP* adenosine triphosphate, *cAMP* cyclic adenosine monophosphate

cells induced by a red wine polyphenolic extract requires calcium in the extracellular medium and is associated with an increased intracellular calcium signal [48]. However, the calcium signal is rather small and short lasting; therefore, it has been suggested that the calcium signal is most likely acting in synergy with the Src/PI3-kinase/Akt pathway to induce a rapid and sustained eNOS-dependent NO formation in endothelial cells.

The activation of eNOS in endothelial cells by red wine polyphenols involves several classes of phenolic compounds (Fig. 76.3). Indeed, the fractionation of a red wine polyphenolic extract indicated that the activation of eNOS and subsequent formation of NO are mainly associated with anthocyanins and procyanidins [49, 50]. Moreover, the activation of eNOS by anthocyanins appears to be strongly related to the structure of the compounds. Indeed, two closely related anthocyanins have been isolated from a red wine extract, malvidin-3-coumaroylglucoside and petunidin-3-coumaroylglucoside, which differ only by a single substituent: a methoxyl function on position 5′ for the malvidin-3-coumaroylglucoside and a hydroxyl function on position 5′ for the petunidin-3-coumaroylglucoside. While petunidin-3-coumaroylglucoside strongly activated eNOS by phosphorylation on serine 1177, malvidin-3-coumaroylglucoside did not have such an effect, indicating a critical role for the hydroxyl function on position 5′ of the B ring for eNOS

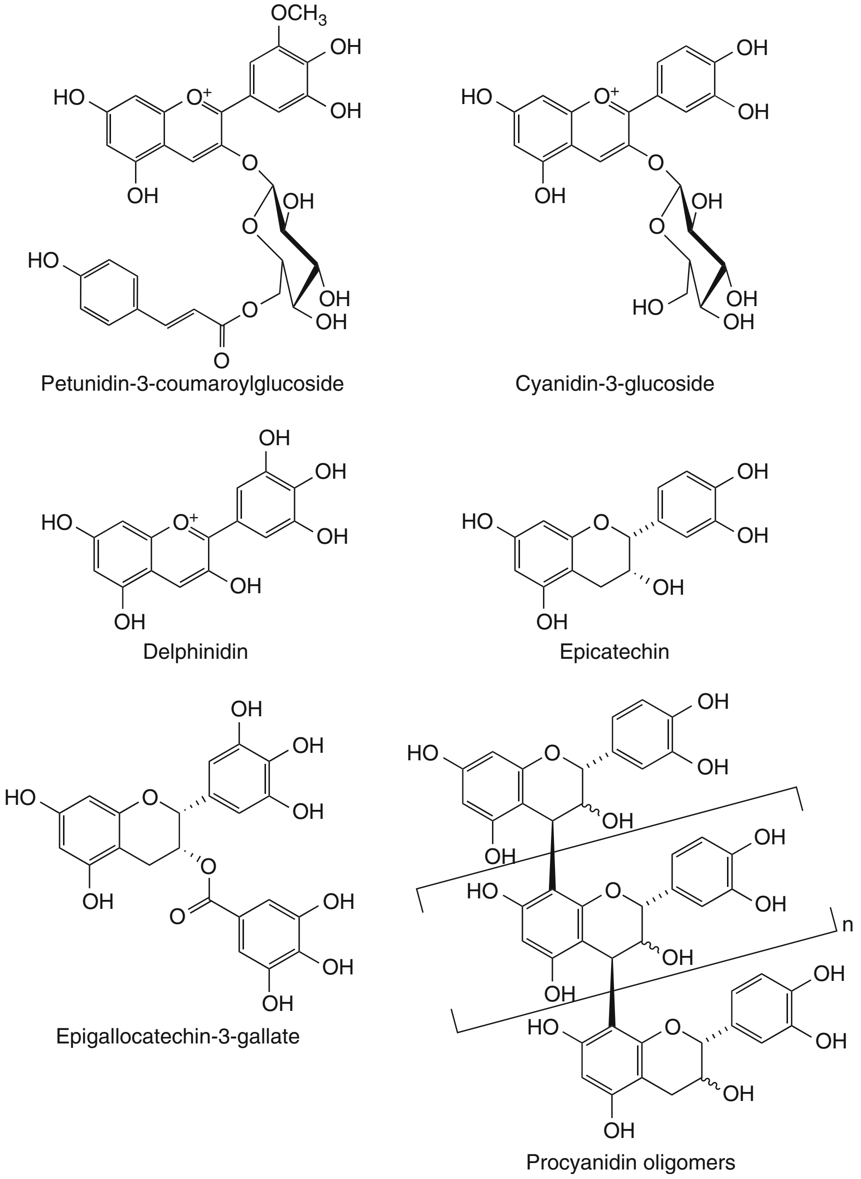
Petunidin-3-coumaroylglucoside
Cyanidin-3-glucoside
Delphinidin
Epicatechin
Epigallocatechin-3-gallate
Procyanidin oligomers

Fig. 76.3 Chemical structure of selected polyphenols inducing the activation of eNOS

activation by anthocyanins [50]. In addition, petunidin-3-glucoside and petunidin aglycone, two closely related compounds differing by the nature of the substitution attached in position C3, were not able to induce activation of eNOS in endothelial cells [50]. These results indicate that the nature of the substitution on position C3 is also critical for the induction of eNOS activation by anthocyanins.

3.2 Red Wine Polyphenols Induce Endothelium-Dependent Relaxations Ex Vivo

The study by Fitzpatrick et al. using rat aortic rings suspended in organ chambers indicated that addition of increasing volumes of several wines and grape juices induced pronounced relaxations in precontracted rings with an intact endothelium, while rings where the endothelium was mechanically removed showed little or no relaxation [41]. The endothelium-dependent relaxation was associated with an increased formation of cGMP, and competitive inhibitors of NO synthase (N^{ω}-nitro-L-arginine and N^{ω}-monomethyl-L-arginine) prevented both the relaxation and the increase in cGMP, indicating that red wine and grape polyphenols induce endothelium-dependent relaxations through an increased formation of NO leading to the subsequent vascular formation of cGMP. These results were confirmed by Andriambeloson et al. using a red wine polyphenolic extract, which caused relaxations in endothelium-intact rings with concentrations 1000-fold lower than those inducing relaxations in rings without a functional endothelium [51]. The endothelium-dependent relaxations were strongly inhibited by a competitive inhibitor of NO synthase (N^{ω}-nitro-L-arginine methyl ester) but not those in arterial rings without endothelium. Moreover, the measurement of NO formation by electron paramagnetic resonance provided direct evidence that red wine polyphenols induce the formation of NO in endothelium-intact arterial rings [51]. The ability of red wine to induce endothelium-dependent relaxations in arteries has been attributed to its polyphenolic fraction rather than its alcohol content since it has also been observed with other grape-derived polyphenol-rich products such as red wine without alcohol [52, 53], red wine extracts [54, 55], purple grape juice [56], and grape extracts [57–61]. However, the comparison of different wines for their ability to induce endothelium-dependent relaxations has indicated that some red wines are potent inducers while others have little effects [62–64]. The phenolic content of red wine is strongly correlated to the ability to induce vasorelaxation [62]. The identification of the molecules responsible for the vasorelaxant effect of red wines is a difficult task since wines are complex mixtures of several hundreds of polyphenolic compounds [65]. However, fractionation of red wine polyphenolic extracts has indicated that a great variety of compounds, in particular procyanidins and anthocyanins, are able to stimulate the endothelial formation of NO [49, 50]. The importance of polyphenols, and in particular procyanidins and anthocyanins, is also suggested by the fact that grape skin and grape seed extracts, products rich in such polyphenolic compounds, induce strong endothelium-dependent relaxations [41, 59–61, 66] while white wines, which contain less than 10 % of the phenolic content of red wines and only traces of procyanidins and no anthocyanins, cause little or no endothelium-dependent relaxations [64].

Grape-derived products have been shown to induce endothelium-dependent relaxations in a great variety of blood vessels including conductance arteries such as the rat and rabbit aorta [41, 51, 63], the porcine coronary artery [45, 46], the rat mesenteric artery [67], and the human coronary and internal mammary arteries [64, 68]. It has also been reported that grape-derived products are able to induce endothelium-dependent NO-mediated relaxation in resistance arteries such as the rat mesenteric bed and cerebral arterioles [66, 69], and the porcine retinal artery [70].

The investigation of the signaling pathway involved in the endothelium-dependent NO-mediated relaxation induced by red wine polyphenols indicates that, surprisingly, polyphenols act through an intracellular redox-sensitive mechanism. Indeed, in coronary artery rings, the relaxation induced by a red wine polyphenolic extract was markedly reduced by membrane-permeant analogs of either superoxide dismutase (MnTMPyP or polyethyleneglycol-SOD) or catalase (polyethyleneglycol-catalase) but remained unaltered by native superoxide dismutase and catalase which are unable to cross the cell membrane [46]. Interestingly, NO-mediated relaxations to bradykinin, a physiological agonist, were not affected by MnTMPyP indicating that red wine polyphenols and bradykinin activate distinct signaling pathways leading to eNOS activation [46]. The endothelial source of superoxide anions induced by polyphenols remains unclear, as it does not seem to involve major enzymatic sources such as NADPH oxidase, xanthine oxidase, cytochrome P450, or the mitochondrial respiratory chain [61, 71]. Studies using a grape seed extract or an isolated green tea catechin (epigallocatechin-3-gallate) have shown that the methylation of the hydroxyl groups resulted in the loss of the vasorelaxant activity, suggesting a key role of the polyphenol hydroxyl moieties, probably through autoxidation, a mechanism known to generate semiquinones and then quinones with the concomitant formation of superoxide anions [57, 71]. The further characterization of the signaling pathway has indicated that the prooxidant response in endothelial cells triggers the activation of the Src/PI3-kinase/Akt pathway leading to the activation of eNOS by phosphorylation and the subsequent NO-mediated relaxation of isolated arteries [46]. A similar eNOS activation pathway has been identified for other grape-derived products such as grape juice and a grape skin extract [56, 61].

Some studies have investigated the role of estrogen receptors in the polyphenol-induced NO-mediated relaxation of isolated arteries since estrogens have been shown to activate eNOS via the PI3-kinase/Akt pathway and polyphenols can interact with estrogen receptors [72]. A recent study by Chalopin et al. indicated that a red wine extract caused small endothelium-dependent relaxations in aortic rings from estrogen receptor alpha wild-type mice but not in estrogen receptor alpha knockout mice [73]. However, other studies have reported that fulvestrant, a selective antagonist of estrogen receptor alpha, did not affect endothelium-dependent NO-mediated relaxations to a different red wine extract and also to two major red wine polyphenols, kaempferol and rutin, in the rat aorta [47, 74]. Taking together, these findings suggest that the estrogen receptor alpha may contribute to some extent to the NO-mediated relaxation of isolated arteries in response to some red wine polyphenols.

While the NO-mediated relaxation is a predominant event in large-conductance arteries, red wine has also been reported to induce EDH-mediated relaxations in smaller arteries. Indeed, in porcine coronary artery, a red wine polyphenolic extract induced concentration-dependent EDH-mediated relaxations as well as hyperpolarizations of the vascular smooth muscle [45]. A similar effect has been reported in the rat mesenteric bed with an alcohol-free freeze-dried red wine from Brazil [75]. The activation of the EDH-mediated relaxation by red wine seems to involve also the redox-sensitive PI3-kinase/Akt pathway like the NO-mediated relaxation [45, 76].

3.3 Red Wine Polyphenols Have Beneficial Effects on the Endothelial Function In Vivo

In vitro and ex vivo studies are useful for characterizing the effect of red wine on the endothelium. However, the major limitation of such studies is that most of the phenolic compounds applied to cells and artery rings are not those present in the blood circulation after oral absorption. Indeed, most polyphenols are circulating in blood in form of metabolites such as glucuronides, methyl, and sulfo-conjugates [77–79]. Noteworthy, some polyphenols like procyanidin oligomers may be absorbed in the intact form since they have been identified in blood and urine following ingestion of a procyanidin-rich grape seed extract by humans and rats [80, 81]. In addition, procyanidin oligomers have been estimated to reach the nanomolar range in human plasma after consumption of procyanidin-rich cocoa and grape seeds [81, 82].

The in vivo evaluation of the beneficial effect of red wine polyphenols on the endothelium has mostly been assessed in experimental models of hypertension, which are associated with an endothelial dysfunction. Indeed, oral ingestion of red wine has been shown to reduce blood pressure in spontaneously hypertensive rats, a genetic model of hypertension [83]. The same effect has also been reported after ingestion of a grape seed extract [84]. Moreover, red wine has been shown to lower blood pressure in several other experimental models of hypertension including the angiotensin II-induced hypertension [85], the N^{ω}-nitro-L-arginine-induced hypertension [75, 86, 87], and the DOCA salt-induced hypertension [88] in rats. Taken together, these experimental studies indicate that ingestion of red wine products is associated with a beneficial effect in several experimental models of hypertension associated with an improved endothelial function.

Several studies have indicated that the endothelial dysfunction is often associated with an increased oxidative stress in the vascular wall due, at least in part, to an increased expression and activity of the NADPH oxidase, a major vascular source of superoxide anions [89–93]. The vascular oxidative stress is also likely to involve other enzymatic sources such as cyclooxygenases, mitochondria, cytochrome P450, or uncoupled eNOS [93, 94]. Red wine polyphenols have been shown to reduce strongly the level of oxidative stress in the arterial wall of pathological arteries in association with a reduced vascular expression of NADPH oxidase [91–94]. In addition, polyphenols may reduce vascular oxidation stress due to their direct antioxidant properties and also to their ability to inhibit the activity of NADPH oxidase [95]. Moreover, the angiotensin system appears to be a potent inducer of the endothelial dysfunction and vascular oxidative stress by activation of NADPH oxidase [96, 97]. In ageing rats, the endothelial dysfunction is associated with an increased vascular expression of both angiotensin II and its receptors AT1R and AT2R, and these responses are normalized by intake of red wine polyphenols [93, 94]. Taken together, these data suggest that polyphenols are able to exert their beneficial effects on the endothelium, at least in part, by reducing the vascular oxidative stress through several mechanisms including direct antioxidant properties, inhibition and downregulation of NADPH oxidase, and downregulation of the vascular angiotensin system.

4 Clinical Studies on the Beneficial Effects of Wine Polyphenols

Several epidemiological studies have linked a protective effect against cardiovascular diseases and regular consumption of either red wine [7, 8, 98] or polyphenol-rich sources such as green tea [14, 15, 99], cocoa, or chocolate [16, 17]. Since many cardiovascular diseases are associated with an impaired endothelial function, the evaluation of the endothelial function is of great importance. In humans, the endothelial function is often assessed by the flow-mediated dilatation (FMD). For this purpose, reactive hyperemia is induced after a transient ischemia in the upper arm using an inflatable cuff like that used for the measurement of blood pressure, while the diameter of the brachial artery is monitored continuously at the elbow level by ultrasound. The cuff is inflated until the occlusion of the brachial artery, which is maintained for 5 min before the blood flow is released. The reperfusion then induces an endothelium-dependent dilatation of the brachial artery due to the increased shear stress, the most important physiological activator of eNOS. Impaired endothelial function, assessed by FMD, has been reported as an independent predictor of cardiovascular outcome in subjects with cardiovascular risk factors or established cardiovascular diseases [18].

FMD and blood pressure have been often used for the evaluation of the effect of the dietary intake of polyphenols on the protection of the cardiovascular system. Indeed, the basal FMD is increased in healthy subjects after two glasses of red wine with or without alcohol [100] or after consumption of 3 ml/kg of red wine [101]. Similarly, intake of red wine has been shown to restore a normal endothelial function in hypercholesterolemic patients with impaired FMD [99, 102]. A study by Papamichael et al. has also shown that acute intake of two glasses of red wine with or without alcohol is able to reduce the endothelial dysfunction induced by the smoking of one cigarette by healthy nonsmokers [103]. Moreover, acute intake of two glasses of red wine without alcohol improves FMD in patients with coronary artery disease, while intake of regular red wine was less effective, suggesting that the beneficial effect is due to the polyphenol content rather than the alcohol component of red wines [104].

The beneficial effect of polyphenols on the endothelial function has also been observed for other dietary polyphenol-rich sources. Indeed, intake of purple grape juice has been shown to improve the endothelial function in hypercholesterolemic patients with impaired FMD similarly to red wine [102]. Moreover, purple grape juice intake improved FMD in teenagers with metabolic syndrome [98]. In addition, a grape-derived product has been shown to improve the impaired FMD in healthy subjects subsequent to the intake of a high-fat meal [77]. Similarly, intake of cocoa

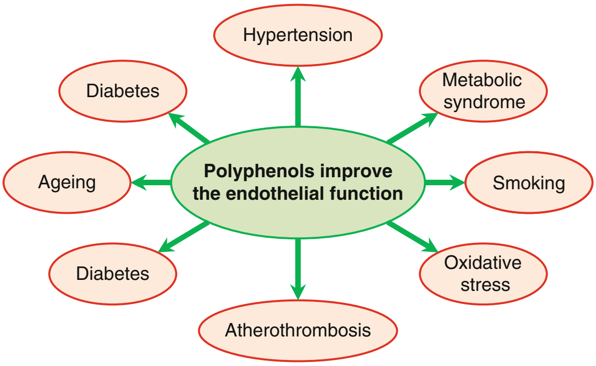

Fig. 76.4 Potential health beneficial effects of polyphenols

and chocolate, sources rich in epicatechin and procyanidin that are also present in red wine, has been associated with an improved FMD. Indeed, basal FMD has been increased after ingestion of a single dose of 46 g of dark chocolate or a low dose of purified epicatechin, 1 or 2 mg/kg body weight in healthy adults [105, 106]. Similarly, intake of dark chocolate improved FMD in patients with heart failure [32], habitual smokers [107], and overweight adults [108]. Taken together, these studies indicate that the intake of polyphenol-rich sources has a beneficial effect on the endothelial dysfunction associated with several pathologies and physiological situations such as ageing, which are associated with an increased prevalence of cardiovascular diseases (Fig. 76.4).

5 Conclusion

The endothelium plays a key role in the maintenance of vascular health, and the alteration of its function will promote the development of cardiovascular diseases. A large number of experimental and clinical studies indicate that polyphenols are potent activators of the endothelial cell leading to the formation of major vasoprotective factors including NO and EDH and that they contribute to delay the development of an endothelial dysfunction and to improve also an established endothelial dysfunction, in part, by reducing the vascular oxidative stress. All these effects will contribute to a better perfusion of target organs and, hence, improve vascular health.

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Isoflavonoids and Phytoestrogenic Activity 77

Catherine Bennetau-Pelissero

Contents

1 Introduction 2385
2 Plant Origin 2388
3 Food Content and Intake 2391
4 Bioavailability and Metabolism 2395
5 Mechanisms of Action 2399
6 Animal Studies 2402
6.1 Breast Cancer 2402
6.2 Colon Cancer 2403
6.3 Prostate Cancer 2404
6.4 Osteoporosis 2405
6.5 Memory 2406
7 Epidemiological Studies 2406
7.1 Menopausal Symptoms 2407
7.2 Breast Cancer 2408
7.3 Colon Cancer 2408
7.4 Prostate Cancer 2409
7.5 Osteoporosis 2409
7.6 Memory 2410
8 Clinical Trials 2411
8.1 Menopausal Symptoms 2411
8.2 Breast Cancers 2412
8.3 Colon Cancer 2412
8.4 Prostate Cancer 2413
8.5 Osteoporosis 2413
8.6 Memory 2414
9 Pharmacological Applications 2415
9.1 Menopausal Symptoms 2415
9.2 Breast Cancer 2416

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9.3 Colon Cancer 2416
9.4 Prostate Cancer 2416
9.5 Osteoporosis 2416
10 Toxicity 2417
10.1 Reproduction 2417
10.2 Estrogen-Dependent Cancers 2421
10.3 Hypothyroidism 2421
10.4 Myeloid Leukemia 2422
11 Conclusion 2422
References 2424

Abstract

In the early 1930s, some isoflavonoid compounds were discovered to exhibit estrogenic activities that were found to be deleterious at high doses for cattle reproduction. Later, several toxicological studies on these compounds were performed between the 1940s and 1980s using mainly rodent models. These same compounds were discovered in edible plants and in human biological fluids, and a large body of scientific work was then dedicated to their putative impact on human health.

Because estradiol is a natural hormone with ubiquitous effects in mammals, nearly all biological functions can be influenced by isoflavonoids that have estrogenic activities. Their effects are significantly affected by their biological concentrations in body fluids and by their affinity to known estradiol receptors. Our knowledge of the biological effects of estrogenic isoflavonoids progresses the more we learn about the effects of estradiol and of endocrine disruptors. In this respect, the latest information concerns the discovery of both new pathways for cellular action and epigenetic effects. In addition, the two latest issues involve the factors influencing the metabolism of phytoestrogenic compounds and their bioavailability, and this confirms the importance of the efficient doses and of their affinities for the estradiol receptors.

After a general introduction on the classical molecular effects of estradiol, this chapter presents the main isoflavones with estrogenic activities that have been investigated so far, with their plant sources and forms as well as their role in plants. Their presence in human food and their bioavailable forms in human and in animal models are then evoked. Next, this chapter presents the latest data concerning their mechanisms of action and explains how these processes can influence more highly integrated physiological functions. The data from animal studies and epidemiological studies are discussed as an introduction to pharmacological applications demonstrated in clinical trials. Because estradiol in mammals can have both beneficial and harmful effects, depending on the dose, the physiological status, and the target organ, the dark side of the story of phytoestrogenic compounds is also exposed.

Keywords

Bioavailability • bone health • breast cancer • colon cancer • food sources • isoflavones • leukemia • mechanism of actions • menopause • prostate cancer • reproductive disruption • thyroid

Abbreviations

17β-E_2	17β-Estradiol
3-PBA	3-Phenoxybenzoic acid
5-AR	5α-Reductase
8-PN	8-Prenyl naringenin
AFP	α-Fetoprotein
AKt	Protein kinase B
AMH	Anti-mullerian hormone
AR	Androgen receptor
ARKO	Aromatase knock out
Bioch	Biochanin A
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BTM	Bone turn-over markers
CCa	Colon cancer
C_{max}	Maximum concentration
CREB	c-AMP response element binding protein
DLD-1	Colon cancer cell line
DMBA	Dimethylbenz[a]anthracene
DNA	Deoxyribonucleic acid
DPX	Deoxypyridinoline (Bone resorption marker)
E_2	Estradiol
EGFR	Epidermal growth factor receptor
Eq	Equol & its isomer *S*-Equol ***S*-Eq**
ERE	Estrogen responsive element
ERK	Extracellular related kinase
ERR	Estrogen related receptor with isoforms α, β, γ
ERs	Estradiol receptors
ERα	Estradiol receptor α
ERβ	Estradiol receptor β
ESR1	Gene coding for estradiol receptor alpha
ESR2	Gene coding for estradiol receptor beta
Form	Formononetin
FSH	Follicle stimulating hormone
Gapd-s	Glyceraldehyde 3-phosphate dehydrogenase gene
Gen	Genistein
Gly	Glycitein

GnRH	Gonadotropin releasing hormone
GPCR	G-protein coupled receptor
GPR-30	G protein Receptor-30
HRT	Hormone replacement therapy
IC_{50}	Inhibiting concentration for 50 %
ICI 182,780	Estradiol receptor specific inhibitor
IGF-1	Insulin like growth factor-1
IL-6	Interleukin 6
Isofl	Isoflavones
JNK	c-Jun N-terminal kinase
LBD	Ligand binding domain
LH	Luteinizing hormone
LNCap	Prostate cancer cell line
MAPK	Mutagen activated protein kinase
MCF-7	Breast cancer cell line
MDM2	Proto-oncogene in estrogen-dependent tissues
MDR	Multi-drug resistance
NTx	*n*-Telopeptide (Bone resorption marker)
OECD	Organization for economic cooperation and development
OPG	Osteoprotegerin
OR	Odd Ratio
PC-3	Prostate cancer cell line
PCa	Prostate cancer
PI3K	Phosphatidyl inositol 3 kinase
PIP	Phosphatidyl inositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PSA	Prostate serum antigen
PTH	Parathyroid hormone (Hypercalcemic hormone)
PTK	Protein tyrosine kinase
RANK	Receptor activator for nuclear factor κB
RANK-L	Receptor activator for nuclear factor κB Ligand
RR	Relative risk
SDN-POA	Sexually dimorphic nucleus of the preoptic area
SF-1	Steroid factor 1
SHBG	Sex hormone binding globulin
SPE	Soy protein extract
Src	Proto-oncogenes coding for a family of tyrosine kinase
T	Testosterone
$T_{1/2}$	half-life time to reach half concentration
T_{max}	Time for maximum concentration
UDPGT	Uranyl di-phosphate Glucuronosyltransferase
UGT	Uranyl di-phosphate Glucuronosyltransferase
WHI	Women's health initiative (large American interventional study to assess the effects of HRT on cardiovascular diseases)

1 Introduction

The isoflavonoid family represents a distinct group among the flavonoid compounds. Within this group, some of the most potent estrogenic molecules of the plant kingdom can probably be found. Only one coumestane, namely coumestrol, and one prenyl-flavanone, namely 8-prenyl naringenin (8-PN), are known to be more estrogenic in some in vitro tests [1]. Coumestrol is present in alfalfa [2], sometimes in soy, and in some fruits and beans together with isoflavones (**Isofl**) [3], whereas 8-PN has been found so far only in hop (*Humulus* sp.) [4] and in *Sophora flavescens*, a fabaceae used in Chinese traditional medications [5]. However, although coumestrol and 8-PN can be more estrogenic in vitro, their bioavailability in plasma is usually low [6, 7]. Meanwhile, the **Isofl** usually exhibit a higher plasma bioavailability, resulting in a higher estrogenic effect in vivo. This is particularly the case in humans [8].

The estrogenic effects of all these compounds are based on their chemical structures, which share some similarities with that of the estradiol molecule. Indeed, there are several natural estrogens in vertebrate animals, among which 17β-estradiol (17β-E_2) is the most potent. 17β-E_2 is an aromatized C18 steroid with hydroxyl groups at the 3- and 17-β positions (Fig. 77.1a). In humans, it is produced primarily by the cyclic ovaries and the placenta. It is also produced by the brain, the adrenal cortex, and the adipose tissue of men and postmenopausal women. As shown in Fig. 77.1a, 17β-E_2 is a three-dimensional molecule with its two hydroxyl groups at a distance of 10 Angstroms (Å) and bearing a phenolic ring. The hydroxyl groups form an angle allowing the best interaction with the specific estradiol receptors (ERs) [9]. These receptors, namely ERα and ERβ, derive from two different genes: ESR1, located in humans on chromosome 6, and ESR2, located on chromosome 14 (Fig. 77.1b). These receptors have nuclear transcriptional effects and present a ligand binding domain (LBD) with a hydrophobic pocket constituted by the E/F domain (C-terminal domain) of the protein and a part of the A/B domain (N-terminal domain) folded in its vicinity (Fig. 77.1b). The E/F domain is constituted of several helix structures (Fig. 77.1c, d).

The position of the H12 helix is crucial for the transcriptional effect of the ERs (Fig. 77.1c, d). Whether or not the transcription factors are recruited depends on the spatial position of the H12 helix in the LBD. This, in turn, allows or does not allow the estrogen-dependent gene transcription [10]. The 17β-E_2 spatial structure is so relevant for the ligand–receptor interaction that the natural 17-α-isomer of estradiol binds only weakly to the ERs and exhibits little estrogenic genomic activity in estrogen-responsive tissues [11]. 17β-E_2 physiological plasma concentrations in women usually vary from 10 to 20 pg/mL (i.e., from 36 to 72 pM) in the metestrus phase of the female cycle to 500–600 pg/mL (i.e., 2 to 4 nM) in the estrous phase of the female cycle. During pregnancy, these 17β-E_2 levels can reach values over 30 ng/mL (i.e., over 110 nM). In the meantime, 17β-E_2 levels in men are below 50 pg/mL and are below 40 pg/mL in postmenopausal women (i.e., below 200 pM in both cases).

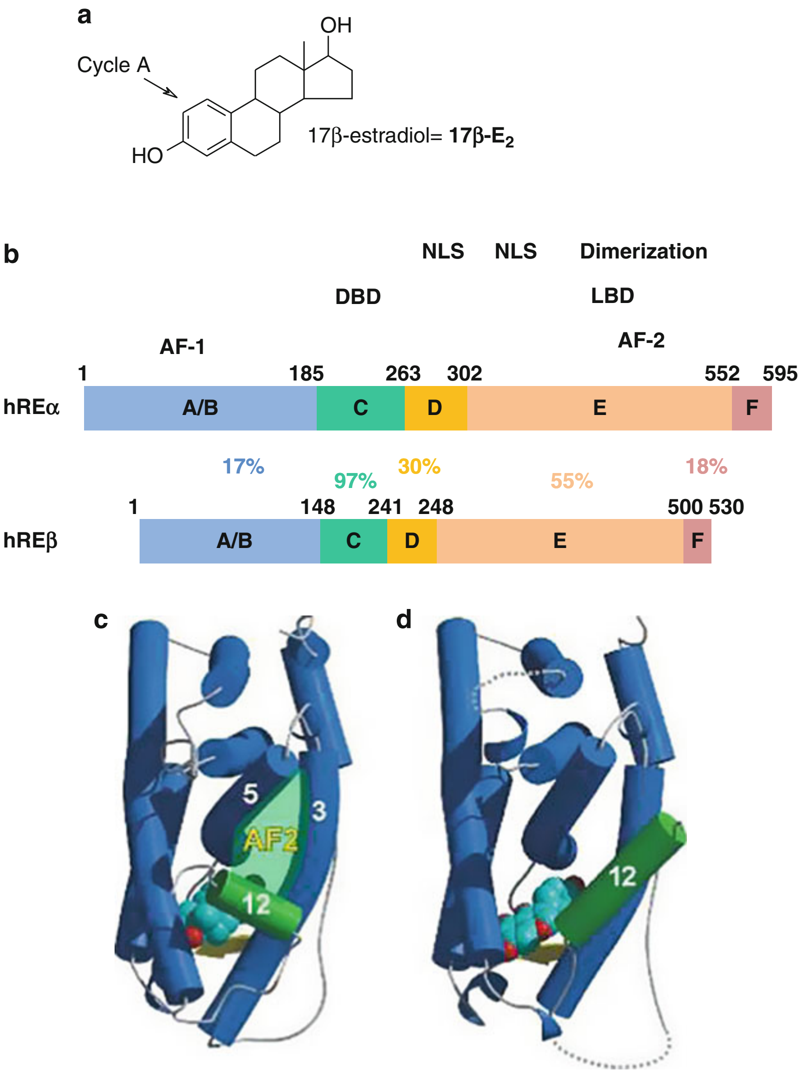
a
Cycle A
OH
HO
17β-estradiol= 17β-E2
b
NLS
NLS
Dimerization
DBD
LBD
AF-1
AF-2
1
185
263
302
552
595
hREα
A/B
C
D
E
F
17%
97%
30%
55%
18%
1
148
241
248
500
530
hREβ
A/B
C
D
E
F
c
5
3
AF2
12
d
12

Fig. 77.1 Estradiol and estradiol receptor structures. (**a**) 17β-estradiol chemical structure, (**b**) Comparative structures of ERα and ERβ. (**c**) Conformation of ERα bound to 17β-E_2 [11], (**d**) Conformation of ERα bound to **Gen** [11]

Despite the great specificity of the ERs for their natural steroidal mammalian ligands, since the 1970s, many molecules have been found to be able to bind to the ERs [12]. They were first called xeno-estrogens and now are regularly encompassed under the general name of endocrine disruptors [13]. Isoflavones with estrogenic activities can be classified in this category [14]. Their chemical structures are presented in Fig. 77.2a. As for 17β-E_2, the molecules exhibit two

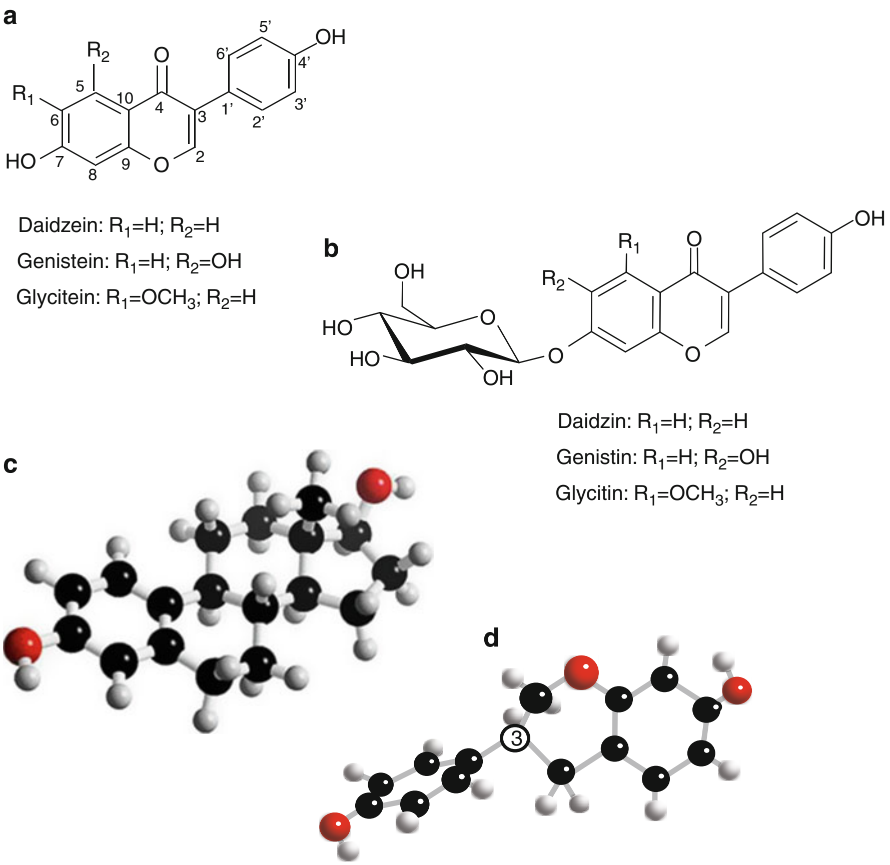
a
R$_2$
O
R$_1$
5
10
4
3
6
HO
7
8
9
O
2
1'
2'
3'
4'
5'
6'
OH
Daidzein: R$_1$=H; R$_2$=H
Genistein: R$_1$=H; R$_2$=OH
Glycitein: R$_1$=OCH$_3$; R$_2$=H
b
OH
HO
HO
O
OH
O
R$_2$
R$_1$
O
O
OH
Daidzin: R$_1$=H; R$_2$=H
Genistin: R$_1$=H; R$_2$=OH
Glycitin: R$_1$=OCH$_3$; R$_2$=H
c
d
3

Fig. 77.2 (**a**) Structure of the main Isoflavones with estrogenic activities. (**b**) Structure of the main Isoflavone glucosides. (**c**) Tri-dimensional structure of 17β-E2. (**d**) Tri-dimensional structure of *S*-Equol

hydroxyl groups on the opposite sides of the molecule at a distance of 10 Å and they also exhibit phenolic rings. Their main differences reside in their spatial shape. Indeed, whereas 17β-E_2 is a three-dimensional molecule (Fig. 77.2b), **Isofl** are planar compounds because of the double bond existing between the C_2 and C_3 carbons and therefore the spatial position of their hydroxyl groups into the hydrophobic pocket of the LBD are not strictly stackable to those of 17β-E_2. As a result, the affinities of **Isofl** for the ERs are much lower than that of 17β-E_2. Usually, the affinities of the **Isofl** with estrogenic activities are considered to be better for ERβ and to range from genistein (**Gen**) to kievitone according to the following order: **Gen**>daidzein >glycitein>biochanin A>formononetin>phaseolin>kievitone [12, 15]. Other compounds like glyceolin were also found to exhibit estrogenic activity. However, these activities are not yet fully characterized [16].

The affinity of **Isofl** with estrogenic activities for ERs can change according to the system used. Indeed, the solubilized receptors extracted from an estrogen target tissue usually exhibit lower affinity to non-steroidal estrogens than recombinant proteins (1/100–1/10,000 vs. 1/10–1/1,000, respectively) and even less than the recombinant LBD of those receptors [15, 17] (1/1–1/1,000). Although, these affinities are low, especially in the most physiological systems, the **Isofl** with estrogenic properties can reach plasma concentrations 10,000–100,000 times higher than those of 17β-E_2. Namely, **Isofl** can reach micromolar concentrations in plasma, whereas 17β-E_2 is currently found at picomolar to nanomolar concentrations in those same plasmas. In vitro tests, performed on estrogen reactive cells, can also show discrepancies in their response to phytoestrogens according to their equipment in ERs [18], to their molecular context (i.e., their ability to synthesize the transcription factors) [19], and to their proper enzymatic equipment [20]. Indeed, according to their function and tissue origin, cells are either able or unable to transform conjugated estrogens (steroidal or not) into free estrogens based on their equipment in phase 1, 2, and 3 enzymes, ABC-binding cassettes, and multidrug resistance (MDR) enzymatic complexes [20]. To add another level of complexity, it must be mentioned that the affinity of xeno-estrogens for the ERs does not strictly reflect their transcriptional activities. It was shown that, although soy estrogenic **Isofl** exhibit a greater affinity for ERβ, their nuclear transcriptional efficiency is greater via ERα [19]. However, in vivo, both ERs can possibly act together, forming heterodimers [21].

2 Plant Origin

The estrogenic isoflavonoids including **Gen**, daidzein (**Daid**), formononetin (Form) biochanin A (Bioch), and glycitein (**Gly**) are secondary metabolites from plants. Isoflavones can mainly be found in great concentrations in Leguminosae. Some of them contain high amounts of **Isofl** with estrogenic properties (i.e., up to several hundred mg/100 g of dry matter [22]). The most concentrated Leguminosae are red kidney beans (*Phaseolus vulgaris*) [15], soy (*Glycine max*) [23], kudzu (*Pueraria lobata* or *tuberosa*) [24], alfalfa (*Medicago* sp.) [3], clover (*Trifolium* sp.) [25], and so on. Estrogenic **Isofl** are also found in a smaller quantity in Leguminosae traditionally edible in Western countries like beans, lentils, favas, chickpeas, and potatoes, but in these vegetable sources the concentrations are about 500 times lower than in the plants previously cited [26, 27]. See Table 77.1 for more details.

In addition, Farnsworth et al. [28, 29] also detected these compounds in more than 300 different plants, including many species of Leguminosae like *Baptisia*, *Cytisius*, *Dalbergia*, *Genista*, *Lupinus*, *Medicago*, *Phaseolus*, *Teline*, *Trifolium*, and *Ulex* and in several species of Rosaceae like *Prunus*. In Western countries, these plants were traditionally used as sources of antifertility agents.

There is evidence that **Isofl** act as phytoalexin [15]. They are antimicrobial and may have a role in plant protection. As an example, the antifungal activity of lupin **Isofl** has been demonstrated [30]. In soybean cultivars, an increase in **Isofl** concentrations was shown to be a specific response to the attack of a saprophytic fungi

Table 77.1 Plant Sources of isoflavonoids with estrogenic properties (content in µg/100g)

	Biochanin A	Daidzein	Formononetin	Genistein	Glycitein	Author
Plants						
Soybean seeds	nd	27,126.7	nd	19,787.7	na	[3]
Soybean sprouts	nd	24,24.4	186.7	1,974.9	na	[3]
Soybean	30	47,000	70	74,000	na	[26]
Vegetables and legumes						
Alfalfa sprouts	66.9	151.7	3,898.9	117.6	na	[3]
Alfalfa sprouts	nd	nd	340	nd	na	[47]
Alfalfa sprouts	nd	nd	2,610	nd	na	[50]
Alfalfa sprouts	152	67	3,899	118	na	[3]
Asparagus	nd	57.8	nd	tr	na	[3]
Broccoli	nd	5	nd	7	na	[46]
Broccoli	1	nd	nd	nd	na	[3]
Broccoli sprouts	nd	43.7	nd	nd	na	[3]
Chickpeas	1,700	40	140	60	na	[26]
Carrots	nd	2	nd	2	na	[46]
Carrots	5	nd	nd	nd	na	[3]
Clover sprouts	751.2	71.3	4,019.9	70.9	na	[3]
Garbanzo beans	1,394.1	nd	52.0	67.2	na	[3]
Garbanzo beans dried	1,520	nd	nd	nd	na	[47]
Garbanzo beans dried	1,780	80	140	120	na	[50]
Garbanzo beans dried	2,822	33	258	82	na	[3]
Garbanzo beans	838	11	215	73	na	[46]
Garlic	nd	2	nd	1	na	[46]
Garlic	nd	nd	23	1	na	[3]
Mung bean sprouts	tr	387.2	26.1	424.1	na	[3]
Mung bean sprouts	nd	nd	tr	nd	na	[47]
Mung bean sprouts	nd	745	nd	1,902	na	[46]
Pinto beans dried	560	nd	nd	nd	na	[47]
Pinto beans dried	nd	20	nd	520	na	[50]
Pinto beans dried	4	42	nd	98	na	[3]
Pinto beans	196	nd	nd	9	na	[49]
Pinto beans	nd	10	nd	5	na	[3]
Fruits						
Grapefruit	tr	35.6	tr	27.1	na	[3]
Other						
Coffee	nd	50.3	nd	tr	na	[3]
Licorice (black)	nd	293.0	1,493.1	599.3	na	[3]
Nuts and Seeds						
Almond, kemel only	25	<1	<1	1	<1	[2]
Brazil nuts	13	6	<1	85	<1	[2]
Cashews plain	7	nd	2	2	1	[2]
Coconut fresh	6	nd	nd	nd	4	[2]
Hazelnuts	12	<1	<1	9	<1	[2]

(*continued*)

Table 77.1 (continued)

	Biochanin A	Daidzein	Formononetin	Genistein	Glycitein	Author
Peanuts fresh	8	<1	4	48	10	[2]
Pecans	30	nd	2	2	nd	[2]
Pine nuts	27	<1	<1	4	<1	[2]
Walnuts	17	1	<1	11	<1	[2]

na not analysed, *nd* not detected, *tr* trace

Mucor ramosissimus. In the soybean strains resistant to the fungus, **Isofl** (including glyceollins I, II, and III; glycinol; glyceocarpin; **Gen**; isoformononetin; and N-acetyltyramine) were induced by the fungal attack, all compounds possessing antifungal activity with the exception of **Gen** [31]. A contamination of a soy strain with the fungus *Diaporthe phaseolorum f.* sp. *Meridionalis*, induced the accumulation of **Isofl** (**Gen**, **Daid**), pterocarpans (glyceolins), and flavones (apigenin and luteolin) via a nitric oxide synthase pathway [32]. To go on with interactions of the plants with microorganisms, there is some evidence that the symbiotic relationship between *Rhizobium lupine* and *Lupinus albus* stimulates an increase in production of prenylated **Isofl** in the root nodules [33]. These prenylated **Isofl** possess in vitro activities against a number of other *Rhizobium* species. Zhang and Smith [34] also showed that **Gen** plays a major role in the establishment of the symbiosis between *Bradyrhizobium japonicum* and its host, that is, soy (*Glycine max*). **Gen** is the soy recognition molecule inducing the greatest plant-to-bacterium signal. In fact, the binding of **Gen** to *B. japonicum* activates many of the bacteria nod genes.

There is solid proof that farming practices directly influence the levels of **Isofl** in soy. As a matter of fact, irrigation was shown to enhance **Isofl** content in soybeans by as much as 2.5 fold [35]. A deficit in nitrogen fertilizer increases the estrogenic activity of a clover pasture [36]. On the other hand, a supplementation in the same fertilizer decreases the occurrence of **Isofl** with estrogenic activity in a clover pasture [36]. A study where soy cultivars (*Glycine max*) were bred for 3 years demonstrated that levels of **Isofl** were related to both environmental and genetic characteristics and could be susceptible to selection [37]. The genomic regions implicated in this process have been identified [38]. Vyn and coworkers [39] showed that **Gen**, **Daid**, and **Gly** contents in soy are correlated with potassium in roots and leaves and with crop management using potassium-rich fertilizers. Lindner in [22] gave an evolutionist theory about the ability of Leguminosae to produce estrogenic **Isofl**. Indeed, it appears that isoflavonoid compounds are synthesized by the plants in response to bacterial or fungal attacks or to water stress. Estrogenic **Isofl** can then be considered as protective compounds. In the case of over-grazing of a pasture by mammalian predators, the production of great quantities of estrogenic compounds that could impair predator reproduction would result in the reduction of the predator pressure. Finally, because estrogenic **Isofl** are also specific attractants for symbiotic bacteria of the *Rhysobium* gender, and because these bacteria can fix the atmospheric nitrogen, human selection against the production of **Isofl** in Leguminosae would result in the loss of one of their greatest interests in crop management.

3 Food Content and Intake

Isoflavones with estrogenic activities are present in significant amounts in food processed from soy (see Table 77.2 and [40]) and in food supplements containing soy, clover, alfalfa, and kudzu extracts [41–44] and (see Table 77.3). Isoflavones, under a glycosylated form in plants (Fig. 77.2b), are generally soluble in water and uncovalently linked to proteins rather than present in the lipid phase of the crude matter. Consequently, there is almost no **Isofl** in soy oil or soy lecithin preparations (see [40, 41, 44] and Table 77.2). Isoflavones with estrogenic activities are present in several mg per 100 g of processed soy-based food, or several tenths of mg per 100 mL of liquid soy-based food [3, 40, 45–50]. This leads to food intakes from a few milligrams of **Isofl** up to 50 mg per portion of soy-based food [40, 41]. Soy-based food-supplements usually contain **Isofl** in the same range of quantities, even though several laboratories producing food supplements have developed formula containing up to 100 mg per capsule [40, 41, 44]. In parallel, several soy-based infant formulas were designed and commercialized to address lactose intolerance in babies. Although they are generally prepared from the best quality concentrated soy protein, and because soy is used as the major protein source, these formula contain large amounts of **Isofl** [48, 51–53].

In this specific case, infants, before diet diversification, can be exposed to **Isofl** amounts 5–11 times higher than those encountered in adulthood in a diversified regimen [51] or by menopausal women on soy-based food supplements [44]. Cooking or other food transformation processes can generate acetyl or malonyl conjugates of the phytoestrogenic glycosylated **Isofl** [54]. However, the aglycone compounds are thermally highly resistant except in highly acidic conditions. Therefore, common cooking transformations including extrusion do not considerably affect the **Isofl** content of soy-based-food [55]. It should be noted that crude soy was usually not considered to be edible by humans or animal species because it contains several anti-nutritional factors including anti-trypsin agents, saponins, and sapogenins [56]. Initially, in Asian countries, soy was essentially used in crop rotations based on its ability to fix atmospheric nitrogen in the soil. Asian people learned subsequently how to use it as an edible crop, empirically submitting it to traditional Asian food transformation processes. These traditional processes include cooking, precipitation with magnesium chloride from Nigari, and pressing after seven aqueous extractions (tofu, miso, and traditional Asian-derived products), or fermentation with specific bacteria (tempeh, soy sauce, and traditional Asian-derived products) [57]. These traditional forms of processing reduce the amount of anti-nutritional agents and of **Isofl** with estrogenic activities in the food matrix. Western processing used in the preparation of soy “milk” (i.e., tonyu) and derived products (including yogurts, ice creams, desserts, cheese, etc.), but also Western-made tofu and derived products do not follow the traditional Asian recipes. Consequently, the **Isofl** with estrogenic activities may remain, in some cases, in the diet at high concentrations [41, 45, 55]. However, as mentioned earlier, the **Isofl** concentration in a plant varies greatly depending on its genetic background and on its cultivating conditions and/or fungi or bacterial attacks. Therefore, the

Table 77.2 Processed food as Sources of isoflavonoids with estrogenic properties (content in µg/100g or µg/100mL)

	Biochanin A	Daidzein	Formononetin	Genistein	Glycitein	Author
Traditional soy-based food						
Chinese black bean sauce	nd	9,617.4	nd	5,576.9	na	[3]
Honzukuri miso	na	7,993.2	na	17,891.5	3,800	[47]
Miso soup	nd	1,127.1	32.8	437.3	na	[3]
Miso	na	27,157.2	na	24,619	7,730	[47]
Soybean seeds (edible)	nd	27,126.7	nd	19,787.7	na	[3]
Soybean sprouts	nd	2,424.4	186.7	1,974.9	na	[3]
Soy sauce	nd	233.1	nd	59.9	na	[3]
Soy sauce	na	1,127	na	627	na	[40]
Tempeh	na	27,715.2	na	32,289.4	3,282	[47]
Tofu	nd	10,342.8	nd	12,281.0	na	[3]
Tofu	na	14,900.6	na	16,473.9	3,386	[47]
Tofu	na	3,400	na	4,200	na	[41]
Tofu	na	11,340	na	16,640	na	[47]
Tofu	na	7,580	na	16,640	na	[48]
Tofu	na	7,450	na	20,000	na	[46]
Tofu	na	7,440	na	16,200	na	[49]
Tofu	na	10,343	na	12,281	na	[3]
Tofu chain grocery stores	na	5,984	na	5,881	na	[3]
Tofu Chinese markets	na	11,775	na	15,988	na	[3]
Tofu Ferm	na	9,440	na	13,350	na	[50]
Tofu Soft	na	11,990	na	18,230	na	[50]
Western diet adapted as soy-based food						
Chocolate cream	na	40,000	na	30,000	na	[41]
Chocolate cream	na	11,000	na	10,000	na	[41]
Croq soy	na	22,000	na	32,000	na	[41]
Croque tofu	na	31,000	na	29,000	na	[41]
Fermented beans	na	14,300	na	22,300	2,300	[47]
Hyperproteinated meal	na	27,000	na	46,000	na	[41]
Roasted soy	na	57,928.4	na	89,485.5	19,216	[47]
Soy-based hot-dog	na	3,629	na	8,414.2	3,352	[47]
Soy bacon	na	2,600	na	6,988.3	2,418	[47]
Soy-based Cheddar A	na	nd	na	400	2,658	[47]
Soy-based Cheddar B	na	4,492	na	4,246.8	3,437	[47]
Soy-based creamy cheese	na	2,800	na	3,400	na	[41]
Soy-based Mozarella	na	1,325	na	3,781.7	2,947	[47]
Soy-based parmesan	na	1,352	na	600	4,088	[47]
Soy-based noddle	na	780	na	3,670.8	3,861	[47]
Soy breakfast	na	10,000	na	19,000	na	[41]

(*continued*)

Table 77.2 (continued)

	Biochanin A	Daidzein	Formononetin	Genistein	Glycitein	Author
Soy cream	na	12,000	na	13,000	na	[41]
Soy sausages	na	23,000	na	26,000	na	[41]
Soy soup	na	14,000	na	22,000	na	[41]
Soy “veggie” burgers	nd	3,048.7	nd	2,020.3	na	[3]
Soy Yoghurt	na	8,000	na	9,000	na	[41]
Soy yoghurt	na	13,000	na	13,000	na	[41]
Soy yoghurt	na	5,818	na	9,702.4	1,256	[47]
Smoked tempeh	na	1,120	na	1,653	na	[40]
Tempeh burger	na	6,968	na	1,986.5	293	[47]
Tofu pancake	na	15,000	na	23,000	na	[41]
Vanilla cream	na	19,000	na	29,000	na	[41]
Liquid Soya-based foodstuffs (µg/100 mL)						
Soy milk	nd	5,022.8	nd	6,298.9	na	[3]
Soy milk	na	1,810	na	2,530	na	[48]
Soy milk	na	1,650	na	2,555	na	[49]
Soy milk	na	4,450	na	6,060	na	[50]
Soy milk	na	5,023	na	6,290	na	[3]
Soy milk chain grocery stores	na	4,585	na	6,247	na	[3]
Soy milk Chinese markets	na	6,375	na	6,505	na	[3]
Tonyu, chocolate	na	31,400	na	55,800	na	[41]
Soymilk nature	na·	138,200	na	143,300	na	[41]
Soymilk, vanilla	na	46,300	na	82,700	na	[41]
Soya drink	na	63,700	na	78,300	na	[41]
Biosoya	na	31,700	na	51,500	na	[41]
Instant beverage A	na	32,090	na	63,592	10,822	[47]
Instant beverage D	na	41,850	na	68,535	11,071	[47]
Western Food with hidden soy flour or protein (USA)						
Bread (white)	nd	606.0	nd	830.8	na	[3]
Bread (whole grain)	nd	155.8	nd	141.8	na	[3]
Buns, English muffins	nd	177.1	nd	229.6	na	[3]
Canned chili	34.5	543.3	tr	713.5	na	[3]
Canned tuna	tr	412.8	nd	730.0	na	[3]
Diet shakes	tr	88.7	nd	88.7	na	[3]
Doughnuts	tr	1,973.3	nd	3,214.7	na	[3]
Ice cream	nd	47.8	nd	43.5	na	[3]
Non-dairy creamer	nd	61.1	nd	144.9	na	[3]
Pancakes, waffles	nd	1,300.4	nd	1,357.9	na	[3]
Pizza	nd	230.8	nd	241.6	na	[3]
Powder for instant drink	na	50,340	na	67,290	na	[40]
“Power”-type bars	nd	1,803.8	nd	3,269.4	na	[3]

(*continued*)

Table 77.2 (continued)

	Biochanin A	Daidzein	Formononetin	Genistein	Glycitein	Author
Nuts and Seeds						
Coconut, desiccated	nd	<1	<1	2	<1	[2]
Peanuts, dry roasted	22	<1	22	58	12	[2]
Peanuts, roasted, salted	21	4	3	70	56	[2]
Peanut butter, smooth	1	<1	3	36	21	[2]
Pistachios, roasted & salted	27	nd	2	2	2	[2]
Pumpkin seeds	7	<1	3	5	2	[2]
Sunflower seeds	nd	<1	nd	1	<1	[2]
Coffee						
Coffee	nd	50.3	nd	tr	na	[3]
Coffee, instant powder	nd	153.0	4	594.0	162.0	[2]
Coffee, instant, decaffeinated						
powder	nd	nd	1	4	nd	[2]
Tea						
Tea, chamomile	3	<1	<1	3	<1	[2]
Alcoholic Beverages						
Lager, canned	6	<1	2	2	4	[2]
Beer, brown ale	4	<1	2	1	<1	[2]
Stout, 4–5%	2	<1	1	2	3	[2]
Cider, dry	1	<1	<1	nd	4	[2]
Cold cereal	nd	53.7	nd	nd	na	[3]
Oils						
Flaxseed	6	nd	1	nd	2	[2]
Roasted pumpkinseed	<1	<1	1	<1	3	[2]
Rapeseed	4	<1	3	2	3	[2]
Other						
Eggs	tr	27	tr	tr	na	[3]
Soy Lecithin	na	68.6	na	17.9	na	[40]
Soy based infant formula µg/100mL of reconstituted milk						
Allsoy concentrate[a]	na	862.3	na	1,824.2	163.8	[52]
Gallia soja ready to feed	na	1,120	na	2,220	na	[40]
Isomil ready to feed[a]	na	1,113.42	na	2,630.67	384.3	[52]
Modilac soja ready to feed	na	1,310	na	1,740	na	[40]
Mead Johnson, Prosobee	na	1,710	na	2,180	na	[51]
Nutrilon soja ready to feed	na	1,160	na	2,650	na	[40]
Prosobee soja ready to feed	na	635	na	1,115	na	[40]
Prosobee concentrate[a]	na	1,946.2	na	4,195.64	390.6	[52]
Ross Isomil reconstituted	na	780	na	1,580	350	[51]

(*continued*)

Table 77.2 (continued)

	Biochanin A	Daidzein	Formononetin	Genistein	Glycitein	Author
Ross Isomil ready to feed	na	1,910	na	2,260	na	[51]
Wyeth-Ayert, Nursoy ready	na	750	na	1,600	280	[51]
Soy based infant formula µg/100g not reconstituted milk powder						
Enfamil, Next Step	na	7,230	na	14,750	3,000	[51]
Mead Johnson, Gerber	na	8,080	na	13,900	3,120	[51]
Mead Johnson, Prosobee						
Liquid concentrate	na	1,100	na	2,220	na	[51]
Mead Johnson, Prosobee	na	7,050	na	14,940	2,950	[51]
Ross Isomil	na	6,030	na	12,230	2,730	[51]
Wyeth-Ayert, Nursoy	na	1,020	na	2,820	na	[51]
Formula A	na	5,500	na	9,200	na	[53]
Formula B	na	5,000	na	8,100	na	[53]
Formula C	na	4,800	na	9,100	na	[53]
Formula D	na	4,400	na	8,300	na	[53]
Nursoy[a]	na	4,513.04	na	11,626.21	831.6	[52]
Isomil[a]	na	4,766.94	na	11,626.13	989.1	[52]

na not analysed, *nd* not detected, *tr* trace
[a] Recalculated from complete formula analysis

amounts of **Isofl** with estrogenic activities are highly variable from one batch of the same food trademark to another. Soy consumption is regular and moderate in Asia, currently 2–7 soy dishes/week. The **Isofl** intake in China is 25 mg/day on average [58] and in Japan from 45 to 60 mg/day [59]. However, there is a great variability in the intake, especially in the various regions of China. In Western countries, soy is usually consumed anecdotally and European **Isofl** intake is considered to be below 2 mg/day [60–62]. However, in the past few years there has been effective publicity on the health benefits of soy consumption. As a consequence, some Western consumers have exaggerated their soy intakes, leading to cases of over-consumption of **Isofl** with estrogenic properties. The health effects of such behaviors will be detailed below.

4 Bioavailability and Metabolism

In plants and vegetables, isoflavones are present mainly as glycosides (Fig. 77.2b). Glycosidases linked to the enterocyte layer are present in the gut and can quantitatively hydrolyze the **Isofl** glycosides from the food. This has been deduced since no glycosides can usually be found in human feces, urine, or blood [63]. Once deglycosylated into aglycone compounds, estrogenic **Isofl** can enter the enterocyte barrier, where they can be subjected to the action of detoxification enzymes. These enzymes, called phase

Table 77.3 Isoflavone contents in some food supplements freely available in 2005 on the European market

Soy based supplements	Manufacturer	Genistein (mg per tablet)	Daidzein (mg per tablet)	Total isoflavones (mg per tablet)	Intake per day (mg)
Anacaps	Ducray	0.76 ± 0.07	0.63 ± 0.07	1.39 ± 0.15	2.8 ± 0·30
Biopause	Monin Chanteaud	1.4 ± 0.2	1.0 ± 0.1	2.45 ± 0.22	4.9 ± 0·44
Biopause Fort	Monin Chanteaud	0.4 ± 0.1	1.50 ± 0.2	1.9 ± 0.22	3.8 ± 0·44
Bioptimum Soja	Boiron	3.1 ± 0	5.9 ± 1.3	12.1 ± 0.55	24.2 ± 1.10
Compleal	Besins Int. Nutraceutique	28.5 ± 2.9	13.9 ± 0.7	42.4 ± 0.77	84.8 ± 1.5
Cybestron	Vital	0.07 ± 0.01	<0.001	0.07 ± 0.01	0.07 ± 0·01
Efodyne	Yves Ponroy	1.5 ± 0.2	6.7 ± 1.0	8.20 ± 0.45	8.20 ± 0.45
Elugyne	Dolisos	2.7 ± 0.4	10.6 ± 1.6	13.30 ± 0.58	13.30 ± 0.58
Estrofort	Rotapharm	21.5 ± 1.9	10.0 ± 0.14	31.5 ± 0.58	31.5 ± 0.58
Estronat	Lescuyer	6.6 ± 1.0	6.5 ± 1.0	13.10 ± 0.58	39.3 ± 1.74
Evestrel	Theramex	11.6 ± 1.7	9.2 ± 1.4	20.80 ± 0.72	41.6 ± 1.44
Evestrel jour/nuit	Theramex	20.4 ± 2.3	6.6 ± 0.3	27.0 ± 0.66	54.0 ± 1.22
Feminibiane	Pilege	6.5 ± 0.2	5.2 ± 0.2	11.7 ± 0.26	11.7 ± 0.26
Feminine	Medikem	3 ± 0.5	1.5 ± 0.2	4.8 ± 0.34	9.6 ± 0.68
Feminite Soja D3	Œnobiol	4.2 ± 0.4	5.8 ± 0.7	10.0 ± 0.43	20.0 ± 0.86
Gydrelle	Iprad Sante	4.5 ± 0.7	13.3 ±2	17.7 ± 0.67	35.0 ± 1.34
Gydrelle phyto fort	Iprad Sante	17.9 ± 1.4	9.4 ± 0.2	27.3 ± 0.52	27.3 ± 0.52
Gynalpha fort	CCD	28.6 ± 4.3	64.2 ± 9.6	92.8 ± 15.1	92.8 ± 15.2
Gynalpha	CCD	12.1 ± 1.8	15.0 ± 2.2	27.1 ± 0.82	54.2 ± 1.64
Gynalpha plus	CCD	12.0 ± 0.7	10.1 ± 0.8	22.1 ± 0.5	22.1 ± 0.5
Gynosoya	Codifra	27.0 ± 4.0	7.4 ± 1.1	34.4 ± 0.92	68.8 ± 1.84
Inneov fermete	Inneov	4.9 ± 0.7	3.9 ± 0.4	8.8 ± 0.43	17.6 ± 0.86
IF super concentres	Solgar-SoyLife	5.1 ± 0.3	18.5 ± 3.3	23.6 ± 0.77	23.6 ± 0.77
Isoflavone de soja	Vitarmonyl	10.7 ± 1.9	5.0 ± 0.6	15.7 ± 0.65	31.4 ± 1.30
Isopro	Eko Bio	4.4 ± 0.1	21.1 ± 1.7	25.5 ± 0.55	25.5 ± 0.55
Isoyam	Starvital	1.7 ± 0.2	1.5 ± 0.2	3.2 ± 0.26	9.6 ± 0.78
Macasoyam	Fenioux	2.2 ± 0.3	4.8 ± 0.7	7.0 ± 0.41	42.0 ± 2.46
Menocomplexe	Biotechnie	18.3 ± 0.2	9.3 ± 0.2	27.6 ± 0.26	55.2 ± 0.52
Menoflore	Floressance	<0·001	<0·001	<0·001	<0·003
Menolig	Vichy	11.3 ± 1.7	5.6 ± 0.8	16.9 ± 0.65	67.6 ± 2.60
Menopause	Juvamine	7 ± 0.1	1.2 ± 0.13	9 ± 0.18	15.6 ± 0.72
Oligoforme 50	IDO	0.4 ± 0.06	0.2 ± 0.03	0.6 ± 0.12	2.4 ± 0.48

(*continued*)

Table 77.3 (continued)

Soy based supplements	Manufacturer	Genistein (mg per tablet)	Daidzein (mg per tablet)	Total isoflavones (mg per tablet)	Intake per day (mg)
Pausanorm	Alkimson	0.04 ± 0.04	0.2 ± 0.02	6.2 ± 0.55	0.24 ± 0.04
Preluzelle	LPF	8.6 ± 1.3	10.7 ± 1.6	19.4 ± 0.70	38.8 ± 1.4
Promensil	Novogen	0.7 ± 0.02	0.3 ± 0.005	44.4 ± 2.16§	44.4 ± 0.06
Phytofemme (iso)	Superdiet	4.9 ± 0.7	4 ± 0.6	8.9 ± 0.47	17.8 ± 0.94
Phytosoya	Arkopharma	1.7 ± 0.2	7.1 ± 0.9	8.7 ± 1.0	17.4 ± 2.00
Sojacal	Novagyn	9·5 ± 1·4	6·3 ± 0·9	15·8 ± 0·62	31·6 ± 1·24
Sojalia	Biocentury	6·6 ± 0·5	3·7 ± 0·7	10·0 ± 0·45	20·0 ± 0·90
Sojamag	Novagyn	10·7 ± 1·6	6·1 ± 0·9	16·8 ± 0·65	33·6 ± 1·30
Sojapause	Oligo pharma	14·7 ± 0·4	7·2 ± 0·25	21·9 ± 0·33	43·8 ± 0·66
Sojyam	Tonipharm	5·9 ± 0·9	2·8 ± 0·4	8·7 ± 0·47	26·1 ± 1·41
Soya femme 24 jour	Forte Pharma	12·7 ± 0·5	9·8 ± 1·2	22·5 ± 0·53	22·5 ± 0·53
Soya femme 24 nuit	Forte´ Pharma	13·0 ± 0·5	9·9 ± 1·9	22·9 ± 0·63	22·9 ± 0·63
Soya Menopause	Nutrisante	20 9·2 ± 0·9	4·9 ± 0·5	14·1 ± 0·48	28·2 ± 0·96
Soyolig	Vichy	11·9 ± 1·8	6·7 ±1	18.6 ± 0·68	37·2 ± 1·36
Thalassovital	Dietetique et Sante	2·1 ± 0·1	7·4 ± 0·5	9·5 ± 0·32	9·5 ± 0·32
Ymea	Chefaro-Ardeval	0·7 ± 0·02	2·5 ± 0·04	3·2 ± 0·10	6·4 ± 0·20
Ysoflavone complexe	Ysonut	5·0 ± 0·5	12·9 ± 0·5	17·9 ± 0·58	36·8 ± 1·16

1, 2, and 3 enzymes, are, respectively, responsible for the hydroxylation, glucuronidation or sulfatation, and finally expulsion of the conjugated polyphenols from the cells. The actions of these enzymes are incomplete at the gut level. They are completed in the liver and kidney [63]. The phase 2 enzymes encompass, among others, sulfotransferase (SULT) and glucuronosyltransferase linked to uranyldiphosphate molecules, namely UDPGT or UGT. There are many isoforms of these enzymes, which can be highly specific of certain substrates. However, again the structural similarities between $17\beta\text{-}E_2$ and **Isofl** lead to cross activities of the UGT enzymes. Indeed, UGT1A is responsible for the glucuronidation of $17\beta\text{-}E_2$ in the C3 position in men's liver microsomes, whereas UGT2B7 is responsible for the glucuronidation of $17\beta\text{-}E_2$ in the 17β position [64]. It was shown that UGT1A can also use **Gen**, **Daid**, Bioch, Form, and **Gly** as substrate [65]. In addition, the same study showed that, at physiological doses, UGT1A9 is also able to conjugate **Isofl**. Isoflavone sulfation was demonstrated in the gastrointestinal tract and in the liver [66]. For **Gen** at least, SULT1A is involved. Usually, a substrate can also modulate the activity of its proper enzyme. Consequently, it was shown in rodents that high doses of **Isofl** can modulate the activities of several phase 2 enzymes known to be implicated in the 17β-E_2 conjugation [67, 68]. Because the soy **Isofl** can act differently on the different enzymes studied, they can influence 17β-E_2 bioavailability in a complex manner, including at the tissue level [20]. As an example, Sun and coworkers [69] showed that **Isofl** can modulate the activity of these enzymes in the LNCap cells (prostate cancer cell line) probably playing a role on 17-βE_2 bioavailability in tissues. The relative affinity of **Isofl** and 17β-E_2 is crucial when considering the respective effects of one to the other. Isoflavones are likely to exert an estrogenic activity if 17β-E_2 is absent or only present at low concentrations, that is, in the young, in men, and in post-menopausal women. On the other hand, in premenopausal or in pregnant women where 17β-E_2 is high in plasma, **Isofl** are likely not to exhibit an effect unless **Isofl**-containing-foods are consumed in exaggerated amounts. This will be discussed later. Because of this, there are small differences of **Isofl** bioavailability between men and women [41].

Isoflavones undergo metabolic transformation in the gut. Namely, **Daid** is known to be transformed into *o*-desmethyl-angolensin and **Gen** into *p*-ethylphenol [70] by gut bacteria. These compounds can enter the blood stream but are not estrogenic. In contrast, **Daid**, can be biotransformed by the gut bacteria into *S*-equol (***S*-Eq**) [71, 72]. Unlike in humans, this conversion is highly efficient in animal models including mice [73], rats [74], monkeys [75], and hamsters [76], but not in pigs, which seem to be rather close to humans on this point [77]. In human consumers, ***S*-Eq** occurs only in about 20–45% of the tested populations [78].

Contrary to the **Isofl**, ***S*-Eq** possesses an asymmetric carbon in position C_3. This gives it a three-dimensional shape closer to that of 17β-E_2 (Fig. 77.2c, d). Thanks to this, ***S*-Eq** exhibits a greater affinity for the ERs than its precursor **Daid** [72], and has a greater effect on bone resorption prevention [79]. This also raises the question of the relevancy of animal models when considering the putative effect of soy or a soy extract on human health.

Considering the data mentioned above, isoflavonoids in plasma are mainly under a conjugated form and, in humans under moderate soy-food intake, essentially under a glucuronide conjugated form [80]. As mentioned earlier, **Isofl** exhibit the best bioavailability among the known polyphenols [8]. At a dietary relevant rate, their absorption ranges between 60 and 80 % according to the compounds and the studies [8, 81]. Their T_{max} is usually obtained between 6 and 8 h after ingestion depending on the compound considered [41]. Their elimination half-life ($T_{½}$) is rather long, from 10 to 24 h, since it can sometimes be influenced by a recirculation phenomenon involving biliary excretion of liver-targeted absorbed **Isofl** [41]. The residence time of **Isofl** in plasma increases from **Daid** to **Gen** with **Daid**<**Gly**<**Gen**, and chronic ingestion can lead to plasma steady-state levels. As a consequence, under chronic ingestions, although **Gen** is usually ingested in a greater amount via soy-food, the urinary levels contain more **Daid** and do not reflect the ingestion rate of **Isofl**. They do not reflect the plasma levels either [78]. The T_{max} of ***S*-Eq** is usually reported to be between 16 to 18 hours since its absorption occurs in the colon. It was also shown that **Isofl** with estrogenic activities can bind to the specific proteins binding 17β-E_2 and testosterone (T) in plasma, with a low affinity and in a nonspecific manner. These sex hormone binding globulins, namely SHBG, are present in plasma from adults and α-fetoprotein (AFP) of fetuses and neonates [82]. This binding can influence **Isofl** and 17β-E_2 excretion. This is particularly relevant since it was shown in vitro that **Gen** significantly induces SHBG production in HepG2 cells [83]. Several authors reported plasma **Isofl** concentrations ranging from 150 ng/mL to 219 ± 330 ng/mL in Asian consumers under a traditional diet [84–86]. In the meantime, other authors reported plasma **Isofl** levels ranging from 177 ± 104 ng/mL to 941 ± 370 ng/mL in Western consumers with a significant soy-food intake [41, 87, 88]. The measures were performed in similar conditions (doses and time between ingestion and collection). These differences can be linked to the ethnic origin of the subjects and possibly to a peculiar effect of the respective bacterial flora of the two groups [89]. Isoflavones from food supplements are more bioavailable than those from a soy-food matrix [41]. In the following paragraphs, the in vitro tests cited will be those involving molecules known to be present in plasma and therefore susceptible to being in contact with the target cells. The glycosylated precursors of plants will not be considered.

5 Mechanisms of Action

Isoflavones with estrogenic activities can exhibit nuclear actions involving the classical ER pathways. They have been shown to bind to the classical receptors ERα and ERβ and also to induce transcriptional activities via these receptors [19]. Via these pathways, their activity is between 1/100 and 1/10,000 that of 17β-E_2. This depends on the compounds considered. Genistein is by far the most studied **Isofl** with estrogenic effects. The investigations were mainly undertaken to prove the beneficial effects of isoflavonoids on breast cancer risk after having found the presence of **Isofl** and **Eq** in the human diet and in human biological fluids [90, 91] after soy consumption. Recently, it was shown that the natural form of **Eq** found in humans is the isomer S, that is, ***S*-Eq**, which presents in vitro a strong affinity for ERβ, almost 50 times higher than for ERα [92]. Many of the mechanisms of action described below will be illustrated with **Gen**, although all its effects are not strictly stackable to the other members of the **Isofl** family. In the last decade, classic ERs were shown to be present in the cell membranes under a palmitoylated form [93]. Under this form they are also embedded in other membranes such as those of endoplasmic reticulum [94] and those of mitochondria [95]. The cell membrane estradiol receptors (mERs) can be blocked by ICI 182,780, as can nuclear ERs [96]. Membrane classical ERs are known to establish a cross-talk with the insulin-like growth factor 1 (IGF-1) receptor [97] and via these pathways to induce breast cancer cell proliferation. The actions of **Gen** on ERs and mERs are blocked similarly by ICI 182,780 [96]. Genistein is also known to increase IGF-1 receptor gene expression via an IGF-1 dependent mechanism, and this mechanism is probably involved in the proliferative effect of **Gen** at low concentrations (from 0.5 to 10 μM) on breast cancer cell lines. However, there are discrepancies in the literature concerning the effect of **Gen** on breast cancer cell growth. While Chen and coworkers [96] say that **Gen** induce the ER/IGF-1 cross-talk, Anastasius and coworkers [98] showed that long-term exposures to very low doses of **Gen** (i.e., 10 nM) reduce the expression of AKt and phosphorylated AKt in MCF-7 when **Gen** is applied for 10–12 weeks. This reduces proliferation of MCF-7 cells. The AKt pathway is induced via the IGF-1 receptor. According to these two studies, **Gen** could then act in opposite ways on the same pathways. Indeed, the authors of these two studies argue for different doses of exposure of the same line of cells. To highlight this issue, one must remember the following: (1) When soy is ingested, **Gen** is present with other compounds including **Daid**, **Gly**, and in some cases ***S*-Eq**; these three compounds do not exhibit the same mechanisms of action. (2) **Gen** plasma concentrations vary widely and plasma concentrations can reach up to 1–2 μM at C_{max} after a soy-based food intake, however, these concentrations do not remain for long. (3) Breast contains adipose tissue known to fix **Isofl** differently with a predominance of ***S*-Eq** [99]. (4) The E-screen test involving MCF-7 cells conducted in vitro and in which **Isofl** with estrogenic activities induce cell proliferation is the one chosen by the OECD to check for potential adverse effects of chemicals on breast cancer [100]. In the last decade, a new membrane estrogen receptor, a G-protein coupled ER, the so-called GPCR or GPR-30, has been discovered. It belongs to the rhodopsin-like receptor family and is coupled to a G protein. It mediates very rapid estrogenic effects, that is, effects occurring within a delay ranging from a few seconds to several minutes. Its estrogenic action is mediated by cytoplasm cascades of phosphorylations and de-phosphorylations. These cascades can involve the PI3K – MAPK – ERK 1/2 and SF-1 pathway. Recently, **Gen** was found to bind GPR-30 and to induce the kinase activations induced by both 17β-E_2 and tamoxifen [101]. This led to activation of endometrial cell proliferation. As far as we know, neither **Daid**, ***S*-Eq**, nor the other **Isofl** with estrogenic properties have been tested yet on this newly discovered membrane ER. In addition to the membrane receptor GPR-30, orphan estrogen-related receptors (ERRα, ERRβ, and ERRγ) were discovered in the nuclear compartment [102]. These receptors, whose physiological ligands have not yet been identified, are closely related to classical ERs but they do not respond to 17β-E_2. The ligand-binding pockets of ERRα and ERRγ are extremely small compared with those of the classical ERs because the ERR ligand-binding pockets are partly filled by lipophilic side chains. As a consequence, the classic agonists or antagonists of the ERs act differently on ERRs. 4-OH tamoxifen binds ERRβ and ERRγ without initiating transcription when diethylstilbestrol (DES, known as a potent estrogen) acts as an antagonist on all ERR isoforms [103–105]. Recently, **Eq** was shown to bind to ERRγ and to significantly induce dependent-gene transcription [106]. Previously, Suetsugi and coworkers [107] showed that Bioch was able to bind and to induce transcription via the three ERR isoforms while **Gen** and **Daid** were active only through ERRα and β. At the same time, **Isofl** were tested on many targets for anti-estrogenic action. Genistein was then shown to exhibit anti-proliferative effects at high doses (>20 μM) via a specific anti-protein tyrosine kinase (PTK) effect [108], affecting Bcl-2 availability in breast cancer cells [109] and inducing cell apoptosis. This effect is not elicited by either **Daid** or **Eq**. Daidzein was shown to exert an anti-protein kinase C (PKC) effect at doses higher than 60 μM [110]. Isoflavones were shown to exhibit anti-angiogenesis effects in vitro at doses higher than 50 μM. Recently, it was shown that **Gen** acts via an anti-PTK route decreasing the activation of JNK and p38MAPK in Huvec cells [111]. **Gen** and **Daid** were also shown to inhibit the 5α-reductase (5-AR) and the 17β-hydroxysteroid dehydrogenase enzymes implicated, respectively, in the biotransformation of T into dihydroT (DHT) and of estrone into 17β-E_2. Inhibitors of these enzymes are used in the treatment of breast and prostate cancers. However, the effects are only recorded using doses higher than 10 μM with IC_{50} values over 50 μM [112]. These doses are much higher than the doses available in the plasma of soy consumers. Isoflavones were also shown to have slight anti-aromatase effects [113]. Aromatase is the key enzyme for 17β-E_2 production. Drugs with anti-aromatase activities are used in breast cancer treatments. However, this time again the IC_{50} in vitro are very high (i.e., over 100 μM). On the other hand, **Isofl** at dietary concentrations were shown to antagonize anti-aromatase treatments in athymic nude mice implanted with MCF-7 cells [114]. This leads to a proliferative estrogenic effect. Flavonoids are usually considered as potent antioxidants. In this respect, and because of their specific chemical structure that prevents a total delocalization of the electrons on the carbon skeleton of the molecule, isoflavonoids are poor antioxidants, even though they were shown to have such an effect in vitro at extraphysiological concentrations [115]. In 2002 and 2006, it was reported that **Gen** can have epigenetic effects. The demonstration was obvious since **Gen** altered the epigenome of offspring in viable yellow agouti mice [116, 117]. In this study, involving dietary doses, **Gen** appeared as a methylating agent. Epigenetic effects that result in the modulation of gene expression can be achieved via the DNA methylation of CpG islands and via the acetylation or the methylation of histones. These effects can be transferred from one cell generation to the other. Several studies showed that high doses of **Gen** or other **Isofl** may have demethylating effects on the promoters of metastases or tumor suppressor genes and induce their expression [118–120]. However, the active doses are not always relevant to the nutritional situation and may induce PTK inhibition. Gupta and coworkers [121] studied the effects of 24 nutraceuticals including **Gen** on various epigenetic modulations of genes implicated in the inflammatory process. They also studied their consequences on tumor cell viability, proliferation, invasion, and angiogenesis. However, the active doses were usually extraphysiologic. Epigenetic effects are interesting to consider for **Isofl**, since they fit with epidemiological data showing an environmental protection of Asian populations against some cancers after early, moderate, and continuous exposure to soy food, that is, from childhood to old age. Such a protection is absent if soy is consumed later in life. These epigenetic effects, if achieved with low doses and on genes implicated in early tumorization processes, may be particularly relevant to study in the context of cancer prevention by nutritional factors.

6 Animal Studies

In animals, the bioavailability of **Isofl** is different from that observed in humans. Globally in rodents, **Isofl** bioavailability is 10 times lower than in humans; the oral doses must be 10 times higher to lead to the plasma doses recorded in humans. Indeed, 20 mg/kg/day of **Isofl** leads to 610 ng/mL **Isofl** in rat plasma [122], while 1.4 mg/kg/day leads to 599 ng/mL **Isofl** in human plasma [123]. However, as mentioned earlier, rodents are intensive ***S*-Eq** producers. This is not always the case in humans. Because ***S*-Eq** exhibits specific health effects, soy consumption in animals may not induce the same health effects as in humans. In addition, one must remember that soy consumption leads to significant plasma levels of **Gen**, **Daid**, ***S*-Eq**, and **Gly**. Therefore, the test of a pure compound cannot be transposed to soy consumption.

6.1 Breast Cancer

There are two major types of results that were obtained in rodent models concerning breast cancers. The first category encompasses those obtained on nude athymic mice implanted with human breast cancer cell lines. Globally, these experiments using MCF-7 implanted cells show a proliferative effect induced by ingested soy [124], or by ingested isolated soy **Isofl** [125, 126]. These experiments also show a negation of the effects of the anti-aromatase letrozole [114] or of the effect of the anti-estrogen tamoxifen [127] by the dietary **Isofl**. Note that processed soy does not induce the same response [128]. Recently, Onoda and coworkers [129] tested pure **Eq** and a fermented preparation, namely SE5-OH containing large proportions of ***S*-Eq** on the proliferation of MCF-7 cells transfected with a luciferase gene (MCF-7-E10) in athymic nude mice. They found no proliferation at a dietary dose of 250 and 500 ppm. However, in this paper **Gen** was not active either and this is in discrepancy with previous works. In addition, the same treatments induced proliferation of the cell lines in vitro. Therefore, these results need confirmation. Although MCF-7 is a widely recognized breast cancer cell type it presents specificities (that is, ERα and sometimes ERβ positive with a full context for 17β-E_2 action). Consequently, it is not surprising to find that **Gen** exhibited no proliferative effects on mice implanted with ER-negative tumor cells (MDA-MB-231). In this case, soy inhibited cell growth [130]. This advocates for an estrogenic and a proliferative effect of **Isofl** and their plasma metabolites at dietary relevant plasma concentrations on ER-positive cells. The other type of results show, on the contrary, a preventive effect on chemo-induced tumors. Indeed, **Gen** was shown to prevent proliferation of chemo-induced breast tumor cells [131]. This effect was only obtained when **Gen** was administrated prior to the tumor inductor dimethylbenz[a]anthracene (DMBA) [132]. Daidzein and its metabolite ***S*-Eq** do not exhibit the same pattern of activity [133].

These effects are mediated by the ERs and not by the MEK 1-2 or ERK 1-2 pathways [134]. Taken together, these results were long considered as discrepant. However, in the first case we observed an estrogenic effect on cells already transformed in tumor cells, and in the second case, the effect of **Gen** is examined before cell tumorization and appears to prevent it. It was first thought that the preventive effect of **Gen** on the transformation of normal cells into tumor cells was due to its capacity to induce mammary development and differentiation [135]. That said, the differentiation process is in opposition with the dedifferentiation process occurring in cancer cells. Nowadays, the hypothesis of a protective effect of **Gen** through epigenetic pathways is attracting attention [136]. The epigenetic effects seem probable, although they have to be shown with relevant **Isofl** dietary plasma concentrations.

6.2 Colon Cancer

Although it is hypothesized that soy may have a preventive effect on colon cancer (CCa), the role of **Isofl** in soy is still unclear and the demonstration is not conclusive in animal studies undertaken with males. This time again, two different types of studies were performed. Some tried to check the effects on tumor cell proliferation and others tested the potential role of soy or **Isofl** from soy on the prevention of chemo-induced tumors. Some studies tried to sort out the effect of **Isofl** compared with that of other compounds of soy protein. As an example, Vis and coworkers [137] checked for the effects of several components of soy food on colon cell proliferation.Neither methionine nor **Isofl** were found to influence colon cell proliferation. However, soy saponins may exert a protective effect on colon cell proliferation by reducing the lytic activity of cholic acid. When fish oil and **Isofl** in soy were tested on the proliferation of colon and breast cells [138], it was found that **Isofl** in soy had no effect on apoptosis in either tissue, but reduced mitosis in the colon ($P < 0.001$) while increasing it in the mammary gland ($P = 0.001$). These changes were associated with contrasting modifications in the ER expression. **Isofl** in soy increased ERα and decreased ERβ expressions only in the colon. In parallel, Xiao and coworkers [139] examined the chemo-preventive effects of soy protein with **Isofl** and of **Gen** versus casein on the prevention of CCa induced by azoxymethane (AOM). There were no effects of either treatment when limited to gestational exposure. However, soy protein fed to male rats at weaning and until the end of the AOM exposure reduced the colon tumor incidence by 47 %. It is not clear from this study whether **Isofl** are responsible for this preventive effect. More recently, a study [140] examined the effects of a life-time exposure (including pregnancy and lactation) to soy **Isofl** of male rat treated with AOM to induce CCa. Two doses of **Isofl** were tested (40 and 1,000 mg/kg diet). Soy **Isofl** did not affect incidences or multiplicities of colon aberrant crypts or tumors but the lowest dose of **Isofl** decreased tumor burden and size ($P < 0.05$). ERβ expression in colon tumors was increased by soy **Isofl**.

Controls were made in vitro on DLD-1 cells. Their proliferation was decreased dose dependently by **Isofl** and the expression of ERβ was increased. Note that in mammary tumor cells the ERβ positive cells that are not ERα positive do not grow under **Isofl** treatments [18]. Lately, the epigenetic effects of **Gen** and of a soy protein extract (SPE) were examined on three different cell lines [118] together with their anti-proliferative activity. It was found that **Gen** and SPE regulated the expression of WNT5a. The WNT signaling pathway plays a critical role in normal epithelial regeneration and tumorigenesis in the human colon. This regulation was accompanied by a decrease in DNA methylation in the CpG islands of the WNT5a promoter. However, the doses used (75 μM) were far higher than the dietary relevant doses and can inhibit the PTK pathway. Therefore, these results are not fully convincing and may be reanalyzed with lower and dietary relevant doses.

6.3 Prostate Cancer

Animal studies indicate that **Isofl** and **Eq** can have beneficial effects on prostate growth and cancer since they decrease prostate serum antigen (PSA) production, and they bind and prevent the synthesis of 5α-DHT [141, 142]. Several data would indicate that **Isofl** and especially **Gen** at high doses may prevent and cure prostate cancers. The cell action determined on chemo-induced prostate tumors or in LNCap cells implanted in nude athymic mice leads either to cell apoptosis via the IGF/Src/Bax-BCl_2 pathway already described, or via a direct induction of caspase 3 [143, 144]. The other pathway that is thought to be involved in PCa protection is the prevention of the pro-oncogene MDM2 synthesis, this pro-oncogene being common to several cell types including breast cells. However, it was shown using reporter transgenic mice models coupling androgen responsive elements and luciferase that **Gen** administrated orally at a dose of 5 mg/kg is agonist of the androgen receptor (AR) in the prostate of intact males and antagonist in castrated males [145]. Because androgens are tumorotrophic agents in PCa, this result indicates that the effect of **Gen** on PCa is far from being simple. To add to the controversy, Nakamura and coworkers [146] recently developed a patient-derived prostate cancer xenograft model. They grafted clinical prostatectomy samples into nude athymic mice fed 0, 2, or 10 mg/kg **Gen**. For these doses they showed dose-dependent increases in lymph nodes and secondary organ metastases (liver, lungs). They also showed the aggregation of invasive malignant cells in the secondary organs of the **Gen**-treated groups and not in the control. Their data suggest that this effect is due to the enhanced activities of PTK, EGFR, and their downstream Src in **Gen**-treated groups. They underlined that the effect of **Gen** may depend on the kind of tumor considered. In addition, Hillman and coworkers [147] reported that **Gen** combined with irradiation was remarkably efficient in the induction of PC-3 cell apoptosis in nude athymic mice when ingested at a rate of 5 mg/day (PC-3 are human prostate cancer cells). However, **Gen** alone significantly induces PC-3 proliferation and lymph node dissemination in secondary organs.

6.4 Osteoporosis

Bone is in constant remodeling, with accretion and resorption phenomena occurring at the same time [148]. During growth the accretion overcomes resorption. During adulthood the two mechanisms are balanced. In women, during and after menopause, the resorption mechanisms take precedence over bone formation. Two cell types are involved in these phenomena: osteoblasts take part into bone formation while osteoclasts are responsible for bone resorption. At the physiological level, bone health can be measured directly by physical measurements of bone mineral density (BMD) or bone mineral content (BMC). Other indirect measurements can be performed on urine or plasma samples for resorption or accretion biomarkers, that is, BTM (for Bone Turn-over Markers) like deoxypyridinoline (DXP) or *n*-telopeptide (NTx). Hormonal, mineral, and vitamin measurements including parathyroid hormone (PTH), calcium, and vitamin D can also predict the bone turn-over efficiency. However, BMD and BMC are not the best predictors of the bone fracture risk. The bone architecture has to be conserved to avoid fractures. In animal studies, bone resistance to fracture can be measured and is a good marker of osteoporosis. Osteoblasts derive from stromal cells and their differentiation is under the control of estrogens and growth factors. Osteoclasts derive from monocytes. Their differentiation requires the activation of the RANK receptor by its ligand RANK-L. RANK-L is produced by the osteoblasts. However, osteoblasts also produce osteoprotegerin (OPG), which interferes with RANK-L in its binding to RANK. OPG, when present, prevents osteoclast differentiation. Therefore, the bone accretion is the result of a finely balanced synthesis of RANK-L and OPG by the osteoblastic cells. This balance is tightly controlled by endocrine, paracrine, and nutritional factors. Estrogens are one among other factors controlling bone accretion [149]. Isoflavones with estrogenic activities were tested on this function in animal models. All published data are positive with either soy or individual **Isofl** [122, 148, 150, 151]. Equol and ***S*-Eq** seem to exhibit a particularly favorable effect on bone accretion [79, 152]. These effects were examined using diets containing relevant concentrations of **Isofl** on ovariectomized rats, which may mimic the menopausal situation in humans. Bone physiology was assessed by measuring bone markers of resorption in plasma (DXP, TLx), osteocalcin, and BMD and BMC. In animals at sacrifice the bone resistance to fracture was also measured. In all cases the results published in the literature showed a positive effect of **Isofl** or soy on bone physiology. These results are sustained by in vitro data showing that pure **Isofl**, when tested at relevant doses, activate osteoblastic differentiation [153, 154], osteoblastic proliferation, and cytoskeleton protein secretion [155, 156]. Isoflavones also increase alkaline phosphatase production and that of OPG [157–159]. Isoflavones also decrease the production of inflammatory cytokines like IL-6, which are known to activate the osteoclasts [158]. These effects are dependent on the nuclear ERE pathways being blocked by ICI 182,780 [160] and also by membrane pathways leading to CREB activation [161].

6.5 Memory

Estrogens are known to be neuroprotective and neurotrophic agents [162]. Their mechanisms of action include direct effects on neurons, actions on astroglial cells, and vascular effects [162]. On vessels, estrogens were shown to induce vasodilatation, improving oxygen perfusion of the surrounding cells [163]. Estrogens also reduce the production of inflammatory molecules such as IL-6 [164] by glial cells. Finally, in neurons, they increase the dendritic spine density known to be involved in neuronal plasticity [165]. They activate glutamate receptor mobility and target them at the synapses [166]. They are involved in mitochondria maintenance and activity [95]. At physiological doses they are beneficial for the calcium balance involved in the long-term potentiation (LTP) phenomenon [165]. LTP is the best cellular model so far that has been connected to memory functioning. 17β-E_2 is also synthesized in neurons and especially in the pre- and post-synaptic compartments [167]. ERs, mER, and GPR-30 [168] are present in many brain territories, including some involved in the memory process like the hippocampus [169], amygdala, striatum, and prefrontal cortex [170]. They are also present in other brain areas projecting onto or into these territories like median septum or basal forebrain [170], which are involved in the cholinergic activation of the hippocampus. In rodents, seven studies describing the effects of **Isofl** used either pure compounds or soy-based-food [171–177]. All studies, except that of Lund and Lephart [172], which involved very high doses of **Isofl** (600 mg/kg), showed a positive effect on working, reference, or spatial memory and even anxiety. The study by Lund and Lephart [173] showed beneficial effects on spatial memory in females but deleterious effects in males. These results are sustained by in vitro data obtained using pure **Isofl**. At high doses (>10 μM) **Gen** may induce an inhibition of PTK and exhibit deleterious effects, including the disruption of calcium homeostasis and induction of apoptosis via the Bax-BCl_2 pathway [178]. At lower doses, **Isofl** induce dendritic spine growth [171], cholinergic activation of the hippocampus [176], and neurotrophic effects on hippocampal neurons [179]. They exert anti-inflammatory effects on glial cells [180]. In addition, some of their effects seem to prevent the deleterious effects of β-amyloid [181–183].

In summary, except for bone health, all data obtained on animal models are not fully conclusive. The doses tested must be considered carefully and the compounds available in plasma should be taken into account when a parallel is to be drawn with humans.

7 Epidemiological Studies

Epidemiological studies suggested that soy consumption with **Isofl** presenting estrogenic activities in Asian countries may prevent several disorders and diseases, some of them being hormone dependent, such as menopausal symptoms; cardiovascular diseases; breast, prostate, and colon cancers; fractures; and memory defects. Asian populations, known as regular consumers of soy and **Isofl**, were carefully analyzed for their susceptibility to various diseases and compared with those existing in Western populations. The diseases studied were mainly breast, prostate, and colon cancers as well as osteoporosis. Some recent studies examined the effects of soy intake on memory. The first studies relied on dietary surveys linked to an estimation of phytoestrogen intake, which could be incomplete [184]. Some studies then linked the relative risk or incidence of disease to urinary **Isofl** levels [185]. Then, menopausal, health, BMI, or age status were taken into account [186]. Finally, some environmental, genetic, and cultural factors were considered in these comparisons [187, 188]. There are other factors that can possibly act on diseases incidence: dietary factors, oral contraception and hormone replacement therapies (HRT), morphology and posture, and gene polymorphism. Recent studies have tried to sort out these different factors.

7.1 Menopausal Symptoms

Menopausal symptoms encompass hot flushes, night sweats, vaginal dryness, mammary density, and mood fluctuations. They are considered as adverse effects of menopause but not as a disease. In Western countries, they have been treated for more than 20 years using HRT. However, several studies, including the famous Women's Health Initiative (WHI). showed that the deleterious effects of the US treatments on breast and endometrial cancers as well as on the cardiovascular pathologies may overcome their benefits [189]. A few epidemiological studies tried to examine whether the consumption of soy and **Isofl** with estrogenic activities may prevent hot flushes. This is relevant since it is known that hot flushes and night sweats are controlled by the hypothalamus preoptic area implicated in body core temperature regulation. This area induces shivering or sweating when it records a body temperature over a lower and an upper threshold. Between these thresholds is a thermal neutral zone. The amplitude of this neutral zone is under serotonin and noradrenergic control.17β-E_2 is known to induce the local synthesis of these two neuromediators as well as their respective receptors [190]. The study of Nagata and coworkers [191] showed a reduction in hot flushes with increased soy consumption. Note that, in this case, the highest quartile consumed 50.2 mg of **Isofl** per day (115 g of soy per day) and in this case the Hazard ratio was 0.42 with a $CI_{95\%}$ between 0.25 and 0.72. In Hawaii, Sievert and coworkers [192] showed that the incidence of hot flushes based on self-declarations was higher in the European-American ($n = 203$) versus the Japanese-American ($n = 249$) participants (72% vs. 53% $P < 0.01$). In China, in a population of 1,399 participants, consumption of soy three times a week reduces the incidence of hot flushes but not significantly (OR = 0.67 $CI_{95\%}$ = (0. 38,1.20), P value = 0.18) [193]. A recent study performed in China on 4,842 women with non-metastatic breast cancer and aged between 20 and 75 did not show a correlation between hot flushes, night sweats, and vaginal dryness on one side and soy consumption on the other [194]. The **Isofl** consumption ranged from less than 20 to more than 62.3 mg/day. As far as we know, no observational epidemiological studies were performed on the effect of either soy consumption or of **Isofl** on hot flushes incidence in women from Western countries.

7.2 Breast Cancer

Breast cancer can be a hormone-dependent disease when malignant cells possess ERs (ER-positive tumors, about 80 % of the post-menopausal breast cancers). It has been hypothesized that **Isofl** could exert both estrogenic and anti-estrogenic activities. From the previous discussion it appears that at dietary doses the effects in vitro or in relevant animal models are mainly estrogenic and proliferative in ER-positive cells [124]. However, the two ERs, ERα and ERβ, may play different roles, and according to the cell equipment in ERs, **Isofl** can induce the proliferation of ERα-positive cells and can be anti-estrogenic and anti-proliferative in ERβ-positive cells, which do not express ERα [18]. In the epidemiological studies conducted so far and considering soy or **Isofl** effects, the precise status of the tumor cells are usually not stated in detail. This adds confusion to the results. In addition, we will see later that cycles and 17β-E_2 production in premenopausal women can be impacted by **Isofl** consumption. This would suggest a difference between the premenopausal status when 17β-E_2 is synthesized under the pituitary stimulation, and post-menopause when **Isofl** with estrogenic activities can be the major estrogens in plasma. A recent review, presenting a meta-analysis of the relative risk of breast cancer with **Isofl** consumption, showed that Asian women may be protected to a slight extent but not Caucasian women living in Western countries [195]. This can be explained by two main facts. Firstly, in Western countries the **Isofl** intake is usually low (below 5 mg per day). Secondly, a high **Isofl** consumption is mostly related to food-supplement intake, which usually occurs late in life at peri-menopause and during menopause and post-menopause. Another recent study established that differences exist between menopausal and postmenopausal women regarding soy **Isofl** effect on breast cancer [196]. When soy is consumed moderately from childhood in an Asian context, protection is observed in premenopausal women who generally mainly present ER-negative tumors. It is not the case in menopausal women [197]. Other recent analyses tried to find a link between soy intake and breast cancer recurrence. The relationship remains unclear, with some studies showing a reduction in the recurrence of breast cancer [198] and others no effect [199]. However, the study by Shu [198] has to be considered with caution since it included volunteers who all had bilateral breast surgery. Finally, when the intake duration is taken into account, it seems that a limited protection can be associated with early, regular, and moderate soy intake from childhood to menopause [200]. In very few studies the ER status of breast tumors was considered. When it was done it appeared that **Isofl** could be slightly protective as could other polyphenols on ER-negative tumors, but that this "protection" by estrogenic **Isofl** is lost when ER-positive tumors are considered [201].

7.3 Colon Cancer

Colon cancer (CCa) is thought to be influenced by estrogens. However, a recent re-analysis of the WHI results has shown that HRT may not be beneficial, but vitamin D may be [202]. A meta-analysis was recently done analyzing the results of studies performed to associate soy or **Isofl** consumption to the incidence of CCa [203]. Eleven studies tried associating soy intake with CCa incidence, and four looked at CCa mortality with soy consumption. The incidence was reduced by soy consumption only in two studies and only in women. The mortality rate was never associated with soy consumption except for one inverse correlation in Chinese women from Hong Kong. Six case–control studies have analyzed the intake of **Isofl** with CCa risk. Among them, three showed no effect and three showed a significant reduction of the risk. Among the positive studies, only one showed the effect of significant **Isofl** intake (20 mg/day) [204]. In the others, the intake was below 1.5 mg/day [205, 206] raising the question of confounding factors. The authors of the meta-analysis [203] concluded that soy consumption may lead to a reduction of CCa in women but not in men. However, other studies are needed to take into account possible confounding factors.

7.4 Prostate Cancer

The incidence and mortality rate of prostate cancer (PCa) varies largely worldwide. The lowest rates are observed in Japan and other Asian countries [207], but the efficiency of the detection program is not the same as in Western countries [208]. In addition, the incidence of latent PCa discovered at autopsy is not substantially different between Japan and the US. This suggests that the differences are related to cancer progression and not cancer appearance. Several factors, including genetic and environmental, may play a role in the development of PCa. It was shown that the risk for PCa increased dramatically in Japan concomitantly to the introduction of a Western-like diet [207]. Migrant studies have shown an increase in PCa incidence in Asian men after emigration to the US [209]. Western lifestyle presents many differences from the Asian one, including several dietary habits among which soy and tea consumption have drawn much attention so far. In addition, the Mormon and Seventh-day Adventists communities in the US, which are not genetically different from the White Americans but have different lifestyles (no tobacco, no drugs, more vegetables and fiber, more polyphenols with antioxidant activities) exhibit lower incidence of many cancers, including PCa [210]. Recently, a meta-analysis indicated that tofu consumption and urinary **Gen** and **Daid** were associated, in Asian people, with a lower incidence of PCa [211]. The odd ratios (OR, 95 %) were 0.69 for total soy foods with a confidence interval ($CI_{95\%} = 0.57–0.84$) and 0.75 ($CI_{95\%} = 0.62–0.89$) for non-fermented soy foods. The consumption of miso, nato, or tonyu was not associated with this incidence. These data have not been confirmed so far by clinical trials [212].

7.5 Osteoporosis

Ecological studies showed that the incidence of osteoporotic fractures in Chinese women is lower than that observed in Caucasian women in Western countries [213]. This was attributed by some authors to the dietary intake of estrogenic **Isofl** by the Asian populations. Among 10 epidemiological studies that examined the correlation between soy-food consumption and bone loss based essentially on BMD, Lagari and Levis [214] indicated that 7 showed a positive association and 3 no association. It is difficult to draw out a definitive opinion from these data since, in most cases, pre-, post-, and menopausal women were included. In addition, the number of subjects was greatly different (from 24,403 to 15). However, in the three negative studies, two involved Caucasian women from Western countries whose soy consumption is generally low.

7.6 Memory

The WHIM study which dealt with the memory assessment of the US HRT on memory lead to confusion about the role of 17β-E_2 on memory. WHI (1995–2002) was stopped prematurely because a higher rate of vascular incidents was observed in the HRT-treated group. In addition, in the WHIM sub-population, a slight deterioration of the "Minimal Mental Scores" was detected in treated women versus placebo. However, the figures were far from alarming: 40/2,245 versus 21/2,245, respectively [215]. Later, it was understood that the type of HRT combined with the age of the volunteers and with their body weight most probably favors the deleterious effect of HRT on the vascular function and secondarily on the brain function. There are few epidemiological studies connecting soy consumption and memory and none connecting **Isofl** and memory. Previously, one study had reported an adverse effect of high tofu intake on dementia incidence in old men [216]. Another independent study reported a deleterious effect of tofu consumption on memory characteristics in old men but a positive effect of tempeh (containing vitamin B9) [217]. Hogervorst noted that the deleterious effects he observed at first on a large cohort of Indonesian subjects were no more significant on a smaller group of subjects living in the same district and after a stratification based on age [218]. Note that the tofu consumption that was considered in that study varied from 0 servings per week to 21 per week (three times per day). The largest group had one tofu serving per day. From these data no firm conclusion can be drawn on the effect of either soy or **Isofl**.

Epidemiological and ecological studies are of great interest, especially when they involve large populations of subjects. However, when different populations are compared, abusive correlations can be made between different items that are not always relevant because of the many existing confounding factors that are not always considered. Comparisons of Asian and Caucasian populations each in their own contexts must take into account cultural, socio-economic, medical, and environmental factors that include diet. When health disorders are considered it must be remembered that medical surveys are not the same in all countries and that, in some cases, figures can be misestimated for that reason. For example, menopausal symptoms or prostate cancers may not be declared equally in developed and emerging countries. Exposure to persistent pollutants (radioactivity or chemicals) or to specific climate factors (long nights in northern countries) can greatly influence several health parameters. When Asian and Western diet habits are compared it must be remembered that there are great regional variations, especially in emerging countries where local habits based on traditional food availability is a major factor in foodstuff consumption. Therefore, epidemiological and ecological data must be sustained by clinical interventions and mechanistic explanations.

8 Clinical Trials

8.1 Menopausal Symptoms

Many studies exist in the literature dealing with the clinical effects of soy **Isofl** with estrogenic activities on menopausal symptom relief. Two recent reviews were retained here to illustrate this specific point. The first one by Bolaños and co-workers [219] analyzed 19 previous studies. They tried to separate the studies, and their conclusions were based on the source of **Isofl** used. They observed more convincing results than the previous meta-analysis. They sorted **Isofl** sources between soy concentrates, soy-based food supplements, and soy extracts. The general tendency is a reduction of hot flush incidence, but the greatest heterogeneity of the results was observed with soy-based food supplements. The other recent meta-analysis is that of Keeley and Caroll [220]. These authors examined the effects of preparations based on several plant extracts. They selected studies conducted in treated versus placebo clinical trials and using pure extracts not mixed with other plant extracts or with vitamins or minerals. Their conclusions are drawn for each preparation type. Considering **Isofl** preparations, they conclude that a lack of well-designed-studies cannot lead to the conclusion of a beneficial effect of soy-based food supplements on menopausal symptoms. They underlined the great variability in the study designs and the lack of control over the plant extracts. From our point of view, **Isofl** preparations are likely to have a beneficial effect on the occurrence of hot flushes even though the great heterogeneity of the studies did not show it conclusively. We argue for a positive effect of the **Isofl** preparations based on the popularity of such preparations among peri- and menopausal women. Indeed, many other types of preparations were proposed to relieve these symptoms and none have been as popular as the soy-based food supplements so far. It is difficult to believe that women would have wasted money for years without any relief. However, there is a crucial need for well-designed studies testing homogenous groups of women. These studies need to use fully characterized extracts and avoid interaction with other chemicals or plant extracts. **Isofl** bioavailability and metabolism should be evaluated among women during these trials since several metabolites may have greater effects than others. The incidence of hot flushes needs to be evaluated more precisely and to be based on more than self-declaration. The occurrence of placebo effects on such a symptom is undisputed, but several studies showed that **Isofl**-containing preparations may have a better effect than placebo, although the improvement could be moderate [221, 222]. For now, the market is highly heterogenous with a large choice of doses, products not claiming any **Isofl** content, and products making false claims. This anarchic offering [41] combined with the great inter-individual differences in **Isofl** bioavailability and metabolism among women [78] can only result in conflicting opinions on the benefits of **Isofl** on menopausal symptoms.

8.2 Breast Cancers

Some clinical trials designed to examine the effect of **Isofl** on menopausal symptoms, on bone resorption, or on vascular risk factors tried to determine whether these treatments could influence the occurrence of breast cancer. To date, no study has reported an effect of **Isofl** on breast cancer proliferation because no ethics committee would ever authorize the test of such compounds on women with a proliferating ER-positive tumor. The only data that are available were collected some time ago on women with benign breast hyperplasia, and the examination was usually done on samples collected on healthy tissue. Three studies match these characteristics, that of Petrakis and coworkers [223], that of McMickael-Philipps and coworkers [224], and that of Hargreaves and coworkers [225]. All three studies are concordant, which gives their results an undisputed power. The authors used daily administrations of either 45 or 60 mg of **Isofl** in soy-based food supplements or in soy-based-food. These doses are common in the market. The treatment was done from 2 weeks to 1 month prior to the biopsies, on premenopausal women. All studies looked for an estrogenic effect of the **Isofl** on the healthy breast tissue without clear statement on volunteers' ovarian cycles. All found an effect that went from the secretion of breast fluid to the increased production of estrogen-dependent proteins, like PS-2 and progesterone receptors (PR). Some clearly showed breast epithelial cell proliferation. From these studies, it can be said that an oral intake of **Isofl**, at doses from 45 to 60 mg per day, has an estrogenic effect on healthy breast tissue. This means that the **Isofl** bioavailable concentrations and their circulating forms are adequate for an estrogenic effect in the breast tissue. More recently, the study of Qin and coworkers [226] tried to find epigenetic effects of **Isofl** on genes implicated in breast cancer progression. Although their approach is interesting, their results are not fully conclusive. The doses they used were 37.2 and 128.8 mg **Isofl** on premenopausal women aged between 19 and 54 treated for an ovarian cycle. However, contrary to the previous studies, they found no estrogenic effect based on C3 and cell morphology measurements. They interpreted some of their results as anti-estrogenic effects. Conversely, they found different effects according to the doses they used on the hypermethylation of some genes' promoter. As an example, *RAR*β*2* methylation decreased for **Gen** plasma levels below 600 ng.mL^{-1} and increased for **Gen** plasma levels above 600 ng.mL^{-1}. This correlation, which excludes the other **Isofl**, likely needs additional analysis. In addition, the great variability in the age of the recruited subjects probably requires discussion. Because all of these studies were performed involving premenopausal women who presented significant 17β-E_2 plasma levels, the hypothesis of a synergy between **Isofl** and 17β-E_2 cannot be rejected. Therefore, the transposition to the menopausal or post-menopausal situation must be considered cautiously.

8.3 Colon Cancer

There are few clinical trials involving **Isofl** with estrogenic activities and colorectal cancer (CCa). Their results are not consistent. Adams and coworkers [227] found no effect of a 12-month dietary soy intervention with 81 mg/day of **Isofl** on three sites in the colon on the basis of five different measurements in a population of mostly men aged

50–80 with previous adenomatous polyps. Soy consumption on the contrary may have a preventive effect on CCa [203], but this needs to be confirmed by supplemental studies.

8.4 Prostate Cancer

Prostate cancers (PCa) are hormone-dependent diseases. The proliferation of prostate cancer cells is under the positive control of both androgens and estrogens. Testosterone and DHT induce PCa growth, and AR are the main targets of current therapies. However, ERs are differentially expressed in the prostate tumors and during cancer progression and Aromatase KO (ARKO) mice had a lower incidence of PCa showing that the local production of 17β-E_2 from T plays a role in cancer progression. While the proliferative effect of 17β-E_2 seems to be essentially mediated by ERα, ERβ on the other hand seems to be protective since it is either absent or not detectable in 40 % of the tumors [228]. Therefore, the combination of anti-5-AR, ERα antagonist. and ERβ agonist seems to be a promising treatment option for PCas. Although the exact implication of this ERβ receptor is not yet well defined, **Isofl** are thought to prevent clinical PCa because they exhibit a better affinity for ERβ than for ERα. As mentioned above, affinity does not mean transcription activity, and heterodimers can possibly be active. There are unconvincing results from clinical trials involving **Isofl** with estrogenic activities on PCa [143]. One study evaluating red clover **Isofl** supplements on the progression of PCa before surgery found a significant elevation of apoptosis in radical prostatectomy specimens [229]. The doses were 160 mg/day and there were 20 cases versus control. On the contrary, many studies did not find any effect on tumors. The study of de Vere White and coworkers [230] found a partial reduction of PSA in 17 % of their patients (up to 50 % reduction) with the administration of 900 mg of **Isofl** for 6 months. Lazarevic and coworkers found a modest reduction of PSA (7.8 %) with 30 mg of pure **Gen** in 54 % of their subjects treated for 3–6 weeks prior to prostatectomy [231]. Because PSA is not an absolute marker of PCa tumor progression, there is no pure evidence of a protective effect of **Isofl** with estrogenic activities on PCa [232], although soy may be protective [233].

8.5 Osteoporosis

Osteoporosis is a disease characterized by osteopenia and a higher risk of bone fracture. Because bone architecture is as essential for bone resistance as BMD, BMD cannot reflect, on its own, the osteoporosis prevention. In addition, although bone is in constant remodeling, its evolution is slow and a preventive effect cannot be demonstrated in short-term trials. Therefore, at the same time as the epidemiological studies involving soy-based-food, some clinical trials were performed involving soy-based food supplements. They allow compliance with higher-dose-treatments for long periods of time. Among the 17 studies cited by Lagari and Levis [214], 12 were performed in peri- and menopausal women between 40 and 65 years of age. The inclusion of peri-menopausal women is likely to have reduced the significance of the results since these women with significant circulating 17β-E_2 are likely not to respond to the **Isofl** treatments in the same way as menopausal women. Three studies only lasted for several weeks and measured BTM. One involving pure **Gen** did not show any effect and the two involving **Isofl** mixtures showed a reduction of BTM. To complete these data, note that when ***S*-Eq** is analyzed on its own in clinical studies, it seems to exert a specific lowering effect on bone turnover. The other studies lasted from 6 months to 4 years. Generally, when BMD was measured it improved, as well as BTM. It must be emphasized that these effects were all obtained using high doses of either **Gen** or mixed **Isofl**. These doses ranged between 40 and almost 700 mg **Isofl** per day. Efficient doses were commonly over 54 mg per day and their effects were essentially limited to the lumbar spine and sometimes to the femoral neck; effects were not observed on peripheral bones. It must be specified that several studies associated **Isofl** with calcium and/or vitamin D supplements as well as physical exercise. In addition, it is well known now that other dietary factors including alkaline components, proteins, vitamins [148], and polyphenols [234] can prevent bone loss, and that colas and acidified food should be avoided [235].

8.6 Memory

Some studies showed maintenance of several types of memory performance in post-menopausal women under soy supplementations compared with other food supplementations. A moderate intake of **Isofl** produces positive effects and spatial and verbal memory are usually improved or at least maintained, compared with controls [236, 237]. Three other experiments were performed using soy. In young volunteers a 10-week soy consumption with 100 mg of **Isofl** improved cognition [238]. In post-menopausal women, 99 mg of **Isofl** in soy for 12 months did not improve the cognitive performances tested [237]. Conversely, 72 mg of **Isofl** daily in soy milk had no effect on different memory tests and was deleterious to verbal working memory performances [239]. When these results are considered in parallel with the epidemiological studies, soy may not be beneficial to memory. When **Isofl** are considered in soy-based food supplements, eight studies should be included. They were performed on young or aged subjects, on Caucasian or Asian subjects, and on either men or women. Five studies exhibited positive results on either memory tests or on mood tests whereas three studies led to no improvement. Duffy and coworkers [240] showed, in 33 post-menopausal women treated for 12 weeks with 60 mg soy **Isofl**, a significant improvement in recall of pictures and in a sustained attention to tasks. Casini and coworkers [241] treated post-menopausal women for 6 months with 60 mg of soy **Isofl** and showed an improvement of mood disorders. Gleason and coworkers [242] tested 100 mg of soy **Isofl** for 6 months on 34 elderly men and women. They found that **Isofl**-treated subjects improved their performance on tests of visual-spatial memory ($P < 0.01$), construction ($P = 0.01$), and verbal fluency ($P < 0.01$) and speeded dexterity ($P = 0.04$). Islam and coworkers [243] tested a supplementation of 100 mg soy **Isofl** for 3 days at various periods of the menstrual cycle in 28 premenopausal women and compared their effect to that of naturally synthesized 17β-E_2. They showed that **Isofl** supplementation during menses (low 17β-E_2 plasma levels) led to an improvement in working memory and verbal memory when 17β-E_2 in the luteal phase seemed only to affect verbal memory. Thorp and coworkers [244] tested 116 mg of soy **Isofl** on 34 men in a 12-week double-blind, placebo-controlled cross-over trial. They found that **Isofl** supplementation significantly improved spatial working memory ($P = 0.01$) but did not affect auditory and episodic memory. On the contrary, Ho and coworkers [245] found no effect of 80 mg supplementation of soy **Isofl** in healthy Chinese post-menopausal women. However, nothing is said in this study on the soy consumption of the subjects and this may have induced a bias in the results. Maki and coworkers [246] found no effect of 120 mg **Isofl** from clover fed to 14 women for 12 months. Finally, Pilsáková and coworkers [247] tested the mental rotation in 20 men and 16 women. They found a negative effect of 0.34 mg **Isofl** administrated for 1 week. From these studies, it appears that soy **Isofl** as supplements may have a positive effect on spatial working memory. However, the treatment needs to be done at doses from 60 to 120 mg for 12 weeks to 12 months with a correct management of confounding factors including diet factors.

9 Pharmacological Applications

The pharmacological use of **Isofl** with estrogenic activities is an undisputed fact today. In 2005, our team identified 136 different preparations from 67 different laboratories available in French shops or presented in French on the web. Our partial analysis of this market presented notably in [41] led to the same conclusions as those expressed by Setchell and coworkers [248], Nurmi and coworkers [249], and Kelley and Carrols [220]. In all cases, it was considered that the selection was greatly heterogeneous with **Isofl** content claims varying from 2 to 120 mg per units (capsules, tablets, etc.) with preparations with no claimed doses and with discrepancies between the claimed doses and those found after analysis. Moreover, in several cases when different batches were analyzed, variations in the **Isofl** composition were observed, indicating that the soy extract was not the same from one batch and the other. This market must be monitored to avoid misleading consumers. Several preparations like Bonistein® containing pure synthetic **Gen**, vitamin D, K1, and fish oil are sold today. The claim is bone protection, although the results are not yet fully convincing. Soy-germ-based food supplements containing a high amount of **Gly** are also sold by various manufacturers. Note that **Gly** does not induce MCF-7 proliferation in vitro [250]. Recently, a fermented soy extract enriched in ***S*-Eq**, namely SE5-OH, appeared on the market [251].

9.1 Menopausal Symptoms

The major claim for **Isofl** containing food supplements is the relief of menopausal symptoms. There is still much controversy around this effect. However, the popularity of these preparations around the world suggests a positive effect in at least some of the users. Because of the large variability in the preparations available on the market, the placebo effect, and the large inter-individual variations on **Isofl** bioavailability it seems highly logical that there should be a conflict of opinions on this issue.

9.2 Breast Cancer

Because of the controversy around the effects of **Isofl** on breast cancer proliferation and risks, the soy or clover-based food supplements advertised in the 1990s for breast cancer prevention have disappeared from the market today. They were proposed to children and teenagers in a preventive strategy. Because soy contains many compounds that are as interesting as **Isofl**, like phytosterols, soya saponins, abd protease inhibitors, the reduction of the beneficial effect of soy to that of **Isofl**, especially in the case of breast cancer prevention, seems illogical. It is more sensible to advise a regular and moderate soy-food intake from childhood to adulthood as part of a well-balanced and diversified diet.

9.3 Colon Cancer

There is no clear effect of **Isofl** on CCa in men, even though some data would advocate for a protecting role in women. However, soy may be protective through other soy components like saponin, sapogenin, or Bowman-Birk inhibitor [252, 253]. These compounds only have limited blood availability. However, they are in contact with gut cells at a significant rate. Marketing **Isofl** in food supplements for the prevention of CCa does not rely on consistent scientific data.

9.4 Prostate Cancer

There are **Isofl**-containing food supplements on the international market claiming to be healthy for the prostate health. Usually, **Isofl** are associated with other ingredients with anti-oxidant properties. The efficacy of these preparations is unclear.

9.5 Osteoporosis

Despite the concordance of positive results in vitro, in animal models, and sometimes in clinical studies, estrogenic **Isofl** are not considered to exert a clear preventative effect on human osteoporosis. As mentioned earlier, the reduction of the excretion of BTM or maintenance of BMD does not strictly indicate a reduction of the risk of bone fracture. In France and in Europe, the food and health agencies have not so far allowed a health claim on osteoporosis prevention. A claim on the prevention of bone loss with daily doses over 60 mg, preferably in two intakes per day, may be more easily obtained. However, as mentioned earlier, such a preventive effect can be achieved only with high doses of **Isofl**, which are susceptible to affect the proliferation of ER-positive breast cancer cells in menopausal women with breast cancer. Therefore, this use must be monitored carefully.

10 Toxicity

The effects of 17β-E_2 are numerous and tightly regulated through a precise balance of its bioavailable concentrations in fetuses, in infants, during childhood, in adult men and women, and during senescence. Therefore, a slight modification of the estrogens in an individual can lead to so-called endocrine disruptions. The adverse effects presented here are essentially demonstrated in animals with high doses of **Isofl**, and usually only suspected in humans where it is ethically difficult to experiment on toxicological effects.

10.1 Reproduction

The potential adverse effects on reproduction are sustained by the historical data from the 1940s obtained on Australian ewes grazing clover pastures and presenting an infertility syndrome [254] and also by the work of Farnworth and coworkers [28, 29] identifying **Isofl** with estrogenic activities in many plants traditionally used in our countries as sources of anti-fertility agents. In addition, the classical definition of estrogens as having uterotrophic characteristics [255] may lead to a suspicion of adverse effects on women's reproductive system. We are now examining the potential adverse effects of high doses of **Isofl** with estrogenic activities during in utero exposure, during infancy and in adult life.

10.1.1 In Utero Exposures

An effect of **Isofl** with estrogenic activities can be considered if their concentrations combined with their affinity overcome that of 17β-E_2, or if these concentrations induce a modification of the endogenous 17β-E_2 bioavailability in fetuses by interfering with the AFP. In both cases, considering the affinity ratio for the ERs and the AFP of steroidal and non-steroidal estrogens respectively, the **Isofl** concentrations must be very high in the fetus plasma and therefore ingested at very high levels by the mother. In Western women, who do not consume soy significantly, the **Isofl** levels in the amniotic fluid are on average 40 nM [256]. In contrast, it was shown that in Asian women under regular and moderate soy consumption with an intake of **Isofl** in the range of 20 mg per day, the **Isofl** level in the amniotic fluid was 250 nM [257] or about 10 times that of 17β-E_2 [258]. These concentrations are not sufficient to induce an adverse effect on the fetuses as seen in Asian populations.

In parallel, it was shown that 45 mg of **Isofl** per day affect LH secretion in premenopausal women, lengthening the menstrual cycle in American Caucasian women [259] to the duration commonly observed in Japanese women, that is, 30 days instead of 28 days in Western countries. During pregnancy, LH secretion required for progesterone production is essential. An adverse effect of **Isofl** during pregnancy may be observed through this mechanism but only at high doses. This was observed in ewes on clover pastures by Findlay and coworkers [260]. This disruption of LH and progesterone levels was shown to lead to miscarriages. However, according to Sharpe and Skakkebeck [261], an estrogenic contamination can also affect the sex differentiation in males via a complex mechanism. The estrogens can reduce the GnRH secretion of the fetuses affecting their in utero secretion of FSH and LH and consequently their secretion of T by Leydig cells and of 17β-E_2 by Sertoli cells. This can lead to a hyposecretion of anti-Mullerian hormone (AMH) and consequently to cryptorchidism and hypospadias. If a regular and moderate intake of soy is safe in this mechanism, it must be pointed out that two independent studies reported a significant elevation of the incidence of hypospadias in babies whose mothers had a vegetarian diet during pregnancy [262, 263]. The results are remarkably concordant on hypospadias risk with OR = 4.99; $IC_{95\%}$ = 2.10–11.88 in [262] and OR = 4.6; $IC_{95\%}$ = 1.6–13.3 in [263]. However, some confounding factors, like a lack of crucial nutrients or an elevated intake of herbicides or pesticides with estrogenic effects due to the consumption of vegetables, prevent the conclusion that there is a direct effect from soy **Isofl**.

10.1.2 Neonatal Exposure

As mentioned previously, only high **Isofl** intakes can possibly cause adverse effects. However as seen in Table 77.2, babies fed soy-based infant formula exclusively during their first months of life experience a tremendous intake of **Isofl**, 5–11 times higher than that occurring in adults under normal soy consumption or in adults using soy-based food supplements [51] (Table 77.2). The report by Rozman and coworkers [51], who worked on behalf of the US Department of Health and Human Services, mentioned **Isofl** plasma levels in babies under this specific diet ranging from 3.5 to 6.5 μM. As mentioned previously, **Isofl** at a plasma concentration of about 1 μM decrease LH production in premenopausal women. Therefore, it seems sensible to fear a decrease of LH production in babies under an exclusive intake of soy-based infant formulas. As a matter of fact, in male babies it is known that a LH surge occurs naturally around 4 months of age [264, 265]. This is before teething and before diet diversification. This LH surge is responsible for the masculinization of the sexually dimorphic nucleus of the preoptic area (SDN-POA). It must be noted that the masculinization of the SDN-POA is obtained after LH induced T production in the testes and after the aromatization of T into 17β-E_2 in the SDN-POA. This means that the masculinization effect itself is under estrogen stimulation. This process leads to an increase in the size of the SDN-POA in males. If the neonatal LH surge is prevented in male neonate macaque rhesus using a GnRH analog, for example, a smaller SDN-POA is observed, a devirilization of adult behavior [266, 267], a reduction in adult testicular size [266], a reduction of sperm production in adults [266], osteopenia [268], and a partial immunodeficiency [269] affecting specifically the T $CD4^+$ lymphocytes. In female infants the LH surge is not as pronounced and usually occurs later, on average after 6 months of age usually after diet diversification.

Now, what could be expected from an overloading of **Isofl** with estrogenic activities in infants? Isoflavones can exert two major effects an anti-LH effect and an estrogenic effect. In marmosets, it was shown that partial feeding of male infants with human soy-based infant formulas leads to a decrease in the neonatal T levels induced by the normal LH neonatal surge [270]. It seemed that this effect was probably compensated later since the cellular composition of testes with respect to Leydig and Sertoli cells was finally not affected at the adult stage [271]. Note that in that specific experiment the feeding was not exclusive and therefore no rigorous comparison can be made with a human situation where a mother conscientiously feeds her baby several times a day (and night) with a soy-based infant formula. If the feared decrease in LH production is achieved, one could expect a reduction in the testicular size, a reduction in the sperm production later in life, but no disruption of the sexual behavior since at the SDN-POA level, the lack of T and consequently of aromatized 17β-E_2 could be compensated by **Isofl**. On the other hand, in female infants LH is not expected to be produced before 6 months of age or the estrogens induced by such a synthesis. Then the SDN-POA remains normally of a small size. In that context, a contamination by xeno-estrogens, including **Isofl** with estrogenic activities, may induce a masculinization effect on the SDN-POA leading to a masculinization of the behavior later in life. This has been observed in rats using treatments with high doses of coumestrol and **Gen** [272]. Up to now, only one study has given data on the effects of exclusive soy-based formula intake during infancy versus cow's milk-based formula on adult reproductive parameters, including sexual behavior, and that is the study by Strom and coworkers [273]. They claimed that no effect was observed. However, they did not examine testicular size or sperm production in men fed soy formula in their infancy, or the women's social behavior under the same diet. Conversely, they found a significant and higher incidence of acne spots during menstruation and of painful menstruations in women fed a soy-based formula during infancy. In addition, there were 3 stillborns out of 79 births in the soy-formula group versus 0 out of 148 births in the cow's milk group. These results were not significant because the population size was not large enough. Because acne spots and painful menstruations are under hormonal control, it seems abusive to conclude an absence of endocrine disruption with exclusive soy-based infant formula feeding in neonates. Indeed, several pediatric societies around the world, in the United State, Great Britain, France, and New Zealand, clearly advise against the use of these formulas at an early age unless there is a clear health indication (galactosemia or lactase deficiencies, which are very rare) or unless it corresponds to the parents' convictions [51]. Finally, a recent review advises care with soy-based infant formulas considering the recent growing data on the effects of soy **Isofl** on male sperm count and male fertility [274].

10.1.3 Over-loading in Adults

Cases Reported in Women

Chandrareddy and coworkers [275] reported three cases of endometrial disruptions correlated to high soy consumption. The consumers were American women. One was a 56-year-old postmenopausal woman, one was a 43-year-old pre-menopausal woman, and the third was a 35-year-old woman; all declared having high soy consumption for periods varying from 2 to 14 years. The pre-menopausal women were taking contraceptive pills based on norethisterone, which blocks menstrual bleeding, this bleeding being very painful. Despite this treatment, they both exhibited bleeding and associated pains. All three women had endometrial polyps and fibroids. They exhibited endometriosis patterns that were recurrent even after adequate surgery. Pharmacological doses of **Gen** were shown to induce endometriosis patterns in rats [276]. All symptoms disappeared in these women after the interruption of soy consumption. The youngest got pregnant after soy and contraception arrest. In this case, the **Isofl** intake in pre-menopausal women was so high that it counteracted the norethisterone treatment, establishing an estro-progestational contraception where a progestational contraception was intended. In his study, Chandrareddy did not detail the levels of the **Isofl** intake. However, we observed (unpublished data) the case of a 31-year-old pre-menopausal woman under norethisterone contraception who had painful menstruations even when under treatment. In her case, the soy and **Isofl** intake could be evaluated. She declared having a soy-based yogurt, a soy-based-dessert cream, and soy-based cereals every day, 30 mg **Isofl** on average. She also had tofu once a week and also 1 L of soy milk (tonyu) two or three times a week. According to our measurements and considering the great variability of **Isofl** content in tonyu (80–280 mg/L) her **Isofl** consumption could then vary from 30 to 333 mg of **Isofl** per day when she had a tofu and a tonyu drink highly concentrated in **Isofl**. The highest dose is about 15 times higher than the habitual exposure in China and five times higher than the highest consumption considered in Japan. In these circumstances, the symptoms observed could be easily explained.

Cases Reported in Men

Again, only high soy consumption or the specific potentiating effects of **Isofl** are under discussion here. Chavarro and coworkers [277] presented the effect of soy and **Isofl** consumption on sperm count in men. They recruited 99 men who were male partners from subfertile couples with complete dietary and semen analysis data. They constituted four groups according to the soy food and **Isofl** intake. Age, abstinence time, BMI, smoking status, caffeine, and alcohol intakes were considered. Seventy-two percent of the men were overweight, with BMI > 25 kg/m^2. The authors observed that soy-food and **Isofl** intakes were inversely related to sperm concentration. Men with the highest intake level of soy foods (≥ 2 times per week) had 35 million sperm.mL^{-1} less than men who did not consume soy foods ($CI_{95\%} = -67, -3$), and there was a significant trend toward decreasing sperm concentration with increasing soy food intake (P, trend $= 0.03$). The adjusted difference in sperm count was significant, $P < 0.05$ for obese volunteers under the highest soy food intake, with a decrease of 50.45 million sperm.mL^{-1}. There was a strong trend in non-obese volunteers still on the highest soy food intake, with a decrease of 48.2 million sperm.mL^{-1}. The difference between obese and non-obese volunteers can be explained by a synergy in the obese subjects between **Isofl** and 17β-E_2 produced by adipose tissues. More recently in Japan, Toshima and coworkers [278] analyzed the urine concentrations of cadmium, 3-PBA (an insecticide with endocrine disruption effects), and **Daid** and ***S*-Eq** of 42 Japanese men. They were male partners of couples who had infertility problems. Semen parameters (volume, concentration, and motility) were examined. After multiple regression analysis it was found that fruit and coffee consumption as well as urinary **Daid** explained a reduction in sperm concentrations ($P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively). In addition, coffee consumption, ***S*-Eq** production, and 3-PBA detection in urine explained a reduction in sperm motility ($P < 0.001$, $P < 0.05$, and $P < 0.01$, respectively). This pilot study suggested that apyrethroid exposure through fruit consumption and other dietary habits, including coffee and soy intake, were significant contributors to poorer semen quality. These studies may be considered with that of Fisch et al. [279], who reported that sperm count was significantly lower in Asian people than in men from Western populations without fertility disruption. These data are also supported by results recently reported in male mice exposed to soy containing food from conception to adulthood versus a soy free diet [280]. The authors observed a reduction of haploid germ cells in testes with a 25 % decrease in epididymal sperm counts and a 21 % reduction in litter size. Transcripts coding for androgen-response genes in Sertoli cells and Gapd-s, a specific spermatid marker, were significantly reduced. In addition, dietary soy decreased the size of the seminal vesicle without affecting its proteolytic activity. There was no effect on male behavior. From this study it can be said that dietary soy and phytoestrogens may affect male reproductive function resulting in a small decrease in sperm count and fertility.

10.2 Estrogen-Dependent Cancers

There are no data available on humans, except those obtained from studies conducted for other scientific purposes, regarding the incidence of cancer during soy or **Isofl** supplementation. As a consequence, there are no significant results or data. However, the US National Toxicology program checked for the toxicological effects of **Isofl** in a comprehensive 2-year study [281]. Sprague–Dawley rats were exposed for five generations to 0, 50, 100, 500, and 1,250 ppm of **Gen** in their diet. Different conditions of exposure were tested. None revealed a carcinogenic activity of **Gen** in males whatever the dose. On the contrary, the different exposures showed some evidence of the carcinogenic activity of **Gen** in female Sprague–Dawley rats, especially for the 500 ppm dose, based on increased incidences of mammary gland adenoma or adenocarcinoma (combined) and pituitary gland neoplasms. In addition, exposure to **Gen** was shown to accelerate the onset of aberrant estrous cycles. The effects of **Gen** on estrous cycling and the incidence of common hormonally related spontaneous neoplasms in female Sprague–Dawley rats are considered by the authors to be consistent with an estrogenic mechanism of toxicity.

10.3 Hypothyroidism

Links have already been made between alterations in thyroid function and soy consumption. In the 1960s when the first soy-based infant formulas were commercialized, hypothyroidism goiters were observed and led to iodine supplementation in these formulas. However, when hypothyroidism is detected it still is difficult to manage in babies under soy-based infant formulas [282, 283]. In parallel, the case of a woman who was treated with levothyrox for hypothyroidism showed that the intake of soy-based food supplements interacts with drug absorption [284]. As a result, the doses required to balance her thyroid function had to be increased when soy-based food supplement was taken simultaneously with levothyrox treatment. The mechanism of action is not fully characterized but **Gen** was found to bind to thyroid hormone receptors [285] and to compete with T3 at concentrations of 1 μM, which are dietarily relevant. Many other studies, however, showed no adverse effects from soy or isoflavones on thyroid function of healthy subjects [286–291]. In addition, in Japan, where soy is consumed at the highest rate on earth, no adverse effects were consistently reported. However, the traditional Japanese diet is based on a large variety of sea foods that are rich in iodine. They may compensate for the effect of soy and/or isoflavones on an alteration of the thyroid function. Taking all these data together, it seems that the adverse effects of soy or of soy **Isofl** may occur only in persons with previous hypothyroidism. In that case, a balance in the thyroid function may be more difficult to achieve medically.

10.4 Myeloid Leukemia

Gen also exhibit anti-DNA topoisomerase II effects at high doses [292]. DNA topoisomerase II is a nuclear enzyme involved in DNA repair. Its inhibition can lead to increased consequences for DNA damage. This effect was advocated in the late 1990s to favor infant myeloid leukemia via a fetal exposure from mothers consuming soy [293]. Although this effect has not been proved, one study pointed out the highest prevalence of this pathology in Chinese populations with a relation to genetic influence [294].

11 Conclusion

Sometimes the effects of **Isofl** with estrogenic activities are wrongly associated with those of soy. This has to be considered with caution inasmuch as soy contains many other compounds with potential health impacts, like saponins, sapogenins, protease inhibitors, and phytosterols. Because of their low efficiency of transfer through the enterocyte barrier, their actions are most probably relevant for gut health. The effects of **Isofl** with estrogenic activities are relevant to consider for soy extracts enriched in **Isofl** and for pure compounds. Again, because the different **Isofl** do not exhibit the same properties at the cellular level, the effects of a pure compound cannot necessarily be extrapolated to an **Isofl** extract or to the intake of a vegetable source of **Isofl**. That said, soy is a plant that is most probably good for our health because it is rich in protein, low in cholesterol, and contains phytosterols known to prevent cholesterol absorption. However, soy also contains high amounts of **Isofl** with estrogenic activities and high bioavailability in mammal plasmas. Potential estrogenic effects can be expected from high soy consumption. The health effects of **Isofl** with estrogenic activities, although highly probable on bone preservation or on prostate cancer progression, are not yet fully demonstrated because many biases can interfere in the epidemiological or clinical studies undertaken so far, including the effects of other nutrients. Only meta-analysis, taking into account these biases, may help with deciphering what truly happens. Isoflavones with estrogenic activities have demonstrated their estrogenic effects only at high doses in accordance with their low affinities with ERs. The only effects on estrogen-dependent pathologies that could be consensual so far are those related to mammalian reproduction or to interferences with thyroid function in occasional cases of hypothyroidism. As mentioned in the abstract, only chronic intakes of high doses of **Isofl** can lead to significant bioavailable plasma doses because, contrary to anthropoid endocrine disruptors, these molecules only have a short plasma residence time in mammalian organisms. Because of a strong advertising campaign, essentially led by soybean producers and manufacturers, the health effects of soy, which are most probably real, have been largely disseminated. In some cases, they have led to the over-consumption of soy and of **Isofl** with estrogenic activities. If soy consumed on a moderate and regular basis can most certainly lead to disease prevention, high consumption of soy or of **Isofl** with estrogenic activities are most probably not safe. It must be kept in mind that in Western countries, estrogens are only prescribed by doctors via either contraceptive pills or via hormonal replacement therapies. This means that the use of estrogens must be controlled and reserved to particular situations involving specific doses. Indeed, medical drugs are developed for our health and are not designed to be poisonous. However, they are designed to be used in specific doses and for specific symptoms. These considerations must be kept in mind when considering **Isofl** with estrogenic activities. If there is almost a general consensus on their effects on bone health (although strong in vivo scientific evidence is still missing in humans) it is not a reason to administer them in high doses to newborns. Likewise, although **Isofl** with estrogenic activities may be beneficial to prostate cancer, this does not mean that they can be consumed without limit by patients with breast cancer. We think that **Isofl** extracts and **Isofl**-containing-foods are more than simple functional foods. Their estrogenic effects lead them to be classed as food-drugs, because, in Western countries, estrogens are delivered as medical drugs. As such, they must be consumed cautiously, as they are in Asia. This means that they can be consumed on a moderate and regular basis but that consumers must be careful not to over-consume them through frequent daily intake. The limit in humans should be 1 mg/kg/day. According to the food item consumed and to the body weight this should be from one (in children) to a maximum of three normal portions (in adults) of soy items per day. To date, food manufacturers have refused to mention the **Isofl** content of their foodstuffs. Yet, this would be the best way to widen soy food consumption in the community. In the nutrition domain, like in others, excess is never good. In addition, the consumption of **Isofl** or **Isofl**-containing-foods should be mentioned to medical practitioners, who are the people who are best placed to advise about a significant estrogen supplementation.

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Phenolics in Human Nutrition: Importance of the Intestinal Microbiome for Isoflavone and Lignan Bioavailability

78

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Contents

1 Introduction 2434
2 Phenolics in Human Nutrition 2435
3 Bioavailability: Importance of the Intestinal Microbiota 2439
4 Microbial Diversity: Relevance for Phenolic Conversion 2441
5 The Gut Microbiota Influences Health Effects of Phenolics 2442
5.1 Core Bacterial Reactions and Conversion of Isoflavones and Lignans 2442
5.2 Health Effects of Isoflavones and the Bacterial Metabolite Equol 2446
5.3 Health Effects of Enterolignans 2449
6 Impact of Phenolics on Intestinal Microbiota 2451
7 Can We Potentiate Intestinal Microbial Metabolism? 2453
8 Conclusion 2455
References 2456

Abstract

Depending on nutritional habits, our diet may contain a substantial load of phenolics, defined as plant secondary metabolites consisting of one to several phenol groups. Their bioavailability, in other words the active fraction of ingested amounts that reaches targeted cell types or tissues where biochemical

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properties can act, is markedly influenced by metabolism and absorption in the gastrointestinal tract. Indeed, our intestine is the primary metabolically active site of absorption of exogenous factors in our body and harbors trillions of microbial cells with a vast metabolic potential, referred to as the intestinal microbiota. The aim of the present book chapter is to give insights into the role of phenolic compounds in human health. We will focus our attention on two families of polyphenols of importance in human nutrition, namely, the isoflavones and lignans, and will discuss in detail the role of intestinal microorganisms in regulating their metabolism and thereby health effects.

Keywords

Bioavailability • enterolignans • equol • health • human nutrition • intestinal microbiota • isoflavones • lignans • microbiome • phenolics • phytoestrogens

Abbreviations

BMD	Bone mineral density
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ED	Enterodiol
EFSA	European Food Safety Authority
EL	Enterolactone
ER	Estrogen receptor
FOS	Fructooligosaccharides
GI	Gastrointestinal
LDL	Low-density lipoprotein
PCR	Polymerase chain reaction
RCT	Randomized controlled trials
SDG	Secoisolariciresinol diglucoside

1 Introduction

Nutrition, in combination with other environmental factors such as climate shifts and changes in ecosystem structure, has played a key role in evolutionary processes that made us what we are: *Homo sapiens*. Our brain must be constantly fueled with energy in the form of glucose, and essential nutrients such as fatty acids are required for proper brain development. Thus, selectively advantageous eating behaviors have certainly favored essential nutrient supply, efficient energy harvest from food stuff and effective mechanisms of energy storage, contributing to nutritional stability and thereby to more rapid development of cognitive functions and the emergence of our species [1]. Beyond evolutionary issues, it is nowadays acknowledged that nutrition, along with physical activity, are important factors influencing human health. In westernized countries, the long-term deleterious health effects of diets rich in calories, simple sugars, saturated fat, and red meat with respect to the development of cardiovascular diseases, colorectal cancer, and the metabolic syndrome are as much recognized as the virtue of eating enough portions of fruits and vegetables, although underlying mechanisms of actions remain to be described [2–4]. Positive effects of fruits and vegetables are usually attributed to high content of fiber, vitamins, and phenolic compounds (hereon defined as plant secondary metabolites with a backbone structure made of one or several phenol groups). Assuming that a substantial proportion of the dietary intake of common ancestor species consisted of plant materials, it is not surprising that, over millions of years of evolution, our body has inherited an efficient metabolic machinery to dispose of the large quantity of phenolic compounds that we still ingest daily as part of our omnivorous diet. Intestinal microorganisms are intrinsic parts of this metabolic machinery. Indeed, from an evolutionary perspective again, the human body can be considered as a supra-organism made of not only own eukaryotic cells, but also the hundred trillions of microorganisms that colonize various body sites such as the skin and the genital, respiratory and, most importantly with respect to nutrition, the gastrointestinal (GI) tract [5]. The intestinal microbiota is referred to as the assemblage of microbial communities and associated genomes (the metagenome) primarily colonizing the distal GI tract. Due to its highly diverse metabolic potential, the intestinal microbiota greatly alters the fate of phenolics in the human body by changing their structure and absorption rates in the gut, thereby influencing their biological effects.

In that context, the present chapter gives insights into the relevance of phenolic compounds in human nutrition. We will primarily discuss bioavailability and biological properties of isoflavones and lignans in the context of human health and disease, our main focus being the metabolic activities of intestinal bacteria.

2 Phenolics in Human Nutrition

Concoctions of plant products for the purpose of curing disease or sustaining health in human subjects have a long history of use, especially in traditional Chinese medicine. However, molecular mechanisms underlying positive effects have yet to be defined and traditional medicine therefore faces intense criticism [6]. Nevertheless, the emergence of systems biology approaches may help shedding light on host responses toward treatment with plant products that obviously contain a wealth of phenolic compounds [7–9]. Beyond these issues on the role of herbal treatment for improvement of human health, there is a plethora of epidemiological data highlighting beneficial effects associated with intake of food items rich in phenolic compounds. A well-known example of such food items is soy (or soy products) which contain elevated concentrations of the isoflavones daidzein and genistein as well as their glycosylated and methylated precursors (Fig. 78.1).

Biological properties of isoflavones were first coined in the 1940s, after infertility problems started to occur in female sheep grazing on clover pastures containing high amounts of isoflavones, and later in the 1980s in captive cheetahs fed a soy-based diet [10, 11]. These data already suggest that dietary phenolics or corresponding metabolites, such as equol, one of the two end metabolites produced

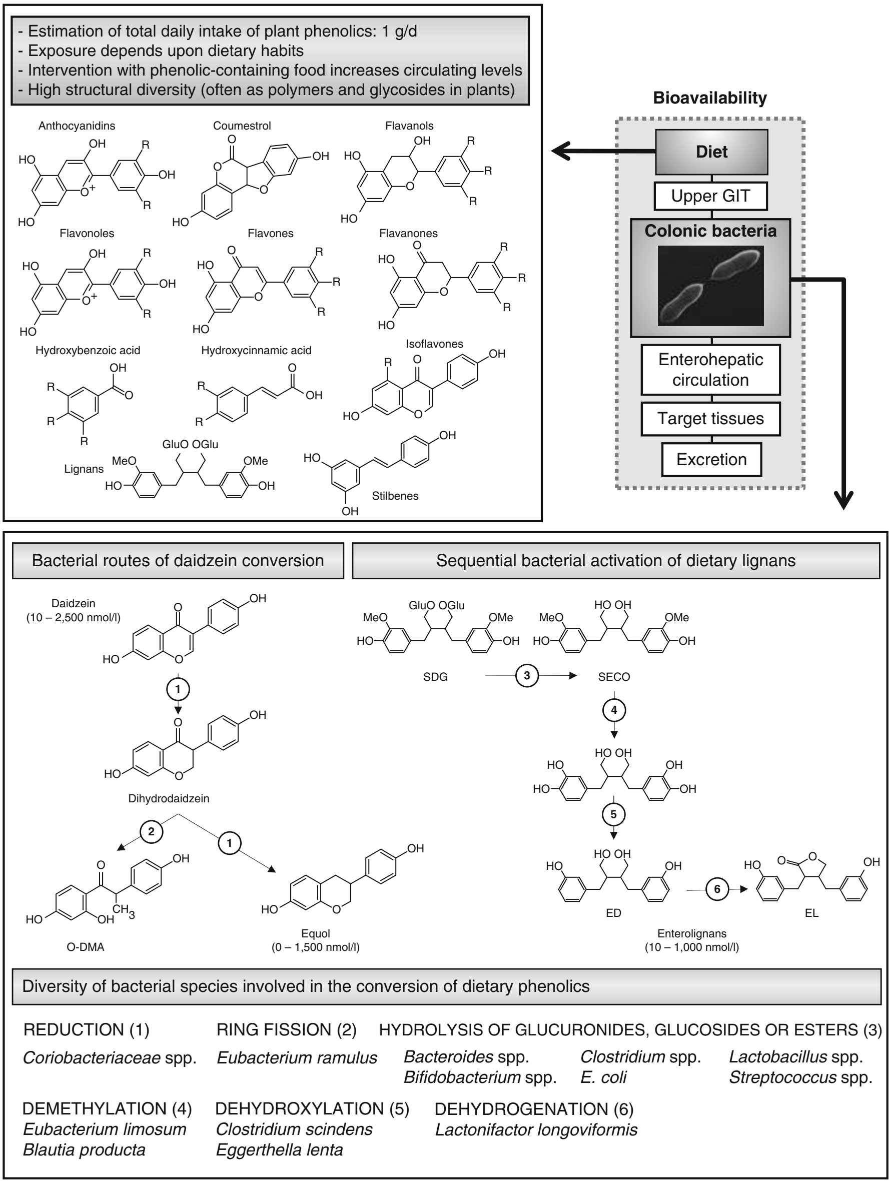
- Estimation of total daily intake of plant phenolics: 1 g/d
- Exposure depends upon dietary habits
- Intervention with phenolic-containing food increases circulating levels
- High structural diversity (often as polymers and glycosides in plants)
Anthocyanidins
Coumestrol
Flavanols
Flavonoles
Flavones
Flavanones
Hydroxybenzoic acid
Hydroxycinnamic acid
Isoflavones
Lignans
Stilbenes
Bioavailability
Diet
Upper GIT
Colonic bacteria
Enterohepatic circulation
Target tissues
Excretion
Bacterial routes of daidzein conversion
Sequential bacterial activation of dietary lignans
Daidzein (10 – 2,500 nmol/l)
Dihydrodaidzein
O-DMA
Equol (0 – 1,500 nmol/l)
SDG
SECO
ED
EL
Enterolignans (10 – 1,000 nmol/l)
Diversity of bacterial species involved in the conversion of dietary phenolics
REDUCTION (1)
Coriobacteriaceae spp.
RING FISSION (2)
Eubacterium ramulus
HYDROLYSIS OF GLUCURONIDES, GLUCOSIDES OR ESTERS (3)
Bacteroides spp.
Bifidobacterium spp.
Clostridium spp.
E. coli
Lactobacillus spp.
Streptococcus spp.
DEMETHYLATION (4)
Eubacterium limosum
Blautia producta
DEHYDROXYLATION (5)
Clostridium scindens
Eggerthella lenta
DEHYDROGENATION (6)
Lactonifactor longoviformis

Fig. 78.1 Phenolics in human nutrition: A microbiological perspective. Bioavailability of dietary compounds depends on the sum of molecular mechanisms underlying liberation of the compounds from dietary matrices, absorption, distribution into body tissues via blood circulation, metabolism (in the GI tract or target tissues), and elimination from the body. The keypad shows parameters of relevance to phenolic bioavailability. The two enlarged windows illustrate the diversity of both phenolics in food and microbial functions involved in phenolic conversion, with a focus on isoflavones and lignans. Estimates of blood concentration of daidzein, equol, and enterolignans are

by bacteria from the plant isoflavone daidzein, have the potential to interfere with highly sensitive host hormonal pathways. Since then, numerous meta-analyses and epidemiological studies including Asian populations consuming soy products on a daily basis have reported positive effects of soy intake on the development of breast cancer, bone disorders, and cardiovascular diseases [12–14].

The lignans, a family of polyphenolic compounds with a dibenzylbutane structure (Fig. 78.1), are another example of major plant phenolics relevant to human nutrition. Dietary lignans are converted to the enterolignans enterodiol (ED) and enterolactone (EL) by bacteria in the GI tract [15]. In contrast to isoflavones that occur in high concentrations almost exclusively in soy, a vast variety of food items such as flaxseeds, sesame seeds, berries (blackberry and strawberry), cereals (rye and wheat), and beverages (coffee, tea, and wine) contain detectable concentrations of lignans, which are therefore of importance in westernized diets [16]. Importantly, lignins have also been shown to be dietary precursors of enterolignans [17]. Of note, researchers originally proposed in 1980 that enterolignans were new mammalian hormones after they detected them in urinary extracts from female primates and human adults via spectrometric measurement [18, 19]. This shows that lignans share structural features with steroid hormones and are, as isoflavones, also referred to as phytoestrogens. Two years later, in 1982, the same authors reported that urinary lignans originate from food precursors [20]. Thereafter, enterolignan production has been associated with positive effects on the incidence of heart diseases as well as breast and prostate cancer [21–23].

Besides isoflavones and lignans, human food contains a wealth of phenolic compounds (Fig. 78.1). Rapid improvement in the sensitivity of analytical tools, together with the development of specific databases such as Phenol-Explorer and the USDA Flavonoid Database, have substantially contributed to the understanding of human exposure to phenolics [24]. Table 78.1 provides a non-exhaustive list of major groups of dietary phenolics and representative food sources. Depending on dietary habits, total intake of phenolics in European populations can reach up to 1 g/day or higher [26, 28, 30]. By studying dietary intake in 4,942 French adults, Scalbert et al. showed that the most dominant dietary phenolics are hydroxycinnamic acids, flavonols, and anthocyanins [30]. However, there is a direct positive association between ingested amounts of specific food products and blood concentrations of corresponding metabolites, showing that one can easily and rapidly modulate exposure to specific phenolics by modulating dietary intake. In Asian populations for instance, isoflavones intake is nearing 100 mg/day due to high intake of soy products [35]. Nevertheless, health effects of dietary phenolics do not depend solely on ingested amounts, but rather on the concentration of active compounds that reaches target tissues. In that respect, what makes lignans and isoflavones outstanding is that plant precursors are usually less biologically

Fig. 78.1 (continued) given in brackets (large interindividual differences are observed due to various dietary habits and ability to metabolize polyphenols). Abbreviations: *ED* enterodiol, *EL* enterolactone, *GIT* gastrointestinal tract, *LARI* lariciresinol, *O-DMA* *O*-desmethylangolensin, *R* residues (−H, −OH, or $-CH_3$), *SECO* secoisolariciresinol, *SDG* secoisolariciresinol diglucoside

Table 78.1 Dietary intake and example food sources of phenolic compounds[a]

Compound	Intake[b]		Food	Content[c]
Phenolic acids				
Hydroxybenzoic acids (e.g., gallic acid)	10–30		Blackberry	8–27
			Tea leaves	Up to 450
Hydroxycinnamic acids (e.g., caffeic and ferrulic acid)	25–800	(68 %)	Coffee	35–175
			Blueberry	Up to 200
Flavonoids				
Anthocyanidins (e.g., cyanidin, peonidin)	20–80		Blackberry	100–400
			Black currant	130–400
Flavanols (e.g., epicatechin)	10–20		Chocolate	45–60
			Apricot	10–25
Flavanones (e.g., naringenin, hesperitin)	25–50		Orange juice	22–69
			Grapefruit juice	10–65
Flavones (e.g., apigenin, luteolin)	5–30		Parsley	24–185
			Celery	2–14
Flavonols (e.g., quercetin, kaempferol)	10–20		Yellow onions	35–120
			Curly kale	30–60
Isoflavones (e.g., daidzein, genistein)	<1–60		Boiled soybean	20–90
		(0–82 %)	Soy milk	3–20
		(3–30 %)	Whole grain bread	0.3–0.8
Lignans[d]	0.5–2	(10 %)	Flaxseed	300
		(3 %)	Sesame seed	39
		(16 %)	Broccoli	1.3
		(0.7 %)	Strawberry	0.3
		(7.4 %)	Wheat bread (whole grain)	0.1
		(3 %)	Beer	0.03
		(11 %)	Coffee	0.02–0.03

[a] Compiled using data from Phenol-Explorer (www.phenol-explorer.eu), the USDA Flavonoid Database (www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.html) and the following Refs. [16, 25–32]

[b] Given as ranges (in mg/day aglycone equivalent) for each of the listed families of phenolics (e.g., isoflavones). Ranges reflect fluctuations of mean dietary intake depending on population origin (Asia, Denmark, Finland, France, Germany, Holland, Italy, Spain, UK, or USA) and dietary habits. Values in brackets indicate mean contribution of some of the given food items to dietary intake in European populations

[c] In mg/100 g or 100 ml. Of note, concentration of phenolic compounds in food can be altered by food processing [33]. During production of tempeh for instance, a traditional soy product from Indonesia, isoflavone glycosides are hydrolyzed to aglycones [34]

[d] Refers to lariciresinol, matairesinol, pinoresinol, and secoisolariciresinol

active so that enterolignans and equol can be seen as paradigm metabolites highlighting the relevance of bacterial activation of dietary components in the intestine. Hence, no matter which phenolics are of interest and what health effects they have, bioavailability and bacterial metabolism are matters of primary importance.

3 Bioavailability: Importance of the Intestinal Microbiota

Bioavailability refers to the proportion of absorbed doses of a molecule, and eventually metabolites thereof, which reaches sites of physiological activity. Our GI tract is of course at the front line of metabolic events regulating bioavailability due to its primary role in nutrient absorption and because oral intake is the major voluntary route of exchange with our environment (compared with passive exposure to exogenous factors via the skin and the respiratory tract). The liver and kidneys play a central role in bioavailability as well. Efficient conjugation of phenolics for the purpose of increasing water solubility, and eventually excretion, occurs in all three organs (gut, liver, kidneys) mainly via the activity of *O*-methyl transferases, UDP-glucuronosyltransferases, and sulfotransferases [26]. The bioavailability of dietary phenolics is thus tuned by the sum of molecular mechanisms underlying liberation from dietary matrices, absorption, metabolism (by both host and microbial cells), distribution, and excretion (Fig. 78.1).

In upper parts of the GI tract, there is a paucity of data on the fate and role of polyphenols. Their effects have been discussed in the context of oral cancer prevention [36]. Their fate in the stomach has not yet been systematically studied. Quercetin has been shown to be absorbed in the rat stomach, but only as aglycone [37]. Fast plasma appearance of anthocynins may also be explained by rapid absorption in the stomach [38]. Concerning lignans, we found that secoisolariciresinol diglucoside (SDG) (the main enterolignan precursor in flaxseed) is resistant to acid hydrolysis in vitro [39], which confirmed previous findings [40]. In the jejunum, there is good evidence that isoflavones and flavonols can be deglycosylated via lactase-phlorizin hydrolase activity and rapidly absorbed in the brush border membrane of enterocytes [41, 42]. Rat in vitro perfusion models have also been useful in demonstrating absorption of phenolic acids as well as quercetin and phloretin in the small intestine [43, 44]. However, the flavanol epigallocatechin-3-gallate can inhibit hydrolase activity in vitro, yet this inhibition is regulated by salivary proline-rich proteins [45]. This raises the question of the effect of chewing on polyphenol bioavailability via indirect or direct mechanisms such as salivary hydrolysis [46]. Plant phenolic substrates can be detected in blood and urine samples shortly after intake, which speaks in favor of rapid absorption, albeit, in low amounts. For instance, only about 2 % of the ingested dose of plant lignans was found in plasma of four individuals 1 h after intake of 50 g sesame seeds [47]. In some individuals however, plant lignans may occur in higher concentrations than enterolignans in blood samples [48]. This is also true for the isoflavone daidzein, which occurs at higher concentrations than its metabolite equol in blood samples [49], most likely because bacterial production of equol in the gut is a limiting reaction (see details in Sect. 5.1). Altogether, characterization of the metabolic network regulating phenolic bioavailability in the upper GI tract requires further investigation. In particular, very little is known about phenolic transport from gut lumen into blood stream. A recent pharmacokinetic study in human adults based on the use of equol isotopes revealed peak plasma concentrations 2–3 h after oral intake of the isotopes (350–500 ng/ml after administration of a single bolus of 20 mg) [50]. One may interpret that transport mechanisms in the gut are not region-specific, since equol is supposed to be primarily produced in distal parts of the intestine. Absorption rates of phenolics and kinetics of appearance in blood vary greatly depending on chemical structure. For instance, glucosides of quercetin (but not rhamnoglucosides) are more efficiently absorbed than aglycones [51]. However, underlying molecular mechanisms of absorption are not known. So far, only monocarboxylic acid transporters and the plasma membrane carrier bilitranslocase have been discussed for transport of phenolic acids and anthocynins, respectively [52–55]. Independently of what exactly happens in the upper GI tract, it is acknowledged that a substantial proportion of ingested polyphenols can reach the colon, where lower transit time favors bacterial conversion.

The first piece of evidence demonstrating that distal parts of the GI tract are crucial for the metabolism of phenolics is the so-called second plasma peak observed after 6–8 h postprandial when measuring phenolic metabolites in plasma samples overtime after ingestion of plant substrates [56]. Indeed, a substantial proportion of absorbed phenolics is efficiently conjugated in enterocytes and later in the liver prior to secretion back into the small intestine via the bile (enterohepatic circulation) [57]. Enterohepatic circulation thereby contributes to bacterial "re-feeding" since the bulk of glucuronidated and sulfated phenolic metabolites released in the bile can be hydrolyzed by various bacterial species [58]. Bacterial hydrolysis thus allows reabsorption of otherwise lost conjugated phenolics to be excreted in feces and thereby to delayed appearance of phenolic metabolites in the blood (second plasma peak). Another piece of evidence showing that distal gut microorganisms are crucial for phenolic metabolism is the drop in plasma and urinary concentrations of phenolics associated with alteration of intestinal microbial communities following oral antibiotic treatment [56, 59, 60]. Finally, the use of germfree mice, that is, mice that are bred in isolators under sterile conditions and are thus deprived of any living microorganisms, has provided major insights into the important role of intestinal microbial communities in shaping host physiology, including the ability to metabolize food substrates such as phenolics. To some extent, one can consider germfree mice as knockout mice, in which a multifunctional set of genes (the microbiome) has been disrupted, leading to loss of functions. Indeed, besides alteration of immune cell development [61], the absence of microorganisms in germfree animals has major impacts on energy balance [62], nutrient supply via production of short-chain fatty acids, and degradation of mucin [63] as well as phytoestrogen conversion. Enterolignans and equol, for instance, are not detectable in the intestine and body fluids of germfree rats fed phenolic-rich diets, yet gnotobiotic rats colonized with fecal suspensions from phenolic-converting human donors or with isolated active bacterial consortia regain the ability to produce active metabolites [64–67]. Taking into account that the intestinal microbial ecosystem in mammals harbors a total of up to 10^{14} cells belonging to more than 1,000 different species per host, each bearing approximately a few thousands of genes, it is not surprising that the absence of such diverse microbial communities is linked to disturbances in metabolic functions. In the following two sections, we will highlight specific features of intestinal microbiota that are of importance for phenolic conversion.

4 Microbial Diversity: Relevance for Phenolic Conversion

As seen above in Sect. 2, a broad array of food items contain various phenolic compounds in a wide range of concentrations (from a few micrograms up to a few hundred milligrams per 100 g), which highlights the rationale for "eating a little of everything each day" to cover supplies yet avoid adverse effects due to long-term excessive intake of a limited number of food items. With respect to chemical structure, the variety of phenolics is also quite large and the amounts ingested are driven by dietary habits, which differ markedly between individuals (Fig. 78.1). Hence, the mixture of phenolics in the intestinal lumen is determined by multiple levels of complexity and is thus highly diverse and variable.

Diversity is also a major attribute of intestinal microbial communities in mammals. Indeed, although our intestinal microbiota consists dominantly of only four of the 30 known bacterial phyla (highest taxonomic level within the superkingdom *Bacteria*; www.ncbi.nlm.nih.gov/Taxonomy; www.bacterio.cict.fr), namely, the *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*, the diversity at low taxonomic levels (≤ genus) is very high. Most recent molecular studies refer to a few thousands different bacterial species being present in the human gut, and accordingly even more individual strains [68]. Although bacteria make up the majority of intestinal microbial populations, our intestine harbors also *Archaea* (two dominant methane-producing species, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, have been described to date), eukaryotic microorganisms, such as fungi and protozoa, as well as viruses. However, the role of these microorganisms in the metabolism of phenolics is unknown (bacteriophages may, for instance, influence phenolic conversion by regulating the density of specific active bacterial populations).

As often in biology, the efficacy of one complex system (the intestinal microbiota) is greater than the sum of its biologically active parts (bacterial strains). Indeed, one key asset of the high diversity of our intestinal microbiota is that several different bacterial species can carry out one given function, such as cleaving glucose moieties of phenolics. This is referred to as "functional redundancy" (one bacterium can take over the function of another if for some reason the latter disappears). This ensures flexibility and is crucial to achieve stability and ecosystem equilibrium over time upon influence of various environmental stimuli [69]. Hence, the high diversity of our intestinal microbiota helps us cope with the high diversity of exogenous chemical compounds that we ingest. Nevertheless, in spite of this high diversity, there are a few bacterial species (50–100), and by extension a few associated bacterial functions, that make up the so-called core microbiome [70], that is, the assemblage of species/functions that are dominant (occur in high numbers) and show a high prevalence (they are found in most individuals).

Despite the notion of a core microbiome and the stability of the gut microbial ecosystem over time without major changes in dietary habits, each individual person harbors their own characteristic intestinal microbiota (in the sense of a personalized fingerprint). Indeed, there are large interindividual differences in both intestinal bacterial composition (proportion of taxa) and diversity (qualitative pattern of taxa); there is marked quantitative variation and low similarity indexes between gut samples from different individuals, even for dominant bacterial groups [68, 71, 72]. Individualized intestinal microbial patterns in adulthood are highly dependent upon a dynamic sequence of events affecting the ecosystem throughout life, especially in early life. At birth, the human body is colonized by microorganisms from the environment. Primary colonizers (aerobic or facultative anaerobic bacteria) help establish a reduced environment that is suitable for subsequent colonization by strictly anaerobic species, which largely dominate the ecosystem in adulthood. In infants below 1 to 2 years of age, the human intestinal microbiota is unstable and composition fluctuates greatly [73]. The infant gut microbiome seems not to be well equipped for efficient conversion of polyphenols. For example, equol is not detected in urine and blood samples from infants below the age of 12 months who are fed cow or breast milk [74, 75]. Delivery mode at birth (vaginal delivery vs. caesarian section) and breast versus formula milk feeding have been shown to influence microbial colonization patterns [76–79]. In early life, and very often thereafter, the intestinal ecosystem is challenged by infectious agents and antibiotic therapies. In most cases, the ecosystem shows resilience, thanks to its diversity, that is, it rapidly returns to its original state after a challenge. However, in some cases, and more likely and frequently during infancy where microbial populations are not yet fully stabilized, the ecosystem or at least specific community niches can be permanently affected [80]. Altogether, this variety of colonization and challenging events can partly explain why certain individuals harbor specific bacteria and others do not, and why the latter group therefore lacks the functions expressed by absent or subdominant bacterial species. For instance, it is well known that only about 30–50 % of human subjects produce equol from the isoflavone daidzein, meaning that one half to two thirds of human populations do not harbor equol-producing bacteria in their intestine, at least not in high enough densities [81, 82]. Likewise it has been shown that bacteria capable of catalyzing the production of enterolactone from plant lignans belong to subdominant populations, that is, they occur at densities below 10^8 cell/g content (compared to a total cell density of approximately 10^{12} cell/g) [83, 84].

In summary, our intestinal microbiome encodes numerous core functions of importance for the conversion of dietary phenolics, yet interindividual differences in the makeup of bacterial species that colonize our gut underlie interindividual differences in phenolic metabolism and thereby in possible health effects. In Sect. 5, we will give more details on active bacterial members and central metabolic reactions involved in phenolic conversion prior to focusing on health effects of isoflavones and lignans.

5 The Gut Microbiota Influences Health Effects of Phenolics

5.1 Core Bacterial Reactions and Conversion of Isoflavones and Lignans

Exceptions prove the rules: the functional diversity of intestinal microbiota implies that all plant phenolics that we ingest can be converted by microorganisms.

However, there are exceptions, such as the isoflavone irilone, which seems to be resistant to bacterial conversion [85]. Gut bacteria catalyze an array of dominant core reactions that play key roles in the metabolism of a large panel of phenolic compounds, including isoflavones and lignans: (I) hydrolysis of esterified and conjugated bounds, (II) deglycosylation (removal of sugar moieties), (III) demethylation (substitution of a methyl by a hydroxyl group), (IV) dehydroxylation (reduction of hydroxyl groups), (V) dehydrogenation, (VI) reduction. Figure 78.1 gives a brief overview of the so far identified bacterial species that catalyze these reactions [86, 87]. It is noteworthy that several species occur in proximal parts of the bowel (*Enterobacteriaceae*, lactobacilli, lactococci, and streptococci in the stomach and small intestine), showing that bacterial metabolism of phenolics may be crucial not only in the colon, but already before, for example, for hydrolysis of conjugated metabolites secreted in the bile. Most dietary phenolics occur as biologically inert polymers or glycosides, meaning that reaction type I and II are crucial for phenolic activation and influence downstream reactions such as demethylation. As a matter of fact, the production of one given active metabolite often results from sequential reactions involving several bacterial species. For instance, production of enterolignans from SDG requires four reactions, among which demethylation and dehydroxylation are catalyzed only if the substrate has been previously deglycosylated and demethylated, respectively [15, 88]. Reaction type III (demethylation) is also crucial with respect to biological activities since most plant phenolics are methylated and are less active than hydroxylated metabolites. This is obvious, for instance, in the case of caffeic acid phenyl ester (an active phenolic constituent of honeybee propolis), for which we found that methylation of catechols markedly reduces anti-inflammatory activities [89].

The physiological advantage for bacteria to convert phenolic compounds is easily understandable in the case of deglycosylation (active species can utilize released glucose moieties as carbon and energy sources) or demethylation (acetogenic bacteria, for instance, are capable of producing energy by incorporating methyl groups into the Wood-Ljungdahl pathway of acetogenesis). In contrast, it is more difficult to identify driving forces that led to the establishment of complex phenolic-converting metabolic chains involving various distantly related bacterial species. One simplistic way to assess such a complex system is to try gaining access to individual bacterial components of the metabolic chain by means of anaerobic cultivation for subsequent in vitro characterization. Indeed, the isolation of pure bacterial cultures, in combination with the use of biochemical techniques (high performance liquid chromatography and mass spectrometry), for the purpose of metabolite identification allows description of key bacterial players in phenolic metabolism, including subdominant bacterial populations [15, 90]. Microbiologists have been culturing microorganisms for a long time, rapidly leading to major breakthroughs in biomedical research such as the identification of *Mycobacterium tuberculosis* by Robert Koch in 1876 or the discovery of the antibiotic penicillin by Alexander Fleming in 1928. In contrast, it is only from the 1950s onwards that the development and use of anaerobic tools by pioneers such as René Dubos, Sydney Finegold, Lillian Holdeman, Robert Hungate, Edward Moore, and Russel Schaedler

Table 78.2 Cultivable bacteria capable of converting the isoflavone daidzein[a]

Bacterial strain	End metabolite	Origin	References
Adlercreutzia equolifaciens FJC-B9^T	Equol	Human feces	Maruo et al. [96]
Asaccharobacter celatus do03^T	Equol	Rat cecum	Minamida et al. [97]
			Minamida et al. [98]
Eggerthella sp. YY7918	Equol	Human feces	Yokoyama et al. [99]
Enterorhabdus mucosicola Mt1-B8^T [a]	Equol	Mouse ileal mucosa	Matthies et al. [100]
			Clavel et al. [101]
Eubacterium ramulus wK1	*O*-desmethylangolensin	Human feces	Schoefer et al. [102]
Lactococcus sp. 20-92	Dihydrodaidzein	Human feces	Shimada et al. [103]
Slackia equolifaciens DZE^T [a]	Equol	Human feces	Jin et al. [104]
			Jin et al. [105]
Slackia isoflavoniconvertens HE8^T [a]	Equol	Human feces	Matthies et al. [106]
Slackia sp. NATTS	Equol	Human feces	Tsuji et al. [107]
Strain D1 and D2	Equol	Pig feces	Yu et al. [108]
Strain HGH6	Dihydrodaidzein	Human feces	Hur et al. [109]
Strain HGH136	*O*-desmethylangolensin	Human feces	Hur et al. [110]
Strain Julong 732	Equol [b]	Human feces	Wang et al. [95]
Strain Niu-O16	Dihydrodaidzein	Bovine rumen	Wang et al. [111]
			Zhao et al. [112]
Strain SY8519	*O*-desmethylangolensin	Human feces	Yokoyama et al. [113]
Strain TM-40	Dihydrodaidzein	Human feces	Tamura et al. [114]

[a] These strains are also able to produce 5-hydroxy equol from the isolflavone genistein
[b] From dihydrodaidzein only (this strain does not convert daidzein)

gave rise to extensive culture-based work dealing with commensal bacterial communities from human intestinal samples [91–93].

In 1985, Borriello et al. were the first to study the conversion of plant lignans by fecal slurries in detail [94], yet active bacterial strains were first isolated in 2000 [88]. In the case of phenolic acids, which as mentioned above are dominant phenolics in human diet, knowledge of bacterial conversion and involved species is scant. Hydroxycinnamates (e.g., *p*-coumaric, ferulic, and sinapic acid) as well as benzoic acids (e.g., gallic, syringic, and vanillic acids) are rapidly degraded by intestinal bacteria and a few members of the *Firmicutes* are known to demethylate a variety of phenolic acids [39, 86]. Actually, this is the case of isoflavones that rapidly drew most of the attention of microbiologists working in the field of polyphenols. Reasons for this are the low proportion of equol producers among humans (30–50 %) and the fact that equol is the most potent known isoflavone metabolite. Researchers have thus embarked on a microbial “Gold Rush” attempting to isolate and identify those rare equol-producing bacteria that colonize the human gut. The first evidence for microbial equol production was published in 1995 [81], however the first equol-producing bacterium, strain Julong 732, was isolated in 2005 (and so far this isolate is still not taxonomically classified) [95]. To date, a total of 16 daidzein-converting strains have been identified (Table 78.2).

From this listing, it is obvious that proper taxonomic description is needed, as some of the isolates could belong to the same species. It is also striking that all equol-producing bacteria with a validly published name are members of the family *Coriobacteriaceae*. This hints at functional specialization in the gut, maybe contributing to the better survival of this bacterial group in the competitive intestinal milieu. Interestingly, some *Coriobacteriaceae*, such as *Eggerthella* spp., are dominant intestinal bacteria and can convert steroid hormones and biliary acids [58]. This shows again that core functions such as dehydroxylation are relevant to various substrates and raises the question on the influence of host hormonal status on polyphenol metabolism [115].

A major advantage of culture-based approaches is that isolated strains can be used in vivo to assess physiological roles of phenolic-converting bacteria (in e.g., germfree mice) or in vitro for isolation and characterization of active enzymes. So far, very few corresponding data have been published. Crude enzyme extracts from *Asaccharobacter celatus* converts daidzein to dihydrodaidzein under anaerobic conditions and a dihydrodaidzein-producing reductase from lactococci has already been cloned (UniProtKB E1CIA4 and E7FL40/1) [103, 116]. However, culturing is per definition restricted to the study of microorganisms able to be isolated and to grow in the laboratory (most recent estimation refers to a proportion of 60 % cultivable bacteria in the mouse intestine) [62]. Again, it is important to remember that one given reaction can be catalyzed by several phylogenetically distantly related bacteria, which highlights the notion of functional bacterial groups and the importance of considering intestinal microbiota as a dynamic pool of functions rather than an assemblage of taxonomic entities. A more comprehensive way to assess the bacterial conversion of phenolics at the level of the entire ecosystem (the pool of microbial functions) than culturing is to use metagenomic techniques, i.e., molecular tools dedicated to the study of the metagenome (the sum of genomes originating from the thousands of bacterial species colonizing the intestine) [68, 117]. For instance, culture- or PCR-based screening of gut metagenomic clone libraries can give direct access to bacterial genomic information involved in conversion of phenolics, metagenomic libraries being defined as collections of >10,000 *Escherichia coli* clones where each clone expresses functions encoded on one large DNA fragment (commonly 40,000 bp) from the gut metagenome. As an example, metagenomic clones can be cultured on agar plates containing a glucosylated phenolic substrate as sole carbon and energy source, an approach that has been already used with other kinds of substrates such as β-glucans [118]. In such an assay, only clones capable of utilizing the substrate would grow and could be further analyzed by sequencing for determination of active gene sequences. Alternatively, colorimetric reactions may also be used for detection of for instance phenolic-demethylating clones [119].

One additional key issue in the field of bacterial enzymatic conversion of polyphenols is enantiospecificity. Many polyphenols, such as isoflavones and lignans, are optically active molecules that display several asymmetric carbon atoms. So far, only *S*-equol has been detected as a bacterial product of daidzein conversion [95, 120]. In the case of lignans, both (+)- and (-)-enantiomers occur in plants and bacterial conversion in the gut seems to be enantiospecific and preserve absolute configuration [121]. There is strong evidence that biological activity depends upon chirality of equol [122, 123], stressing the need for stereochemical analysis of other phenolic metabolites produced by intestinal bacteria. This serves as further proof of the necessity to isolate phenolic-converting bacterial enzymes for potential biotechnological production of active metabolites [103]. Finally, the search for new bacterial metabolites (and determination of corresponding biological properties) is also of primary interest. Considering the diversity of both dietary phenolics and intestinal bacterial species, it is likely that the panel of intermediate and end metabolites produced by intestinal bacteria is much larger than hitherto observed. For instance, we have found that the lignan-dehydrogenating bacterium *Lactonifactor longoviformis* does not only produce enterolactone, but also the novel metabolite 2,3-bis(3,4-dihydroxybenzyl)butyrolactone, the occurrence of which in vivo along with biological activities is still to be determined [121].

In summary, the array of enzymatic reactions catalyzed by the gut microbiome alters the structure of ingested phenolics. In view of the notion of structure/activity relationship, we conclude that intestinal bacteria greatly influence the biological activities of dietary phenolics. In the case of the isoflavone daidzein the route of bacterial conversion (i.e., the production of equol or *O*-desmethylangolensin depending on gut bacterial composition), is key to downstream health effects (Fig. 78.1). In the following two sections, we will give detailed information on biological activities and potential health effects of isoflavones and lignans.

5.2 Health Effects of Isoflavones and the Bacterial Metabolite Equol

In recent reports, the European Food Safety Authority (EFSA) refuted claims about the role of isoflavones in body function effects (article 13.1) such as maintenance of normal blood LDL-cholesterol concentrations in the general population [124, 125]. This has two main implications (also true beyond the sole case of isoflavones): first, even when scientific rationale is sound and there is a substantial number of well-conducted studies showing an overall significant trend toward positive effects of a defined dietary compound, a major problem in nutrition research is that intake of definite food stuff may need to stretch over long life periods before one can observe significant effects, when compared, for example, with pharmacological products usually associated with instant target effects (even though long-term effects of pharmacological therapies are often also not determined, yet beneficial immediate effects indeed prevail). Thus, the preventive aspect of nutritional strategies implies to carry out studies at scales (both in terms of time and cohorts) virtually impossible to manage in order to substantiate beneficial effects. This very often hampers closing the gap between scientific evidence and clear recommendations for consumers. The second implication is that, whereas it is very difficult to corroborate findings for the "general population," it makes sense to look at health effects of isoflavones in sensitive target groups, like infants. For these reasons, this is not our intention to provide here an exhaustive review of possible health effects of isoflavones. Instead, we will focus our attention on osteoporosis affecting menopausal women and on the effect of early exposure to isoflavones, thereby highlighting the biological properties of the bacterial metabolite equol.

Infants make up a study population of particular interest for several reasons: (1) they have not yet necessarily acquired a fully functional phenolic-metabolizing machinery (at least from a microbiological perspective), (2) the use of soy-based infant formula has become a rather common feeding alternative in westernized countries, and (3) a growing human body may be particularly sensitive to the biological properties of isoflavones. There are several published papers showing that early exposure to isoflavones has the potential to influence hormone levels and organ differentiation in the offspring of various animal species [126–129]. For instance, male marmoset twin monkeys fed soy formula milk for 30–40 days from the age of 5 days were characterized by lower mean testosterone levels in blood samples [129]. However, long-term effects must be further investigated. Furthermore, caution must be taken when interpreting results obtained using doses higher than the estimated intake of 2–10 mg isoflavones per day per kilogram body weight in infants fed soy-based formula [130, 131]. Exposure of human infants to dietary isoflavones has drawn attention of researchers since the mid-1990s. Depending on studies, isoflavone concentrations in soy-based infant formula range from 30 to 280 mg/kg [131–133]. Setchell et al. found that mean plasma concentrations of both genistein and daidzein in seven infants fed soy-based formula were 979 ng/ml (approximately 4 μmol/l) [131]. This concentration was markedly higher than in infants fed either cow-milk formula (5.3 ng/ml) or human breast milk (4.2 ng/ml), and is also higher than in adults on their usual diet. Interestingly, infants can also be exposed to isoflavones via breast milk during lactation. In seven breastfeeding mothers, ingestion of 55 mg/day isoflavone glucosides for 2–4 days increased isoflavone concentrations significantly in breast milk (from ca. 5 to 70 nmol/l) and in infant urine (from ca. 30 to 110 nmol/mg creatinine) [134]. Hence, it is clear that infants can be exposed to relatively high isoflavone concentrations and experimental work shows some significant effects of early exposure to isoflavones in animals. However, there is an obvious lack of physiological evidence in humans, as underlined in recent review papers and human infant trials [135–139].

The rationale for considering possible health effects of dietary isoflavones in infants is substantiated by in vitro and in vivo work on their biological properties. Especially, the estrogenic-like properties of isoflavones have been studied as early as in the 1950s based on the mouse uterine weight method [140], 30 years before equol was first detected in human urine [141]. Among daidzin metabolites, equol has the strongest binding affinities to estrogen receptors (ER), especially for ER-β [122, 142, 143]. Nevertheless, 17β-estradiol is 10–100 times more potent than equol. Interestingly, the *R*- and *S*-enantiomer of equol exhibit different binding affinities for ER-α (0.5 vs. 2 % of 17β-estradiol binding, respectively) or ER-β (1 vs. 20 %) [122]. Beyond binding affinities, equol can also modulate ER transcriptional activity [142, 144, 145]. Very recently, induction of estrogenic responses by equol has been demonstrated in vivo using the 3xERE-luciferase mouse model, which allows detection of estrogen activity by light production [146]. On the other hand, isoflavones have the potential to reduce estradiol bioavailability by increasing levels of circulating sex hormone–binding globulin [147, 148]. Obviously, the pro- or anti-estrogenic activities of equol depend on circulating concentrations of estradiol, which markedly vary during puberty, menstrual cycle, and menopause. Isoflavones concentrations in blood may reach up to a maximum of 10 μmol/l after ingestion of phenolic-rich food, which exceeds blood concentration of estradiol by a factor of $> 10{,}000$ [149]. Interestingly, tissue accumulation of polyphenols (including isoflavones and lignans) has been reported, which likely contributes to modulation of biological properties in target tissues [150–153].

In spite of the aforementioned properties of equol, its direct contribution to health effects is unclear. From the complex metabolite mixtures found in blood and target tissues after soy intervention, it is impossible to relate effects to only one specific molecule. Still, discoveries from the last decade may form the basis of future research to assess the exact role of equol in mediating health effects. Indeed, the fact that single equol-producing bacterial strains are now available allows the design of gnotobiological experiments using animal model of diseases. In such experiments, germfree animals colonized with an equol-producing or non-producing bacterium (a closely related inactive species or a mutant strain in which active enzymes have been knocked-out) could be compared with respect to the development of, for instance, tumors in various tissues or bone disorders in response to ingestion of daidzein-rich diets. In addition, large-scale production of pure enantiomers of equol for use in experimental or even clinical studies will surely help in deciphering direct health effects and underlying molecular mechanisms (US Patent no. 7528267 and 6716424).

To follow up on phytoestrogenic activities of isoflavones in vivo, a number of studies have looked at the effect of soy consumption on fertility parameters in adults. Again, there is evidence in animal species [154, 155], but very few data in human [156]. Alteration of semen quality by soy food or isoflavones is questionable [157, 158] and a recent meta-analysis of 15 placebo-controlled studies concluded that soy or isoflavone consumption is not associated with changes in testosterone levels in healthy men [159]. In contrast, peri- and postmenopausal women represent a target population of particular relevance. We will here focus only on the effect of isoflavones on osteoporosis, which has been intensively studied in postmenopausal women and represent a major public health problem [160]. Readers interested in the effects of isoflavones on cardiovascular risks and breast cancer may refer to already published comprehensive papers [13, 14, 161–163]. Osteoporosis is characterized by low bone mass, deterioration of bone tissue, and disruption of bone microarchitecture resulting in compromised bone strength and increased fracture risk [160]. The diagnosis of osteoporosis is primarily established by measurement of bone mineral density (BMD) [164]. Of course, genetic factors determine peak bone mass. However, studies involving twins indicate that environmental factors, including dietary habits, play a substantial role in the pathogenesis of osteoporosis [165].

Again, the EFSA refuted claims related to the use of soy isoflavones for maintenance of BMD [125]. This highlights the difficulty to reach consistency in experimental setups required for drawing conclusion on definite intake of isoflavones associated with long-term health benefits. Nevertheless, there is a growing body of valid scientific data showing overall that beneficial effects of isoflavones on bone disorders in elderly women are promising [161]. In two recent meta-analyses [166, 167], Ma et al. selected randomized controlled trials (RCT) investigating the effects of soy isoflavones on BMD and markers of bone turnover in peri- and postmenopausal women. Based on a total of 19 RCT with an intervention period of 1–24 months and isoflavone intake of 4–150 mg/day, the authors concluded that isoflavone intervention significantly attenuates bone loss of the spine in menopausal women, inhibits bone resorption, and stimulates bone formation. These results were confirmed by even more recent meta-analyses [168, 169]. However, it must be acknowledged that most studies are not appropriate for assessment of soy isoflavone consumption for more than 1 year [12]. Thus, one major remaining challenge is to characterize long-term clinically relevant effects of isoflavones prior to making statements on their use in hormone replacement therapies [170]. In a very recent double-blind RCT, Tai et al. found that treatment with 300 mg/day isoflavones for 2 years did not prevent decline of BMD in lumbar spine and proximal femur in postmenopausal Taiwanese [171].

5.3 Health Effects of Enterolignans

As for isoflavones, there is a vast number of studies investigating various biological properties and potential health effects of lignans [115]. There is good experimental evidence that lignans are beneficial with respect to the development of cardiovascular diseases and breast cancer. It is obvious however that RCT in human subjects are lacking. Interventions based on the use of flaxseeds as main lignan source have revealed promising effects with respect to reduction of prostate cancer proliferation [172, 173], tumor growth in breast cancer patients [174–176], and low-density lipoprotein (LDL) cholesterol levels [22, 177]. In addition, recent data from the EPIC study (European Prospective Investigation into Cancer and Nutrition) suggested that lignan intake decreases colon cancer risk in women [178]. However, because flaxseeds contain substantial amounts of fibers and oil, it is not possible to distinguish between direct effects of lignans and confounding or synergistic effects of fibers and oil. We will thus focus hereafter only on studies assessing health effects that can be attributed to pure lignans converted in vivo to the enterolignans ED and EL by gut bacteria. Unfortunately, viewed from that perspective, the number of human intervention trials shrinks further away. We found only two different double-blind RCT, in which authors analyzed the effect of flaxseed extracts enriched in SDG (ca. 30 % dry mass). Hallund et al. found that an intervention with 500 mg/day SDG equivalent for 6 weeks in 22 healthy postmenopausal women marginally reduced C-reactive protein concentrations and had no effect on endothelial function and plasma lipid concentrations [179–181].

In another trial involving 78 subjects with benign prostatic hyperplasia, ingestion of a flaxseed lignan extract (>300 mg/day SDG equivalent) over a 4-month period significantly improved International Prostate Symptom and Quality of Life Scores [182]. It is thus again in laboratory animals that most of the beneficial effects of pure lignans have been reported. In rodents, Lilian Thompson and colleagues found that SDG reduces or delays mammary tumor growth [183–185], affects mammary gland structure [186, 187], reduces metastasis in the lung [188] as well as colon carcinogenesis (number of aberrant crypt foci after azoxymethane treatment) [189]. In contrast, matairesinol and secoisolariciresinol did not protect against intestinal tumor formation in Min mice [190]. More recently, lariciresinol was found to attenuate mammary tumor growth in xenograft- and carcinogen-induced rat models [191]. With respect to cardiovascular risks, SDG was found to reduce the incidence of atherosclerosis in rabbits and to induce neovascularization-mediated cardioprotection in rats [192–194].

Biological properties underlying the aforementioned protective effects of lignans are not well characterized, especially in vivo. As stated in Sect. 2, plant lignans are usually less active than enterolignans, which are thus seen as paradigm metabolites for the relevance of bacterial conversion. In vitro studies showed that EL has slightly higher binding affinity for the human pregnane X receptor, which mediates induction of enzymes involved in steroid metabolism and xenobiotic detoxification, than its precursor secoisolariciresinol [195]. Moreover, EL binds to estrogen receptors, with a preference for ER-α [142, 196], and can activate estrogen responsive elements [197]. Both ED and EL modulate ER-α mRNA and protein contents and compete dose dependently with estradiol and the unsaturated fatty acid arachidonic acid for binding site on rat and human α-fetoprotein, an estradiol-binding protein [198, 199]. However, binding affinities of enterolignans appear to be 10–10,000-fold lower than those of other phytoestrogens or sex hormones. Both enterolignans and plant lignans also bind to sex hormone–binding globulin, with possible consequences on circulating levels of the sex hormones testosterone and estradiol [200]. The estrogen-dependent properties of ED and EL include as well inhibition of aromatase, 5α-reductase, and 17β-hydroxysteroid dehydrogenase, three enzymes involved in the metabolism of growth-promoting steroid hormones [201–204]. Besides, EL was found to induce the expression of the estrogen-responsive protein pS2 in human breast cancer MCF-7 cells [205]. This and other in vitro studies showed that ED and EL alter cell proliferation of various breast, colon, and prostate cell lines, as well as endothelial cells derived from bovine brain capillaries [206–210]. In vitro, both ED and EL have also higher antioxidant activities than plant precursors [211, 212]. In vivo, short-term feeding of SDG to rats only led to minor changes in the antioxidant status of hepatic tissue [213].

To conclude on the last two sections on health effects, one can say that polyphenols are generally regarded as safe and there are only a few reports on possible toxic effects (yet not in the case of isoflavones and lignans in humans) [214–217]. However, polyphenols have the potential to interact with sensitive hormonal systems. Moreover, as implied above when discussing bioavailability, efficient conjugation and excretion mechanisms as well as relatively low phenolic concentrations in blood

(<200 nmol/l without intervention [51, 218, 219]), when compared with other molecules of dietary origin (sugars, amino acids, acetate, etc ...), are hallmarks of efficient host metabolism dedicated to the elimination of exogenous molecules. Thus, one should not presume that biological properties of phenolics are solely synonyms of beneficial effects, for example, equol may trigger hyperplasia of rat uterine tissue [220] and lignans have been shown to affect pregnancy outcome, reproductive development, and estrous cycling in rats and women [221–223].

Isoflavones are promising with respect to improvement of osteoporosis in postmenopausal women, but long-term effects and dose/activity relationship must be further investigated. Regarding lignans, data obtained using animal models of cancer and cardiovascular disorders are promising too. However, there is a paucity of data in human subjects. In both cases (isoflavones and lignans), direct in vivo effects of bacterial metabolites is a future research area of particular interest.

6 Impact of Phenolics on Intestinal Microbiota

One fundament of intestinal ecosystems is the trialog between dietary components, intestinal microorganisms, and the host. Over the last century, medical microbiology had been a dominant field of research and the focus was mainly placed on the study of bacteria-host interactions. However, over the last 20 years, the impact of nutrition on human health and the intestinal microbiome has gained a lot more attention in westernized countries [5]. This is mainly due to: (1) research-founded breakthroughs (molecular mechanisms underlying benefits or deleterious effects of specific dietary molecules are being described); (2) shifts in public health challenges and mentalities (while many bacterial infections are no major threat anymore, chronic disorders such as allergies, obesity, and inflammatory diseases in an ever-aging population represent an increasing social and economical burden; meanwhile, many people are concerned about self-improvement of well-being via nutrition); and (3) market-driven issues (global food companies are lured by profits associated with massive consumption of functional foods and nutraceuticals).

There is nowadays strong evidence that diet greatly influences the composition of intestinal microbiota. The most studied dietary components having striking effects on microbial diversity are fat and fibers [224, 225]. In contrast, the effect of dietary microcomponents like polyphenols on intestinal microbiota is much less known, in spite of various possible mechanisms of actions. First of all, the fact that the conversion of phenolics is under the control of bacterial metabolic chains means that any substrate affecting one chain link has the potential to alter the entire system. Secondly, there is good indication that phenolic extracts and pure phenolics have antimicrobial properties and may thereby alter the growth of intestinal bacteria like clostridia, bacilli, and members of the *Enterobacteriaceae* [226–229]. In addition, since gene expression of enzymes catalyzing, for instance, dehydroxylation can be induced by matching substrates [230], it is possible that polyphenols directly influence core gut microbial functions. At the same time, the growth of phenolic-metabolizing bacteria may be favored if conversion provides a net energy input for the bacteria. This could lead in parallel to increased competitive advantage and thus indirect growth inhibition of other bacterial groups. Finally, certain polyphenols or metabolites thereof may interfere with quorum sensing, a molecular system that coordinates gene expression of, for instance, virulence factors according to bacterial cell density [226–229]. In spite of these mechanisms, which must still be substantiated by further investigations, there is to the best of our knowledge only 11 papers reporting effects of phenolic compounds on intestinal microbiota. In vitro experiments showed that incubation of fecal slurries with tea extracts prevented growth of clostridia [231]. Possemiers et al. showed that the hop prenylflavonoid isoxanthohumol increased the abundance of members of the *Clostridium* cluster XIV as well as bifidobacteria in a continuous culture system [232]. In rats, Hanske et al. found that xanthohumol does not affect the diversity of dominant fecal microbial communities, as analyzed by denaturing-gradient gel electrophoresis [233]. Smith et al. reported that a diet rich in proanthocyanidins increased the occurrence of *Enterobacteriaceae* and *Bacteroides* in rat feces [234]. The remaining papers relate to human intervention trials. Tea polyphenols increased viable counts of bifidobacteria and decreased counts of *Clostridium perfringens* in eight Japanese healthy adults [235], but had no major impact on fecal microbiota in six hypercholesterolemic volunteers [236]. We found in 2005 that a dietary treatment with 100 mg isoflavones per day for 1 month altered the bacterial diversity and composition in fecal samples from 39 postmenopausal women [71]. Very recently, Tzounis et al. found that a diet rich in cocoa-derived flavanols (494 mg/day) consumed for 4 weeks by 22 healthy human volunteers increased the proportion of lactic acid bacteria by a factor of two, as measured by in situ hybridization [237]. The same authors had previously reported that 150 mg/l of the flavanol monomers epicatechin and catechin stimulated growth of bifidobacteria, *E. coli* and members of the *Firmicutes* in vitro [238]. With respect to bacterial activities, Wiseman et al. found that soy consumption for 10 weeks increased beta-glucosidase activity in feces from 76 healthy young adults [239]. Finally, Hoey et al. reported two- to tenfold lower counts of bacteria in feces from ten infants (aged 4–12 months) fed a soya- versus milk-based formula [74]. However, the fecal concentration of total short chain fatty acids (ca. 45 μmol/g) as well as beta-glucosidase and glucuronidase activities (both ca. 10–25 μmol/h per g) were unchanged.

Bottom line is that the amount of data is too limited to draw firm conclusions on the impact of phenolics on intestinal microbiota. The task ahead is challenging due to the diversity of phenolics in food as well as interindividual intestinal microbial profiles. The use of next generation molecular approaches will be crucial for the identification of core responses to dietary phenolics at the level of the entire gut microbial ecosystem. High-throughput 16 S ribosomal RNA sequencing allows for instance in-depth characterization of changes in bacterial diversity. However, it will be essential to translate the meaning of such structural changes for host health development, since microbial functions are the driving force of bacteria-host interactions and changes in diversity are not necessarily linked to changes in ecosystem functions. Ecological approaches, such as metatranscriptomic or metabolomic, for gene or metabolite expression profiling could be used for identification of core microbial functional markers under the influence of dietary phenolics [240].

7 Can We Potentiate Intestinal Microbial Metabolism?

The pace of research involving phenolic compounds has rapidly increased over the last two decades (Fig. 78.2). One obvious underlying reason is the wish to prevent or cure diseases by means of natural products. Since intestinal bacteria are essential for phenolic bioavailability and associated health effects, nutritional strategies favoring production of active metabolites via the microbiome look very attractive. As seen above, the use of antibiotics is the best proof-of-concept that influencing metabolite production by targeting intestinal microbial communities is promising [56, 60, 241–244]. However, there is to date no valid data substantiating the theory of diet-driven optimization of microbial phenolic conversion.

The link between intake of specific dietary components and phenolic metabolite production is unclear. Although increased excretion of equol has been associated with increased consumption of fat, meat, and fruits, for instance [82, 152, 245–247], and enterolignans excretion seems to correlate well with dietary intake of fibers [248–250], more work is needed to reach consensus in results. Nonetheless, it is clear that ingestion of isoflavone and lignan food substrates enhance production of equol and enterolignans [33, 251–253]. An intriguing question is however to know whether the activity or growth of phenolic-activating bacteria can be specifically induced, that is, in the case of equol, for example, whether non-equol producers on

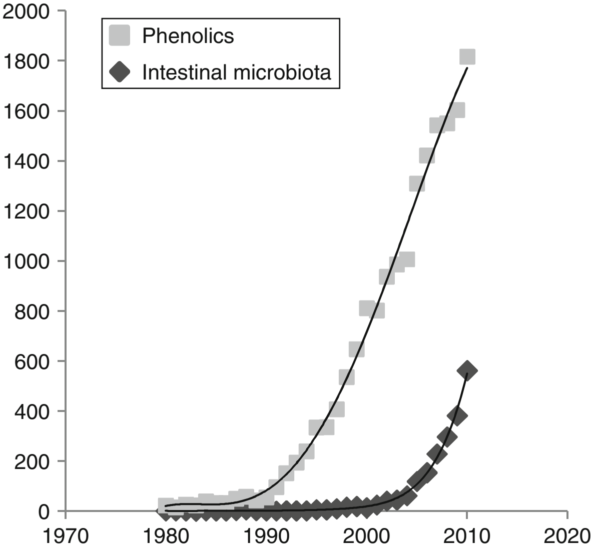

Fig. 78.2 Publication output in the field of phenolic research and intestinal microbiota. The PubMed database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/pubmed) was searched for the number of articles per year (from 1980 until 2010) responding to the following queries: "intestinal microbiota" (*black diamond*) and "phenolics OR phytoestrogens OR polyphenols" (*gray squares*)

their usual diet can become producers, thanks to ingestion of appropriate plant substrates. In 12 Caucasian postmenopausal women, Védrine et al. found that isoflavone intervention (100 mg/day) increased plasma equol concentrations from 0.31 to 0.99 μmol/l in equol producers, but that the seven volunteers classified as non-equol producers did not acquire the ability to produce equol after 1 month exposure [251]. In contrast, another study in China revealed a higher proportion of equol producers among 200 healthy adults challenged with a soy-isoflavone supplement for 3 days (60 % equol producers after supplementation vs. 27 % at baseline) [254]. Here too, more work is needed to draw firm conclusions on equol phenotype changes and influence of demographic origin on phenolic bioavailability [245, 255, 256].

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host [257]. Their use has drawn quite some attention for improvement of phenolic activation in the gut based on the rationale that glucosidases from probiotic lactic acid bacteria (mainly lactobacilli and bifidobacteria) may enhance phenolic bioavailability by increasing concentrations of aglycones. However, the dominance of endogenous phenolic-deglycosylating *Bacteroides*, *Bifidobacterium*, and *Clostridum* spp. in the intestine suggests that deglycosylation is not a limiting step in the in vivo production of active metabolites. Moreover, while there are many reports on the fermentation of soy products by probiotic bacteria, all ten intervention trials based on soy and probiotic treatment in human subjects failed to demonstrate any positive effects of probiotic bacteria [258–267]. Concerning lignans, the only one study available also failed to show any beneficial probiotic effects [268]. More interestingly, researchers in the group of Willy Verstraete at Ghent University have successfully used phenolic-converting bacteria originating from the human intestine, such as *Eubacterium limosum* catalyzing demethylation, to enhance the activation of isoflavones and isoxanthohumol in continuous culture systems and in rats [269, 270].

Functional food products also include prebiotics like fructooligosaccharides (FOS) and inulin, which are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon [271]. The prebiotic concept was first coined in 1995 by Glenn Gibson and the bifidogenic effect of FOS and inulin has since then been confirmed by many studies. In contrast, there are only a few reports on the influence of prebiotics on isoflavone bioavailability. Steer and colleagues showed in vitro that 10 g/l FOS in combination with soyabean isoflavones significantly prevented genistein breakdown in continuous culture system vessels [272]. Similar results were obtained by Piaza et al. using inulin in a randomized double-blind crossover study enrolling 12 healthy postmenopausal women [273]. The authors found increased plasma concentration of daidzein and genistein after inulin treatment (approximately 7 g/day) for 21 days. Possible synergistic effects of combined isoflavone and prebiotic intervention are of particular interest with respect to health parameters such as blood lipid profiles or bone density and calcium homeostasis [274–276].

8 Conclusion

The issue of phenolics in human nutrition bears resemblance to industrialized production factories, where input of raw materials is important, yet processing strategies determine the quality of final products. That is, the amount of phenolics that we eat makes of course a difference, but metabolism within the body determines their fate and health effects. Future prospects related to phenolic bioavailability (especially bacterial metabolism) that have been evoked throughout the chapter are summarized in Table 78.3.

Intestinal microbial functions are essential for conversion of a vast majority of dietary phenolics, for example, isoflavone and lignan activation. The main future

Table 78.3 Take home messages and future challenges[a]

Facts	Perspectives
Human diet contains a wealth of highly diverse phenolic compounds	Implementation of phenolic databases is crucial for good estimation of intake depending on dietary habits
Plant phenolics can be absorbed in the upper GI tract	Transport mechanisms and kinetic of appearance in blood must be characterized in detail, especially in relation to chemical structure
The intestinal microbiota is highly diverse and has a vast metabolic potential	Functional metagenomic screening is a promising approach for characterization of bacterial genes involved in phenolic conversion
Bacterial culture allows isolation of phenolic-converting bacteria	Identified strains can now be used for colonization of experimental animal models and for large-scale production of pure phenolics
Isoflavones may improve osteoporosis in postmenopausal women, and lignans can protect against tumor growth and atherosclerosis in animal models	More clinical data are needed; long-term effects must be defined
	Studies in gnotobionts or using pure substances are required for assessing direct health effects of equol and enterolignans in vivo
Health effects depend on the type of bacterial metabolites produced	Effort must be put into studying enantiospecificity of bacterial conversion
There are large interindividual differences in the ability to metabolize plant phenolics in the gut	High-throughput sequencing and metabolite analysis will allow dynamic characterization of the gut microbial ecosystem in human intervention trials
Intestinal microbiota is sensitive to dietary changes	Impact of phenolics on gut microbial diversity and activities must be further studied
	Use of pre- and probiotics to increase bacterial production of active metabolites is not yet scientifically founded
Infants can be exposed to substantial amounts of phenolics and colonization events determine the metabolic potential of intestinal microbiota	Epidemiological data on the impact of chronic early exposure to phenolics are warranted and effort should be put in characterizing the establishment of intestinal microbiota in large infant cohorts

challenge for microbiologists working in the field of phenolics and human nutrition is to characterize metabolic networks at the level of the entire intestinal ecosystem, in relation to host functions. New generation molecular techniques will certainly help taking on this challenge, although computer analysis of the colossal amounts of data generated by high-throughput methods is a high hurdle for most microbiologists. It would be valuable, for instance, if large-scale human intervention trials on phenolics were designed so as to include microbiological analysis of intestinal samples via, for instance, sequencing or spectrometry analysis to characterize bacterial diversity and identify core functions of relevance to phenolics. This could lead to the discovery of phenolic-specific enterotypes, as in the sense of specific clusters of microbial species associated with functional profiles of relevance [277], thereby allowing detailed characterization of interindividual differences. The long-term objective is the ability to generate personalized meta-metabolic profiling for development of individualized nutritional strategies [278].

In view of individualized nutritional strategies, we must also say that, even though the focus of the present chapter is the metabolic potential of intestinal microorganisms, host genotype strongly determine health effects of phenolics too. Thus, a challenging task is also to assess the role of host genotype in controlling phenolic health effects, either directly via differential expression of specific key genes (coding for ER or intestinal transporters for instance) or indirectly via alteration of microbiota [279].

Finally, it is important to remember that early life periods are critical for shaping the intestinal microbiome. More effort should be put into characterizing intestinal microbiota development in infants and the implication of early dietary exposure to phenolics for health homeostasis later in life. To date, it is also not possible to provide clear recommendations with respect to dietary intake of isoflavones or lignans for treatment or prevention of diseases. Nevertheless, good evidence has been accumulating regarding improvement of bone disorders in postmenopausal women by isoflavones and cardiovascular risks as well as breast cancer by lignans. More clinical and epidemiological data are mandatory and effort should be put into performing experiments in gnotobiotic animal models of disease to draw firm conclusions on the direct role of bacterial metabolites, such as equol and enterolignans, in host health.

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Bioavailability of Anthocyanins

Ana Faria, Iva Fernandes, Nuno Mateus, and Conceição Calhau

Contents

1 Introduction 2466
2 Anthocyanin Chemistry and Stability 2467
2.1 Occurrence and Intake 2468
3 Bioavailability: Basic Concepts 2470
3.1 Factors Affecting Bioavailability 2472
4 Bioavailability of Anthocyanins 2473
4.1 Oral Cavity, Stomach, Small Intestine, and Colon 2473
4.2 Tissue Distribution of Anthocyanins 2478
4.3 Mechanisms of Anthocyanin Absorption 2478
4.4 Microbiota Impact on Anthocyanin Availability and Bioactivity 2479
4.5 Considerations Regarding Anthocyanin Bioavailability 2480
5 Conclusion 2481
References 2482

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Abstract

Anthocyanins are naturally occurring compounds widespread in plant-derived foodstuffs and therefore abundant in our diet. There are evidences regarding the positive association of their intake with healthy biological effects displayed in vivo. This chapter aims to review some concepts regarding anthocyanins' bioavailability. It summarizes the latest advances on the ingestion, absorption, bioavailability, and biotransformation of these compounds through different approaches. Attention is also given to the role of microbiota in anthocyanin metabolism and bioavailability.

Keywords

Absorption • anthocyanins • bioavailability • metabolism • microbiota

1 Introduction

Anthocyanins belong to the large group of flavonoids that occur in nature arising from plant secondary metabolism. They constitute the largest group of water-soluble pigments widespread in the plant kingdom, being responsible for the colors displayed by many flowers, fruits, and leaves of angiosperms. These pigments are usually associated with fruits, but they also occur in vegetables, roots, legumes, and cereals [1, 2].

The scientific community has become more aware of the importance of anthocyanins in a regular diet as different experiments have been performed aiming to support and explain some biological and health-promoting features attributed to these molecules.

The antioxidant capacity of anthocyanins has been demonstrated by radical scavenging of reactive oxygen species, reducing capacity, inhibition, or delaying of lipoprotein oxidation and platelet aggregation. Anthocyanins as individual compounds or present in a more complex extract have been found to play a role in the prevention of cardiovascular diseases and to be involved in several different events like the prevention of DNA damage, estrogenic activity, enzymatic inhibition, anti-inflammation response, lipid peroxidation inhibition, etc. [3–7].

Over the last years, more attention has been paid to the putative antitumoral properties of anthocyanins and anthocyanin extracts [8]. The induction of apoptose in tumor cells as well as the suppression of cell proliferation and angiogenesis have been pointed out as possible mechanisms where anthocyanins may play their anticarcinogenic role [9–11]. Although there are several works reported in the literature dealing with in vitro antioxidant and biological properties of anthocyanins, there is a lack of more in vivo evidences. This is mostly due to bioavailability issues as it will be further discussed in this chapter.

2 Anthocyanin Chemistry and Stability

Polyphenolic compounds share the presence of an aromatic ring with two or more hydroxyl groups. Currently, more than 8,000 compounds belonging to this family are known. Depending on the presence of the flavan nucleus, polyphenols are usually divided in flavonoids and non-flavonoids. The basic flavonoid structure, the flavan nucleus, consists of a C6-C3-C6 structure constituted by two aromatic rings linked by an heterocyclic ring, labeled A, B, and C (Fig. 79.1).

The various classes of flavonoids differ in the level of oxidation and substitution pattern of ring C, while individual compounds within a class differ in the arrangements of hydroxyl, methoxyl, and glycosidic side groups. Dietary flavonoids exist primarily as 3-*O*-glycosides and polymers [12].

Anthocyanins, a particular class of flavonoids, naturally occur as glycosides of flavylium (2-phenylbenzopyrylium) salts but differ from them by structural variations in the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, the nature and number of sugar moieties attached to the phenolic molecule, and the position of the attachment, as well as the nature and number of aliphatic or aromatic acids attached to the sugars [1]. The sugar moieties vary but are usually a mono- or disaccharide unit, frequently glucose, galactose, rhamnose, arabinose, or xylose [13].

Most commonly known anthocyanins are based on six anthocyanidins: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin (Fig. 79.2), but there are 539 anthocyanins reported to be isolated from plants [14].

The more widespread anthocyanins in fruits are glycosylated in the 3-OH position (3-O-monoglycosides) and, in less extension, in both position 3-OH and 5-OH (3,5-*O*-diglycosides).

Physicochemical properties of anthocyanins, particularly their color, structural characteristics, and stability, are unique. Anthocyanins are highly reactive molecules and thus sensitive to degradation reactions. Several factors such as oxygen, temperature, light, enzymes, and pH affect anthocyanin chemistry and consequently their stability and color. The degradation of anthocyanins may occur during their extraction, food processing, and storage. A critical factor that is strictly related to the color displayed by anthocyanins is the fact that they coexist in equilibrium

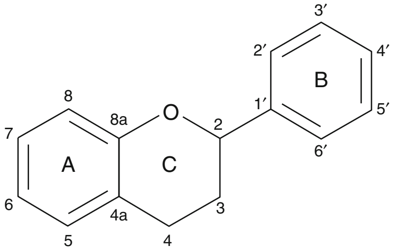

Fig. 79.1 Basic structure of flavan nucleus

Anthocyanin	R1	R2
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3
Cyanidin	OH	H
Peonidin	OCH_3	H
Pelargonidin	H	H

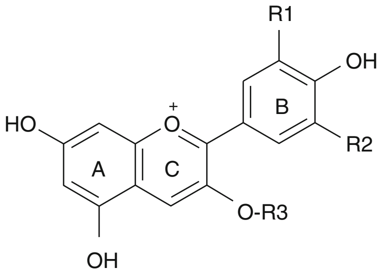

Fig. 79.2 Structures of the major anthocyanin 3-*O*-glucoside present in fruits

between five species: flavylium cation, carbinol base, chalcone, quinonoidal base, and anionic quinonoidal base (Fig. 79.3). In aqueous solution, anthocyanins occur between those different structures depending on pH [15–17].

As a result of these features, in solution, these natural compounds present little color expression above pH 3.5. On the other hand, in nature, the same pigments may display more color as a result of copigmentation phenomena [18, 19]. This ability of anthocyanins to establish noncovalent interactions with colorless copigments is extremely relevant as it has been considered to be the first step in the formation of new anthocyanin-derived pigments [20].

Concerning temperature, anthocyanins become paler after heating as the equilibrium is displaced toward the colorless carbinol and chalcone forms. The stability of anthocyanins (or other derived pigments) regarding pH and temperature variations and their degradation pathways are reported in the literature [21].

The complex biochemistry of these compounds is a particular important point during passage through gastrointestinal (GI) tract where the exposition of anthocyanins to different pH and temperature conditions may alter anthocyanin chemical forms and, consequently, their potential bioactivity.

2.1 Occurrence and Intake

Polyphenols are widespread, virtually present in all foods and beverages of plant origin, such as fruits, vegetables, tea, cocoa, and wine. It is extremely difficult to estimate the daily average intake of polyphenols for several reasons such as the extensive diversity of chemical structures that makes the estimation of polyphenol content in foods complex, the analytical methods used for these estimations, variation of content with geographical region, cultivar, and season, as well as dietary habits. Progresses are currently being made through the construction and application of a database on polyphenol content in foods [22].

Scalbert and Williamson have proposed for the total dietary intake about 1 g/day, which is much higher than that of all other known dietary antioxidants,

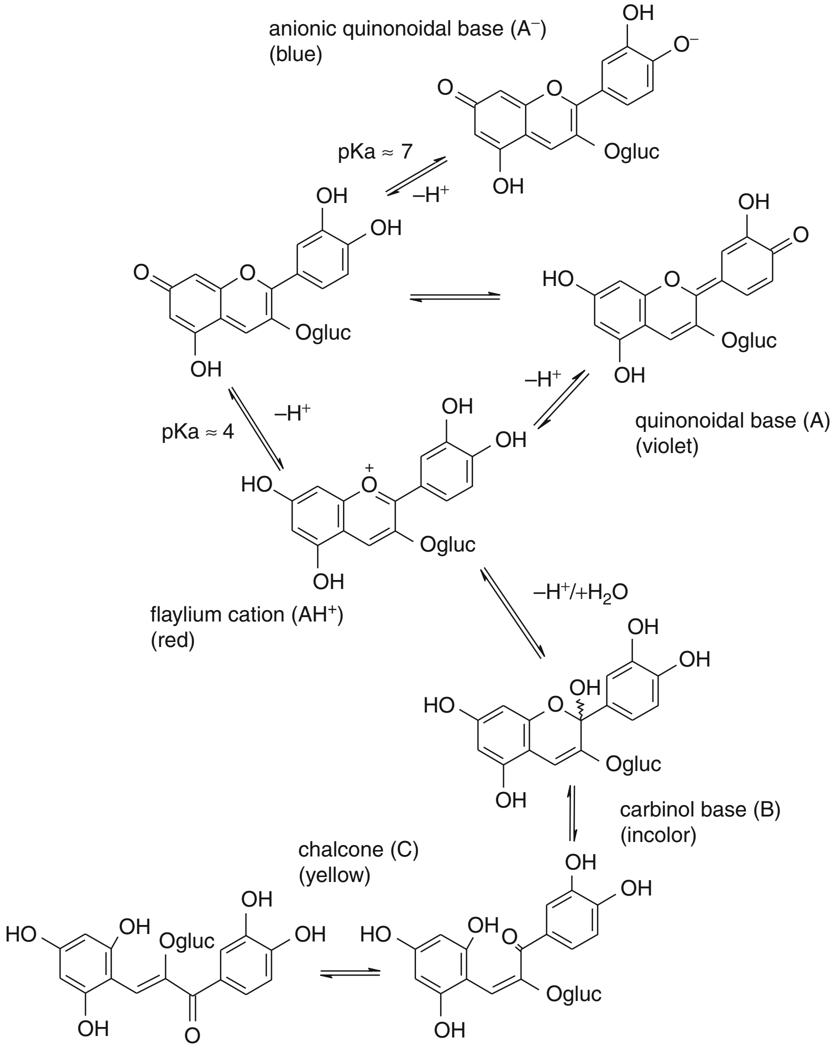
anionic quinonoidal base (A⁻)
(blue)
pKa ≈ 7
−H+
quinonoidal base (A)
(violet)
−H+
pKa ≈ 4
−H+
flaylium cation (AH+)
(red)
−H+/+H2O
carbinol base (B)
(incolor)
chalcone (C)
(yellow)
OH
O⁻
O
HO
Ogluc

Fig. 79.3 Structural transformations of anthocyanins (cyanidin-3-*O*-glucoside) in aqueous solutions depending on pH (Modified after Brouillard R [17])

about 10 times higher than that of vitamin C and 100 times higher than those of vitamin E and carotenoids [23]. Recently, Pérez-Jiménez and colleagues, using a French cohort and a phenolic food content database, have confirmed that the daily intake of polyphenols is about 1 g/day. Nevertheless, the authors point out a possible underestimation due to insufficient data or lack of accurate data on the content in foods of some complex polyphenols [24].

Anthocyanins are found in the human diet as part of red wine, certain varieties of cereals, and certain leafy and root vegetables (aubergines, cabbage, beans, onions,

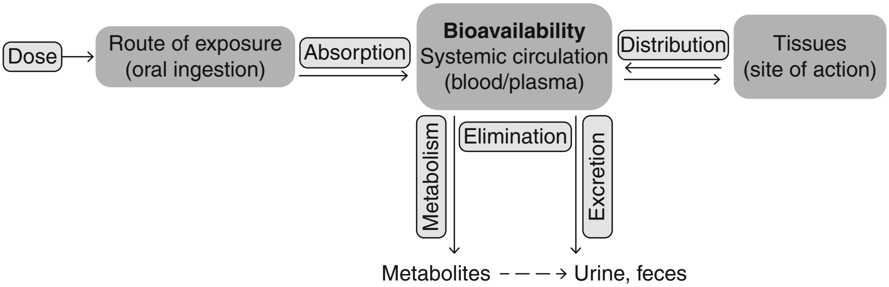

Fig. 79.4 Simplified diagram illustrating common biokinetic concepts (Adapted from [32])

radishes), but they are most abundant in red fruit such as cherries, plums, strawberries, raspberries, blackberries, grapes, red currants, and black currants. Cyanidin is the most common anthocyanidin in foods. Food contents are generally proportional to color intensity and reach values up to 2–4 g/kg fresh wt in black currants, black elderberry, or black chokeberry [25–27]. These values increase as the fruit ripens. Anthocyanins are found mainly in the skin, except for certain types of red fruit, in which they also occur in the flesh (cherries and strawberries). Red wine contains up to 1 g anthocyanins/L, and these anthocyanins can be transformed into various complex structures as the wine ages [26–31].

3 Bioavailability: Basic Concepts

The basic kinetic concepts in the body system initially came from the study of drug actions, or pharmacokinetics. Nowadays, these principles are usually applied to study or predict the movement of a substance in the organism, such as a toxin, an environmental pollutant, or even a phytochemical, constituting what is called biokinetics.

There are some general but essential concepts that are important to remind and are illustrated on Fig. 79.4 [32].

Dose is the amount of chemical delivered to the body. The means and location by which the chemical is delivered to the body is the *route of administration*. The most common routes of administration include intravenous (IV), oral ingestion (po), subcutaneous (sc or sq), and intramuscular (IM).

Absorption is the movement of chemical molecules from the site of delivery to the systemic circulation. For some routes of administration, a portion of the chemical dose may never reach the systemic circulation. The fraction of the administered dose that reaches the systemic circulation is called the bioavailable fraction or *bioavailability*.

Once absorbed into the systemic circulation, blood flow delivers the chemical to the body tissues. In some cases, specific body tissues act as storage depots, with chemical initially collecting in the tissues while plasma concentrations are high, then later being released back into the bloodstream after plasma concentrations lower with time. This back and forth movement of a chemical between circulating plasma and tissues (including the target tissue) is called *distribution*.

Enzymatic conversion of the drug molecule into a different chemical form is called metabolism, or *biotransformation*, with the converted molecules labeled metabolites. *Excretion* is the process by which chemicals or metabolites are discharged into body wastes, typically in the urine or feces. *Elimination* is a collective term for all processes that decrease the amount of chemical in the body, which includes both biotransformation and excretion.

Biokinetic, or *disposition*, is the term for everything that happens to a chemical in the body. Thus, disposition of a chemical, or xenobiotic, is defined as the composite actions of its absorption, distribution, biotransformation, and elimination.

Throughout all these processes, there are tissue barriers made of one or more cell types that collectively form a distinct boundary between two different physiological environments. Chemicals movement through tissue barriers implies passage through biological membranes. Biological membranes are composed by a phospholipid bilayer, the polar head groups of phospholipids being oriented toward the outer and inner surfaces of the membrane. Numerous proteins are embedded at irregular intervals, but only some are transmembrane proteins, traversing the entire lipid bilayer. At physiologic temperatures, membranes have a fluid character which is largely determined by the structure and relative abundance of unsaturated fatty acids. Unsaturated fatty acids and cholesterol are positively and negatively related with membrane fluidity, respectively.

There are several mechanisms by which a chemical can cross biological membranes. It can pass by passive processes; it can involve a carrier (carrier-mediated process) or a specialized transport process. Passive processes are only possible if chemicals are small, lipid soluble, and nonionized and if there is a concentration gradient between the two sides of the membrane (principle of Fick's law). Chemicals pass from regions of higher concentration to regions of lower concentration, without energy expenditure, according to a concentration gradient. Carrier-mediated transport involves one or more transporter(s), and it can be either influx or efflux of substances. The transport can be uniport (transport of a single molecule), symport (transport of two or more different molecules or ions in the same direction), or antiport (transport of two or more different molecules or ions in opposite directions), and because it uses a transporter, it is saturable and specific. Specialized transport includes endocytosis, with and without the interaction with membrane receptors. This form of chemical distribution is more likely for larger molecules and proteins. Endocytosis includes phagocytosis, pinocytosis, and potocytosis, depending on the nature of transported compound.

The bioactivity of a substance is directly dependent on the concentration, which makes the disposition of any compound a major contributor to its potential bioactivity. Therefore, because the disposition of a chemical determines its concentration at the site of action, the concerted action of absorption, distribution, and elimination dictates the potential for biological events to occur.

3.1 Factors Affecting Bioavailability

Regarding phytochemicals, polyphenols and flavonoids in particular, their major route of entry is by oral ingestion, since they are consumed as part of a normal diet. Their bioavailability, similarly to what happens with other xenobiotics ingested orally, can be influenced by several factors. The physicochemical characteristics of the compound, such as the size of the molecule, its lipid/water solubility, or its pKa, can dictate its ability to cross a membrane by simple diffusion or not. For example, due to its size, larger molecules may imply other transport processes rather than diffusion. If the compound is hydrophilic, it is unlikely to cross a membrane freely, but if it is too lipophilic, it may not be completely soluble in gastric secretions which can also make its absorption difficult. In addition, if the compound has ionic charge, it is not probable to cross biological membranes by diffusion and may involve other process to enter the cell.

There are several additional factors relating to the GI tract itself that influence the absorption of xenobiotics such as pH, the presence of food, digestive enzymes, bile acids, bacterial microflora of the GI (microbiota), and the motility and permeability of the GI tract.

Gastric motility and emptying time may influence bioavailability since a higher contact time is usually related with higher absorption. The same principle applies to intestinal motility and transit time. Higher permanence time of the compounds on absorptive surfaces is usually related with higher absorption and consequently with higher bioavailability.

Considering flavonoids, food matrix is a very important issue because it can influence the availability of the compounds to be absorbed in several ways: first, the flavonoid must be liberated from the food matrix where it is inserted, and the difficulty of this process is dependent on the type of matrix; second, if the food matrix has a more lipophilic environment, it can facilitate flavonoids' solubilization and absorption. Ethanol seems to exert crucial effects on anthocyanin intestinal bioavailability, favoring its transport across intestinal epithelia. Also, interaction between different compounds may occur and interfere with absorption; as well, competition or interactions for specialized transport systems are likely to occur due to the variety of molecules present in GI tract after a meal. Another important concept should be the frequency of the consumption. For anthocyanins, it is known that intestinal epithelial cells chronically exposed to anthocyanins are more prone to their own transport [33]. This is an important finding, justifying dietary recommendations, highlighting chronic consumptions of fruits and vegetables as healthy food habits.

Additionally, there are other factors such as age, interindividual and sex differences, biotransformation, and protein binding that may insert variations in xenobiotic absorption between two different individuals. Here, the role of microbiota on biotransforming flavonoids has to be emphasized because it is critical for flavonoid bioactivity. This issue is developed later in this chapter.

When taken together, these factors can cause large interindividual and intraindividual variations in bioavailability, but the knowledge about disposition of flavonoids and their metabolites is a critical determinant to understand its biological effects upon a particular organism. Although flavonoid antioxidant activity in vitro is well established and supported under different circumstances of oxidative stress, antioxidant potential of these compounds in vivo is still an unclear issue. Flavonoids from different classes have different chemical properties which make the study of flavonoid bioavailability difficult as a whole. Each flavonoid class has particular features that make it unique and with characteristics that can make a difference on the extent of the absorption and on their bioactivity. Anthocyanins are a class of flavonoids with very special features, for example, in nature, anthocyanins are glycosylated with different types of sugar moieties, have a positive charge at lower pHs, and are chemically unstable with pH variations [34]. These particular features affect anthocyanin absorption, biotransformation, bioavailability, distribution, and, as a consequence, their bioactivity.

Several animal and human studies have already associated biological effects with anthocyanin consumption, but for that to happen, the compounds or their metabolites must reach target tissues. Bioavailability is sometimes taken as guaranteed, and the mechanisms by which molecules are available, the factors that modulate this bioavailability and the chemical forms that may have bioactivity are not adequately explored. These questions still remain with few, unclear, and broad informations as an answer.

4 Bioavailability of Anthocyanins

Despite the remarkable progress achieved in the field of flavonoid bioavailability and flavonoid-mediated cellular effects, the whole task remains quite complex [35].

From the perspective of preventive nutrition, dietary flavonoids are expected to act at low concentrations following daily intake and in long term. Cell studies typically make use of much higher concentration of flavonoids and often in the biologically irrelevant form of aglycones or native glycosides so that the relevance of the conclusions as to their nutritive effects remains speculative.

In the case of anthocyanins, this is even more complex as they coexist under different structural forms at physiological pH. Extensive knowledge of the bioavailability of anthocyanins is thus essential if their health effects are to be understood (Fig. 79.5).

4.1 Oral Cavity, Stomach, Small Intestine, and Colon

Studies involving individual anthocyanins revealed that their amount in plasma is generally 1% of consumed quantities, due to limited intestinal absorption, although additional factors may contribute to the proposed low anthocyanin bioavailability, such as high rates of cellular uptake, metabolism, and excretion [36].

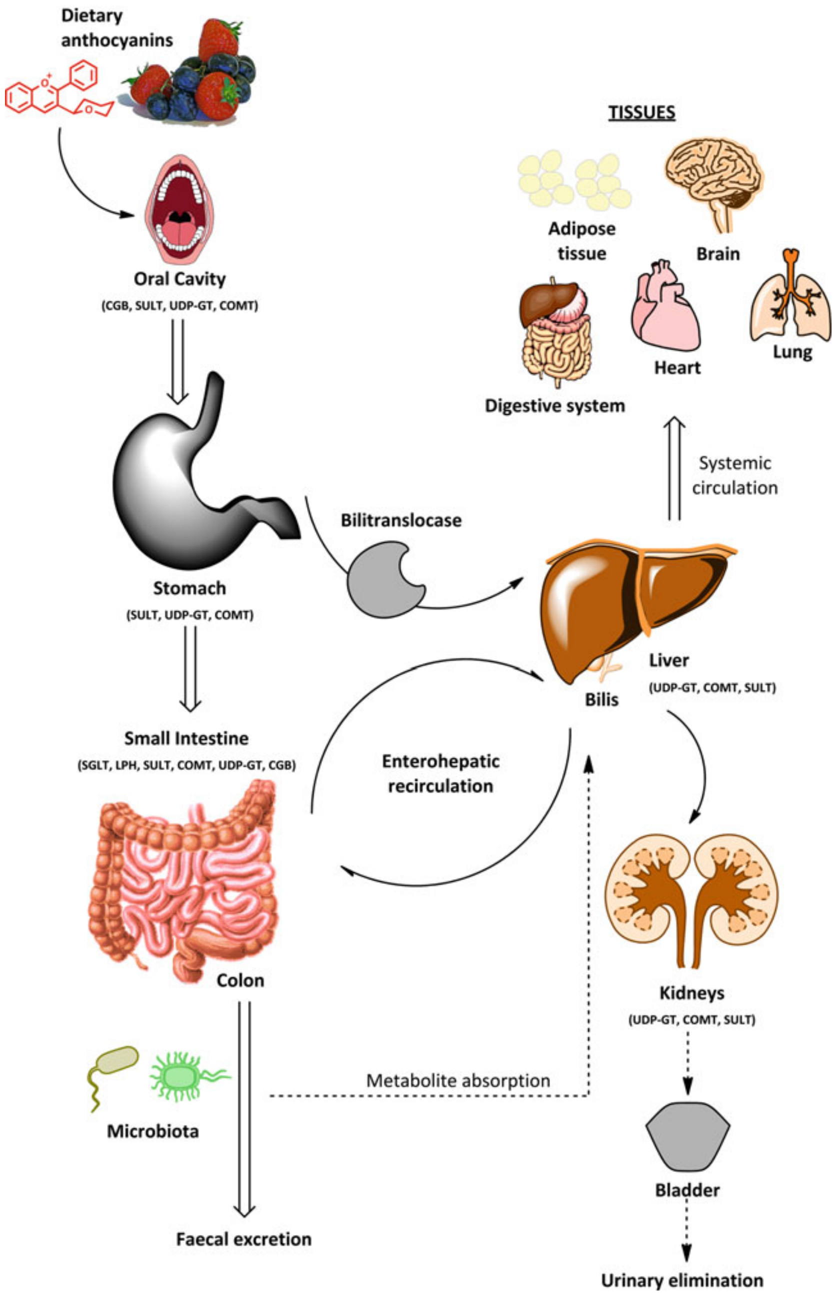
Dietary
anthocyanins
TISSUES
Adipose
tissue
Brain
Oral Cavity
(CGB, SULT, UDP-GT, COMT)
Heart
Lung
Digestive system
Systemic
circulation
Bilitranslocase
Stomach
(SULT, UDP-GT, COMT)
Liver
(UDP-GT, COMT, SULT)
Bilis
Small Intestine
(SGLT, LPH, SULT, COMT, UDP-GT, CGB)
Enterohepatic
recirculation
Colon
Kidneys
(UDP-GT, COMT, SULT)
Microbiota
Metabolite absorption
Bladder
Faecal excretion
Urinary elimination

Fig. 79.5 Hypothetic pathways of anthocyanin absorption, distribution, metabolism, and excretion based on current information (CGB-cytosolic beta-glucosidase; SULT-sulfotransferase; UDP-GT-glucuronosyltransferase; COMT-catechol-O-methyl transferase; SGLT-sodium-dependent glucose cotransporters; LPH-lactase-phlorizin hydrolase)

Upstream to gastrointestinal absorption, a variety of binding processes can take place, namely, interaction with food proteins or with salivary proteins and digestive enzymes [37–39].

In a recent work with healthy volunteers, black raspberry anthocyanins could be detected under their hydrolyzed aglycone form in the oral cavity, resulting from the activity of ß-glycosidase derived both from bacteria and oral epithelial cells [40]. In the same study, parent anthocyanins and cyanidin-3-glucoside microbiota metabolite, protocatechuic acid, were detected in the saliva. Furthermore, saliva samples contained glucuronidated anthocyanin conjugates, consistent with intracellular uptake and phase II conversion of anthocyanins [40].

Still, whether these oral transformation reactions will be of importance for local effects in the oral epithelium is difficult to assess considering the relatively short residence time of most foods in the oral cavity.

Anthocyanins, for consumers that eat berries and drink red wine on a routine basis, are major dietary components. The key difference compared to the other flavonoid glycosides, however, is that anthocyanins undergo rearrangements in response to pH and temperature [15]. Physiological temperatures are highly suitable both thermodynamically and kinetically for observing the chalcone tautomer [15]. The limited available experimental evidence indicates that in the acidic conditions that prevail in an empty gastric compartment, anthocyanins are in the red flavylium form, while all the other dietary flavonoids remain neutral.

After ingestion, anthocyanins are readily detected in plasma, in their parent forms, possibly as a result of their absorption through the gastric wall [41–44].

Oppositely to other extrahepatic organs, such as the intestine and kidneys, the stomach has been widely ignored as a metabolizing organ although it has been identified as a site of absorption for different compounds [45, 46].

The contribution of the gastric mucosa to the metabolism of anthocyanins should not be ruled out because the stomach possesses conjugative enzyme activities (UDP-glucuronosyltransferase, sulfotransferase, and catechol-*O*-methyl transferase) [47–50]. Besides, in vitro studies showed that some flavonoids could be metabolized into glucuronidated and sulfated metabolites by the gastric wall [45, 51].

The anthocyanin fraction that is not absorbed in the stomach reaches the small intestine. Once anthocyanins enter more basic conditions in the small intestine, the carbinol pseudobase and the chalcone forms are likely to predominate. Unlike flavonoids where glycosides are hydrolyzed, anthocyanin glycosides are rapidly and efficiently absorbed from the small intestine [52–54]. Furthermore, anthocyanins are quickly metabolized and appear in the circulation or are excreted into bile and urine as both intact and metabolized forms (glucuronidated, sulfated, or methylated derivatives) [52, 53, 55–59].

Unabsorbed anthocyanins reach the colon where they undergo substantial structural modifications. Previous studies have suggested that this is likely due to the spontaneous degradation under physiological conditions [60] or following microbial metabolism. In fact, colonic microbiota hydrolyzes glycosides into aglycones and degrades them to simple phenolic acids.

According to Vitaglione and coworkers, protocatechuic acid is the major human metabolite of cyanidin-3-glucoside in humans [61]. In particular, protocatechuic acid accounts for almost 73% of the ingested anthocyanins. This metabolite was detected in plasma 2 h after blood orange juice ingestion, indicating that perhaps it was formed through chemical degradation at the physiological conditions of the systemic circulation or in the intestinal mucosa. This metabolite is recovered in fecal samples, which suggests that the gut extensively metabolizes anthocyanins [62].

The metabolism of berry anthocyanins resulting in phenolic acids in humans was recently studied [63]. The main anthocyanin metabolites detected were homovanillic and vanillic acids.

In another recent study, blueberry anthocyanin absorption and metabolism in rats was accomplished, and the main metabolite detected in urine was hippuric acid, which may be produced in liver through a conjugation of glycine with aromatic phenolic acids [64].

Since anthocyanin phenolic acids can be further absorbed in colon [65], it is possible that they are additionally metabolized by hepatic cells [66]. Health benefits associated with anthocyanin-rich foods may also be explained by a slow and continuous release of phenolic compounds through the gut into the bloodstream.

Despite low apparent bioavailability, plasma concentrations of anthocyanins appear sufficient to induce changes in signal transduction and gene expression in vivo [67, 68] in a manner that suggests their putative role in physiological functions and health outcomes.

Some of the human studies investigating anthocyanin bioavailability are summarized in Table 79.1.

In the studies listed, anthocyanins are administrated from different sources varying from beverages (red wine, fruit juices) to the ingestion of fruits, like blood orange, grapes, and different berry extracts or concentrates. The data values from all studies are summarized in the next data ranges:

- Anthocyanin dose administrated, 56–3.570 mg
- Duration of the assay, 3–48 h
- % urinary recovery, 0.003–5
- Maximal plasma or serum concentration, 1.4–592 nmol/L
- Time to reach maximal plasma concentration, 0.3–4 h
- Half-life of elimination, 1.5–3.0 h

The overall analysis of the biokinetic parameters has facilitated some main assumptions in what anthocyanin bioavailability is concerned. The most important one is that although there is a considerable variability in the values for the biokinetic parameters, anthocyanins appear to be rapidly absorbed and eliminated, reaching low maximal concentrations in plasma and urine.

Several factors including variations in the dose, anthocyanin chemical composition in the different sources, food or beverage matrix or processing, age and gender of the individuals, and the analytical methodology used can have a huge effect on the bioavailability and metabolism of anthocyanins.

Table 79.1 Biokinetics of anthocyanins following oral consumptions in humans (Adapted and updated from [88])

Source	Anthocyanin dose (mg)	Duration (h)	(%) Urinary recovery	C_{max} (nmol/L)	t_{max} (h)	$t_{1/2}$ (h)	References
Cranberry	95	3	0.79	4.64	3		[89]
Bilberries and lingonberries	650	48	–	138	1.5	–	[63]
Blood orange juice	71	24	1.2	1.9	0.5	–	[61]
Cranberry	651	24	5.00	–	–	–	[90]
Chokeberry	721	24	0.15	96.1	2.8	1.5	[91]
Blackberry	431	24	0.16	–	–	–	[92]
Hibiscus extract	147	7	0.018	7.6	1.5	2.6	[93]
Red grape juice	283	7	0.23	222.7	0.5	1.8	[94]
Red wine	280	7	0.18	95.5	1.5	2.0	
Elderberry	3.570	5	0.053	–	–	–	[95]
Blackcurrant	145	7	0.04	–	–	1.7	[96]
Elderberry	147	7	0.37			1.7	
Chokeberry	1.300	24	–	592	–	–	[97]
Red wine	280	7	0.23	222.7	0.5	1.83	[98]
Red grape juice	283	7	0.18	95.5	1.5	1.52	
Blackcurrant	345	7	0.029	–	–	–	[56]
Boysenberry	189	7	0.064				
Blueberry	439	7	0.020				
Strawberries	77	24	1.9	–	–	–	[99]
Blackcurrant juice	1.239	4	0.07		0.7	–	[100]
	716	4	0.05		0.7		
Elderberry	1.900	6	0.03	–	–	–	[44]
Blueberry	1.200	4	0.003	29.2	4.0	–	[101]
Blueberry	690	6	0.004	–	–	–	[102]
Elderberry	720	24	0.08	97.4	1.2	–	[43]
Red wine	68	6	0.03	1.4	0.3	–	[103]
De-alc red wine	56	6	0.03	1.7	1.5		
Red grape	117	6	0.03	2.8	3.0		
Elderberry	720	24	0.05	97.4	1.2	2.2	[42]
Blackcurrant	236	8	0.11	60.0	1.5	3.0	[104]
Elderberry	500	–	0.05	–	–	–	[105]
Blackcurrant	153	5	0.03	–	–	–	[106]
Mixed berries	162	–	–	29.0	1.0	–	[53]
Red wine	218	12	3.3	–	–	–	[107]

C_{max} maximum concentration, t_{max} time to reach maximum concentration, $t_{1/2}$ half-time of elimination

4.2 Tissue Distribution of Anthocyanins

It has been only recently that studies were conducted to determine tissue concentrations of anthocyanins. The stomach exhibited only native anthocyanins, while in other organs (jejunum, liver, and kidney), native and methylated anthocyanins as well as conjugated anthocyanidins (monoglucuronides) were identified [69].

In another work, pigs were fed diets supplemented with blueberries for 4 weeks. Although no anthocyanins were detected in the plasma or urine of the fasted animals, intact anthocyanins were detected in liver, eye, cortex, and cerebellum. The results suggest that anthocyanins can accumulate in tissues, including tissues beyond the blood–brain barrier [70].

In a more recent work, proportions of anthocyanin derivatives (methylated anthocyanins and glucurono-conjugated derivatives) were identified in various organs (bladder, prostate, testes, heart, and adipose tissue) in rats fed with a blackberry anthocyanin-enriched diet for 12 days [71]. In this study, the bladder contained the highest levels of anthocyanins, followed by the prostate. Prostate, testes, and heart contained native cyanidin-3-glucoside and a small proportion of cyanidin monoglucuronide. Cyanidin-3-glucoside and methylated derivatives were present in adipose tissue.

Moreover, two recent works report the capacity of dietary anthocyanins from grape and berries to reach the brain [69, 72].

4.3 Mechanisms of Anthocyanin Absorption

The mechanisms responsible for the gastrointestinal absorption of anthocyanins are not fully understood. Whether anthocyanins are absorbed through passive diffusion or via a facilitated transport remains to be elucidated.

Recently, Passamonti and coworkers have suggested that an organic anion carrier, bilitranslocase expressed in the gastric epithelium, could be involved in the absorption of anthocyanins at the gastric level [73]. The administration of high amounts of anthocyanins, far from diet levels, could induce saturation of this transport and contribute to the lower anthocyanin bioavailability reported in those particular studies [55].

The potential mechanisms of anthocyanin glycosides absorption in the small intestine may involve a specific glucose transporter, such as SGLT1, as previously suggested for the flavonoids [74].

A recent work points for the putative involvement of GLUT2 transporter in anthocyanins' absorption at the intestinal level [33].

Another possible mechanism may involve the hydrolyzation of anthocyanins by brush border enzymes such as lactase phloridzin hydrolase, prior to passive diffusion of the aglycone, as already proved for other flavonoids [74, 75].

The health benefits associated in epidemiologic studies with the consumption of anthocyanin-rich foods contradict the apparent low bioavailability of these compounds. More subtle scenarios may exist, and detailed information is unlikely to be forthcoming until ring-labeled ^{14}C-anthocyanins [76] and anthocyanin metabolite conjugate standards become available [77].

4.4 Microbiota Impact on Anthocyanin Availability and Bioactivity

Microbiota keeps an important role in human metabolism. It must be considered a metabolizing "organ," with impact on endo- and xenobiotic metabolism, beyond its metabolic relevance for vitamin B12 synthesis, and carbohydrate breakdown, between other important functions.

As it has been known, no systematic reviews have been undertaken to assess the role of xenobiotic biotransformation in colon, in particular in what concerns anthocyanins.

Colonic microbiota could now be considered as a "microbial organ" placed within a host organism. In addition to the obvious role of the intestine in the digestion and absorption of nutrients, the human GI tract contains a huge collection of microorganisms. Microbiota has not been fully described, but it is clear that the human gut is home for an ecosystem of around 10^{13}–10^{14} bacterial cells. As a whole, the microorganisms that live inside humans are estimated to outnumber human cells by a factor of ten. And the microbiome represents overall more than 100 times the human genome [78].

The diet anthocyanins (parent compounds) that reach the microbiota are those not absorbed in the upper GI level and, also, their metabolites excreted in the bile and/or from the enterohepatic circulation (Fig. 79.5). In the microbiota, anthocyanins are extensively metabolized, especially by genera and species able to do that.

Anthocyanins are metabolized since part of the absorbed amounts have been found as methylated, sulfated, or glucuronidated forms. Anthocyanin metabolization includes the cleavage of glycosidic linkages and breakdown of the anthocyanin heterocycle [79–81].

For this kind of biotransformation, β-D-glucosidases, β-D-glucuronidases, and alpha-L-rhamnosidases that release aglycones from their glycoside or glucuronidate forms will be necessary [82–84]. There is a lack of information about this, but, probably, Bacteroides could be the genus mostly involved in these biotransformation reactions since this genus expresses those enzymes. For this reason, one could also consider, based on a specificity for substrate, that anthocyanins have a prebiotic effect upon Bacteroides, allowing them to grow. This is a new concept but important to consider, especially in an occidental diet pattern that needs to increase Bacteroides number. There is evidence from studies that compare microbiota from lean and from obese individuals that the last ones have an increase on Firmicutes genera and a Bacteroides decrease (about 50% reduction) in comparison with lean ones [85].

Furthermore, it is important to highlight that anthocyanin bioavailability may vary according to: (1) the food source and, consequently, by the matrix of the food source, and (2) the individual characteristics of the microbiota that strongly depends on dietary habits [86].

Depending on food source, anthocyanins can be chemically different, mainly as glucosides or rutinosides. However, the structure of the metabolites is not dependent on sugar moieties naturally attached to polyphenol but on the structural characteristics of the polyphenol. Protocatechuic acid is the main metabolite detected after anthocyanin consumption [79, 81, 84].

4.5 Considerations Regarding Anthocyanin Bioavailability

Anthocyanins are compounds with a particular chemistry that is responsible for their attractive colors but also for their instability and easy degradation. As previously referred, these compounds are pH sensitive, being more stable at acidic pHs. When the pH rises, chemical transformations of the anthocyanin structure occur and the molecule is no longer a cation (Fig. 79.3), becoming more unstable and losing the red color. The most common methodologies for detection of anthocyanins are based on their color as a flavylium cation form and rely on their ability to convert back into the cationic form after acidification. The fact is that this conversion may not be complete, which could lead to an underestimation of anthocyanins in the analyzed samples. Also, it should be taken into account that at physiologic pH, the main anthocyanin structure is the hemiacetal/chalcone and if these structures are metabolized/biotransformed, it may not be converted to the cationic form, contributing once again to the underestimation of these compounds on biological samples. Besides, the biologic role of these hemiacetal forms or their metabolites is not clear, but it is likely that these structures play a major function on the biologic effects attributed to anthocyanin consumption.

In another perspective, considering that anthocyanin absorption is reduced, perhaps due to its chemical properties, certain biological actions could still occur at the membrane level. As a consequence of a direct interaction with membrane lipids, flavonoids can alter membrane physical properties, modulating enzyme activity, ligand–receptor interactions, ion fluxes, signal transduction, transport, and other membrane-associated functions [87]. These membrane–flavonoid interactions can also affect the absorption of other dietary components, such as macronutrients, vitamins, or even other flavonoids. Flavonoids can also interact with membrane proteins modifying or altering their biological function. This is a point to bear in mind since flavonoids may trigger biological effects that do not depend on their absorption by the cells. Furthermore, interactions with microbiota should be considered, including flavonoid metabolism, as well as flavonoid effects on the microbiota and consequently effects on the balance between a myriad of species that constitute the microbiota. It means that anthocyanin healthy effects could result, at least in part, from these interactions, since microbiota metabolism could markedly interfere with human metabolism.

The scientific community has enriched its knowledge through the widespread use of in vitro techniques. This type of studies has provided valuable biochemical information. However, conclusions, interpretation, and extrapolation should be rigorous and careful in several aspects: (1) regarding the use of cell cultures, one should bear in mind the species from where the cell line is originating: different species may express, for example, different transporters that could have a role in flavonoid absorption; (2) tumor-derived cell lines often have a cellular machinery different from normal cells, thus influencing the measured outcome.

Also, when working with flavonoids, dose is always a concern. Ideally, doses should reflect in vivo physiologic situations, but most of the times, the detection and quantification tools available are not sensible enough for these doses. This leads to the use of higher doses which could compel a particular effect that would not happen or could be less pronounced if lower doses were used. Furthermore, in vitro studies often use the aglycones or native glycosides that, as discussed before, could be biologically irrelevant to the tissue or cell line used in the study; subsequently, the relevance of the conclusions as to their nutritive effects remains speculative.

Nevertheless, the knowledge of the pathways where these compounds may interfere, as well as their affinities to transporters, receptors, or enzymes, is valuable since it allows recognition of new putative therapeutic targets.

On the other hand, in vivo studies have increased the awareness of anthocyanin tissue distribution, have given an insight about anthocyanin biokinetic behavior, and allowed to relate anthocyanin consumption with several beneficial health effects. Even so, these types of studies have some limitations given that, generally, there is not a mechanistic approach about the movement of anthocyanins through the cells or the pathways responsible for the biological effects observed.

All these issues make the study of anthocyanin disposition a difficult task that is not fully understood, regardless the scientific advances that have been made in this field.

5 Conclusion

Overall, anthocyanins found in diverse food sources, with berries, and red wine as main sources, are intestinally absorbed, but mainly reach the colon and are submitted to microbiota metabolization. Content of anthocyanins may vary considerably between food sources and environmental conditions, as a direct sun exposure, cultivars, etc. This fact is often neglected in studies, probably justifying result variability and the, still existing, gap in knowledge.

Anthocyanins seem to be absorbed in the upper GI (stomach and small intestine) mainly in the parent forms. In addition, they, themselves and/or their metabolites seem to be absorbed after a microbial action, and microbiota seems to constitute the main responsible factor for anthocyanin bioavailability.

Healthy known effects associated with consumption of anthocyanin-rich foods should be attributed to: (1) direct effects of the absorbed parent compounds (or their metabolites) and (2) indirect effects mediated by nonabsorbed entities that, probably, induce modifications on microbiota environment and, consequently, on human metabolism.

More studies should be carried out in what concerns anthocyanin transport across biological membranes. There are studies announcing neuroprotective effects of anthocyanin-rich foods, but there is a gap in the knowledge concerning, for example, anthocyanin (and metabolites) transport across blood–brain barrier. On top of this, it is urgent to know dietary factors able to modulate anthocyanin bioavailability, helping health professionals to make dietary recommendations. These recommendations will be relevant for a healthy life but also to alert medical doctors as to possible pharmacological interactions with anthocyanins.

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Part IX

Phenolics: Nutraceuticals and Functional Foods

Prospects of Functional Foods/ Nutraceuticals and Markets

80

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Contents

1 Introduction 2492
1.1 Definition of Functional Foods, Nutraceuticals, and Related Terms 2492
1.2 Socioeconomic Context of Functional Foods and Nutraceuticals 2495
2 Market Prospect of Functional Foods 2500
2.1 Global Sales: A Steady Progression 2500
2.2 Situation of Specific Product Launches, Ingredients, and Claims 2502
2.3 New Perspectives 2503
3 Functional Foods Under Scrutiny: Situation of Natural Products Relating to the Substantiation of Evidence 2504
3.1 Regulation of Functional Foods and Nutraceuticals Market 2504
3.2 EFSA Opinions on Phytochemicals and Plant Extracts 2509
3.3 Lessons Learned: Key Scientific Aspects to be Controlled for Obtaining a Health Claim 2509
4 The Impact of New Methodologies for the Assessment of Functional Foods 2512
4.1 Hyphenated Methods for the Characterization of Ingredients 2512
4.2 Bioinformatics 2513
4.3 Omics Data 2513
5 Conclusions 2514
Annexes 2515
References 2522

Abstract

Functional foods and nutraceuticals are reported as one of the top trends of the food industry, but because of the different definitions of the terms, it is uneasy to calculate their global market size. With a broad definition, this value is well over

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$40 billion, and is showing steady annual increases both in sales and new products launched. However, there are differences according to the ingredients and the claims used.

Several factors are considered crucial for the future market evolution: the degree of acceptance and awareness of functional foods by consumers, the association between manufacturers and academic researchers, and the effects of new regulations for nutrition and health claims. Concretely, the Regulation 1924/2006 will have a great impact on the number of products bearing a claim. We have analyzed European Authority of Food Safety (EFSA) opinions on plant extracts and phytochemicals, including phenolic compounds, since they can provide lessons for the development of functional foods all over the world.

Scientific research is indispensable for the substantiation of the evidence for functional foods. Advances in the characterization of plants ingredients by hyphenated MS and NMR technologies, standardization of human clinical trials, and emerging methodologies like bioinformatics or nutrigenomics can be crucial for the development of new functional products.

Keywords

Claims. EFSA • consumers • functional foods • Market

Abbreviations

EFSA	European Food and Safety Authority
FDA	Food and Drug Administration
ILSI	International Life Science Institute

1 Introduction

1.1 Definition of Functional Foods, Nutraceuticals, and Related Terms

The advent of functional foods and nutraceuticals on the market has blurred the distinction between pharma and nutrition [1]. Obviously, the concept of foods promoting health is not new. In 400 b.c., Hippocrates already sentenced “Let food be thy medicine and medicine be thy food” [2], and in the countries of the far East, influenced by Chinese culture, foods such as glutinous rice, wheat, sesame, jujube, gingiber, or leek were included in Chinese medicine books for their traditional use for chronic diseases [3]. Nowadays, the development of functional foods is one of the most intensive areas of food product development worldwide, opening multiple challenges for countries with a vast biodiversity and historical use of plant extracts [4].

However, estimating the market size values for functional foods can be difficult, because of the ambiguity of the term “functional food,” the lack of an official or univocally accepted term (in many countries there is no regulatory

Table 80.1 Concepts related to functional foods and nutraceuticals

Functional foods	A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet. They are not pills or capsules, but part of a normal food pattern	[6]
Nutraceuticals	A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease	[7]
Dietary supplements	A product that contains one or more of the following dietary ingredients: vitamin, mineral, herb, or other botanical, and amino acid (protein). Includes any possible component of the diet as well as concentrates, constituents of extracts or metabolistes of these compounds"	[8]
Food supplements	foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities	[9]
Parnuts	Foodstuffs for particular nutritional uses are foodstuffs suitable for their claimed nutritional purposes and which are marketed in such a way as to indicate such suitability. They can include: – Infant and follow-on formulae – Processed cereal-based foods and baby foods for infants and young children – Food intended for use in energy-restricted diets for weight reduction – Dietary foods for special medical purposes – Foods intended to meet the expenditure of intense muscular effort, especially for sportsmen. – Foods for persons suffering from carbohydrate metabolism disorders (diabetes).	[10]
Herbal medicinal product	Any medicinal product, exclusively containing as active ingredients one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations	[11]

definition) [5], and the diffused borders between concepts for commercial products also related to health-promoting functions such as "functional foods," "nutraceuticals," "superfoods," "dietary supplements," or "cosmeceutics," many of which respond to marketing criteria. Table 80.1 compiles some of the terminologies currently associated to functional foods and nutraceuticals.

Despite the ancestral knowledge of the link between diet and health, the appearance of specific terms related to foods promoting a health benefit is more recent.

In 1984, the concept of functional food was first promoted in Japan by scientists who were studying the relationships between nutrition, sensory satisfaction, fortification, and modulation of physiological systems [12]. The Japanese Ministry of Health and Welfare then introduced "Foods for Other Specific Health Use" (FOSHU) in 1991, promoting its use as a strategic action to reduce healthcare costs. The success of the initiative (in 2000, the total number of approvals under the FOSHU label reached 174, with an estimated market value of around $2 billion [13], while more than 500 products were labeled as FOSHU in 2005 [5]), united to the success of products with health claims in the USA, paved the way for the development of the functional foods market. However, consensus on a definition or categorization of these foods was established by heterogeneous criteria by manufacturers or scientists, not by regulatory agencies.

In Europe, the International Life Science Institute (ILSI) established an operational definition of functional foods: "a food product can only be considered functional if, together with its basic nutritional impact, it has beneficial effects on one or more functions of the human organism, either improving the general and physical conditions and/or decreasing the risk of evolution of diseases. The amount of intake and form of the functional food should be as it is normally expected for dietary purposes. Therefore, it could not be in the form of pill or capsule but only as a normal food form." This definition would establish a clear separation from nutraceuticals, which can be considered as diet supplements that deliver a concentrated form of a presumed bioactive from a food, presented in a nonfood matrix, and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods [14]. Nutraceuticals are sold in presentations similar to drugs: pills, extracts, tablets, etc. [15]. However, in many cases, the functional food market is referred as the "nutraceutics" market [16], and then, it is difficult to separate the exact values for each. A reason for this interconvertibility of terms is that much of the early development of the nutraceutical concept and products was driven from the USA, where the Dietary Supplement and Health Education Act (DSHEA) allowed considerable flexibility and blurred the boundaries between foods and medicines that can be found in other parts of the world [17]. Anyway, there is no definition for nutraceuticals in neither the UE nor the USA, although both have a definition for supplements, which could be considered equivalent to nutraceuticals.

In opposition, the federal Department of Canada has proposed, via Health Canada, differentiated definitions for nutraceuticals and functional foods. A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. It is demonstrated to have a physiological benefit or provide protection against chronic disease. A functional food is similar in appearance to, or may be, a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions. In this case, the difference of formulation between both kinds of products is well established [7].

Most functional foods definitions do not intend to exclude natural foods in the functional foods category. However, many manufacturers and the perception of the general public would disagree, since for many of them, the functional food should imply an inherent fortification of the food with a bioactive ingredient. And from the pragmatic point of view of manufacturers concerned about regulation accomplishments, functional foods would be those that bear or intend to bear a nutritional or a health claim.

Another conflictive concept is the one for cosmeceuticals. Neither the FDA nor the EFSA recognizes this term, which is widely used by the cosmetic industry to refer to cosmetic products that have medicinal or drug-like benefits. Like cosmetics, cosmeceuticals are applied topically but differ in that they contain potent ingredients that can influence the biological function of the skin and deliver nutrients to promote healthy skin [18]. Nearly universally around the world, this has become the catchword for the millennium change in cosmetics to retard aging [19].

Other marketing concepts have arisen like "superfoods," which are referred to natural products that have a low glycemic index and provide key nutrients that are lacking in the typical western diets, like beans, dark green leafy vegetables, citrus, sweet potatoes, berries, tomatoes, fish high in omega-3 acids, whole grains, nuts, fat-free milk, and yogurt [20]. A derivative concept, "superfruits," has emerged to design natural fruits (including blueberries, blackberries, cranberries, pomegranate and exotic ones such as açai or goji) that have a high antioxidant capacity. However, neither of those terms has been recognized by the Food and Drug Administration or the European Food Safety Authority.

Finally, other terminologies that used to be popular such as vitafoods, alicaments, or pharmafoods have become obsolete and fallen out of use.

1.2 Socioeconomic Context of Functional Foods and Nutraceuticals

To understand the success in the functional foods and nutraceuticals sector and the reasons behind their expansion in the market as well as the menaces that can compromise their growing, it is necessary to describe the socioeconomic context, which includes many interactions between consumers, scientists, food manufacturers, and legislative bodies.

1.2.1 Functional Foods as a Vector for Health

In the last decades, the social economic development has induced profound changes in consumer behavior, involving, in particular, food consumption dynamics. The elements that have most influenced and still influence consumer eating habits are the lengthening of life expectancy, the progressive ageing of population, the health economic and social costs rising, the widespread desire for a better quality of life, and media and advertising [21].

Major current problem areas for population health include obesity, cardiovascular health, age-related cognitive decline, metabolic syndrome, insulin resistance, and diabetes [22]. All these concerns are expected to grow because of the change of the age pyramid to older societies. People are living longer, and so incidence of specific diseases or conditions of elder people, such as cardiovascular diseases, diabetes type 2, osteoporosis, or neurodegenerative diseases, will increase. The biggest health concern nowadays is obesity and overweight, especially because of the highest increase in developing countries [23]. The WHO estimated in 2003 that over one billion people are obese and over 400 million people are clinically obese [22]. Cardiovascular diseases are also a big concern: They represent 30–50 % of cause of mortality in developed countries. High blood pressure and cholesterol levels have reached pandemic dimensions, and it is predicted that the worldwide incidence of diabetes will exceed 450 million people by 2025 [22]. The prevalence of arthritis and osteoporosis in the aging populations of developed nations is also growing: In 2010, more than 5.5 million people in the seven major markets were forecast to be suffering from rheumatoid arthritis (RA) [24].

Many of the major chronic diseases are caused substantially by poor diets. The consumption of whole grains and better lifestyle choices could represent a reduction of over 100,000 deaths per year in the UK [25]. Since a major issue for public research is targeting prevention of diet-related diseases [26], there is a growing interest about the links between food and health by public health officials [27]. Functional foods have the potential to improve population health in line with the objectives identified by national public health strategies [28], promoting the prevention and risk reduction of disease, and thus reducing mortality rates and medicinal costs associated to therapeutic treatments. For example, it was estimated that in the USA, total direct and indirect costs associated to diabetes and related disorders reached $98 billions in 1997 [22]. In 2005, $466 m were spent on pharmaceutical medication to treat obesity in the seven major markets, and these sales were set to triple to $1.5 billion by 2010. In 2005, $84 billion were spent in the USA and the 5 major European markets on the pharmaceutical treatment of CVD conditions. By 2010, sales were expected to rise to $105 billion. In 2005, it was estimated that around $22.5 billion were spent worldwide on the sale of drugs used to treat rheumatoid arthritis (antirheumatics), osteoporosis (osteoporosis agents), and inflammatory conditions, with $14 billion of this generated from sale of medicines in the top seven countries [24].

In this context, public health organizations and governments share a mutual interest in promoting health through nutrition and adequate lifestyles, thus reducing medical costs. For example, the consumption of whole grains and better lifestyle choices could represent a reduction of over 100,000 deaths per year in the UK [25]. In fact, this was the key reason why the Japanese government introduced the FOSHU system: to keep the aging population healthy through functional foods and to keep the health care costs down. Similar policies are spread all over the world. For example, agriculture, food, and health are significant themes in the European Union's current Seventh Framework Research Programme (FP7) [26].

1.2.2 The Perspective of the Consumer

Consumer acceptance of the concept functional foods, together with a better awareness of its determinants, is widely recognized as key success factor for market orientation, consumer-led product development, and successfully negotiating market opportunities [29]. However, little research has been conducted to analyze the perspective of the consumers [30].

In fact, one of the main reasons of the success of functional foods has been the role of consumers undertaking new trends to a healthier lifestyle. Many objective data show this tendency. For example, between 2006 and 2007, vegetables and fruits were the top 2 products whose use increased in North America, Western Europe, and Nordic Europe, while processed foods, salty snacks, and sugars were some of the products with the biggest decrease in use. In the last 25 years, butter has decreased from around 70 % of the yellow fat market to 25 %, while low fat spreads have captured half this market. In the cooking fat sector, vegetable oils have taken over the animal fats. Skimmed and semi-skimmed milks have copped 2/3 of milk sales, while low calorie soft drinks have increased to 20 % of the soft drink market [31].

Apart from these examples of healthier choices taken from objective market data, several surveys are periodically conducted in developed countries to follow the public attitude toward health. In 2011, according to the International Food Information Council Foundation Food & Health Survey, 59 % of Americans declared that they were attempting to make changes to improve the healthfulness of their diets, in order to ameliorate their overall well-being (65 %), lose weight (56 %), improve their physical health (56 %), because of a specific health condition (32 %), and/or maintain weight (20 %) [32]. Similar tendencies can be found in European countries.

However, these pronouncements have to be considered carefully, because some data have reported lower frequencies of healthy food consumption in American consumers, despite their intention of eating healthily more often [33]. This distance between the consumers' aspirations and the real consumption is a clear menace to the success of the functional foods niche and should be overcome by providing functional products that are attractive to the potential buyer, both in price, and taste and efficacy.

Acceptance of Functional Foods and Willingness to Buy New Products

In the process of developing new functional ingredients or foods, there must be a consumer need or a problem that requires solution, and there must be self-awareness of the problem from the consumer. But it is also crucial that consumers must be willing to spend money to solve the problem or satisfy the need they have identified [23].

The Functional Foods/Foods for Health Consumer's trending survey is being conducted every 2–3 years since 1998 and provides ongoing American consumers insights into their interest and perceptions about food and beverages and the roles they have in promoting health and wellness [34]. This study summarizes some of the points that can explain the reasons for the success of functional foods. A first aspect to be considered is the acceptance by consumers that functional foods can have added health and wellness benefits. Most data from other surveys in other countries show conformity with this topic. According to the 2009 IFIC Functional Foods/Foods for Health Consumer Trending survey, between 68 % and 85 % of Americans agree that foods or beverages can provide specific health benefits such as improving heart health, contributing to healthy grown and development in children, or improving bone health [34]. In Europe, even when consumers had not heard the term “functional foods,” more than 50 % agreed to fortify functional ingredients in specific food products [35]. Japanese consumers have traditionally been aware of the importance of certain foods for the promotion of health.

Obviously, general health orientation varies systematically as a function of age and gender. According to the results obtained from different questionnaires, rational food consumers are the primary potential target of functional food producers. Women tend to be substantially more health-oriented than men, which explains the females’ stronger purchase interest toward functional foods, an observation that has met consensus in different studies. In general, women have shown to be more reflective about food and health issue. Another relevant socio-demographic factor is the presence of children in the family. On the other hand, middle-aged and elderly consumers tend to be substantially more health-oriented than young consumers. In truth, middle-aged and elderly are the largest group who uses functional foods to target a specific health concern. In this case, it is hypothesized that this group is more likely to have confronted relatives’ loss of good health, and this experience with illnesses and associated economic and social consequences increase probability of functional food acceptance [29]. In overall, the hypothesized effects of socio-demographic determinants are that acceptance of functional foods increases with higher age, being female, having young children, and having an ill family member.

At the same time, there are discrepancies about the role of education in the acceptance of functional foods. In Europe, it is biased toward the higher socioeconomic groups, reflecting a higher willingness or ability to pay a premium price; while in the USA, there is a higher acceptation among the lower educated. Another difference between American and European consumers is the far more critical attitude toward new product and technologies, and therefore, it can be hypothesized that European’s acceptance of functional foods is less unconditional [5].

In conclusion, it seems to be a global acceptance of functional foods, but this must not be taken for granted and cannot reflect the perception of concrete functional foods. In this sense, another of the aspects addressed in consumers’ research is the awareness of functional foods.

Concerning the awareness of the terminology of functional foods, different surveys show that consumers are not greatly informed about this concept, although there is a steady tendency to increase these levels of awareness in all the countries. For example, in an Italian quantitative survey conducted in 400 consumers, 24 % were unable to give a definition for functional foods, 20 % confused them with light and dietary products, and 16 % incorrectly associated them with food for those who have specific health problems [36]. In Belgium, 49 % of consumers were familiar with the term “functional food,” but only 30 % in Hungary and 4 % in Poland [37].

Given the positive acceptance showed for functional foods in general, it should come to no surprise that most studies have shown a significant willingness to pay for this kind of products. For example, Mintel Oxygen 2010 reported that more than half of consumers had bought a functional food or beverage in the 3 months previous to the study. More than half of the Americans reported in 2009 the consumption of foods or beverages for overall health and wellness (56 %), heart health benefit (55 %), or to contribute to a healthy body weight [34]. In a recent survey in Europe, 15 % of consumers reported a daily consumption of functional foods, 24 % reported a high-frequency consumption, and 28 % considered themselves occasional buyers. Only 21 % of respondents stated that they had never consumed these products [36].

Again, regardless of the global willingness to pay for healthy products, the acceptance of a specific functional ingredient is linked to the consumers' knowledge of the health effects. Functional ingredients which are in the mind of the consumers for a relatively long period of time (e.g., vitamins, minerals, fiber, low sugars, etc.) achieve considerably higher rates of acceptance than ingredients that are used for a short period of time. This can have a great impact in the context of the economic recession. Although it can be demonstrated that consumers are willing to spend for relevant functional benefits even in times of financial crisis, consumers are less likely to experiment with new functional foods and beverages: In this sense, the credibility of the health effect and the perceived effectiveness of the product, as well as a correct knowledge of the ingredient and a correct association between the ingredient and the health benefit, is crucial. For example, fiber, calcium, iron, and vitamin D are well-known ingredients and consumers are correctly aware of the kind of health benefit they can expect from them. Omega-3, probiotics, and phytosterols were less known, but thanks to the advertising policies communicating their health effects, consumers have a good knowledge of their benefits. In opposite, products like oligosaccharides, lutein, or peptides are less popular. It is of great importance to have well-informed consumers, conscious of the effects of the ingredients, in order to avoid incorrect expectancies that can further lead to deception and lack of trust in future products.

In sum, for those products with limited consumers' knowledge, there are strong needs for specific information and communication activities. Doctors, nutritional advisers, and public entities are the sources in which the consumers have most confidence (42–45 % of trust), while a lesser degree of confidence is given to producers and product labels [13, 36]. The implementation of specific regulations restricting the use of nutrition and health claims to products with a solid scientific evidence will also offer a reinforcement of the trust of consumers in the long term. But in the short term, the withdrawal of many health claims for top products, some of them very popular among consumers, because of insufficient scientific evidence, can have the contrary effect. So, it is advisable to reinforce the transparency on the information to consumers to avoid a bad global perception of the functional foods products.

1.2.3 Association of the Industry and the Scientific Community

It must not be forgotten that because of its innovative component, functional foods have been possible, thanks to the arousal of new knowledge about the relationship between ingredients and health, which has mainly been provided by the advances in the scientific research on the subject.

Innovative concepts in the area may thus emerge from academic researchers, although they often are not aware of how to translate these ideas into final marketable food products [38]. In consequence, most frequently it is the industry that initiates the cycle of development, but working closely with academics (for example, through the formation of scientific advisory boards) to provide a solid ground to design a new functionality/ingredient, as well as to conduct the series of safety, efficacy, and bioavailability tests to prove the applicability of the new designs. This cycle of innovation can lead to breakthrough products, which can be defined as products, which provide a company with greater profits and longer-lasting competitive advantage in the marketplace in comparison to minor product development or line extensions [39], that can be as well a good and easier way to include a health claim in food products. But while the first case would in general be early-movers, in the second case, normally, it would be companies deciding to approach the functional food market in response to its astonishing growth [40]. This difference in the degree of innovation is well recognized in the UE Regulation 1924/2006, which distinguishes innovative products (Article 13.5), while at the same time provides a list (Article 13.1) that already states more usual ingredients-activities relationships.

2 Market Prospect of Functional Foods

2.1 Global Sales: A Steady Progression

Many market studies of global sales of functional foods differ in the data, depending on the criteria used for the inclusion of products in the analysis. For example, under a strict definition, according to Leatherheadfood, the functional food and drinks market had a combined value of $19.4 billion in 2007, whereas with a broader definition, the market raised to $41.9 billion [41]. In 2011, the global market for a strict definition of functional products as those offering specific health claims was estimated at $24.2 billion [42]. BCC research established a Nutraceuticals Global Market for nutraceutical foods, nutraceutical beverages, and nutraceutical supplements of $40 billion each.

Another source of confusion for the precise definition of the market size is that many functional ingredients were initially included in foods for reasons other than their health-promoting values. For example, the use of antioxidants for food preservation purposes was prior to their commercialization as health-enhancing functional additives, and bioactive compounds such as anthocyanins from red berries or lycopene from tomato were more appreciated because of their application as natural colorants rather than because of their biological activity against free radicals. Obviously, the higher added-value obtained because of their healthy properties has meant a faster growing development of these products. In 2007, global sales of antioxidants used in the manufacture of foods amounted to $788 million, representing a constant yearly increase of 3 %. Functional antioxidants in 2007 already accounted for the majority of sales, holding a value share of almost 56 %, with antioxidants used for food preservation purposes making up the remainder [43].

Despite this lack of precision concerning the data, functional foods have undeniably been reported as one of the top trends facing the food industry. They have been especially active since the last decade, when the annual growth rate of the functional foods market ranged from 15 % to 20 % at the end of the 1990s [29]. The global functional food market in 2002 was roughly estimated to be between $10 billion and $40 billion with an annual increase of about 8 % [39]. In 2008, when using a definition of functional foods that comprised all "products bearing a health claim," this group of products had arisen as the fastest-growing sector of the food market, with estimates forecasting an expected annual growth rate of 10 % for functional foods as compared to an average 2–3 % for the food industry as a whole [44]. Although growth rates estimates have decreased over time, the numbers remain impressive compared to growth rates for the food industry as a whole.

Even in the actual situation of economic recession, which should compromise the willingness of consumers to buy more expensive foods, recent estimates remain optimistic. Consumer interest in functional foods remains strong, and the rising costs of healthcare and the needs of an aging population should still encourage the consumers' commitment to health and wellness pursued through the diet. Recent data from 2007 by Pricewaterhouse Coopers [45] predicted that functional foods in the USA could grow by up to 20 % or five times that of the food industry as a whole [46], which is consistent with other reports by Leatherhead [47] that products making specific health claims –not including neither energy and mood drinks, nor food supplements – are predicted to grow at 4–5 % for the next few years [48]. According to market analyst Freedonia, demand for nutraceutical ingredients like botanicals, vitamins, minerals, and omega-3 s will grow 7.2 % annually until at least 2015 to be worth €18.5 billion with newer markets like Mexico and South Korea helping drive growth in the sector [49]. And a report by Global Industrial Analysts projected the global nutraceuticals market projected to exceed US$243 billion by 2015 [50].

Figure 80.1 shows this evolution in the functional foods and beverages market size.

Concerning differences between countries, according to Datamonitor, some of 90 % of total sales occurs in Europe, the USA, and Japan [51]. In Japan, according to a Leatherhead report utilizing tight functional food definitions, global sales in 2010 reached $24.22 billion, which would represent the 38.4 % of the global functional market, followed by the USA with 31.1 % and Europe (28.9 %) [48].

In the USA, with between $20 billion and $30 billion in sales a year, functional foods comprise about 5 % of the entire US food market [52].

In Europe, Germany, France, United Kingdom, and the Netherlands represent the most important countries within the functional foods market, but many other European markets are experiencing high growth rates, such as the Netherlands and Spain.

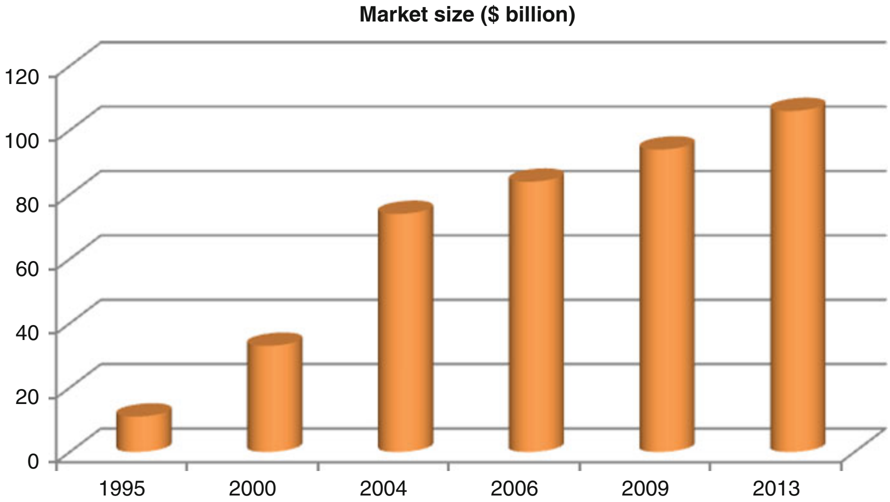


Fig. 80.1 Global market size of functional foods (Sources: New Nutrition Business (1995, 2000) [53]; Euromonitor International (2004, 2006, 2009) [54]; BCC Research (2013) (http://www.bccresearch.com/report/nutraceuticals-processing-markets-fod013c.html))

In addition, newly emerging markets like Hungary, Poland, and Russia are also well positioned [36].

2.2 Situation of Specific Product Launches, Ingredients, and Claims

The growth of the functional food market has been correlated with a steadying increase of the number of functional food products launches. According to Mintel analysts, the global launches of functional products between 2005 and 2009 were more than doubled, from 904 to 1859. Between 2008 and the first half of 2009, USA was the leader in healthy product launches (881 products), followed by Japan (314), Italy (325), UK (237), Germany (235), and France (150) [46].

The main functional foods category correspond to dairy products, that account for the 38 % of the market, followed by bakery and cereals (22.7 %), beverages (12.5 %), meat, fats and oils (8.1 %), fish and eggs (7.4 %), and soy products (5.8 %). In most cases, there has been a significant growth in the market. For example, according to Euromonitor the global market for pre- and probiotic spoonable yogurt had a growth of 128 % between 2004 and 2009, from $3.3 billion to $7.6 billion, while for drinking yogurt, it grew a 44 % to $11.2 billion [55]. Sales of functional spreadable oils and fats grew 54 %.

Classified according to health claims made on the product, digestive health has been the most used claim for new products (Fig. 80.2) [56].

It is relevant to state that food traditions and cultural heritage influence the interest of consumers in functional foods. For example, despite dairy is the biggest

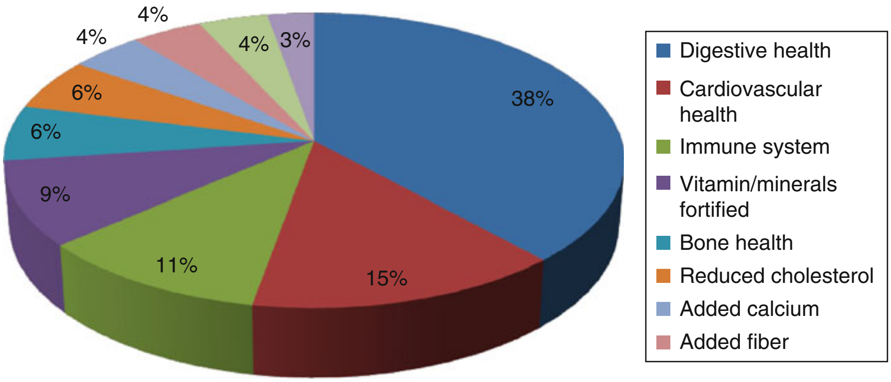


Fig. 80.2 Global distribution of health claims in functional products launches between 2005 and 2009 (Source: Mintel's Global New Products Database)

segment in the global market, in the USA, this predominance is for functional beverages, including energy and mood beverages. According to Leatherhead Food International, functional drinks account for 50 % of the US functional food market, followed by cereal products [48]. Another characteristic of the USA is that consumer expenditures on nutraceuticals are especially active, having reached a reported $20.50 billion in 2004, more than double the amount spent in 2004. Supplementation is also quite popular in Japan, whereas European consumers are less driven to these kinds of products. Another example of regional particularities is the fact that Japan is the world's largest market for green tea sold in leaf, which accounts for nearly 65 % of total leaf tea sales in the domestic market [43].

Concerning claims, digestive health is not the top claim in the USA, this honor belonging to cardiovascular health. On the opposite side, cardiovascular health claims are quite reduced in Japan (less than 10 % of the new functional foods launches). In the UK, cardiovascular health claims have represented a 20 % of the 530 new products between 2005 and 2009 [58].

2.3 New Perspectives

Despite the continuous introduction of new functional food or beverage products to the market, in the last few years, a certain reduction in the number of launches for some of the functional ingredients has been observed. Mintel reported in 2010 a slowdown in the market for energy drinks and fortified waters in the USA [57]. Figure 80.3 shows the reduction on the number of new products with claims related to the polyphenols family, according to Datamonitor. This reduction that can be attributed to different factors such as the development of new legislations such as the recent implementation of the UE 1924/2006 Regulation, that is limiting the use of nutrition and health claims by establishing the need of a strong substantiation of the evidence of the claimed effect. This has led to the dismissal of several health

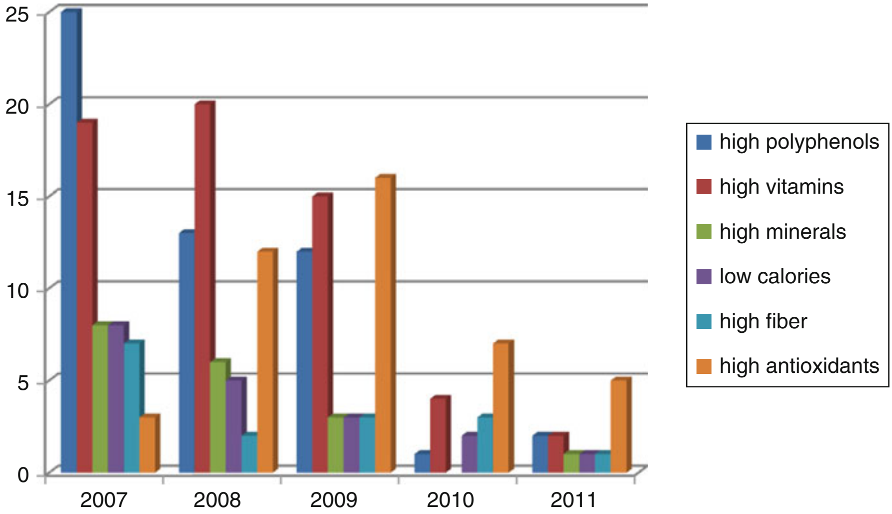


Fig. 80.3 Number of global launches of ingredients with polyphenols associated to different claims (Source: Datamonitor)

claims for many products, especially in areas like natural plant extracts or probiotics. This will mean a reduction on both the number of functional foods as well as their market value, at least according to the definition of functional foods as those wearing a nutrition or health claim. For example, there was an 11 % drop in the number of health claim-bearing launches between 2009 and 2010, even if the transition period for many products was still applied in the UE.

Additionally, in a global economic situation of recession, food companies can find difficulties to assume the costs associated to the development and scientific substantiation of evidence for new products, especially for cases that could imply expensive research.

The difference of the economic impact between products with health claims compared to those without them is uncertain. For example, in the USA in 2000, the market for functional foods with specific health claims achieved a turnover of around $0.5 billion, while functional foods without claims had an annual turnover of at least $15 billion [13].

3 Functional Foods Under Scrutiny: Situation of Natural Products Relating to the Substantiation of Evidence

3.1 Regulation of Functional Foods and Nutraceuticals Market

To ensure that the products are not misleading consumers, governments from various countries have developed regulation systems. Normally there exist associated organizations that take care of the accuracy of the claims used for functional foods and nutraceuticals. Some of these organizations are the European Authority of Food Safety (EFSA) in Europe, the Food and Drug Administration (FDA) in the United States, or the Ministry of Health, Labour and Welfare (MHLW) in Japan. We will discuss the current situation of the USA and Europe in relation to the approval of claims.

3.1.1 USA

In the USA, claims on foods and dietary supplements can belong to three different categories: nutrient content claims, structure/function claims and health claims.

Structure/function claims can be used for dietary supplements, and describe the effect on the structure or function of the body [8, 58].

Nutrient content claims can be considered as "expressed nutrient content claims," which is any direct statement about the level (or range) of a nutrient in the food, or as "implied nutrient content claims," which denotes any claim describing the food or an ingredient therein in a manner that suggests that a nutrient is absent or present in a certain amount or suggests that the food, because of its nutrient content, may be useful in maintaining healthy dietary practices. Annex Table 80.3 shows the nutrient content claims of the USA.

Health claim means any claim (including statements, symbols, vignettes, etc.), made on the label or in labeling of a food or dietary supplement, that characterizes the relationship of any substance to a disease or health-related condition.

In their origin (1990), health claims had to be based on a very high standard of scientific evidence, evaluated by the FDA. However, after the result from a 1999 Court of Appeals Decision, Pearson v. Shalala, the *Qualified Health Claims* were introduced for substance/diseases relationships with lower standards of evidence, that is considered credible but without reaching a significant scientific agreement standard. In the case of Qualified Health Claims, the proposed claim has to include qualifying language that identifies limits to the level of scientific evidence to support the relationship [59].

Table 80.2 compiles the Health Claims and Qualified Health Claims approved by the FDA.

3.1.2 European Union

The EU Regulation 1924/2006 [60] distinguishes two types of claims:

Nutrition claims are claims that state, suggest, or imply that a food has particular beneficial nutritional properties due to the energy it provides or the nutrients it contains [61]. Annex Table 80.4 compiles these claims.

Health claims are any claim that state, suggest, or imply that a relationship exists between a food category, a food, or one of its constituents and health. *Reduction disease risk claims* are health claims that state, suggest, or imply that the consumption of a food category, a food, or one of its constituents significantly reduces a risk factor in the development of a human disease.

The regulation classifies health claims into three main types. Article 13.1 claims pertain to "general function" claims relating to growth, development, and functions

Table 80.2 Health claims and qualified health claims approved by the FDA

Health claims	
Calcium, Vitamin D	Osteoporosis
Dietary lipids (fat)	Cancer
Dietary saturated fat and cholesterol	Risk of coronary heart disease
Dietary non-cariogenic carbohydrate sweeteners	Dental caries
Fiber-containing grain products, fruits and vegetables	Cancer
Folic acid	Neural tube defects
Fruits and vegetables	Cancer
Fruits, vegetables and grain products that contain fiber, particularly soluble fiber,	Risk of coronary heart disease
Sodium	Hypertension
Soluble fiber from certain foods (whole oat, barley, psyllium seed husk)	Risk of coronary heart disease
Soy protein	Risk of coronary heart disease
Oatrim	Risk of coronary heart disease
Stanols/Sterols	Risk of coronary heart disease
Qualified health claims	
Tomatoes and/or tomato sauce	Prostate, ovarian, gastric, and pancreatic cancer risk
Calcium	Colon/Rectal cancer & calcium and recurrent colon/ Rectal polyps risk
Green tea	Cancer risk
Selenium	Cancer risk
Antioxidant vitamins	Cancer risk
Nuts	Heart disease
Walnuts	Heart disease
Omega-3 fatty acids	Coronary heart disease
B vitamins	Vascular disease
Monounsaturated fatty acids from olive oil	Coronary heart disease
Unsaturated fatty acids from canola oil	Coronary heart disease
Corn Oil	Heart disease
Phosphatidylserine	Cognitive dysfunction and dementia
Chromium picolinate	Diabetes
Calcium	Hypertension, pregnancy-induced hypertension, and preeclampsia
0.8 mg folic acid	Neural tube birth defects

of the body. They should be based on generally accepted evidence and could be used by any manufacturers as long as the conditions of use are kept. Article 13.5 claims pertain to general function claims based on new and/or proprietary data. This type of claim is particularly relevant for manufacturers who have invested in innovation and wish to protect their claim and/or underpinning scientific data [62].

Article 14 claims refer to risk reduction claims or claims related to children's health and development.

According to the Regulation, the European Food and Safety Authority (EFSA) is the consulting organization for the analysis of the scientific substantiation of evidence of Health Claims. Its evaluating process, which started after the implementation of the Regulation, will imply a change in the allowed health claims for functional foods in Europe. The Fig. 80.4 compares, for some of the most popular ingredients, the successes and failures on obtaining a positive opinion about the scientific substantiation of evidence for the ingredient's intake and health relationship.

As it can be inferred from EFSA opinions, while there are some ingredients with well-established relationships of cause and effect between their intake and the claimed effects, for many other compounds, this association is much less proven.

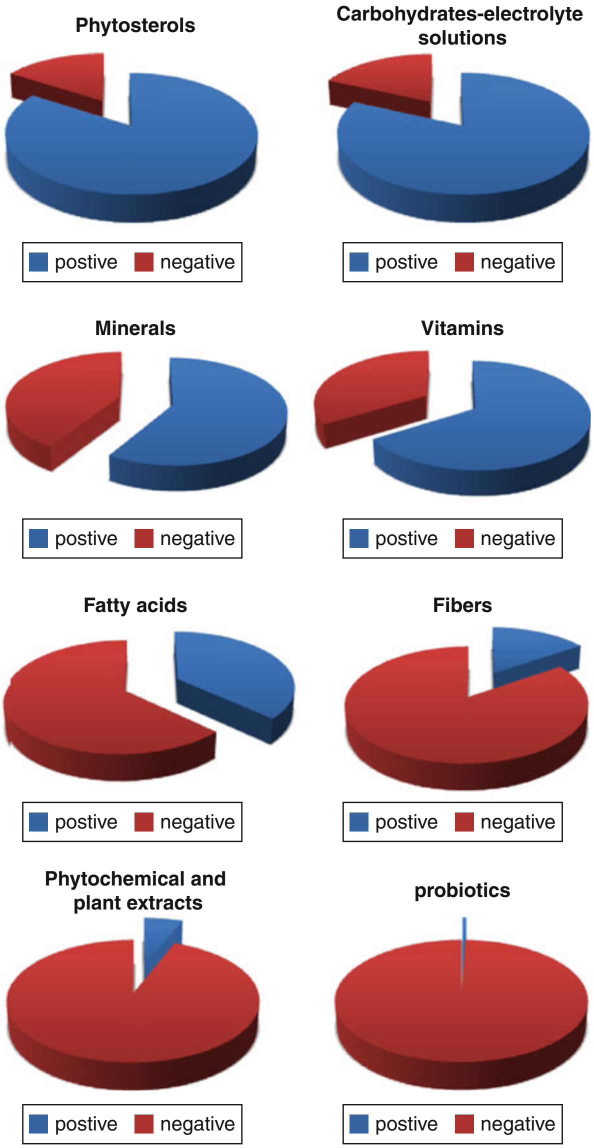
On the positive side, with a high percentage of success, the cholesterol-lowering effect of phytosterols, the role of carbohydrate-electrolyte solutions for the enhancement of water absorption during physical exercise, or the importance of vitamins and minerals toward different processes is well accepted.

In opposition, the cause-effect relationship of probiotics, prebiotic fiber, and phytochemicals (including plant extracts), among other ingredients, has commonly been considered as insufficiently substantiated. For probiotics, an inadequate identification of the probiotic strains, as well as the difficulty to correlate the changes in microflora with a beneficial physiological outcome, can be stated as the main reasons for most of the negative opinions emitted. In the case of phytochemicals, the characterization is also a main reason of rejection, together with the quality of the studies presented and the relevance of biomarkers used to sustain the effect.

Other ingredients show a more equilibrated balance between positive and negative opinions. With reference to fatty acids, positive opinions, for example, in the case of some omega-3 fatty acids like docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), or linolenic acid and their role in maintenance of triglycerides levels, and for long-chain omega-3 fatty acids maintenance and development of vision and brain function have been published. In contrast, the evidence for conjugated linolenic acids or gamma-linolenic acids has been considered unsatisfactory. Concerning fibers, the importance of distinguishing the effects depending on the concrete fiber has been established. For example, the maintenance of normal blood cholesterol levels is well accepted for beta-glucans, glucomannans, or guar gum, but not for acacia gum or isomalto-oligosaccharides. The role of soluble and insoluble fibers is well known to be different, and in consequence, concrete claims related to non-well characterized fibers usually lack enough scientific evidence. For example, reduction on many claims for various foods related to glycaemic index control has been dismissed because of an inadequate definition of the type or carbohydrates.

It is out of the scope of this chapter to make an exhaustive analysis of the scientific gaps that have been found by the EFSA panel. However, they provide

Fig. 80.4 Percentage of positive and negative opinion for different families of ingredients published by EFSA (Source: EFSA Journal, 2008–July 2012)



valuable lessons for the future development of future functional foods, and will undeniably have an impact on the market as well as on the approach to scientific evidence substantiation studies. In consequence, we will analyze some of the key aspects, focused on the families of plant extracts and phytochemicals, that scientists must take into account in order to assist in these developments.

3.2 EFSA Opinions on Phytochemicals and Plant Extracts

In the case of plant extracts and bioactive compounds deriving from them, the levels of success throughout the process of evaluation of health claims by EFSA have been modest at the best. Concerning phytochemicals, between 2008 and July 2012, very few compounds have obtained a positive opinion:

- Caffeine (from guarana, tea, chocolate, coffee, or as a pure form) and increased attention/alertness, reduction in the perceived effort, and increase in endurance capacity and physical performance during short-term high-intensity exercise.
- Polyphenols in olive and protection of LDL particles from oxidative damage.
- Monacolin K from red yeast rice and maintenance of normal LDL-cholesterol concentrations.
- Beta-carotene and maintenance of the normal function of the immune system.
- Cocoa flavanols and maintenance of normal endothelium-dependent vasodilation.

Other popular compounds such as lycopene, lutein, resveratrol, quercetin, catechins from tea, grapes, or cranberries, or soy isoflavones have not obtained a favorable opinion, except in cases where they were actually associated with other compounds that had already proven to have an effect like vitamin E or C.

Concerning plant extracts, many of them have been kept on hold by the European legislation. Prior to this reschedule, all those evaluated by the provisions of Article 13.1 obtained a negative opinion. But at the same time, stories of success have also arisen. It is an illustrative example the case of the tomato WSC extract, whose effect over platelets aggregation was well established by pertinent and company-proprietary clinical studies and was worth of being the first accepted ingredient via the Article 13.5.

This example illustrates how, although there have been more deceptions than triumphs in the evaluation of scientific evidence provided by plant extracts and phytochemicals, these results, rather than being seen as a disappointment, can be considered an excellent opportunity for manufacturers to invest in scientific research in order to complete standardized quality studies that clearly establish a cause-relation effect between the ingredient intake and the claimed effect, and thus obtaining specific claims according to the article 13.5 of the 1924/2006 Regulation.

3.3 Lessons Learned: Key Scientific Aspects to be Controlled for Obtaining a Health Claim

Following the lessons learned by the EFSA evaluation of health claims, 3 different aspects have to be assessed for obtaining a health claim:

1. Characterization of the ingredients
2. Relevance of the sustained claimed effect
3. Scientific evidence provided by efficacy studies

3.3.1 Characterization

Although many of the functional ingredients (vitamins, minerals, omega-3 fatty acids, etc.) possess reliable methodologies of quantification, this aspect is sometimes incomplete for plant extracts. In some cases, there was no reference to the content of bioactive compounds, the part of the plant used for the preparation, no indication of diary doses, or the extracts were used as multibotanical combinations without specification of the other components.

One of the main reasons for this lack of concretion is the complexity of plant matrices, whose diversified secondary metabolism includes a vast number of different compounds with close structures that can be hard to identify. Some of these families with a well-documented bibliography related to health effects comprise alkaloids, phenolic compounds (including phenolic acids, stilbenes like resveratrol, or flavonoids such as anthocyanins, procyanidins, or isoflavones), terpenoids, carotenoids, sulfur compounds (such as glucosinolates and isothiocyanates), etc. Their presence and amount in the plant source depend on multiple factors including variety, organ of the plant, soil, sun exposure, climate, or even ways of cultivation.

There are several fast tests that estimate the content of these compounds as a whole by spectrophotometric methodologies. However, it is well stated that many of these compounds interact with the metabolic pathways and exert their effect on a structure-dependent manner. So, for understanding the mechanism of action of an extract, and more important, to obtain standardized extracts on the bioactive principles, the development of validated methodologies of identification, analysis, and quantification of individual components should be mandatory.

3.3.2 Relevance of the Claimed Effect

Another aspect that must not be overlooked is the nature of the intended claim. The use of clinical claims should be avoided (these could fall under the scope of the Directive 2004/24/EC for traditional herbal medicinal products, but not for health claims on foods). Claims too vague and unspecific also fall out of the consideration of the Regulation. It would be the case of "energy and vitality," "tonic," or "detoxification," often used in plant extracts submissions.

Finally, it has to be assessed that the claimed effect has significance to human health. In this aspect, it is worthy to make a detailed analysis of the claims related to antioxidants.

"Antioxidant" Claims

More than half of the claimed effects for phytochemicals and plant extracts are related to their protective effect as antioxidants, and many products include this term on their presentations for marketing purposes.

In fact, a first discussion should contemplate the adequateness of the use of the term "antioxidants" as a nutrition claim. There are main differences between UE and US legislations.

In the USA, it has been finally included in the US Food Labeling Part of the Code of Regulations, § 101.54 (f), which states that "a nutrient content claim that characterizes the level of antioxidant nutrients present in a food may be used on the label or in the labeling of that food when: An RDI has been established for each of the nutrients; The nutrients that are the subject of the claim have recognized antioxidant activity; that is, when there exists scientific evidence that, following absorption from the gastrointestinal tract, the substance participates in physiological, biochemical, or cellular processes that inactivate free radicals or prevent free radical-initiated chemical reactions; The level of each nutrient that is the subject of the claim is sufficient to qualify for the claim The names of the nutrients that are the subject of the claim are included as part of the claim (e.g., – high in antioxidant vitamins C and E||)."

In contraposition, the term "antioxidants" as a nutrition claim has not been included in the corresponding Annex for Nutrition Claims of the UE 1924/2006 Regulation or its amendments. In consequence, for submitting an antioxidant claim, it should be submitted as a health claim, and thus, the significance of the effect for human health has to be considered. And, in this case, the terminology used is of importance.

- "Antioxidant activity, antioxidant capacity, antioxidant properties": These claims would refer to the capacity of food/constituents to scavenge free radicals and/or to their reducing capacity, normally in in vitro models. Since there is no evidence that having antioxidant activity/content and/or antioxidant properties is a beneficial physiological effect on human health (factors such as bioavailability would affect the effects in human), it cannot be considered an acceptable claim [63].
- "Protection of DNA, proteins, and lipids from oxidative damage." This wording reflects correctly and effect that is significant for human health, and so it is acceptable.

3.3.3 Providing the Evidence from Science

The important objective for the development of health claims is to ensure that claims for food components and nutraceuticals are properly justified and they are scientifically substantiated [64]. The evidence provided has to be sufficient to establish a cause and effect relationship between the consumption of the ingredient and the claimed effect. For phytochemicals and plant extracts, it is often not the case. One of the main problems is that in many cases, only in vitro or animal studies have been conducted to sustain the evidence of the claim, or the human data consist of epidemiological studies. Information related to the pharmacopeia alone, often used for the plant extracts submissions, is also insufficient.

Substantiation of a claim should be based on human data, primarily from well-designed intervention studies considering target population, appropriate controls, adequate duration of exposure, and follow-up to demonstrate the intended effect [17]. Randomized clinical trials are the standard trials for providing evidence.

The design and quality of the human studies conducted is a key point to obtain a positive opinion about the scientific substantiation of the effect. Common mistakes include an inadequate choice of outcomes measured to assess the effect or the use of nonvalidated biomarkers, recruiting individuals not representative of the target population, or conducting studies with products that differ from the ingredient object of the claim.

The major factors involved in the design, conduct, and reporting of human studies can be adapted from the Consolidated Standards of Reporting Trials (CONSORT) checklist for medical trials [65]. Recently, the ILSI has published their own guidelines for the design, conduct, and reporting of human intervention studies to evaluate the health benefits of foods [66].

4 The Impact of New Methodologies for the Assessment of Functional Foods

4.1 Hyphenated Methods for the Characterization of Ingredients

The role of advances in chromatographic techniques has been a step point in the development of phytochemistry [67]. Because of the complexity of crude herbal extracts, various online hyphenated techniques have been developed for the analysis of the complex mixtures. These techniques include liquid chromatography (LC), mass spectrometry (MS), LC nuclear magnetic resonance (NMR), and LC-NMR-MS [68]. They facilitate the structure determination of unknown constituents in crude extracts. For example, they are of great applicability in the analysis of flavonoids and other phenolic compounds [69, 70].

Regarding MS instruments, time-of-flight detectors provide elevated mass resolution and accuracy over a broad mass of range, and thus help structural elucidation of nontargeted compounds based on accurate mass measurements and isotopic patterns. Triple quadrupole instruments are particularly well suited for targeted analysis and provide excellent sensitive and selectivity by performing tandem MS/MS analysis [71].

Despite the prominence attributed in scientific research to these hyphenated methodologies, other techniques are worth to be considered in the identification of bioactive vegetal compounds. High-performance thin-layer chromatography (HPTLC) is an evolution of thin layer chromatography (TLC) that remains the sole technique in which all the components of the sample are included in the chromatogram and presents the results as an image [67]. In HPLC, irreversible adsorption of some compounds can occur in the stationary phase, which in consequence cannot be eluted or detected, while TLC and HPTLC avoid this problem. Another technique avoiding this irreversible adsorption is countercurrent chromatography, which is an all-liquid separation technique which relies on the partition of a sample between two immiscible solvents [67], and can be used for the fractionation of crude plant extracts (in multigram quantities) or for final purification steps.

4.2 Bioinformatics

In several aspects, the approach toward finding a new functional ingredient is quite similar to the development of new pharmaceuticals. Bioinformatics tools have been largely employed as the first steps for drug design and recently, they are beginning to be used in connection with food or food-related components in several areas of food chemistry [72]. It is widely reported that natural compounds in the diet can improve health conditions and prevent disease by direct interaction with key proteins in metabolic pathways. For example, pure monacolin K (lovastatin) has been shown to be effective in reducing total cholesterol and LDL-cholesterol concentrations in individuals with hypercholesterolemia and is a well-known inhibitor of HMG-CoA reductase.

Studies of molecular similarity, pharmacophore modeling, molecular docking, and quantitative structure-analysis relationships (QSAR), based on in silico calculations, can be good screening methodologies for selecting those ingredients with the best predicted probability to interact with those targets and to predict their activity. At the same time, they can assist in formulating theoretical plausible mechanisms of action for the ingredients.

Molecular similarity searches are based on the hypothesis that similar molecules will have similar properties. In pharmacophore modeling, the chemical features and conformation of the modulating ligands in relation to the target proteins is extracted from 3D structures, and in consequence the response of multiple ingredients can be screened depending on their fitting with this model. Molecular docking actually calculates the best conformation of a molecule to fit into the target-binding pocket. In the case of QSAR models, a prediction of the activity of molecules according to 2D or 3D descriptors is obtained after establishing a model with experimentally measured outcomes.

4.3 Omics Data

One of the biggest challenges in nutrition is the establishment of adequate biomarkers that are able to predict health benefits, as well as early indicators for disease risk [73]. Nowadays, new -omics technologies are used in nutrition research, giving access to holistic discovery of efficacy biomarkers by transcriptomics, proteomics, and metabolomics data. They are considered by the ILSI as emerging technologies for efficacy demonstration [74].

Transcriptomic studies, which analyze gene expression, have improved the understanding of the complex interaction between genetic and environmental factors, such as lifestyle and nutrition. Transcriptomic technologies have arguably achieved the highest level of technical maturity of any of the functional genomics. Production of very high-quality, genome-wide expression profiling data by DNA microarrays is now a routine matter.

Proteomics is a central platform in elucidating the molecular events in nutrition: It can identify and quantify bioactive proteins and peptides and address questions of nutritional bioefficacy. The advances in methods of separation of peptides by microflow and nanoflows or chip detections, as well as mass spectrometry–rooted proteomic techniques like MALDI-TOF for protein identification and quantification have been pivotal in the application of these methodologies to understand nutritional effects of ingredients [75].

Metabolomics is the comprehensive analysis of metabolites and has gained a strong impact on nutritional research [76]. The great asset of these methodologies is the quantitative, noninvasive analysis of easily accessible human body fluids like urine, blood, saliva, and tears. The metabolome is complex, and thus requires multiple highly sophisticated techniques of separation and identification, such as NMR and MS. Additionally, the use of chemometrics for analyzing the complex data obtained is mandatory [73].

5 Conclusions

The functional foods and nutraceuticals market is one of the healthiest sectors in food industry, and is experiencing a continuous growth even in the context of an economic recession. However, to keep being successful, it is mandatory to assure the acceptance and awareness of consumers. The presence of many ingredients whose efficacy can be doubted can first mislead consumers and later compromise the credibility of the whole functional foods concept.

In consequence, manufacturers have to collaborate actively with academic researchers in order to provide the scientific evidence to substantiate health claims. This substantiation has to address three main points: the characterization of the ingredient, the relevance of the claimed effect, and the establishment of a cause and effect relationship between the ingredient’s intake and the claimed effect by efficacy studies on human intervention trials. These trials have to follow strict criteria of quality and design in order to be successful, especially by choosing adequate participants, outcomes, and biomarkers.

At the same time, scientific research is very important for the development of new functional foods. Discoveries provided by holistic techniques like -omics methodologies are allowing a better comprehension of the effects of nutrition and the interaction with the human metabolism. This will allow to define new biomarkers, especially early indicators of disease risk, that will contribute to designing new functional products.

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Annexes

Table 80.3 Nutrient content claims approved In the USA

Nutrient content claims for protein, vitamins, minerals, dietary fiber, antioxidants in relation to the reference intake value or daily reference value	
"High," "Rich in," "Excellent source of"	20 % or more of the Reference Daily Intake (RDI) or the Daily Reference Value (DRV) per reference amount customarily consumed
"Good source," "Contains," "Provides"	10–19 % of the RDI or the DRV per reference amount customarily consumed
"High in fiber," "Good source of fiber," "more fiber"	If the food is not "low" in total fat, then the label shall disclose the level of total fat per labeled serving.
"More," "Fortified," "Enriched," "added," "Extra," "Plus" for Protein, Vitamin, Minerals, Dietary Fiber, Potassium" (Realtive claims)	10 % more of the RDI (Vitamins, minerals) of the RDI or the DRV per reference amount customarily consumed/per 100 g of food than an appropriate food
	10 % more of the DRV (Protein, Dietary fiber, potassium) of the RDI or the DRV per reference amount customarily consumed/per 100 g of food than an appropriate food
"High potency"	Individual vitamins or minerals at 100 % or more of the RDI per reference amount customarily consumed
High/Good source/More "Antioxidant"	An RDI has to be established for antioxidant nutrients
Nutrient content claims for "light" or "lite"	
"Light," "Lite" when the food derives 50 % or more of its calories from fat	Fat content reduced by 50 % or more per reference amount customarily consumed compared to an appropriate reference food
"Light," "Lite" when the food derives less than 50 % of its calories from fat	The number of calories is reduced by at least 33.33 %
	The fat content is reduced by 50 % or more per reference amount customarily consumed compared to an appropriate reference food
"Light," "Lite" for a product whose reference food contains 40 cal or less and 3 g fat or less per reference amount customarily consumed	Sodium content reduced by 50 % or more
"Light in sodium," "Lite in sodium" for a product whose reference food that contains more than 40 cal or more than 3 g fat or less per reference amount customarily consumed	Sodium content reduced by 50 % or more
"lightly salted"	50 % less sodium than which is normally added to the reference food, indicating when the product is "not low in sodium"
Nutrient content claims for the calorie content of foods	
"Calorie free," "Free of calories," "No calories," "Zero calories," "Without calories," "Trivial source of calories," "Negligible source of calories," "Dietarily insignificant source of calories"	The food contains less than 5 cal per reference amount customarily consumed and per labeled serving.

(*continued*)

Table 80.3 (continued)

"Low calorie," "Few calories," "Contains a small amount of calories," "Low source of calories," "Low in calories"	(a) The food has a reference amount customarily consumed greater than 30 gram (g) or greater than 2 tablespoons and does not provide more than 40 cal per reference amount customarily consumed (b) The food has a reference amount customarily consumed of 30 g or less or 2 tablespoons or less and does not provide more than 40 cal per reference amount customarily consumed and per 50 g (except for sugar substitutes) (c) For mails, if the product contains 120 cal or less per 100 g
"Reduced calorie," "Reduced in calories," "Calorie reduced," "Fewer calories," "Lower calorie," "Lower in calories"	The food contains at least 25 % fewer calories per reference amount customarily consumed than appropriate reference food
Sugar content claims	
"Sugar free," "free of sugar," "No sugar," "Zero sugar," "Without sugar," "Sugarless," "Trivial source of sugar," "Negligible source of sugar," "Dietarily insignificant source of sugar"	The food contains less than 0.5 of sugars per reference amount customarily consumed and per labeled serving
"No added sugar"; "Without added sugar"; "No sugar added"	No amounts of sugars, or any other ingredient that contains sugars that functionally substitute for added sugars is added during processing or packaging[. . .] The food that it resembles and for which it substitutes normally contents added sugars[. . .]
"Reduced sugar," "Reduced in sugar," "less sugar," "lower sugar," "Lower in sugar"	The food contains at least 25 % less sugar per reference amount customarily consumed than an appropriate reference food
Nutrient content claims for the sodium content of foods	
"Sodium free"; "Free of sodium," "Zero sodium," "Without sodium," "Trivial source of sodium," "Negligible source of sodium," "Dietary insignificant source of sodium"	The food contains less than 5 mg of sodium per reference amount customarily consumed and per labeled serving
"Very low sodium," "Very low in sodium"	The food has a reference amount customarily consumed greater than 30 g and contains 35 mg or less sodium per reference amount customarily consumed
"Low sodium," "Low in sodium," "little sodium," "contains a small amount of sodium," "low source of sodium"	The food has a reference amount customarily consumed greater than 30 g and contains 140 mg or less sodium per reference amount customarily consumed
"Reduced sodium," "Reduced in sodium," "Sodium reduced," "Less sodium," "Lower sodium," "Lower in sodium"	The food contains at least 25 % less sodium per reference amount customarily consumed than an appropriate reference food
"Salt free"	Only if the food is "sodium free"

(*continued*)

Table 80.3 (continued)

“Without added salt,” “Unsalted,” “No salt,” “No salt added”	No salt is added during processing; the food that it resembles and for which it substitutes is normally processed with salt; and if the food is not sodium free, it includes a statement “not a sodium free food” or “not for control of sodium in the diet”
Nutrient claims for fat, fatty acid, and cholesterol content of foods	
“Fat free,” “Free of fat,” “No fat,” “Zero fat,” “Without fat,” “negligible source of fat,” “Dietarily insignificant source of fat”	The food contains less than 0.5 g of fat per reference amount customarily consumed and per labeled serving or, in the case of a meal product or main dish product, less than 0.5 g of fat per labeled serving; and
	The food contains no added ingredient that is a fat or is generally understood by consumers to contain fat unless the listing of the ingredient in the ingredient statement is followed by an asterisk that refers to the statement below the list of ingredients, which states “adds a trivial amount of fat,” “adds a negligible amount of fat,” or “adds a dietarily insignificant amount of fat;” and
	If the food meets these conditions without the benefit of special processing, alteration, formulation, or reformulation to lower fat content, it is labeled to disclose that fat is not usually present in the food (e.g., “broccoli, a fat-free food”)
“Low fat”; “Low in fat,” “Contains a small amount of fat”; “Low source of fat”; “Little fat”	The food has a reference amount customarily consumed greater than 30 g or greater than 2 tablespoons and contains 3 g or less of fat per reference amount customarily consumed; or
	The food has a reference amount of 30 g or less or 2 tablespoons or less customarily consumed and contains 3 g or less of fat per reference amount customarily consumed and per 50 g of food (for dehydrated foods that must be reconstituted before typical consumption with water or a diluent containing an insignificant amount of all nutrients per reference amount customarily consumed, the per 50-g criterion refers to the “as prepared” form); and
	If the food meets these conditions without the benefit of special processing, alteration, formulation, or reformulation to lower fat content, it is labeled to clearly refer to all foods of its type and not merely to the particular brand to which the label attaches (e.g., “frozen perch, a low fat food”).
“Reduced fat”; “Reduced in fat”; “Fat reduced”; “Less fat”; “Lower fat”; “Lower in fat”	The food contains at least 25 % less fat per reference amount customarily consumed than an appropriate reference
“X % fat free”	The food meets the criteria for “low fat.” A “100 % fat free” claim may be made only on foods that meet the criteria for “fat free,” that contain less than 0.5 g of fat per 100 g, and that contain no added fat

(*continued*)

Table 80.3 (continued)

"Saturated fat free," "free of saturated fat," "no saturated fat," "zero saturated fat," "without saturated fat," "trivial source of saturated fat," "negligible source of saturated fat," or "dietarily insignificant source of saturated fat"	The food contains less than 0.5 g of saturated fat and less than 0.5 g trans fatty acid per reference amount customarily consumed and per labeled serving and
	The food contains no ingredient that is generally understood by consumers to contain saturated fat (unless the listing of the ingredient in the ingredient statement is followed by an asterisk that refers to the statement below the list of ingredients which states, "adds a trivial amount of saturated fat," "adds a negligible amount of saturated fat," or "adds a dietarily insignificant amount of saturated fat," and
	If the food meets these conditions without the benefit of special processing, alteration, formulation, or reformulation to lower saturated fat content, it is labeled to disclose that saturated fat is not usually present in the food.
"Low in saturated fat," "low saturated fat," "contains a small amount of saturated fat," "low source of saturated fat," or "a little saturated fat"	The food contains 1 g or less of saturated fatty acids per reference amount customarily consumed and not more than 15 % of calories from saturated fatty acids.
	If a food meets these conditions without benefit of special processing, alteration, formulation, or reformulation to lower saturated fat content, it is labeled to clearly refer to all foods of its type and not merely to the particular brand to which the label attaches (e.g., "raspberries, a low saturated fat food").
"Reduced saturated fat," "reduced in saturated fat," "saturated fat reduced," "less saturated fat," "lower saturated fat," or "lower in saturated fat"	The food contains at least 25 % less saturated fat per amount customarily consumed than an appropriate reference food
"Cholesterol free," "free of cholesterol," "zero cholesterol," "without cholesterol," "no cholesterol," "trivial source of cholesterol," "negligible source of cholesterol," or "dietarily insignificant source of cholesterol"	The food contains less than 2 mg of cholesterol per reference amount customarily consumed and per labeling serving and
	The food contains no ingredient that is generally understood by consumers to contain cholesterol (unless the listing of the ingredient in the ingredient statement is followed by an asterisk that refers to the statement below the list of ingredients, which states "adds a trivial amount of cholesterol," "adds a negligible amount of cholesterol," or "adds a dietarily insignificant amount of cholesterol") and
	The food contains 2 g or less of saturated fatty acids per reference amount customarily consumed
	If the food contains less than 2 mg of cholesterol per reference amount customarily consumed without the benefit of special processing, alteration, formulation, or reformulation to lower cholesterol content, it is labeled to disclose that cholesterol is not usually present in the food (e.g., "applesauce, a cholesterol-free food").

(*continued*)

Table 80.3 (continued)

"Low in cholesterol," "low cholesterol," "contains a small amount of cholesterol," "low source of cholesterol," or "little cholesterol"	The food contains 20 mg or less of cholesterol per reference amount customarily consumed
	The food contains 2 g or less of saturated fatty acids per reference amount customarily consumed and
	If the food meets these conditions without the benefit of special processing, alteration, formulation, or reformulation to lower cholesterol content, it is labeled to clearly refer to all foods of that type and not merely to the particular brand to which the label attaches (e.g., "low fat cottage cheese, a low cholesterol food").
"Reduced cholesterol," "reduced in cholesterol," "cholesterol reduced," "less cholesterol," "lower cholesterol," or "lower in cholesterol"	The food has been specifically formulated, altered, or processed to reduce its cholesterol by 25 % or more from the reference food it resembles and
	The food contains 2 g or less of saturated fatty acids per reference amount customarily consumed
"Lean" and extra lean claims	
"Lean"	The food is a seafood or game meat product and as packaged contains less than 10 g total fat, 4.5 g or less saturated fat, and less than 95 mg cholesterol per reference amount customarily consumed and per 100 g
"Extra lean"	The food is a discrete seafood or game meat product and as packaged contains less than 5 g total fat, less than 2 g saturated fat, and less than 95 mg cholesterol per reference amount customarily consumed and per 100 g

Table 80.4 Nutrition claims approved in UE

Nutrition claims for energy	
"Low Energy"	The product does not contain more than 40 kcal (170 kJ)/100 g for solids or more than 20 kcal (80 kJ)/100 ml for liquids. For table-top sweeteners the limit of 4 kcal (17 kJ)/portion, with equivalent sweetening properties to 6 g of sucrose (approximately 1 teaspoon of sucrose), applies.
"Energy-reduced"	The energy value is reduced by at least 30 %, with an indication of the characteristic(s) which make(s) the food reduced in its total energy value
"Energy-free"	The product does not contain more than 4 kcal (17 kJ)/100 ml. For table-top sweeteners the limit of 0,4 kcal (1,7 kJ)/portion, with equivalent sweetening properties to 6 g of sucrose (approximately 1 teaspoon of sucrose), applies.
Nutrition claims for fat	
Low fat:	The product contains no more than 3 g of fat per 100 g for solids or 1.5 g of fat per 100 ml for liquids (1.8 g of fat per 100 ml for semi-skimmed milk).
Fat-free:	The product contains no more than 0.5 g of fat per 100 g or 100 ml. However, claims expressed as "X % fat-free" shall be prohibited.
Nutrition claims for saturated fat	
Low saturated-fat:	The sum of saturated fatty acids and trans-fatty acids in the product does not exceed 1.5 g per 100 g for solids or 0.75 g/100 ml for liquids and in either case the sum of saturated fatty acids and trans-fatty acids must not provide more than 10 % of energy.

(*continued*)

Table 80.4 (continued)

Saturated fat-free:	The sum of saturated fat and trans-fatty acids does not exceed 0.1 g of saturated fat per 100 g or 100 ml.
Nutrition claims for omega-3 fatty acids	
Source of:	The product contains at least 0.3 g alpha-linolenic acid per 100 g and per 100 kcal, or at least 40 mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100 g and per 100 kcal.
High:	The product contains at least 0.6 g alpha-linolenic acid per 100 g and per 100 kcal, or at least 80 mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100 g and per 100 kcal.
Nutrition claims for monounsaturated fat	
High:	At least 45 % of the fatty acids present in the product derive from monounsaturated fat under the condition that monounsaturated fat provides more than 20 % of energy of the product.
Nutrition claims for polyunsaturated fat	
High:	At least 45 % of the fatty acids present in the product derive from polyunsaturated fat under the condition that polyunsaturated fat provides more than 20 % of energy of the product.
Nutrition claims for unsaturated fat	
High:	At least 70 % of the fatty acids present in the product derive from unsaturated fat under the condition that unsaturated fat provides more than 20 % of energy of the product
Nutrition claims for sugar	
Low Sugar:	The product contains no more than 5 g of sugars per 100 g for solids or 2.5 g of sugars per 100 ml for liquids.
Sugars-free:	The product contains no more than 0.5 g of sugars per 100 g or 100 ml.
With no added sugar	The product does not contain any added mono- or disaccharides or any other food used for its sweetening properties. If sugars are naturally present in the food, the following indication should also appear on the label: "CONTAINS NATURALLY OCCURRING SUGARS."
Nutrition claims for sodium/salt	
Low sodium/salt:	The product contains no more than 0.12 g of sodium, or the equivalent value for salt, per 100 g or per 100 ml. For waters, other than natural mineral waters falling within the scope of Directive 80/777/EEC, this value should not exceed 2 mg of sodium per 100 ml.
Very low sodium/salt:	The product contains no more than 0.04 g of sodium, or the equivalent value for salt, per 100 g or per 100 ml. This claim shall not be used for natural mineral waters and other waters.
Sodium-free or salt-free	The product contains no more than 0.005 g of sodium, or the equivalent value for salt, per 100 g.
Nutrition claims for fiber	
Source of fiber:	The product contains at least 3 g of fiber per 100 g or at least 1.5 g of fiber per 100 kcal.
High fiber:	The product contains at least 6 g of fiber per 100 g or at least 3 g of fiber per 100 kcal.
Nutrition claims for protein	
Source of protein:	At least 12 % of the energy value of the food is provided by protein.
High protein:	At least 20 % of the energy value of the food is provided by protein.

(*continued*)

Table 80.4 (continued)

Nutrition claims for vitamins/minerals	
Source of:	The product contains at least a significant amount as defined in the Annex to Directive 90/496/EEC or an amount provided for by derogations granted according to Article 6 of Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods
High:	The product contains at least twice the value of "source of [NAME OF VITAMIN/S] and/or [NAME OF MINERAL/S]."
Nutrition claims for nutrients or other substances	
Contains:	The product complies with all the applicable provisions of this Regulation, and in particular Article 5. For vitamins and minerals, the conditions of the claim "source of" shall apply.
Increased:	The product meets the conditions for the claim "source of" and the increase in content is at least 30 % compared to a similar product.
Reduced:	The reduction in content is at least 30 % compared to a similar product, except for micronutrients, where a 10 % difference in the reference values as set in Directive 90/496/EEC shall be acceptable, and for sodium, or the equivalent value for salt, where a 25 % difference shall be acceptable.
Nutrition claims for omega-3 fatty acids	
Source of:	The product contains at least 0.3 g alpha-linolenic acid per 100 g and per 100 kcal, or at least 40 mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100 g and per 100 kcal.
High:	The product contains at least 0.6 g alpha-linolenic acid per 100 g and per 100 kcal, or at least 80 mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100 g and per 100 kcal.
Nutrition claims for monounsaturated fat	
High:	At least 45 % of the fatty acids present in the product derive from monounsaturated fat under the condition that monounsaturated fat provides more than 20 % of energy of the product.
Nutrition claims for polyunsaturated fat	
High:	At least 45 % of the fatty acids present in the product derive from polyunsaturated fat under the condition that polyunsaturated fat provides more than 20 % of energy of the product.
Nutrition claims for unsaturated fat	
High:	At least 70 % of the fatty acids present in the product derive from unsaturated fat under the condition that unsaturated fat provides more than 20 % of energy of the product.
Other Nutrition claims	
Light/lite	Shall follow the same conditions as those set for the term 'reduced'; the claim shall also be accompanied by an indication of the characteristic(s) which make(s) the food "light" or "lite."
Naturally/Natural	Where a food naturally meets the condition(s) for the use of a nutritional claim, the term "naturally/natural" may be used as a prefix to the claim.

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Food Sources of Phenolics Compounds 81

Wende Li and Trust Beta

Contents

1 Introduction 2528
2 Food Sources 2529
2.1 Alcoholic Beverages 2529
2.2 Cereals and Cereal Products 2531
2.3 Cocoa 2533
2.4 Fruits and Fruit Products 2534
2.5 Nonalcoholic Beverages 2540
2.6 Oils 2543
2.7 Seasonings 2545
2.8 Seeds 2549
2.9 Vegetables 2552
3 Conclusion 2557
References 2557

Abstract

Phenolic compounds are a diverse group of phytochemicals classified into flavonoids, phenolic acids, lignans, coumarins, phenols, phenylpropanoids, quinines, stilbenoids, and xanthones. Flavonoids, which make up the largest cadre among phenolics, are further subdivided into anthocyanins, flavanols including proanthocyanidins, flavonols, dihydroflavonols, flavones, isoflavonoids, flavonones, chalcones, and

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dihydrochalcones. Phenolic acids are further subclassified as hydroxybenzoic acids, hydroxycinnamic acids, hydroxyphenylacetic acids, and hydroxyphenylpropanoic acids. Other phenolics include alkylmethoxyphenols, alkylphenols, curcuminoids, furacoumarins, hydroxybenzaldehydes, hydroxybenzoketones, hydroxycinnamaldehydes, hydroxycoumarins, hydroxyphenylpropenes, methoxyphenols, naphthoquinones, phenolic terpenes, and tyrosols. Food databases specifically on phenolic compounds include Phenol-Explorer, USDA-Iowa State University database for isoflavones, USDA database for flavonoids, and USDA database for proanthocyanidins. The phenolic content in foods can be obtained from these accessible databases. The occurrence of phenolics as well as concentration in foods is summarized based on the current literature. Cloves, cocoa powder, dried peppermint, star anise, dried Mexican oregano, celery seed, black chokecherry, dark chocolate, flaxseed meal, and black elderberry are among the top foods abundant in phenolics.

Keywords

Food sources • phenolic compounds • phenolic acids • flavonoids • total phenolic content

Abbreviations

FW	Fresh weight
SIF	Sum of individual flavonoids
SIPA	Sum of individual phenolic acids
TAC	Total anthocyanin content
TPC	Total phenolic content

1 Introduction

Phenolic compounds widely existing in plants and plant-derived foods have been shown to impact human health [1, 2]. Several hundreds of molecules having a polyphenol structure have been found in edible plants [3]. Their abundant presence in our diets coupled with the recognition of their antioxidant properties and purported role in the prevention of various diseases associated with oxidative stress [3] mandates an in-depth understanding of phenolic compounds as constituents of edible plants and foods for researchers and consumers. In addition to numerous literature sources on phenolics, systematic databases such as Phenol-Explorer database on polyphenol content of foods [4], USDA database for flavonoids [5], USDA-Iowa State University database for isoflavones [6], and USDA database for proanthocyanidins [7] exist. The databases are accessible to researchers and consumers providing useful information. Phenolic compounds which are present in food sources are reviewed primarily on the basis of the Phenol-Explorer database. The total phenolic content (TPC, also total polyphenol content), obtained using the Folin assay, is an indirect indication of antioxidant capacity since the method provides a crude estimation of the total antioxidant content [8]. A high TPC is associated with a high antioxidant capacity. Data on TPC, phenolic acids, flavonoids, other phenolic compounds, and total anthocyanin content (TAC) were summarized from the Phenol-Explorer database [4] unless explicitly indicated otherwise. Food sources have been classified into nine groups including nonalcoholic beverages, alcoholic beverages, fruits and fruit products, vegetables, cereals and cereal products, seeds, cocoa, seasonings, and oils for comparison of the levels of their phenolic compounds [9]. All content values are expressed as mg per 100 g fresh weight (FW) for solid foods and as mg per 100 ml for liquid foods according to Phenol-Explorer database [4] unless indicated otherwise. The ten foods most abundant in polyphenols among 452 sources examined were cloves (15,188.0 mg 100 g^{-1}), dried peppermint (11,960.0 mg 100 g^{-1}), star anise (5,460.0 mg 100 g^{-1}), cocoa powder (3,448.0 mg 100 g^{-1}), Mexican oregano, dried (2,319.0 mg 100 g^{-1}), celery seed (2,094.0 mg 100 g^{-1}), black chokeberry (1,756.0 mg 100 g^{-1}), dark chocolate (1,664.0 mg 100 g^{-1}), flaxseed meal (1,528.0 mg 100 g^{-1}), and black elderberry (1,359.0 mg 100 g^{-1}) [8] according to their polyphenol content.

2 Food Sources

2.1 Alcoholic Beverages

Phenolics found in alcoholic beverages include 33 phenolic acids, 75 flavonoids, 7 lignins, 13 stilbenes, and 19 other polyphenols [9]. The major phenolics in alcoholic beverages are summarized in Table 81.1.

2.1.1 TPC

It is evident that TPC has a highly positive relationship with antioxidant capacity [10]; therefore, the antioxidant potential of food sources can be evaluated according to their TPC. Table 81.1 shows TPC in decreasing order according to the maximum values reported in alcoholic beverages [4]. TPC (mg 100 ml^{-1}) in alcoholic beverage ranged from 33 to 70 for ale beer, 7–60 for dark beer, 4–49 for regular beer, 5–23 for alcohol-free beer, 41–42 for antho-beer [11], 13–130 for cider, 24–388 for walnut liquor, 41–83 for black currant wine, 74–418 for red grape wine, 34–130 for rosé grape wine, 10–85 for white grape wine, and 18–20 for champagne. Values of 93 for anthograin liqueur, 7 for Canadian whisky, and 6 for special old rye whisky were recently reported for TPC [12].

2.1.2 Phenolic Acids

The existence of different molecular structures of phenolic acids results in differences in antioxidant activity, for example, protocatechuic acid, chlorogenic acid, caffeic acid, *p*-hydroxybenzoic acid, gentisic acid, ferulic acid, vanillic acid, syringic acid, and *p*-coumaric acid [13]. Such rankings make the information on the composition of phenolic acids in foods imperative. Phenolic acids are divided into hydroxybenzoic, hydroxybenzoic, hydroxycinnamic, hydroxyphenylacetic, and hydroxyphenylpropanoic acids. Phenolic acids exist as three forms in food sources, namely, free phenolic acids, soluble phenolic esters, and insoluble-bound phenolic acids [14]. Since bound phenolic acids are the major forms present in various foods, individual phenolic acids can be released from the bound forms through hydrolysis and then quantified. The sum of

Table 81.1 TPC range (mg/100 ml) and major phenolics in alcoholic beverages

Name	TPC	Major phenolics
Wine [red]	74–418	Malvidin *3-O*-glucoside, (+)-catechin
Walnut, liquor	24–388	Gallic acid, (+)-catechin
Wine [rosé]	34–130	*3*-Caffeoylquinic acid, caffeic acid
Cider	13–130	(−)-Epicatechin
Wine [white]	10–85	(−)-Epicatechin, (+)-catechin
Black currant, wine	41–83	Myricetin, quercetin
Beer [ale]	33–70	(+)-Catechin, ferulic acid
Beer [dark]	7–60	Vanillic acid, isoxanthohumol
Beer [regular]	4–49	*4*-Hydroxybenzoic acid, ferulic acid
Beer [alcohol-free]	5–23	Protocatechuic acid, (+)-catechin
Champagne	18–20	Caffeoyl tartaric acid, *2-S*-glutathionyl caftaric acid

individual phenolic acids (SIPA) indicates the level of phenolic acids in foods given their significant role as contributors to total antioxidant activity of dietary food sources.

Alcoholic beverages with a high maximum SIPA (mg 100 ml^{-1}) were red grape wine (3.0–60.0), walnut liquor (15.1–28.4), white grape wine (2.3–9.0), sherry (6.0–7.2), rosé grape wine (6.0–7.0), and regular beer (0.3–6.6). Others had maximum SIPA of less than 5.0.

The major phenolic acids (mg 100 ml^{-1}) in the above alcoholic beverages with high maximum SIPA (>10 mg 100 ml^{-1}) were identified as caffeoyl tartaric (0.1–17.9) and gallic (0–12.6) acids in red grape wine and gallic (11.2–21.7) and syringic (2.4–3.6) acids in walnut liquor. The SIPA were obtained without performing a hydrolysis step.

After hydrolysis, SIPA of alcoholic beverages were 0.8–11.0 for red grape wine, 4.0 for rosé grape wine, and 1.3–3.8 for regular beer. The major phenolic acids present were *p*-coumaric acid (0.8–11.0) in red grape wine, caffeic (1.8) and *p*-coumaric (0.8) acids in rosé grape wine, and ferulic (0.4–1.7) and gallic (0.3–0.9) acids in regular beer.

2.1.3 Flavonoids

Flavonoids are also important antioxidants in many food sources. Flavonoids are divided into anthocyanins, flavanols/flavans, flavanones, flavones, flavonols, and isoflavones/isoflavonoids [15]. The sum of individual flavonoids (SIF) can be a simple indicator of flavonoid levels in dietary food sources which provides useful information for their potential benefits in human health.

Alcoholic beverages with high maximum SIF (mg 100 ml^{-1}) were red grape wine (21.0–211.9), white grape wine (0.7–15.5), and sherry (8.2) without hydrolysis being conducted on the samples. The maximum SIF was less than 4.0 for the other alcoholic beverages.

The major flavonoids (mg 100 ml^{-1}) in the above alcoholic beverages with a high maximum SIF (>10 mg 100 ml^{-1}) were (+)-catechin (1.4–39.0), malvidin *3-O*-glucoside (0–38.2), (−)-epicatechin (0–16.5), procyanidin dimer B1 (2.2–14.0), procyanidin dimer B3 (0–12.0), procyanidin dimer B4 (0.1–11.3), malvidin

3-*O*-(*6*-acetyl)-glucoside (0.5–11.3), and procyanidin dimer B2 (0.4–9.0) in red grape wine and (−)-epicatechin (0–6.0) and (+)-catechin (0–4.6) in white grape wine.

After hydrolysis, SIF of alcoholic beverages were 0–7.8 for red grape wine, 1.0–3.7 for black currant wine, and 1.5 for black crowberry wine. The major flavonoids identified in the beverages were quercetin (0–3.2), myricetin (0–2.0), and isorhamnetin (0–1.9) in red grape wine; myricetin (0.7–2.3) and quercetin (0.3–1.2) in black currant wine; and quercetin (0.7) and myricetin (0.7) in black crowberry wine.

2.1.4 Other Phenolic Compounds

Other phenolic compounds included proanthocyanins (02–10-mers and >10 polymers), lignins, and stilbenes. The other phenolic compounds (mg 100 ml^{-1}) with high maximum values in alcoholic beverages were tyrosol (0.6–4.5), syringaldehyde (0–4.5), resveratrol *3*-*O*-glucoside (0–4.4), >10 polymers (11.0), 04–06-mers (6.7), 07–10-mers (5.0), and 02-mers (4.0) in red grape wine, resveratrol (1.4–4.4) in red muscadine grape wine, and tyrosol (5.7) in sherry.

2.2 Cereals and Cereal Products

Phenolics reported in cereals and cereal products include 29 phenolic acids, 33 flavonoids, 6 lignans, and 2 other polyphenols [9]. The major phenolics in cereals and cereal products are summarized in Table 81.2.

2.2.1 TPC

Table 81.2 shows TPC in decreasing order according to the maximum values reported in cereals and cereal products [4]. Cereal products had TPC (mg 100 g^{-1} FW) of 131–315 for whole grain flour bread, 111 for purple wheat flour bread [16], 52 for wheat white flour bread [16], 26–156 for purple wheat bran muffin [17], 35–114 for wheat bran muffin [17], 17–277 for infant cereals [18], 77–153 for uncooked whole wheat pasta [19], 72–93 for uncooked regular pasta [19], and 117–271 for whole meal oat breakfast cereals.

TPC of cereals ranged from 2 to 185 for barley whole grain flour, 8–3,300 for buckwheat whole grain flour, 127–783 for thermally treated buckwheat groat, 314–1,096 for common wheat germ, 1–344 for common wheat whole grain flour, 0.1–81 for common wheat refined flour, 171–199 for hard wheat whole grain flour, 25–686 for maize whole grain, 2–390 for oat whole grain flour, 0.8–106 for rye whole grain flour, and 345 (17.8 μmol/g in pearl, sum of soluble, and bound) to 2,214 (114.0 μmol/g in kodo, sum of soluble, and bound) for millet whole grains [20]. Values of 158 for buckwheat refined flour, 56 for hard wheat refined flour, 102 for maize refined flour, 26 for rolled oat, 95 for rice whole grain, 45 for rye refined flour, and 413 for sorghum whole grain were also reported for TPC.

2.2.2 Phenolic Acids

Cereals and cereal products with high maximum SIPA (mg 100 g^{-1} FW) were hard wheat whole grain flour (72.2), oat whole grain flour (9.5–15.1), hard wheat refined flour (14.1), common wheat refined flour (0.1–9.8), oat refined flour (7.5–9.2),

Table 81.2 TPC range (mg/100 g FW) and major phenolics in cereals and cereal products

Name	TPC	Major phenolics
Buckwheat, whole grain flour	8–3,300	Quercetin *3-O*-rutinoside
Common wheat, germ	314–1,096	Ferulic acid, sinapic acid
Buckwheat, thermally treated groats	127–783	Quercetin *3-O*-rutinoside
Maize, whole grain	25–686	Ferulic acid, *o*-coumaric acid
Sorghum, whole grain	413	Polymers (>10 mers), 07–10 mers, 04–06 mers
Oat, whole grain flour	1–390	Ferulic acid, avenanthramide 2c
Common wheat, whole grain flour	1–344	Apigenin galactoside-arabinoside, ferulic acid
Bread, whole grain flour	131–315	*5*-Heneicosylresorcinol, *5*-nonadecylresorcinol
Breakfast cereals, oat, whole meal	117–271	
Hard wheat, whole grain flour	171–199	Ferulic acid, *5*-heneicosylresorcinol
Barley, whole grain flour	2–185	Prodelphinidin dimer B3, procyanidin dimer B3
Buckwheat, refined flour	158	Quercetin *3-O*-rutinoside, *p*-coumaric acid
Rye, whole grain flour	1–106	Ferulic acid, *5*-heptadecylresorcinol
Maize, refined flour	102	Ferulic acid, *5-8′*-dehydrodiferulic acid
Rice, whole grain	95	Ferulic acid
Common wheat, refined flour	50–81	Apigenin galactoside-arabinoside, ferulic acid
Hard wheat, refined flour	56	Ferulic acid
Rye, refined flour	45	Ferulic acid, *5*-nonadecylresorcinol
Oat, rolled	26	Avenanthramide 2c, avenanthramide 2f

sorghum whole grain (0.7–7.9), and rye whole grain flour bread (5.2). Other cereal-based sources had maximum SIPA of less than 3.0. SIPA in whole grain flour is higher than that of the derived refined flour since the bran removed during the milling process contains higher total phenolics than the refined flour [21].

The major phenolic acids (mg 100 g^{-1} FW) in the above cereals and cereal products with high maximum SIPA (>10 mg 100 g^{-1} FW) were ferulic acid in hard wheat whole grain flour (72.2); oat whole grain flour (35.8); hard wheat refined flour (14.1); and avenanthramide 2c (3.8–3.9), avenanthramide 2p (1.4–3.1), avenanthramide 2f (2.6–2.7), and avenanthramide K (1.2–2.7) in oat whole grain flour without conducting a hydrolysis step.

After hydrolysis, cereals and cereal products with high maximum SIPA were maize whole grain (111.0–330.0), maize refined flour (88.6–218.8), hard wheat semolina (1.4–176.6), common wheat germ (164.8–168.9), rye whole grain flour (61.9–167.2), rye whole grain flour bread (120.7), hard wheat whole grain flour (56.7–74.6), common wheat whole grain flour (64.7), oat whole grain flour (35.8), rye refined flour (26.0–35.1), and rice whole grain (29.8). The maximum SIPA was less than 8.0 for other cereal sources. Phenolic acids, which mainly exist as bound forms in cereals such as 80–90 % bound phenolic acids in brown rice [22], need to be released from their bound states through hydrolysis. The SIPA is significantly higher after subjecting cereals to hydrolysis than values obtained without inclusion of a hydrolysis step.

After hydrolysis, the major phenolic acids in cereals and cereal products with high maximum SIPA (>100 mg 100 g^{-1} FW) were identified as ferulic (90.6–255.3), *o*-coumaric (10.9–49.5), and *p*-coumaric (8.4–18.1) acids in maize whole grain; ferulic (62.2–184.9) and *5-8′*-dehydrodiferulic acids (8.3) in maize refined flour; ferulic (0.9–120.0), *8-O-4′*-dehydrodiferulic (0.1–20.8), and *5-8′*-benzofuran dehydrodiferulic (0.1–15.5) acids in hard wheat semolina; ferulic (123.5) and sinapic (27.1–29.6) acids in common wheat germ; ferulic (29.9–105.7), *8-O-4′*-dehydrodiferulic (11.5–18.0), and sinapic (1.6–13.8) acids in rye whole grain flour; and ferulic (109.9) and *p*-coumaric (3.2) acids in rye whole grain flour bread.

2.2.3 Flavonoids

The SIF (mg 100 g^{-1} FW) of cereals and cereal products were 51.5–108.4 for common wheat whole grain flour, 16.4–63.4 for buckwheat whole grain flour, 32.7–39.0 for barley whole grain flour, 13.2–26.3 for common wheat refined flour, 1.7–17.0 for buckwheat refined flour, and 4.0–13.4 for buckwheat thermally treated groats.

The major flavonoids (mg 100 g^{-1} FW) present in the above cereals and cereal products were apigenin arabinoside-glucoside (6.8–50.9) and apigenin galactoside-arabinoside (44.7–57.5) in common wheat whole grain flour; quercetin *3-O*-rutinoside (15.5–62.3) and apigenin *6-C*-glucoside (0.9) in buckwheat whole grain flour; prodelphinidin dimer B3 (22.9–23.4), procyanidin dimer B3 (8.8–14.2), and (+)-catechin (1.0–1.4) in barley whole grain flour; apigenin galactoside-arabinoside (7.2–16.3) and apigenin arabinoside-glucoside (6.0–10.0) in common wheat refined flour; quercetin *3-O*-rutinoside (1.7–16.8) in buckwheat refined flour; and quercetin *3-O*-rutinoside (3.8–13.0) in buckwheat thermally treated groats.

2.2.4 Other Phenolic Compounds

The other phenolic compounds in cereals and cereal products with high maximum values (mg 100 g^{-1} FW) were *5*-heneicosylresorcinol (92.8), *5*-heneicosenylresorcinol (81.5), and *5*-nonadecylresorcinol (43.1–53.5) in bran breakfast cereals; *5*-nonadecylresorcinol (0.4–33.4), *5*-heptadecylresorcinol (0.4–33.1), and *5*-heneicosylresorcinol (0.3–23.9) in rye whole grain flour; *5*-heneicosylresorcinol (9.6–32.6), *5*-nonadecylresorcinol (5.6–24.6), and *5*-heneicosenylresorcinol (9.9–23.7) in common wheat whole grain flour; *5*-nonadecylresorcinol (3.6–34.2), *5*-heneicosylresorcinol (1.9–24.2), and *5*-heptadecylresorcinol (1.3–22.2) in rye whole grain flour bread; and >10 polymers (1307.3–1767.0), 07–10 mers (150.0–325.0), and 04–06 mers (85.1–253) in sorghum whole grain.

2.3 Cocoa

Phenolics found in cocoa include 13 phenolic acids, 25 flavonoids, 2 stilbenes, and 5 other polyphenols [9]. The major phenolics in cocoa are summarized in Table 81.3.

2.3.1 TPC

Table 81.3 shows TPC in decreasing order according to the maximum values reported in cocoa [4]. TPC (mg 100 g^{-1} FW) ranged from 1,173 to 4,437 for dark chocolate, 325–2,439 for milk chocolate, and 1,005–1,204 for cocoa powder.

Table 81.3 TPC range (mg/100 g FW) and major phenolics in cocoa

Name	TPC	Major phenolics
Chocolate, dark	1,173–4,437	04–06-mers, 02-mers, 03-mers, (−)-Epicatechin, cinnamtannin A2, procyanidin dimer B2, (+)-catechin, procyanidin trimer C1
Chocolate, milk	325–2,439	Polymers (>10 mers), 04–06-mers, 07–10-mers, 02-mers, 03-mers, (−)-epicatechin, (+)-catechin
Cocoa, powder	1,005–1,204	Polymers (>10 mers), 04–06-mers, 07–10-mers, 03-mers, 02-mers, protocatechuic acid, (−)-epicatechin, procyanidin dimer B2, (+)-catechin, procyanidin dimer B1, cinnamtannin A2, caffeoyl aspartic acid, procyanidin trimer C1

2.3.2 Phenolic Acids

The SIPA (mg 100 g^{-1} FW) of cocoa powder and dark chocolate were 37.0 and 24.0, respectively. The major phenolic acids (mg 100 g^{-1} FW) were identified as caffeoyl aspartic acid (37.0) in cocoa powder and ferulic acid (24.0) in dark chocolate without performing any hydrolysis. After hydrolysis, SIPA of cocoa powder was 47.8 and its phenolic acid constituents comprised of protocatechuic (40.0), syringic (4.1), and vanillic (3.7) acids.

2.3.3 Flavonoids

The SIF (mg 100 g^{-1} FW) ranged from 256.0 to 1005.0 for cocoa powder, 135.5–392.0 for dark chocolate, and 15.2–36.0 for chocolate milk. The major flavonoids (mg 100 g^{-1} FW) were identified as (−)-epicatechin (63.0–330.0), procyanidin dimer B2 (13.0–262.0), (+)-catechin (61.0–202.0), procyanidin dimer B1 (112.0), cinnamtannin A2 (0–56.0), and procyanidin trimer C1 (5.0–36.0) in cocoa powder; (−)-epicatechin (32.7–125.0), cinnamtannin A2 (29.0–86.0), procyanidin dimer B2 (21.0–54.0), (+)-catechin (10.8–50.0), procyanidin trimer C1(13.0–44.0), and quercetin (25.0) in dark chocolate; and (−)-epicatechin (12.5–24.0) and (+)-catechin (2.7–12.0) in chocolate milk.

2.3.4 Other Phenolic Compounds

The other phenolic compounds with high maximum values (mg 100 g^{-1} FW) in cocoa were 04–06-mers (55.5–1605.0), 02-mers (31.2–1463.0), 03-mers (21.1–1170.0), >10 polymers (68.2–697.0), and 07–10-mers (38.5–295.0) in dark chocolate; >10 polymers (980.0–1685.0), 04–06-mers (736.0–1094.0), 07–10-mers (440.0–745.0), 03-mers (69.0–183.0), and 02-mers (70.0–178.0) in cocoa powder; and >10 polymers (32.8–114.0), 04–06-mers (38.0–68.0), and 07–10-mers (17.0–41.0) in chocolate milk.

2.4 Fruits and Fruit Products

Phenolics reported in fruits and fruit products include 47 phenolic acids, 112 flavonoids, 7 stilbenes, 6 lignans, and 1 other polyphenol [9]. The major phenolics in fruits and fruit products are summarized in Tables 81.4 and 81.5.

Table 81.4 TPC range (mg/100 g FW) and major phenolics in fruits and fruit products

Name	TPC	Major phenolics
Black chokeberry	690–2,556	Cyanidin *3-O*-galactoside, polymers (>10 mers)
Black elderberry	1,950	Cyanidin *3-O*-glucoside, cyanidin 3-O-sambubioside
Skunk currant	1,790	
Black currant	498–1,410	Delphinidin *3-O*-rutinoside, cyanidin *3-O*-rutinoside
Plum, fresh	88–1,400	*3*-Caffeoylquinic acid, cyanidin *3-O*-rutinoside
Black huckleberry	163–1,286	*5*-Caffeoylquinic acid, Quercetin
Plum, prune	1,195	*3*-Caffeoylquinic acid, *5*-caffeoylquinic acid
Evergreen huckleberry	283–1,169	*5*-Caffeoylquinic acid, ferulic acid
Grape, raisin	1,065	Caffeoyl tartaric acid
Blackberry	193–1,056	Cyanidin *3-O*-glucoside, ellagic acid
Ovalleaf huckleberry	261–1,054	Quercetin, myricetin
Black raspberry	980	Ellagic acid, quercetin *3-O*-rutinoside
Rabbiteye blueberry	48–961	Gallic acid, *4*-hydroxybenzoic acid,
Fig, dried	960	
Buffalo berry	958	
Highbush blueberry	20–868	*5*-Caffeoylquinic acid, *5*-caffeoylquinic acid
Lowbush blueberry	295–795	Polymers (>10 mers), malvidin *3-O*-glucoside
Apple [dessert], peeled	16–690	*5*-Caffeoylquinic acid, procyanidin dimer B2
Date, dried	172–667	Ferulic acid, *p*-coumaric acid
Canada blueberry	656	
Lingonberry	652	Cyanidin *3-O*-galactoside, quercetin
Gooseberry	191–630	Polymers (>10 mers), 07–10-mers
Apple [cider], peeled	43–600	*5*-Caffeoylquinic acid, procyanidin dimer B2
Red currant	372–540	Polymers (>10 mers), cyanidin *3-O*-sambubioside
Bilberry	525	Quercetin, *p*-coumaric acid, ferulic acid
Red raspberry	56–517	Ellagic acid, cyanidin *3-O*-sophoroside
Cascade huckleberry	140–491	*5*-Caffeoylquinic acid, caffeic acid
Strawberry	73–443	Pelargonidin *3-O*-glucoside, myricetin
Apple [dessert], whole	66–430	*5*-Caffeoylquinic acid, procyanidin dimer B2
Sour cherry	254–407	Cyanidin *3-O*-glucosyl-rutinoside
Sweet cherry	75–339	Cyanidin *3-O*-rutinoside, 3-*p*-coumaroylquinic acid
Jostaberry	301–338	Caffeoyl glucose, feruloyl glucose
Orange [blond]	9–337	Hesperetin, naringenin
American cranberry	315	Benzoic acid, *p*-coumaric acid
Peach, whole	23–300	*5*-Caffeoylquinic acid, *3*-caffeoylquinic acid
Fig, whole, fresh	49–281	
Date, fresh	2–280	Ferulic acid, caffeic acid
Kiwi	61–278	04–06-mers, 07–10-mers
Mango	48–266	04–06-mers, (+)-catechin
Peach, peeled	25–240	Procyanidin dimer B1, *5*-caffeoylquinic acid

Table 81.5 TPC range (mg/100 g FW) and major phenolics in fruits and fruit products

Name	TPC	Major phenolics
Banana	12–231	(+)-Catechin, 04–06-mers
Red huckleberry	74–228	*4*-Hydroxybenzoic acid, caffeic acid
Pear, peeled	38–220	*5*-Caffeoylquinic acid, (−)-epicatechin
Red raspberry, jam	219	Quercetin *3*-O-glucoside, cyanidin *3*-*O*-glucoside
Grapefruit	14–214	Naringenin, ferulic acid
Grape [black]	175–209	Malvidin *3*-*O*-glucoside
Sour cherry, jam	133–200	Cyanidin *3*-*O*-glucosyl-rutinoside
Half-highbush blueberry	110–199	*5*-Caffeoylquinic acid, caffeic acid
Tangerine	192	
Pear, whole	43–190	*5*-Caffeoylquinic acid, (−)-epicatechin
Pineapple	41–174	Syringaresinol, lariciresinol
Loquat	82–174	*5*-Caffeoylquinic acid, *3*-caffeoylquinic acid
Bog bilberry	151–171	Myricetin, quercetin
Grape [green]	29–145	Caffeoyl tartaric acid, polymers (>10 mers)
Plum, jam	141–144	
European cranberry	135–144	Quercetin, myricetin, *p*-coumaric acid
Star fruit	143	
Apricot	133	*5*-Caffeoylquinic acid, (−)-epicatechin
Guava	126	
Cantaloupe	9–124	
Fig, peeled, fresh	37–101	(+)-Catechin, (−)-epicatechin
Lemon	38–82	(−)-Epicatechin, hesperetin
Nectarine, whole	29–80	Procyanidin dimer B1, (+)-catechin
Nectarine, peeled	27–77	Procyanidin dimer B1, *5*-caffeoylquinic acid
Honeydew melon	6–72	
Watermelon	6–59	Luteolin
Papaya	58	
Passion fruit	57	
Lichee	29	
Longan	15	

2.4.1 TPC

Tables 81.4 and 81.5 show TPC in decreasing order according to the maximum values reported in fruits and fruit products [4].

Dried fruits had TPC (mg 100 g^{-1} FW) of 1,195 for prune plum, 1,065 for raisin grape, 960 for dried fig, and from 172 to 667 for dried date.

Berries had TPC of 690–2,556 for black chokeberry, 1,950 for black elderberry, 1,790 for skunk currant, 498–1,410 for black currant, 163–1,286 for black huckleberry, 283–1,169 for evergreen huckleberry, 193–1,056 for blackberry, 261–1,054 for ovalleaf huckleberry, 980 for black raspberry, 48–961 for rabbiteye blueberry, 958 for buffalo berry, 20–868 for highbush blueberry, 295–795 for lowbush blueberry, 656 for Canada blueberry, 652 for lingonberry, 191–630 for gooseberry, 372–540 for red currant, 525

Fig. 81.1 Saskatoon berry

for bilberry, 57–517 for red raspberry, 140–491 for cascade huckleberry, 73–443 for strawberry, 301–338 for jostaberry, 315 for American cranberry, 74–228 for red huckleberry, 175–209 for black grape, 110–199 for half-highbush blueberry, 151–171 for bog bilberry, 29–145 for green grape, and 135–144 for European cranberry.

Values of 2,283 and 3,791 mg 100 g^{-1} dry weight basis were reported for TPC of sea buckthorn without stone and saskatoon berry (Fig. 81.1), respectively [23].

TPC of citrus ranged from 9 to 337 for blond orange, 14–214 for grapefruit, 192 for tangerine, and 38–82 for lemon.

Drupes had TPC of 88–1,400 for fresh plum, 254–407 for sour cherry, 75–339 for sweet cherry, 23–300 for whole peach, 25–240 for peeled peach, 133 for apricot, 29–80 for whole nectarine, and 27–77 for peeled nectarine.

TPC of gourds ranged from 9 to 124 for cantaloupe, 6–72 for honeydew melon, and 6–59 for watermelon.

Pomes had TPC ranging from 16 to 690 for peeled dessert apple, 43–600 for peeled cider apple, 66–430 for whole dessert apple, 38–220 for peeled pear, and 43–190 for whole pear.

Tropical fruits had TPC of 61–278 for kiwi, 48–266 for mango, 12–231 for banana, 41–174 for pineapple, 82–174 for loquat, 143 for star fruit, 126 for guava, 58 for papaya, 57 for passion fruit, 29 for lichee, and 15 for longan.

The TPC in other fruits ranged from 49 to 281 for fresh whole fig, 2–280 for fresh date, and 37–101 for fresh peeled fig.

The TPC of red raspberry jam was 219 while the content in drupe jams was 133–200 for sour cherry jam and 141–144 for plum jam.

2.4.2 Phenolic Acids

Fruits and fruit products with high maximum SIPA (mg 100 g^{-1} FW) were peeled cider apple (19.0–417.1), sweet cherry (18.3–266.2), fresh plum (5.2–248.7), prune plum (128.8–234.4), black fox grape (227.6), highbush blueberry (68.6–214.1), red raspberry (109.8–161.1), black chokeberry (141.1), loquat (48.0–138.4), lowbush blueberry

(59.0–110.0), blackberry (28.6–90.2), sour cherry (25.0–75.0), dried date (11.5–73.9), American cranberry (51.8–62.0), whole dessert apple (3.1–57.0), whole pear (2.0–52.4), fresh date (5.2–49.6), peeled dessert apple (4.3–47.1), peeled nectarine (3.5–46.0), whole peach (8.1–41.6), black raspberry (38.0), peeled peach (1.6–33.6), peeled quince (0.7–30.0), strawberry (2.5–28.6), apricot (3.5–26.1), cloudberry (25.8), quince jam (1.1–23.0), black currant (11.3–19.3), lingonberry (12.2–16.6), jostaberry (16.2), whole nectarine (5.3–14.8), green grape (0.5–12.6), raisin grape (4.1–11.9), and apricot jam (1.3–10.8). The maximum SIPA was less than 9.0 for the other fruit-based sources.

The major phenolic acids (mg 100 g^{-1} FW) in the above fruits and fruit products high max SIPA (>100 mg 100 g^{-1} FW) were *5*-caffeoylquinic (15.8–400.6) and *4*-*p*-coumaroylquinic (0.1–13.5) acids in peeled cider apple; *3*-*p*-coumaroylquinic (7.2–131.5) and *3*-caffeoylquinic (8.2–128.2) acids in sweet cherry; *3*-caffeoylquinic (2.7–215.4) and *5*-caffeoylquinic (0.9–21.0) acids in fresh plum; caffeoyl tartaric (185.9) and *p*-coumaroyl tartaric (41.8) acids in black fox grape; *5*-caffeoylquinic acid (64.6–207.5) in highbush blueberry; sanguiin H-6 (75.9–89.8), lambertianin C (30.8–44.6), and ellagic acid (0–22.0) in red raspberry; caffeic acid (141.1) in black chokeberry; *5*-caffeoylquinic (32.9–90.7), *3*-caffeoylquinic (9.6–20.7), *5*-feruloylquinic (2.8–14.5), and *4*-hydroxybenzoic (2.2–8.2) acids in loquat; and *5*-caffeoylquinic acid (59.0–110.0) in lowbush blueberry without conducting a hydrolysis step.

After hydrolysis, fruits and fruit products with a high maximum SIPA were American cranberry (568.6), rabbiteye blueberry (0–407.7), highbush blueberry (100.1–203.5), half-highbush blueberry (148.8–176.6), red raspberry (4.5–166.6), red huckleberry (71.2–107.5), strawberry (9.6–71.8), arctic blackberry (0–68.6), evergreen huckleberry (61.0–64.0), cloudberry (0–60.6), dried date (7.5–58.7), blackberry (38.9–49.5), fresh date (2.6–37.2), bog bilberry (0.8–33.2), strawberry jam (19.6–25.4), European cranberry (21.1–25.2), fresh plum (3.1–22.2), cascade huckleberry (12.2–17.8), and black huckleberry (11.9–15.2). Other fruit sources had maximum SIPA of less than 8.0.

After hydrolysis, the major phenolic acids identified in the above fruits and fruit products with high maximum SIPA (>100 mg 100 g^{-1} FW) were benzoic (474.1), *p*-coumaric (25.4), sinapic (21.2), caffeic (15.6), *o*-coumaric (8.9), and ferulic (8.8) acids in American cranberry; gallic (0–258.9), *4*-hydroxybenzoic (0–103.7), ferulic (0–17.0), *p*-coumaric (0–15.8), caffeic (0–6.3), and ellagic (0–6.0) acids in rabbiteye blueberry; *5*-caffeoylquinic (98.1–157.6), caffeic (0–22.3), *p*-coumaric (0–7.2), and ellagic (0–6.7) acids in highbush blueberry; *5*-caffeoylquinic (130.4–152.1), caffeic (13.9–18.6), and ferulic (4.2–5.4) acids in half-highbush blueberry; ellagic (0–160.0) and *4*-hydroxybenzoic (2.6) acids in red raspberry; and *4*-hydroxybenzoic (40.7–68.7), caffeic (14.3–15.5), *p*-coumaric (7.6–11.2), and *5*-caffeoylquinic (5.0–7.7) acids in red huckleberry.

2.4.3 Flavonoids

Fruits and fruit products with high maximum SIF (mg 100 g^{-1} FW) were black elderberry (685.6–2212.7), black chokeberry (394.5–1538.5), black currant (378.6–865.4), sweet cherry (13.3–493.1), lowbush blueberry (55.1–365.4), peeled cider apple (13.4–330.9), blackberry (103.8–315.0), fresh plum (44.4–295.4), highbush blueberry (95.0–264.3), red raspberry (12.5–255.1), black grape (18.0–243.1), strawberry (32.3–151.7), sour cherry (10.2–128.6), peeled peach (8.6–122.8), whole dessert apple (7.2–101.8), peeled dessert apple (4.3–100.7), peeled nectarine (1.3–95.5), lingonberry (91.7), quince jelly (4.4–84.2), American cranberry (78.0), black aestivalis grape (76.6), red currant (24.0–53.3), quince jam (0–35.1), whole nectarine (3.9–31.3), sweet cherry jam (10.2–29.5), custard apple (24.9), lowbush blueberry jam (18.0–21.6), blackberry jam (0.2–21.4), apricot (0.7–20.5), black raspberry (19.0), gooseberry (2.2–15.9), dessert apple puree (15.7), green grape (1.0–12.5), black fox grape (12.2), green currant (11.1), apricot puree (5.8–11.0), and banana (0.1–10.6). The maximum SIF was less than 10.0 for other fruit-based sources.

The major flavonoids (mg 100 g^{-1} FW) in the above fruits and fruit products with a high maximum SIF (>100 mg 100 g^{-1} FW) were identified as cyanidin *3-O*-glucoside (361.0–1266.0), cyanidin *3-O*-sambubioside (269.0–706.4), cyanidin *3-O*-sambubiosyl *5-O*-glucoside (14.0–136.7), quercetin (29.0–60.0), and cyanidin *3,5-O*-diglucoside (5.0–36.0) in black elderberry; cyanidin *3-O*-galactoside (125.6–989.7), cyanidin *3-O*-arabinoside (132.9–372.6), cyanidin *3-O*-xyloside (43.8–48.1), quercetin *3-O*-galactoside (46.5), and quercetin *3-O*-glucoside (42.0) in black chokeberry; delphinidin *3-O*-rutinoside (197.4–409.2), cyanidin *3-O*-rutinoside (93.3–241.1), delphinidin *3-O*-glucoside (54.2–113.2), and cyanidin *3-O*-glucoside (12.5–51.8) in black currant; cyanidin *3-O*-rutinoside (1.6–393.0) and cyanidin *3-O*-glucoside (0–48.0) in sweet cherry; malvidin *3-O*-glucoside (12.6–44.4), malvidin *3-O*-(*6*-acetyl)-glucoside) (0–39.8), malvidin *3-O*-galactoside (1.5–38.6), delphinidin *3-O*-glucoside (5.0–26.0), and delphinidin *3-O*-galactoside (9.5–24.1) in lowbush blueberry; (−)-epicatechin (0–141.0), procyanidin dimer B2 (5.7–87.6), and (+)-catechin (0–58.0) in peeled cider apple; cyanidin *3-O*-glucoside (85.2–190.6) in blackberry; cyanidin *3-O*-rutinoside (0–144.8) and cyanidin *3-O*-glucoside (0–64.1) in fresh plum; delphinidin *3-O*-galactoside (10.7–39.0), petunidin *3-O*-galactoside (5.8–22.9), malvidin *3-O*-galactoside (15.1–19.6), and delphinidin *3-O*-arabinoside (9.1–18.6) in highbush blueberry; cyanidin *3-O*-sophoroside (0–106.2), cyanidin *3-O*-glucosyl-rutinoside (0–30.9), cyanidin *3-O*-glucoside (0–30.5), and cyanidin *3-O*-rutinoside (0–28.4) in red raspberry; malvidin *3-O*-glucoside (0.9–120.3), malvidin *3-O*-(*6-p*-coumaroyl)-glucoside (0–39.0), and peonidin *3-O*-glucoside (2.1–19.3) in black grape; pelargonidin *3-O*-glucoside (20.3–68.3), pelargonidin *3-O*-(*6″*-succinyl)-glucoside (0–31.3), and (+)-catechin (1.6–18.7) in strawberry; cyanidin *3-O*-glucosyl-rutinoside (6.2–109.7) in sour cherry; procyanidin dimer B1 (0.7–68.7), (+)-catechin (0.5–19.7), and (−)-epicatechin (0.7–16.5) in peeled peach; procyanidin dimer B2 (0.9–38.5) and (−)-epicatechin (1.8–19.2) in whole dessert apple; and procyanidin dimer B2 (0–21.3), procyanidin dimer B1 (0–17.3), and (−)-epicatechin (0–19.8) in peeled dessert apple without conducting a hydrolysis step.

After hydrolysis, fruits and fruit products with high maximum SIF were strawberry (0.3–102.0), blackberry (0–67.4), grapefruit (54.5), blond orange (42.0–53.8), lime (46.8), bog bilberry (16.5–40.6), black currant (3.7–39.0), lemon (35.1–38.8), European cranberry (11.4–37.5), American cranberry (12.3–27.9), rabbiteye blueberry (0–22.3), lingonberry (0–21.5), half-highbush blueberry (8.5–17.3), and black crowberry (9.7–10.5). Other fruit sources had maximum SIF of less than 10.0.

The major flavonoids in the above fruits and fruit products with a high maximum SIF (>50 mg 100 g^{-1} FW) were identified as myricetin (0–99.4) in strawberry, myricetin (0–63.6) in blackberry, naringenin (53.0) in grapefruit, and hesperetin (31.0–41.4) and naringenin (11.0–11.9) in blond orange.

Many fruits are rich in anthocyanins which are the major flavonoid compounds present in these fruits that significantly contribute to their total antioxidant activity. The TAC is an indication of anthocyanin levels in fruit. High TAC corresponds to high anthocyanin levels in the fruit. Fruits with high TAC (mg 100 g^{-1} FW) were evergreen huckleberry (336.0–617.0), black raspberry (589.0), rabbiteye blueberry (12.7–515.0), black chokeberry (428.0–460.5), highbush blueberry (35.5–430.0), black currant (128.0–411.0), ovalleaf huckleberry (185.0–400.0), black huckleberry (101.0–360.0), skunk currant (358.0), bilberry (299.0), Canada blueberry (298.0), buffalo berry (273.0), half-highbush blueberry (89.0–265.0), blackberry (67.4–256.0), lowbush blueberry (91.1–208.0), red raspberry (0–206.0), cascade huckleberry (134.0–176.0), bog bilberry (79.0–170.0), jostaberry (43.0–89.0), sour cherry (8.7–67.1), and sweet cherry (0.5–63.4).

2.4.4 Other Phenolic Compounds

The other phenolic compounds in fruits and fruit products with high maximum values (mg 100 g^{-1} FW) were arbutin (0–16.0) in quince jam; >10 polymers (542.6), 07–10-mers (52.9), and 04–06-mers (40.3) in black chokeberry; >10 polymers (260.4), 07–10-mers (27.8), and 04–06-mers (25.7) in lowbush blueberry; >10 polymers (57.3–115.3), 04–06-mers (49.9–64.7), 07–10-mers (33.8–41.2), and 02-mers (16.0–31.5) in fresh plum; >10 polymers (129.0) and 04–06-mers (19.6) in highbush blueberry; >10 polymers (98.7–138.6), 07–10-mers (8.5–10.9), and 04–06-mers (6.7–10.6) in black currant; >10 polymers (75.8), 04–06-mers (28.1), and 07–10-mers (23.9) in strawberry; >10-mers (35.4–113.3) and 07–10-mers (4.49–8.6) in gooseberry; and >10 polymers (27.7–37.6), 04–06-mers (22.8–30.2), and 07–10-mers (19.5–25.4) in whole dessert apple.

2.5 Nonalcoholic Beverages

Phenolics found in nonalcoholic beverages include 49 phenolic acids, 105 flavonoids, 6 stilbenes, 2 lignans, and 12 other polyphenols [9]. The major phenolics in nonalcoholic beverages are summarized in Table 81.6.

2.5.1 TPC

Table 81.6 shows TPC in decreasing order according to the maximum values reported in nonalcoholic beverages [4]. Nonalcoholic beverages had TPC (mg 100 ml^{-1}) of 246–282 for filter coffee beverage and 264–273 for decaffeinated filter coffee beverage. Among the berry juices, a TPC of 68 was recorded for black grape pure juice.

Citrus juices had TPC of 351 for grapefruit juice from concentrate, 180 for lemon juice from concentrate, 122 for lime pure juice, 38–115 for blood orange pure juice, 36–76 for blond orange pure juice, and 54 for grapefruit pure juice.

Table 81.6 TPC range (mg/100 ml) and major phenolics in nonalcoholic beverages

Name	TPC	Major phenolics
Apple [cider], pure juice	52–1,360	5-Caffeoylquinic acid, (−)-epicatechin
Grapefruit, juice from concentrate	351	Naringin, narirutin
Coffee beverage [filter]	246–282	5-Caffeoylquinic acid, 4-caffeoylquinic acid
Coffee beverage [filter], decaffeinated	264–273	5-Caffeoylquinic acid, 3-caffeoylquinic acid
Pomegranate, pure juice	175–257	Punicalagin, cyanidin 3-*O*-glucoside
Lemon, juice from concentrate	180	Hesperidin, eriocitrin
Tea [black], infusion	12–135	(−)-Epigallocatechin 3-*O*-gallate
Lime, pure juice	122	Hesperidin, eriocitrin
Orange [blood], pure juice	38–115	Cyanidin 3-*O*-(6″-malonyl-glucoside)
Tea [green], infusion	29–103	(−)-Epigallocatechin 3-*O*-gallate
Orange [blond], pure juice	36–76	Hesperidin, narirutin
Grape [black], pure juice	68	
Grapefruit, pure juice	54	Naringin, narirutin, phlorin
Common thyme, tea	51	
Pear, pure juice	20–46	5-Caffeoylquinic acid, (−)-epicatechin
Common sage, tea	43	
Pineapple, pure juice	36	
Rosemary, tea	36	
Spearmint, tea	36	
Apple [dessert], pure juice	34	5-Caffeoylquinic acid, procyanidin dimer B2
Peppermint, tea	31	(−)-Epicatechin 3-*O*-gallate
Tarragon, tea	30	
Winter savory, tea	26	
Lemon thyme, tea	25	
Small-leaf linden, tea	23	
Fennel, tea	23	Quercetin 3-*O*-glucuronide, anethole
German camomile, tea	23	(−)-Epigallocatechin 3-*O*-gallate
Roman camomile, tea	18	
Hyssop, tea	17	
Sweet bay, tea	17	
Sweet basil, tea	16	
Coriander, tea	12	
Lovage, tea	11	
Lemongrass, tea	10	
Lemon verbena, tea	8	Verbascoside, luteolin 7-*O*-diglucuronide

TPC of pome juices ranged from 52 to 1,360 for cider apple pure juice, 20–46 for pear pure juice, and 34 for dessert apple pure juice. TPC of tropical fruit juices included 175–257 for pomegranate pure juice and 36 for pineapple pure juice.

TPC was determined in several herbal infusions and values of 51 for common thyme tea, 43 for common sage tea, 36 for rosemary tea, 36 for spearmint tea, 31 for peppermint tea, 30 for tarragon tea, 26 for winter savory tea, 25 for lemon thyme tea, 23 for small-leaf linden tea, 23 for fennel tea, 23 for German camomile tea, 18 for Roman camomile tea, 17 for hyssop tea, 17 for sweet bay tea, 16 for sweet basil tea, 12 for coriander tea, 11 for lovage tea, 10 for lemongrass tea, and 8 for lemon verbena tea were obtained. Tea infusions had TPC ranging from 12 to 135 for black tea infusion and 29–103 for green tea infusion.

2.5.2 Phenolic Acids

Nonalcoholic beverages with a high maximum SIPA (mg 100 ml^{-1}) included decaffeinated filter coffee beverage (32.8–541.9), filter coffee beverage (171.6–256.6), filter robusta coffee beverage (204.6), pomegranate juice from concentrate (168.3), pomegranate pure juice (9.4–147.3), cider apple pure juice (3.3–138.5), filter arabica coffee beverage (87.7), dessert apple pure juice (3.4–83.5), rowanberry pure juice (81.7), green fox grape pure juice (29.9–41.6), plum prune juice from concentrate (41.5), lemon verbena tea (39.4), plum prune pure juice (2.8–37.9), green grape pure juice (3.3–35.2), black tea infusion (6.6–34.0), pear pure juice (7.6–26.2), green tea infusion (11.5–16.5), and cider apple juice from concentrate (1.4–10.2). The other beverages had maximum SIPA of less than 10.0.

The major phenolic acids (mg 100 ml^{-1}) in the above nonalcoholic beverages with high maximum SIPA (>100 mg 100 ml^{-1}) were *5*-caffeoylquinic (11.7–235.6), *3*-caffeoylquinic (7.1–106.1), and *4*-caffeoylquinic (9.9–104.8) acids in decaffeinated filter coffee beverage; *5*-caffeoylquinic (47.9–96.0), *4*-caffeoylquinic (53.0–66.2), and 3-caffeoylquinic (40.0–63.6) acids in filter coffee beverage; *5*-caffeoylquinic (75.8), *4*-caffeoylquinic (36.5), *3*-caffeoylquinic (32.3), and *4*-feruloylquinic (30.1) acids in filter robusta coffee beverage; punicalagin (135.3) and ellagic acid (17.3) in pomegranate juice from concentrate; punicalagin (2.3–126.0) and ellagic acid glucoside (1.8–8.3) in pomegranate pure juice; and *5*-caffeoylquinic (0.7–109.9) and *4-p*-coumaroylquinic (0.5–21.4) acids in cider apple pure juice without conducting a hydrolysis step.

After hydrolysis, SIPA of nonalcoholic beverages were 93.4–207.9 for filter coffee beverage, 5.9–15.9 for blood orange pure juice, 4.6–9.0 for blond orange pure juice, 6.9–8.9 for blood orange juice from concentrate, and 6.2–6.7 for blond orange juice from concentrate. The major phenolic acids identified were caffeic (83.0–96.0), *5*-caffeoylquinic (0–96.0), and ferulic (9.1–14.3) acids in filter coffee beverage; ferulic (3.2–6.4) and *p*-coumaric (1.2–4.5) acids in blood orange pure juice; ferulic (3.0–4.0) and *p*-coumaric (0.7–2.9) acids in blond orange pure juice; ferulic (3.9–4.4) and *p*-coumaric (1.4–2.3) acids in blood orange juice from concentrate; and ferulic (3.5–3.7) and *p*-coumaric (1.6–1.6) acids in blond orange juice from concentrate.

2.5.3 Flavonoids

Nonalcoholic beverages with high maximum SIF (mg 100 ml^{-1}) were green tea infusion (11.5–549.7), soy milk (4.0–376.3), black tea infusion (8.4–359.1), cider apple pure juice (1.3–213.2), oolong tea infusion (0.4–158.8), dessert apple pure juice (21.0–131.9), blond orange pure juice (15.4–111.0), grapefruit pure juice (12.2–101.1), bottled black tea (78.2–94.9), lemon pure juice (10.8–93.5), grapefruit pummelo hybrid pure juice (41.6–92.6), grapefruit juice from concentrate (17.3–86.1), blood orange pure juice (23.1–85.1), blond orange juice from concentrate (57.7–73.9), lemon juice from concentrate (14.7–71.7), tangerine juice from concentrate (15.5–69.7), bottled green tea (8.1–64.3), blood orange juice from concentrate (61.7), plum prune pure juice (10.1–51.0), pomegranate pure juice (0.6–40.9), sea buckthorn berry pure juice (36.5–40.1), chocolate milk beverage (7.9–33.3), red raspberry pure juice (0.1–30.1), bottled oolong tea (13.8–28.0), lime pure juice (16.4–23.9), green grape pure juice (0.5–20.5), plum prune juice from concentrate (17.9), peppermint tea (3.7–16.7), pomegranate juice from concentrate (16.2), pummelo pure juice (3.3–14.4), and lemon verbena tea (10.6). The other beverages had maximum SIF of less than 10.0. The TAC of blood orange pure juice ranged from 9.7 to 103.8 mg 100 ml^{-1}.

The major flavonoids (mg 100 ml^{-1}) in the above nonalcoholic beverages with high maximum SIF (>100 mg 100 ml^{-1}) were identified as (−)-epigallocatechin *3-O*-gallate (0.6–271.4), (−)-epigallocatechin (0.0–100.0), (−)-epicatechin (0–73.9), and (−)-epicatechin *3-O*-gallate (0.1–64.3) in green tea infusion; *6″-O*-malonylgenistin (0.2–87.1), *6″-O*-acetylgenistin (0–82.0), genistin (2.4–71.0), *6″-O*-malonyldaidzin (0.1–69.0), and daidzin (1.3–41.0) in soy milk; (−)-epigallocatechin *3-O*-gallate (0–67.9), (+)-gallocatechin (0–59.2), (−)-epigallocatechin (0–50.9), and (−)-epicatechin *3-O*-gallate (0–31.7) in black tea infusion; (−)-epicatechin (0–82.2), procyanidin dimer B2 (0–55.0), and (+)-catechin (0–40.7) in cider apple pure juice; (−)-epigallocatechin *3-O*-gallate (0.4–85.7) and (−)-epigallocatechin (0–40.0) in oolong tea infusion; procyanidin trimer C1 (19.9–40.0), procyanidin dimer B2 (0.1–22.9), (−)-epicatechin (0.5–22.6), and phloridzin (0.4–19.6) in dessert apple pure juice; hesperidin (4.5–73.0) and narirutin (1.5–15.2) in blond orange pure juice; and naringin (7.3–65.6) and narirutin (2.3–17.9) in grapefruit pure juice without performing a hydrolysis step.

After hydrolysis, nonalcoholic beverages with a high maximum SIF were soy milk (2.5–21.1), blond orange pure juice (9.8–11.2), green tea infusion (0.9–5.1), black tea infusion (0–4.7), and oolong tea infusion (2.7). Other nonalcoholic beverages had maximum SIF of less than 1.0. The major flavonoids in above nonalcoholic beverages with high maximum SIF (>10 mg 100 ml^{-1}) were genistein (1.9–12.2) and daidzein (0.7–8.7) in soy milk and hesperetin (9.0) and quercetin (0–1.3) in blond orange pure juice.

2.5.4 Other Phenolic Compounds

The other phenolic compounds (mg 100 ml^{-1}) with high maximum values in nonalcoholic beverages were phlorin (3.0–10.8) and bergapten (0–1.9) in grapefruit pure juice, phlorin (9.1) in pummelo pure juice, anethole (0–6.7) and *p*-anisaldehyde (0–1.5) in fennel tea, phlorin (4.3) in lemon pure juice, phlorin (1.1–3.7) in blond orange pure juice, and 02 mers (2.2) in chocolate milk beverage.

2.6 Oils

Phenolics found in oils include 28 phenolic acids, 11 flavonoids, 2 stilbenes, 12 lignans, and 22 other polyphenols [9]. The major phenolics in oils are summarized in Table 81.7.

Table 81.7 TPC range (mg/100 g FW) and major phenolics in oils and cereal oils

Name	TPC	Major phenolics
Peanut, butter	536	03-mers, 02-mers
Olive, oil, extra virgin	6–141	*3*,*4*-DHPEA-EDA, *p*-HPEA-EDA
Rapeseed, oil	0–107	*4*-Vinylsyringol, sinapine
Olive, oil, virgin	4–81	*p*-HPEA-EDA, oleuropein-aglycone
Olive, oil, refined	18–20	Oleuropein-aglycone, *3*,*4*-DHPEA-EDA
Sesame seed, oil	2	Sesamin, sesamolin, sesaminol
Soy, oil	0–1	*p*-Coumaric acid, ferulic acid
Sunflower seed, oil	1	

2.6.1 TPC

Table 81.7 shows TPC in decreasing order according to the maximum values reported in oils [4]. Fruit vegetable oils had TPC (mg 100 g^{-1} FW) of 6–141 for extra virgin olive oil, 4–81 for virgin olive oil, and 18–20 for refined olive oil. TPC was 536 for peanut butter among the nut oils. The TPC measured in other seed oils resulted in values of 0–107 for rapeseed oil, 2 for sesame seed oil, 0–2 for soy oil, and 1 for sunflower seed oil.

2.6.2 Phenolic Acids

Oils with high maximum SIPA (mg 100 g^{-1} FW) included maize bran oil (557.0), rapeseed oil (0.8–5.8), and virgin olive oil (0.1–2.6). The maximum SIPA was less than 2.0 in other oils. The major phenolic acids were identified as stigmastanol ferulate (360.0), *24*-methylcholestanol ferulate (100.0), *24*-methylcholesterol ferulate (50.0), and sitosterol ferulate (30.0) in maize bran oil; sinapine (0–2.6) and sinapic acid (0–2.3) in rapeseed oil; and vanillic (0–1.0) and syringic (0–0.4) acids in virgin olive oil.

2.6.3 Flavonoids

Oils with high maximum SIF (mg 100 g^{-1} FW) included extra virgin olive oil (0.1–4.0) and virgin olive oil (0–1.2). Other oils had maximum SIF of less than 1.0. The major flavonoids present were apigenin (0–3.2) and luteolin (0.1–0.8) in extra virgin olive oil and luteolin (0–0.8) and apigenin (0–0.4) in virgin olive oil.

2.6.4 Other Phenolic Compounds

The other phenolic compounds (mg 100 g^{-1} FW) with high maximum values in oils were identified as oleuropein-aglycone dialdehyde (0–163.5), oleuropein-aglycone *mono*-aldehyde (0–60.9), oleuropein-aglycone (0.1–45.9), ligstroside-aglycone dialdehyde (0.1–40.7), ligstroside-aglycone *mono*-aldehyde (0–34.6), hydroxytyrosol acetate (0–24.7), ligstroside-aglycone (0–23.3), and *1*-acetoxypinoresinol (0–11.2) in virgin olive oil; oleuropein-aglycone dialdehyde (0.1–94.2), ligstroside-aglycone dialdehyde (1.2–33.5), and oleuropein-aglycone *mono*-aldehyde (0–25.8) in extra virgin olive oil; *4*-vinylsyringol (0–62.9) in rapeseed oil; sesamin (7.0–1860.0), sesamolin (0–1000.0), sesaminol (6.6–720.0), sesamol (0–337.1), episesamin (192.6), sesamolinol (8.0–124.0), and episesaminol (8.8–81.6) in sesame seed oil; sesamin (13.0–1850.0), sesamolin (34.0–1060.0), sesaminol (16.0–500.0), sesamolinol (0–111.0), and sesamol (0–48.0) in black sesame seed oil; and 03-mers (8.1) in peanut butter.

2.7 Seasonings

Phenolics reported in seasonings include 29 phenolic acids, 62 flavonoids, 1 stilbene, and 25 other polyphenols [9]. The major phenolics in seasonings and herbs are summarized in Tables 81.8 and 81.9.

2.7.1 TPC

Tables 81.8 and 81.9 show TPC in decreasing order according to the maximum values reported in seasonings and herbs [4].

Several herbs have been analyzed to obtain TPC (mg 100 g^{-1} FW) of 1,650–11,500 for dried spearmint, 9,306 for dried pot marjoram, 1,690–8,000 for dried marjoram, 5,452–7,282 for dried wild marjoram oregano, 740–6,550 for dried sweet basil, 1,360–4,767 for common dried sage, 4,512 for dried summer savory, 4,170 for dried sweet bay, 520–4,000 for dried coriander, 2,100–3,196 for dried rosemary, 520–2,726 for dried hyssop, 226–2,580 for fresh peppermint, 2,483 for dried roman camomile, 126–2,253 for fresh lemon balm, 2,250 for dried fenugreek, 960–2,244 for dried parsley, 435–2,221 for fresh wild marjoram oregano, 1,710–1,920 for dried common thyme, 1,880 for dried winter savory, 1,700 for dried lemon balm, 213–1,537 for fresh common thyme, 219–1,377 for fresh rosemary, 850–1,270 for dried German camomile, 1,250 for dried dill, 1,165 for fresh Italian oregano, 854 for fresh marjoram, 155–771 for fresh lemon verbena, 68–701 for fresh fennel leaves, 662 for fresh lemongrass, 570 for fresh tarragon, 402 for fresh sweet bay, 94–400 for fresh spearmint, 350 for dried sorrel, 316 for fresh winter savory, 129–312 for fresh dill, 83–284 for fresh coriander, 263 for fresh lovage, 223–234 for fresh sweet basil, 226 for fresh orange mint, 199–218 for fresh fenugreek, 215 for fresh hyssop, 201 for fresh summer savory, 134–198 for fresh common sage, 180 for dried anise herb, 178 for fresh lemon thyme, 68–152 for fresh parsley, 48–150 for fresh garlic, 131 for fresh pineapple sage, 120 for dried silver linden, 75–105 for fresh chives, and 94 for fresh wild turnip tops.

Spices had TPC ranging from 11,319 to 24,390 for cloves, 7,500–11,900 for Ceylon cinnamon, 230–9,000 for cumin, 610–6,750 for caraway, 3,600 for capers, 148–2,420 for cardamom, 1,610–2,200 for nutmeg, 2,117 for dried turmeric, 1,600–2,020 for star anise, 300–1,700 for black pepper spice, 134–1,250 for coriander seed, 830 for fenugreek seed, 780 for white pepper spice, 317–630 for dried ginger, 380 for green pepper spice, 201–221 for fresh ginger, and 90 for dried horseradish. Curry powder had TPC of 1,075 among spice blends.

2.7.2 Phenolic Acids

Seasonings with high maximum SIPA (mg 100 g^{-1} FW) were dried peppermint (75.9–3860.0), fresh common verbena (840.0–2270.0), dried common sage (277.7–1523.4),

Table 81.8 TPC range (mg/100 g FW) and major phenolics in seasonings and herbs

Name	TPC	Major phenolics
Cloves	11,319–24,390	Rosmarinic acid, *5*-caffeoylquinic acid
Ceylon cinnamon	7,500–11,900	
Spearmint, dried	1,650–11,500	Ferulic acid, caffeic acid, apigenin
Pot marjoram, dried	9,306	Rosmarinic acid, *5*-caffeoylquinic acid
Cumin	230–9,000	Rosmarinic acid, vanillic acid
Marjoram, dried	1,690–8,000	Rosmarinic acid, carnosic acid
Oregano, dried (wild marjoram)	5,452–7,282	
Caraway	610–6,750	
Sweet basil, dried	740–6,550	Quercetin
Common sage, dried	1,360–4,767	Rosmarinic acid, caffeic acid
Summer savory, dried	4,512	Ferulic acid, caffeic acid
Sweet bay, dried	4,170	
Coriander, dried	520–4,000	
Capers	3,600	
Rosemary, dried	2,100–3,196	
Hyssop, dried	520–2,726	Apigenin, bergapten, psoralen
Peppermint, fresh	226–2,580	Apigenin, luteolin
Roman camomile, dried	2,483	Rosmarinic acid, caffeic acid, luteolin
Cardamom	148–2,420	
Lemon balm, fresh	126–2,253	Ferulic acid, caffeic acid
Fenugreek, dried	2,250	Rosmarinic acid, luteolin, hispidulin
Parsley, dried	960–2,244	Carnosic acid, rosmarinic acid, rosmanol
Oregano, fresh (wild marjoram)	435–2,221	*4*-Hydroxybenzoic acid, ferulic acid
Nutmeg	1,610–2,200	Quercetin, vanillic acid
Turmeric, dried	2,117	Rosmarinic acid, hispidulin
Star anise	1,600–2,020	
Common thyme, dried	1,710–1,920	
Winter savory, dried	1,880	*2*-Hydroxybenzoic acid, quercetin
Lemon balm, dried	1,700	
Pepper spice [black]	300–1,700	Kaempferol, quercetin
Common thyme, fresh	213–1,537	
Rosemary, fresh	219–1,377	
German camomile, dried	850–1,270	
Dill, dried	1,250	
Coriander seed	134–1,250	Quercetin, isorhamnetin, kaempferol
Italian oregano, fresh	1,165	Ferulic acid, caffeic acid, gallic acid
Curry, powder	1,075	Quercetin, kaempferol
Marjoram, fresh	854	
Fenugreek seed	830	
Pepper spice [white]	780	Quercetin *3-O*-rutinoside

Table 81.9 TPC range (mg/100 g FW) and major phenolics in seasonings and herbs

Name	TPC	Major phenolics
Lemon verbena, fresh	155–771	
Fennel, fresh leaves	68–701	
Lemongrass, fresh	662	Rosmarinic acid, luteolin
Ginger, dried	317–630	Ferulic acid, gallic acid
Tarragon, fresh	570	
Sweet bay, fresh	402	Apigenin, myricetin, bergapten
Spearmint, fresh	94–400	
Pepper spice [green]	380	
Sorrel, dried	350	*p*-Coumaric acid, vanillic acid
Winter savory, fresh	316	Kaempferol, quercetin, isorhamnetin
Dill, fresh	129–312	Kaempferol, caffeic acid
Coriander, fresh	83–284	Quercetin, luteolin
Lovage, fresh	263	Luteolin *7*-*O*-(*2*-apiosyl-*6*-malonyl)-glucoside
Sweet basil, fresh	223–234	Kaempferol
Orange mint, fresh	226	Verbascoside, luteolin *7*-*O*-diglucuronide
Ginger, fresh	201–221	Caffeic acid, ferulic acid
Fenugreek, fresh	199–218	Jaceosidin, hispidulin
Hyssop, fresh	215	Pinocembrin, naringenin, galangin
Summer savory, fresh	201	Eriocitrin, rosmarinic acid
Common sage, fresh	134–198	
Anise herb, dried	180	Quercetin, kaempferol
Lemon thyme, fresh	178	Caffeic acid, hesperidin
Parsley, fresh	68–152	Genistin, genistein, daidzein
Garlic, fresh	48–150	Tyrosol, (+)-catechin, gallic acid
Pineapple sage, fresh	131	Eugenol, acetyl eugenol, gallic acid
Silver linden, dried	120	Caffeic acid, protocatechuic acid
Chives, fresh	75–105	Kaempferol, caffeic acid, ferulic acid
Wild turnip tops, fresh	94	Caffeic acid, kaempferol
Horseradish, dried	90	Quercetin *3*-*O*-rutinoside

dried spearmint (613.0–1491.0), dried rosemary (918.9–1161.3), dried common thyme (471.4–1075.4), cloves (18.26–802.32), dried wild marjoram oregano (622.0–706.5), dried sweet basil (322.0), fresh rosemary (34.4–219.7), fresh Italian oregano (165.0), fresh common sage (127.5), fresh common thyme (103.5), star anise (52.4), Chinese cinnamon (7.7–35.8), vinegar (0.1–32.3 mg 100 ml^{-1}), Ceylon cinnamon (25.7–29.1), cumin (16.6), caraway (16.4), nutmeg (16.3–16.4), dried ginger (15.5), and dried marjoram (13.0). The other seasonings had maximum SIPA of less than 1.0.

The major phenolic acids (mg 100 g^{-1} FW) in the above seasonings with high maximum SIPA (>100 mg 100 g^{-1} FW) were rosmarinic acid (75.9–3860.0) in dried peppermint; verbascoside (840.0–2270.0) in fresh common verbena; rosmarinic (250.0–1410.0), caffeic (11.0–40.0), and 5-caffeoylquinic (16.7–23.0) acids in dried common sage; rosmarinic (562.0–1430.0), *5*-caffeoylquinic (31.0), and caffeic (20.0–30.0) acids in dried spearmint; rosmarinic (916.0–1100.0) and caffeic (2.9–20.0) acids in dried rosemary; rosmarinic (450.0–1000.0) and caffeic (10.0–30.0) acids in dried common thyme; gallic (17.47–783.5) and *p*-coumaric (0–17.0) acids in cloves; rosmarinic (598.0–600.0), *5*-caffeoylquinic (24.0–33.4), and caffeic (0–21.4) acids in dried wild marjoram oregano; rosmarinic (308.0) and vanillic (14.0) acids in dried sweet basil; rosmarinic (32.8–215.0) and caffeic (1.2–3.0) acids in fresh rosemary; rosmarinic (154.6) and caffeic (10.4) acids in fresh Italian oregano; rosmarinic (117.8) and caffeic (7.4) acids in fresh common sage; and rosmarinic (91.8) and caffeic (11.7) acids in fresh common thyme.

After hydrolysis, seasonings with high maximum SIPA were fennel fresh leaves (142.9), dried lemon balm (66.2), fresh coriander (39.7), fenugreek seed (30.3), fresh tarragon (27.0), dried hyssop (25.1), dried common thyme (16.2), dried marjoram (16.0), dried rosemary (15.8), dried fennel (13.4), dried spearmint (13.2), cumin (11.9), and fresh wild turnip tops (10.3). Others in max SIPA were less than 10.0. The major phenolic acids in the above seasonings high maximum SIPA (>50 mg 100 g^{-1} FW) were *2*-hydroxybenzoic (125.8) and ferulic (7.4) acids in fennel fresh leaves and ferulic (48.0) and caffeic (13.8) acids in dried lemon balm.

2.7.3 Flavonoids

Seasonings with high maximum SIF (mg 100 g^{-1} FW) were dried peppermint (326.9–21763.5), dried Mexican oregano (1,410.5–3,140.1), celery seed (2,094.0), capers (89.0–2,008.0), fresh common verbena (580.0–1,140.0), saffron (318.5–770.5), dried lemon verbena (30.8–215.8), fresh celery leaves (133.4), fresh rosemary (63.1–102.2), fresh common sage (66.4), fresh common thyme (60.3), fresh Italian oregano (52.2), cloves (52.2), cumin (38.6), caraway (16.4), vinegar (0–10.4), and dried marjoram (10.0). Other seasonings had maximum SIF below 6.0.

The major flavonoids (mg 100 g^{-1} FW) in the above seasonings with high maximum SIF (>100 mg 100 g^{-1} FW) were eriocitrin (108.5–14,980.0), luteolin *7-O*-rutinoside (36.5–2,320.0), narirutin (40.0–1,590.0), hesperidin (61.5–1,310.0), isorhoifolin (2.5–500.0), diosmin (30.0–470.0), eriodictyol *7-O*-glucoside (5.5–420.0), and pebrellin (20.9–72.3) in dried peppermint; pinocembrin (53.0–916.0), naringenin (335.0–418.0), galangin (20.0–347.0), luteolin *7-O*-glucoside (256.0–328.0), dihydroquercetin (98.0–149.0), phloridzin (117.0–148.0), hispidulin (85.0–117.5), *6*-hydroxyluteolin (78.1–105.6), sakuranetin (90.0–98.0), eriodictyol (72.0–93.0), cirsimaritin (49.4–83.4), *6*-hydroxyluteolin *7-O*-rhamnoside (23.0–76.0), methylgalangin (0–72.6), and luteolin (51.0–62.0) in dried Mexican oregano; luteolin *7-O*-(*2*-apiosyl-glucoside) (632.0), chrysoeriol *7-O*-apiosyl-glucoside (467.0), luteolin *7-O*-(*2*-apiosyl-*6*-malonyl)-glucoside (427.0), chrysoeriol *7-O*-(*6″*-malonyl-apiosyl-glucoside) (238.0), apigenin *7-O*-apiosyl-glucoside (111.0), luteolin *7-O*-malonyl-glucoside (92.0), and luteolin *7-O*-glucoside (80.0) in celery seed; kaempferol (10.0–280.0), quercetin (3.0–145.0), quercetin *3-O*-rutinoside (76.0–1047.0), kaempferol *3-O*-rutinoside (0–465.0), and kaempferol *3-O*-rhamnosyl-rhamnosyl-glucoside (0–71.0) in capers; luteolin *7-O*-diglucuronide (340.0–700.0) and apigenin *7-O*-diglucuronide (240.0–440.0) in fresh common verbena; kaempferol

3-O-sophoroside *7-O*-glucoside (183.1–321.3), kaempferol *3-O*-sophoroside (60.9–311.5), and kaempferol *3,7,4′-O*-triglucoside (74.5–137.7) in saffron; jaceosidin (9.8–70.2), hispidulin (11.1–52.3), and cirsilineol (3.9–39.0) in dried lemon verbena; luteolin *7-O*-(*2*-apiosyl-*6*-malonyl)-glucoside (35.8), chrysoeriol *7-O*-(*6″*-malonyl-apiosyl-glucoside) (35.6), and apigenin *7-O*-(*6″*-malonyl-apiosyl-glucoside) (31.6) in fresh celery leaves; and naringin (53.1–57.0), cirsimaritin (8.0–24.4), and hispidulin (2.0–19.7) in fresh rosemary without conducting a hydrolysis step.

After hydrolysis, seasonings with high maximum SIF were fresh parsley (0–581.7), fresh lovage (177.0), fresh dill (51.7–163.2), fresh angelica (1.9–138.8), fresh celery leaves (11.1–99.3), fennel fresh leaves (82.5), dried parsley (1.5–63.4), fresh common thyme (56.0), dried common thyme (40.0), dried dill (36.0), fresh chives (6.6–31.7), and fresh tarragon (27.0). Other seasonings had maximum SIF of less than 8.0.

After hydrolysis, major flavonoids in the above high max SIF (>100 mg 100 g^{-1} FW) seasonings were apigenin (0–570.0) and myricetin (0–8.1) in fresh parsley; quercetin (170.0) and kaempferol (7.0) in fresh lovage; quercetin (7.5–79.0), isorhamnetin (43.5), and kaempferol (0–40.0) in fresh dill; and quercetin (1.9–80.9) and luteolin (0–57.9) in fresh angelica.

2.7.4 Other Phenolic Compounds

The other phenolic compounds (mg 100 g^{-1} FW) with high maximum values in seasonings were eugenol (9381.7–14650.0) and acetyl eugenol (2075.1) in cloves; curcumin (580.0–5650.0), demethoxycurcumin (830.0–3360.0), and bisdemethoxycurcumin (420.0–2160.0) in dried turmeric; anethole (5407.9) in star anise; carnosic acid (126.6–1218.0), rosmanol (124.1), and carnosol (53.0) in fresh rosemary; carnosic acid (299.0–716.0) in dried common sage; curcumin (50.0–580.0) in curry powder; *6*-gingerol (187.3) in dried ginger; and coumarin (9.8–43.2) in Chinese cinnamon.

2.8 Seeds

Phenolics found in seeds include 16 phenolic acids, 76 flavonoids, 3 stilbenes, 18 lignins, and 4 other polyphenols [9]. The major phenolics in seeds are summarized in Table 81.10.

2.8.1 TPC

Table 81.10 shows TPC in decreasing order according to the maximum values reported in seeds [4]. TPC (mg 100 g^{-1} FW) measured in nuts included values of 1,580–3,673 for raw chestnut, 1,284–2,016 for pecan nut, 867–1,657 for pistachio, 1,558–1,625 for walnut, 291–835 for hazelnut, 396–420 for peanut, 127–418 for almond, 112–310 for Brazil nut, 137–274 for cashew nut, 46–156 for macadamia nut, 91–114 for dehulled peanut, 0.1–108 for roasted dehulled peanut, 47–71 for dehulled almond, and 32–68 for pines. Common beans had TPC of 55–9,360 for raw whole common bean, 56–4,400 for raw whole black common bean, 42–2,665 for raw dehulled common bean, 62–2,276 for raw dehulled black common bean, 9–490 for raw whole white common bean, and

Table 81.10 TPC range (mg/100 g FW) and major phenolics in seeds

Name	TPC	Major phenolics
Common bean [others], whole, raw	55–9,360	Polymers (>10 mers), 07–10-mers
Adzuki bean, whole, raw	8,970	
Lentils, whole, raw	6,346–6,760	(+)-Catechin *3-O*-glucose
Broad bean seed, whole, raw	55–5,590	(−)-Epicatechin, (−)-epigallocatechin
Common bean [black], whole, raw	56–4,400	Ferulic acid, *p*-coumaric acid
Chestnut, raw	1,580–3,673	Ellagic acid, gallic acid
Common bean [others], dehulled, raw	42–2,665	Ferulic acid, *5*-caffeoylquinic acid
Common bean [black], dehulled, raw	62–2,276	Ferulic acid, *5*-caffeoylquinic acid
Dried pea, whole, raw	2–2,260	
Pecan nut	1,284–2,016	Polymers (>10 mers), 04–06-mers
Pistachio	867–1,657	Polymers (>10 mers), 04–06-mers
Walnut	1,558–1,625	Myricetin, ellagic acid
Pigeon pea, whole, raw	104–1,328	
Hazelnut	291–835	Polymers (>10 mers), 07–10-mers
Common bean [white], whole, raw	9–490	Kaempferol *3-O*-glucoside
Peanut	396–420	Resveratrol, daidzein
Almond	127–418	Polymers (>10 mers), 04–06-mers
Brazil nut	112–310	
Cashew nut	137–274	02-mers, (−)-epicatechin
Macadamia nut	46–156	
Peanut, dehulled	91–114	*p*-Coumaric acid
Climbing bean, whole, raw	109	
Peanut, roasted, dehulled	0.1–108	*p*-Coumaric acid
Almond, dehulled	47–71	
Pines	32–68	
Common bean [white], dehulled, raw	42–53	Ferulic acid, *p*-coumaric acid
Broad bean seed, dehulled, raw	33	*p*-Coumaric acid, ferulic acid
Dried pea, dehulled, raw	16	Ferulic acid, syringic acid

42–53 for raw dehulled white common bean. Other beans had TPC of 8,970 for raw whole adzuki bean, 55–5,590 for raw whole broad bean seed, 109 for raw whole climbing bean, and 33 for raw dehulled broad bean seed. TPC in lentils was 6,346–6,760 for raw whole lentils. Peas had TPC of 2–2,260 for raw whole dried pea, 104–1,328 for raw whole pigeon pea, and 16 for raw dehulled dried pea.

2.8.2 Phenolic Acids

Seeds with high maximum SIPA (mg 100 g^{-1} FW) were raw chestnut (547.0–1959.0), sunflower seed meal (68.5–1265.6), dehulled walnut (21.1–70.9), walnut (25.0–32.0), Japanese walnut (9.0–24.0), and roasted dehulled peanut (2.0–11.7). Other seeds had maximum SIPA of less than 7.0. Major phenolic acids (mg 100 g^{-1} FW) in the above seeds with high max SIPA (>20 mg 100 g^{-1} FW) were ellagic (271.0–1052.0) and gallic (276.0–907.0) acids in raw chestnut, *5*-caffeoylquinic (63.0–1250.0) and caffeic (5.5–15.6) acids in sunflower seed meal, syringic (16.6–57.5) and ellagic (3.3–9.8) acids in dehulled walnut, ellagic acid (25.0–32.0) in walnut, and ellagic acid (9.0–24.0) in Japanese walnut without conducting a hydrolysis step.

After hydrolysis, seeds with high maximum SIPA were flaxseed meal (430.0–820.0), walnut (401.0–497.8), Japanese walnut (191.6–285.3), defatted soy flour (59.9), raw whole white common bean (48.2), raw whole black common bean (24.4–47.2), raw whole other common beans (0–39.3), and raw dehulled cowpea (16.3). The maximum SIPA was less than 10.0 for the other seeds. The major phenolic acids in the above seeds with high maximum SIPA (>50 mg 100 g^{-1} FW) were identified as ferulic acid *4-O*-glucoside (210.0–440.0) and *p*-coumaric acid *4-O*-glucoside (220.0–380.0) in flaxseed meal; ellagic acid (222.0–283.0) and valoneic acid dilactone (179.0–214.8) in walnut; ellagic acid (120.0–167.0) and valoneic acid dilactone (71.6–118.3)in Japanese walnut; and syringic (28.8), ferulic (15.7), and *p*-coumaric (9.4) acids in defatted soy flour.

2.8.3 Flavonoids

Seeds with high maximum values of SIF (mg 100 g^{-1} FW) were soy flour (140.6–887.9), roasted soy nut soybean (136.2–350.4), soy tofu (13.4–324.8), soy tempeh (37.6–298.8), soy paste cheonggukang (245.3–282.1), green soybean edamame (19.1–252.5), soy milk powder (40.0–245.1), soy paste nato (77.1–172.4), soy yogurt (48.8–121.9), raw whole common bean others (54.0–95.5), raw whole white common bean (19.3–82.8), fermented soy tofu sufu (7.5–64.7), raw whole black common bean (54.3), raw whole broad bean seed (49.4), soy pudding (29.1–45.2), soy sausage (19.9–39.4), raw soy burger (0.5–23.8), raw soybean sprout (3.5–17.3), pecan nut (16.7), almond (5.0–14.4), and soy bacon bits (14.4). Other seeds had maximum SIF of less than 9.0.

The major flavonoids (mg 100 g^{-1} FW) identified in the above seeds with high maximum SIF (>100 mg 100 g^{-1} FW) were genistin (1.9–229.0), *6″-O*-malonyldaidzin (26.1–179.3), daidzin (1.3–163.4), *6″-O*-malonylgenistin (102.3–139.5), and *6″-O*-malonylglycitin (5.7–61.1) in soy flour; genistin (55.1–96.8), *6″-O*-acetylgenistin (5.4–74.3), *6″-O*-acetyldaidzin (7.1–68.0), and daidzin (46.0–47.4) in roasted soy nut soybean; *6″-O*-malonylgenistin (4.0–78.8), *6″-O*-malonyldaidzin (1.3–75.3), genistin (4.6–56.2), and daidzin (2.5–45.3) in soy tofu; *6″-O*-malonylgenistin (11.1–75.0), glycitein (0.9–51.8), *6″-O*-malonyldaidzin (6.4–40.4), genistin (6.5–34.6), daidzein (6.9–31.8), genistein (3.1–19.3), and daidzin (0.2–18.2) in soy tempeh; daidzin (79.1–92.6), genistin (86.6–90.8), *6″-O*-acetyldaidzin (34.1), *6″-O*-malonylgenistin (21.8–21.9), and daidzein (3.8–20.4) in soy paste cheonggukang; *6″-O*-malonylgenistin (6.9–85.1), *6″-O*-malonyldaidzin (5.5–51.5), daidzin (1.0–45.1), and genistin (1.3–43.0) in green soybean edamame; genistin (10.3–77.5), daidzin (6.2–52.5), *6″-O*-malonylgenistin (12.8–31.6), and *6″-O*-malonyldaidzin (3.9–26.8) in soy milk powder; genistin (28.3–61.9), daidzin (34.7–56.0), and glycitin (2.3–26.5) in soy paste nato; and genistin (22.9–32.2), *6″-O*-malonylgenistin (5.4–25.1), daidzin (15.7–18.3), and *6″-O*-acetylgenistin (0.7–12.4) in soy yogurt without performing a hydrolysis step.

After hydrolysis, seeds with high maximum values of SIF were walnut (0–456.5), soy bacon bits (6.0–149.8), soy flour (47.3–129.9), raw soybean sprout (0.8–77.0), roasted soybean soy nut (74.7), soy cheese (59.3), soy tofu (3.8–40.9), soy paste miso (4.1–40.1), soy meat (7.4–39.2), green soybean edamame (26.6), raw soy burger (0.3–24.8), soy flakes (21.1), soy tempeh (18.3), soy sausage (0.3–16.7), raw whole common bean others (0.4–15.7), and soy yogurt (10.2). Others in max SIF were less than 8.0. The major flavonoids in the above seeds with high maximum values of SIF (>50 mg 100 g^{-1} FW) were myricetin (0–456.5) in walnut, daidzein (1.0–93.9) and genistein (5.0–45.9) in soy bacon bits, daidzein (30.9–54.1) and genistein (12.4–62.1) in soy flour, genistein (0.5–51.7) and daidzein (0.3–22.5) in raw soybean sprout, genistein (36.3) and daidzein (28.4) in roasted soybean soy nut, and genistein (38.2) and daidzein (21.1) in soy cheese.

2.8.4 Other Phenolic Compounds

The other phenolic compounds (mg 100 g^{-1} FW) with high maximum values in seeds were juglone (6.9–19.2) in dehulled walnut; sesamin (538.1–538.1), sesamolin (133.9), and sesaminol (102.9) in sesame seed meal; >10 polymers (252.9–459.6), 07–10-mers (75.7–135.6), 04–06-mers (80.0–125.9), 02-mers (19.4–32.0), and 03-mers (18.1–29.1) in raw whole common bean others; >10 polymers (322.4), 07–10-mers (74.6), and 04–06-mers (67.7) in hazelnut; >10 polymers (223.0), 04–06-mers (101.4), 07–10 mers (84.2), 02-mers (42.1), and 03-mers (26.0) in pecan nut; >10 polymers (122.5), 04–06-mers (42.2), and 07–10-mers (37.9) in pistachio; >10 polymers (80.3), 04–06-mers (40.0), and 07–10-mers (37.7) in almond; and 04–06-mers (22.1) and >10 polymers (20.0) in walnut.

After hydrolysis, the other phenolic compounds with high maximum values in seeds were secoisolariciresinol (81.7–3236.7), matairesinol (0–52.0), lariciresinol (3.0–36.7), and pinoresinol (3.3–24.6) in flaxseed meal and sesamin (62.72), pinoresinol (29.3–47.1), matairesinol (1.1–39.3), and lariciresinol (9.5–13.1) in sesame seed meal.

2.9 Vegetables

Phenolics found in vegetables include 51 phenolic acids, 92 flavonoids, 6 lignins, and 12 other polyphenols [9]. The major phenolics in vegetables are summarized in Tables 81.11 and 81.12.

2.9.1 TPC

Tables 81.11 and 81.12 show TPC in decreasing order according to the maximum values reported in vegetables [4]. Cabbages had TPC (mg 100 g^{-1} FW) of 254–679 for raw red common cabbage, 91–351 for raw brussels sprouts, 25–337 for raw broccoli, 10–274 for raw cauliflower, 53–224 for raw green common cabbage, 145–206 for raw kale, 183 for raw mustard cabbage, 105–179 for raw savoy cabbage, 109 for raw black cabbage, 108 for raw Italian cabbage, 57–86 for sauerkraut, and 15 for raw white common cabbage. Chinese cabbages had TPC of 119–268 for raw pak choy and 75–158 for raw napa cabbage.

TPC of fruit vegetables included 18–580 for raw green olive, 158–579 for raw red chilli pepper, 114–566 for raw yellow sweet pepper, 101–524 for raw green chilli pepper, 59–424 for raw red sweet pepper, 257–385 for raw yellow chilli pepper, 45–309 for raw green sweet pepper, 21–290 for raw black olive, 24–187 for raw avocado, 24–96 for raw whole tomato, and 57–65 for raw whole purple eggplant.

Table 81.11 TPC range (mg/100 g FW) and major phenolics in vegetables

Name	TPC	Major phenolics
Swiss chard leaves [red], raw	1,320	Kaempferol, quercetin, myricetin
Globe artichoke, heads, raw	731–1,305	*5*-Caffeoylquinic acid, luteolin
Swiss chard leaves [white], raw	830	Syringic acid, ferulic acid
Dandelion, raw	26–745	
Common cabbage [red], raw	254–679	Quercetin, luteolin, apigenin
Olive [green], raw	18–580	Oleuropein, hydroxytyrosol, sinapic acid
Chilli pepper [red], raw	158–579	Quercetin, luteolin
Sweet pepper [yellow], raw	114–566	Luteolin, quercetin
Chilli pepper [green], raw	101–524	Quercetin, luteolin
Spinach, raw	33–484	*5,4′*-Dihydroxy-*3,3′*-dimethoxy-*6*:*7*-methylenedioxyflavone *4′*-*O*-glucuronide
Green bean, raw	5–458	Quercetin *3*-*O*-rutinoside, (−)-epicatechin
Sweet pepper [red], raw	59–424	Luteolin, feruloyl glucose, *p*-coumaroyl glucose
Swiss chard stems [red], raw	410	Syringic acid, ferulic acid, *p*-coumaric acid
Chilli pepper [yellow], raw	257–385	Quercetin, luteolin
Brussels sprouts, raw	91–351	Kaempferol, luteolin, quercetin
Broccoli, raw	25–337	Kaempferol *3*-*O*-sophoroside, *3*-caffeoylquinic acid
Sweet pepper [green], raw	45–309	Quercetin, luteolin, quercetin *3*-*O*-rhamnoside
Swiss chard stems [white], raw	290	Vanillic acid, *p*-coumaric acid, ferulic acid
Olive [black], raw	21–290	Hydroxytyrosol, verbascoside
Cauliflower, raw	10–274	Sinapic acid, quercetin,
Pak choy, raw	119–268	Quercetin, kaempferol, apigenin
Common cabbage [green], raw	53–224	Kaempferol, quercetin
Kale, raw	145–206	Kaempferol, quercetin
Avocado, raw	24–187	04–06-mers, 02-mers, 03-mers
Mustard cabbage, raw	183	
Onion [yellow], raw	17–181	Quercetin *3,4′*-*O*-diglucoside
Savoy cabbage, raw	105–179	Kaempferol, apigenin, quercetin
Kohlrabi, raw	168	Kaempferol, luteolin
Red beetroot, raw	164	Luteolin, quercetin
Potato, raw	17–163	*5*-Caffeoylquinic acid, caffeic acid
Napa cabbage, raw	75–158	Quercetin, kaempferol
Pumpkin, raw	16–157	Syringic acid, *5*-caffeoylquinic acid
Carrot, raw	8–156	*5*-Caffeoylquinic acid, caffeic acid
Burdock root, raw	141	*5*-Caffeoylquinic acid
Asparagus, raw	14–141	Quercetin *3*-*O*-rutinoside, secoisolariciresinol
Arugula, raw	136	
Lettuce [green], raw	4–131	Quercetin *3*-*O*-(6"-malonyl-glucoside), quercetin
Chicory [red], raw	130	*5*-Caffeoylquinic acid, chicoric acid
Onion [red], raw	82–126	Quercetin *3,4′*-*O*-diglucoside, quercetin *4′*-*O*-glucoside

Table 81.12 TPC range (mg/100 g FW) and major phenolics in vegetables

Name	TPC	Major phenolics
Beet greens, raw	37–118	
Shallot [pink], raw	115	
Lettuce [red], raw	114	Quercetin *3-O*-(*6*″-malonyl-glucoside), quercetin
Black cabbage, raw	109	
Italian cabbage, raw	108	
Tomato, whole, raw	24–96	*5*-Caffeoylquinic acid, quercetin
Leek, raw	42–88	Kaempferol, quercetin
Sauerkraut	57–86	Apigenin
Eggplant [purple], whole, raw	57–65	Protocatechuic acid
Radish, raw	29–61	Kaempferol
Celeriac, raw	59	Apigenin, quercetin
Turnip root, raw	55	
Squash, raw	12–51	
Onion [white], raw	46	Quercetin *3,4*′*-O*-diglucoside
Zucchini, raw	26–38	Quercetin *3-O*-rutinoside
Fennel	28	
Cucumber, raw	14–27	Kaempferol, quercetin
Common cabbage [white], raw	15	Quercetin, kaempferol
Celery stalks, raw	13–15	Bergapten, xanthotoxin, isopimpinellin

TPC of 16–157 for raw pumpkin, 12–51 for raw squash, 26–38 for raw zucchini, and 14–27 for raw cucumber were obtained among gourds.

Leafy vegetables had TPC of 1,320 for raw red Swiss chard leaves (Fig. 81.2), 830 for raw white Swiss chard leaves, 26–745 for raw dandelion, 33–484 for raw spinach, 136 for raw arugula, 4–131 for raw green lettuce, 130 for raw red chicory, 37–118 for raw beet greens, and 114 for raw red lettuce.

TPC measured in onion-family vegetables included 17–181 for raw yellow onion, 82–126 for raw red onion, 115 for raw pink shallot, 42–88 for raw leek, and 46 for raw white onion. Among pod vegetables, raw green bean had TPC of 5–458.

Root vegetables had TPC of 168 for raw kohlrabi, 164 for raw red beetroot, 8–156 for raw carrot, 141 for raw burdock root, 30–61 for raw radish, 59 for raw celeriac, and 55 for raw turnip root.

Shoot vegetables had TPC of 731–1,305 for raw globe artichoke heads, 14–141 for raw asparagus, and 28 for fennel.

Stalk vegetables had TPC of 410 for raw red Swiss chard stems, 290 for raw white Swiss chard stems, and 13–15 for raw celery stalks.

TPC of tubers was 17–163 for raw potato.

2.9.2 Phenolic Acids

Vegetables with high maximum values of SIPA (mg 100 g^{-1} FW) were raw red chicory (84.8–555.8), raw black olive (1.4–523.0), raw globe artichoke heads (136.0–305.1), raw green olive (21.3–303.5), raw green chicory (91.1–294.1),

Fig. 81.2 Raw red Swiss chard leaves and stem

raw burdock root (126.8), raw potato (11.1–51.2), raw broccoli (3.2–48.5), and raw carrot (7.0–20.6). Other vegetables had maximum SIPA of less than 10.0.

The major phenolic acids (mg 100 g^{-1} FW) in the above vegetables with maximum SIPA (>100 mg 100 g^{-1} FW) were 5-caffeoylquinic (51.3–432.9), chicoric (14.2–59.7), gallic (8.0–30.9), and protocatechuic (11.2–25.7) acids in raw red chicory; verbascoside (0–320.2), syringic (0–74.4), sinapic (0–40.0), *m*-coumaric (0–25.0), and protocatechuic (0–21.0) acids in raw black olive; 5-caffeoylquinic acid (136.0–305.1) in raw globe artichoke heads; sinapic acid (5.0–83.0), verbascoside (0–66.5), cinnamic (4.0–26.0), and *o*-coumaric (0–20.0) acids in raw green olive; 5-caffeoylquinic (43.2–158.6), chicoric (21.8–61.1), gallic (12.8–38.9), and protocatechuic (13.4–30.2) acids in raw green chicory; and 5-caffeoylquinic acid (126.8) in raw burdock root without performing any hydrolysis step.

After hydrolysis, vegetables with high maximum values of SIPA were raw black olive (151.0–464.0), raw green olive (81.0–360.0), raw red Swiss chard leaves (102.5) (Fig. 81.2), raw white Swiss chard leaves (88.9), raw red Swiss chard stems (27.1) (Fig. 81.2), raw white Swiss chard stems (23.9), and raw carrot (20.7). The maximum SIPA was less than 5.0 for the other vegetables. The major phenolic acids in the above vegetables with maximum SIPA (>50 mg 100 g^{-1} FW) were homovanillic (36.0–111.0), protocatechuic (43.0–74.0), syringic (0–48.0), sinapic (14.0–40.0), *5*-caffeoylquinic (2.0–40.0), vanillic (17.0–32.0), and *m*-coumaric (0–25.0) acids in raw black olive; sinapic (7.0–83.0), *o*-coumaric (31.0–45.0), homovanillic (17.0–43.0), ferulic (4.0–40.0), cinnamic (13.0–29.0), and caffeic (0–28.0) acids in raw green olive; syringic (44.9), caffeic (14.8), and *p*-coumaric (10.4) acids in raw red Swiss chard leaves; and syringic (45.1) and ferulic (10.8) acids in raw white Swiss chard leaves.

2.9.3 Flavonoids

Vegetables with high maximum values of SIF (mg 100 g^{-1} FW) were raw black olive (15.0–552.2), raw red onion (67.2–403.3), raw yellow onion (27.2–290.5), raw spinach (33.6–288.4), raw broad bean pod (169.4–209.7), raw globe artichoke heads (3.2–112.4), raw shallot (112.2), raw curly endive (7.3–40.8), raw red lettuce (12.2–33.1), raw asparagus (0–28.7), raw broccoli (27.8), raw green bean (1.4–25.4), raw escarole endive (18.2), and raw green lettuce (0.1–15.5). Other vegetables had maximum SIF of less than 10.0.

The major flavonoids (mg 100 g^{-1} FW) in the above vegetables with high maximum values of SIF (>100 mg 100 g^{-1} FW) were identified as cyanidin *3-O*-rutinoside (0–320.6), cyanidin *3-O*-glucoside (0–88.2), quercetin *3-O*-rutinoside (11.1–78.7), and luteolin *7-O*-glucoside (0.5–29.2) in raw black olive; quercetin *3,4′-O*-diglucoside (20.2–207.5), quercetin *4′-O*-glucoside (30.0–114.3), and quercetin (0–55.8) in raw red onion; quercetin *3,4′-O*-diglucoside (11.4–135.8), quercetin *4′-O*-glucoside (13.8–83.0), and isorhamnetin (0–53.9) in raw yellow onion; *5,4′*-dihydroxy-*3,3′*-dimethoxy-*6*:*7*-methylenedioxyflavone *4′-O*-glucuronide (6.8–113.1), patuletin *3-O*-glucosyl-(1->6)-[apiosyl(1->2)]-glucoside (7.2–45.0), spinacetin *3-O*-(*2″*-feruloylglucosyl)(1->6)-[apiosyl(1->2)]-glucoside (6.9–26.5), *5,3′,4′*-trihydroxy-*3*-methoxy-*6*:*7*-methylenedioxyflavone *4′-O*-glucuronide (0–22.3), spinacetin *3-O*-glucosyl-(1->6)-glucoside (1.6–22.1), spinacetin *3-O*-glucosyl-(1->6)-[apiosyl(1->2)]-glucoside (5.4–21.9), patuletin *3-O*-(*2″*-feruloyl-glucosyl)(1->6)-[apiosyl(1->2)]-glucoside (4.0–20.2), and jaceidin *4′-O*-glucuronide (1.8–15.4) in raw spinach; (−)-epicatechin (37.6), prodelphinidin dimer B3 (23.5), quercetin *3-O*-galactoside *7-O*-rhamnoside (6.1–22.2), procyanidin dimer B4 (18.5), (−)-epigallocatechin (17.4), (+)-catechin (16.2), procyanidin dimer B2 (12.1), procyanidin dimer B1 (11.3), quercetin *3-O*-(*6″*-acetyl-galactoside) *7-O*-rhamnoside (1.8–10.8), and kaempferol *3-O*-(*6″*-acetyl-galactoside) *7-O*-rhamnoside (2.0–10.0) in raw broad bean pod; luteolin (0–84.2), luteolin *7-O*-glucuronide (0–16.6), and apigenin *7-O*-glucuronide (3.2–11.6) in raw globe artichoke heads; and quercetin *3,4′-O*-diglucoside (74.6) and quercetin *4′-O*-glucoside (35.6) in raw shallot without performing any hydrolysis.

After hydrolysis, vegetables with high maximum values of SIF were raw red lettuce (2.2–91.1), raw spinach (0–88.9), raw yellow chilli pepper (5.8–88.7), raw white onion (0–63.4), raw kale (0.5–59.0), raw red onion (5.6–55.9), raw Pak choy (54.4), raw yellow onion (2.8–52.4), raw green chilli pepper (0–32.8), raw whole cherry tomato (2.1–29.5), raw Swede (0–26.5), raw celery stalks (0–23.1), raw broccoli (4.6–20.9), raw red Swiss chard leave (18.9), raw green lettuce (0–17.0), raw garden cress (14.0), raw whole tomato (0.1–12.9), raw white Swiss chard leaves (11.1), and raw red chilli pepper (0.7–10.0). Other vegetables had maximum SIF of less than 8.0.

After hydrolysis, the major flavonoids in the above vegetables with high maximum values of SIF (>50 mg 100 g^{-1} FW) were identified as quercetin (2.2–91.1) in raw red lettuce, kaempferol (0–55.0) and quercetin (0–27.2) in raw spinach, quercetin (4.2–78.4) and luteolin (1.6–10.4) in raw yellow chilli pepper, quercetin (0–63.4) in raw white onion, kaempferol (0.5–47.0) and quercetin (0–12.0) in raw kale, quercetin (5.6–45.0) and kaempferol (0–4.5) in raw red onion, quercetin (39.0) and kaempferol (9.6) in raw pak choy, and quercetin (2.8–46.3) and myricetin (0–4.1) in raw yellow onion.

2.9.4 Other Phenolic Compounds

The other phenolic compounds (mg 100 g^{-1} FW) with high maximum values in vegetables were hydroxytyrosol (0–413.3), oleuropein (3.6–240.6), oleuropein-aglycone (2.0–199.1), tyrosol (0.9–118.6), demethyloleuropein (1.3–60.0), and oleuropein-aglycone *mono*-aldehyde (1.0–17.0) in raw black olive; oleuropein (0–325.0), hydroxytyrosol (4.3–116.0), oleuropein-aglycone (36.0–81.0), oleuropein-aglycone mono-aldehyde (13.0–22.0), tyrosol (0–21.0), and demethyloleuropein (12.0–14.0) in raw green olive; and 04–06-mers (3.2), 02-mers (1.5), and 03-mers (1.4) in raw avocado.

3 Conclusion

Phenolic compounds are important phytochemicals in foods purported to protect health and prevent diseases. Total phenolic content is a useful tool to predict the antioxidant potential of food sources. To understand the role of dietary sources of plant foods in health, food composition databases of phenolic compounds need to be complete taking into account geographical origin, varietal and environmental factors, and analytical techniques used. Regardless of the challenges, there is a full gamut of dietary foods from plant sources that serve as significant sources of phenolics. Included among the top foods abundant in phenolics are cloves, cocoa powder, dried peppermint, star anise, dried Mexican oregano, celery seed, black chokecherry, dark chocolate, flaxseed meal, and black elderberry.

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Nutraceuticals and Antioxidants in Prevention of Diseases

82

Neelam Jain and Kishan G. Ramawat

Contents

1 Introduction 2560
2 Classification of Nutraceuticals 2564
  2.1 Dietary Supplements 2564
  2.2 Functional Foods 2565
  2.3 Medical Foods 2565
  2.4 Farmaceuticals 2566
3 Phytochemicals as Nutraceuticals 2566
  3.1 Alkaloids 2567
  3.2 Terpenes 2567
  3.3 Phenols 2568
  3.4 Flavonoids 2570
  3.5 Catechins and Stilbenes 2570
  3.6 Anthocyanins 2570
  3.7 Flavanones 2571
  3.8 Isoflavones 2571
4 Use of Antioxidants as Nutraceuticals 2573
5 Drawbacks of Nutraceuticals 2574
6 Market Trends of Nutraceuticals 2574
7 Conclusion 2576
References 2577

Abstract

Drugs and food from natural origin play an important role in public healthcare system throughout the world. The word nutraceutical is a broad term describing foods, food ingredients, and dietary supplements that provide

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specific health or medical benefits, in addition to the basic nutritional value found in the food. A significant source for nutraceutical ingredients is bioactive phytochemicals like alkaloids, various terpenoids, and polyphenols (anthocyanins, flavones, flavanols, isoflavones, stilbenes, ellagic acid, etc.). A variety of these phytochemical compounds form an integral part of the nutraceutical market being antioxidants, and they help in the prevention of many degenerative and chronic diseases. In this review, classification of nutraceuticals, phytochemical classes as nutraceutical ingredients, antioxidants as nutraceuticals, and advantages and drawbacks along with nutraceutical market development are discussed.

Keywords

Antioxidants • dietary supplements • functional foods • nutraceuticals • phytochemicals

Abbreviations

CAGR	Compound annual growth rate
FMCG	Fast-moving consumer goods
ROS	Reactive oxygen species

1 Introduction

Nutraceutical, a term coined by combining the words "nutrition" and "pharmaceutical," is defined by Health Canada as "a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food." A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease [1]. The term nutraceutical was coined in 1989 by Stephen DeFelice, founder and chairman of the Foundation for Innovation in Medicine, an American organization which encourages medical health [2–5]. According to DeFelice, "a nutraceutical is any substance that is a food or a part of food and provides medical or health benefits, including the prevention and treatment of disease." The term nutraceutical is a hybrid term coined from nutrition and pharmaceuticals (Fig. 82.1). Within European Union law, the legal categorization of a nutraceutical is, in general, made on the basis of its accepted effects on the body. Thus, if the substance contributes only to the maintenance of healthy tissues and organs, it may be considered to be a food ingredient. If, however, it can be shown to have a modifying effect on one or more of the body's physiological processes, it is likely to be considered to be a medicinal substance. Within European medicines law, a nutraceutical can be defined as a medicine for two reasons:

1. If it presented for the prevention, treatment, or cure of a condition or disease
2. If it can be administered with a view to restoring, correcting, or modifying physiological functions in human beings. Such products may range from isolated

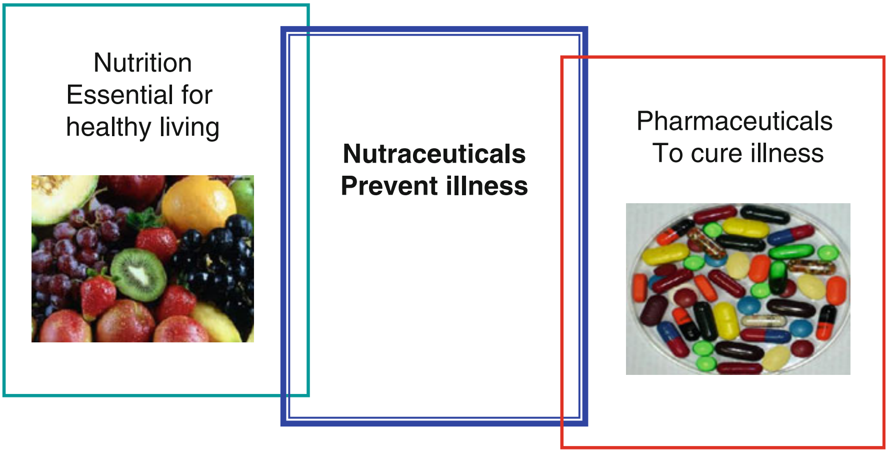

Fig. 82.1 Nutraceutical – term coined from nutrition and pharmaceuticals

nutrients, dietary supplements, and specific diets to genetically engineered designer foods and herbal products [2, 6]. They are natural bioactive, chemical compounds that have health-promoting, disease-preventing, or medicinal properties and found in a mosaic of products. They can be derived from plants (e.g., *Echinacea*, fenugreek), from animals and microorganisms (e.g., essential fatty acids, enzymes), and from marine sources (e.g., glucosamine, chitosan, fish oils). Flaxseed and fish oil are beneficial in cardiovascular diseases. Flaxseeds contain lignans and fiber which are involved in cardiac protection.

The concept of nutraceuticals is not entirely new, although it has evolved considerably over the years. In the early 1900s, food manufacturers in the United States began adding iodine to salt in an effort to prevent goiter (an enlargement of the thyroid gland), representing one of the first attempts at creating a functional component through fortification. Today, researchers have identified hundreds of compounds with functional qualities, and they continue to make new discoveries surrounding the complex benefits of phytochemicals (nonnutritive plant chemicals that have protective or disease-preventive properties) in foods. Table 82.1 represents a sample of available nutraceuticals, their components, their sources, and their potential health benefits. The main attractions of nutraceuticals are their lack of side effects and the possibility that they may be able to affect beneficially the disease course rather than just ameliorating symptoms. They play important role in the treatment of many diseases (Fig. 82.2).

There is a slight difference between the functional foods and nutraceuticals. When food is being cooked or prepared using “scientific intelligence” with or without knowledge of how or why it is being used, the food is called “functional food.” Thus, functional food provides the body with the required amount of vitamins, fats, proteins, carbohydrates, etc., needed for its healthy survival. When

Table 82.1 Functional components of available nutraceuticals, their sources, and their potential health benefits

S. no.	Functional components	Source	Potential health benefits
1.	*Carotenoids*		
	Alpha carotene	Carrots, sweet potatoes, pumpkin, winter squash, broccoli, peas, spinach, turnips, lettuce	Neutralizes free radicals that may cause damage to cells, boosts cellular antioxidant defenses, protects cornea against UV light
	Beta carotene	Carrots, oat, pumpkin, sweet potato	
	Lutein	Green vegetables, corn, egg yolk	Anticancer (colon), reduces risk of macular degeneration
	Zeaxanthine	Eggs, citrus, corn	Maintenance of healthy vision
	Lycopene	Tomatoes, watermelon, guava, papaya	Antioxidant, reduces prostate cancer
2.	*Saponins*	Soybeans, chickpea	Anticancer, lower cholesterol
3.	*Polyphenolic compounds*		
	Flavanones	Citrus fruits	Antioxidant, anticancer
	Flavones	Fruits, vegetables, soybean	Antioxidant, reduce risk of cancer
	Anthocyanins	Blueberries, blackberries, black raspberries	Antioxidant, anticancer, improve visual capacity and brain cognitive performance
	Proanthocyanidins	Fruits (grapes, apples, strawberries), beans, nuts, cocoa	Reduce cardiovascular diseases, improve urinary tract
	Resveratrol	Grapes, raisins, berries, peanuts	Anticancer, reduces cardiovascular diseases and ischemic damage
	Curcumin	Turmeric root	Anticancer, antioxidant, lower blood cholesterol, promotes wound healing, prevents skin wrinkling, inhibits inflammation
	Ellagic acid and ellagitannins	Blackberries, raspberries, strawberries, walnuts, pomegranates	Antiproliferative, antioxidant
	Catechins	Tea, mustard cake, rapeseed some fruits and vegetables and wine	Neutralize free radicals, reduce risk of cancer, protect the skin from UV radiation-induced damage
4.	*Dietary fiber*		
	Soluble fiber (beta glucan)	Oats, barley, peas, beans, apple, psyllium seed husk	Reduce cardiovascular diseases, protects against some cancers, digestive stimulant, lower total cholesterol
	Insoluble fiber	Whole-grain foods, wheat, and corn bran	Reduces the risk of breast or colon cancer, digestive stimulant

(*continued*)

Table 82.1 (continued)

S. no.	Functional components	Source	Potential health benefits
5.	*Fatty acids*		
	Omega-3 fatty acids DHA/EPA	Salmon, flaxseed, olive oil, canola oil	Anti-inflammatory, maintain brain functions, reduce cholesterol deposition.
	Conjugated linoleic acid (CLA)	Milk, cheese, meat products	Improves body composition, reduces cancers
6.	*Phytoestrogens*		
	Isoflavones (genistein, daidzein)	Soybeans, legumes, soy-based food products	Antioxidant, lower LDL cholesterol, reduce menopause symptoms, increase bone strength
	Lignans	Flaxseed, rye, vegetables	Reduce cancer and heart diseases
7.	*Probiotics/prebiotics*		
	Lactobacillus	Yogurt, other dairy products	Improve gastrointestinal health and quality of intestinal microflora
	Fructooligosaccharides	Whole grains, onions, artichokes	
8.	*Glucosinolates*	Cruciferous vegetables	Anticancer, greatest protection against bladder cancer
9.	*Sulfides/thiols*	Garlic and cruciferous vegetables	Improve immune functions
10.	*Tocotrienols*	Grains, palm oil	Inhibit breast cancer cell growth, possess cholesterol-lowering property
11.	*Phytosterols*	Green and yellow vegetables	Anti-inflammatory, block the uptake of cholesterol and facilitate its excretion from the body
12.	*Limonoids*	Citrus fruits	Provide protection to lung tissue
13.	*Nutrients*		
	Vitamins (vitamins A, B, C, E, K)	Food products, fruits, vegetables, milk, eggs, fish	Antioxidant; for growth and development, blood clotting, wound healing, etc.
	Minerals (calcium, selenium, potassium, zinc, copper, cobalt, iodine, iron)	Food products, fruits, vegetables	For growth and development, energy production, etc.
14.	*Sugar alcohols* (xylitol, sorbitol, mannitol, lactitol)	Some chewing gums and other food applications	May reduce risk of dental cavities

functional food aids in the prevention and/or treatment of disease(s) and/or disorder(s) other than anemia, it is called a nutraceutical (Since most of the functional foods act in some way or the other as antianemic, the exception to anemia is considered so as to have a clear distinction between the two terms, functional food and nutraceutical). Examples of nutraceuticals include fortified dairy products (e.g., milk) and citrus fruits (e.g., orange juice) [7].

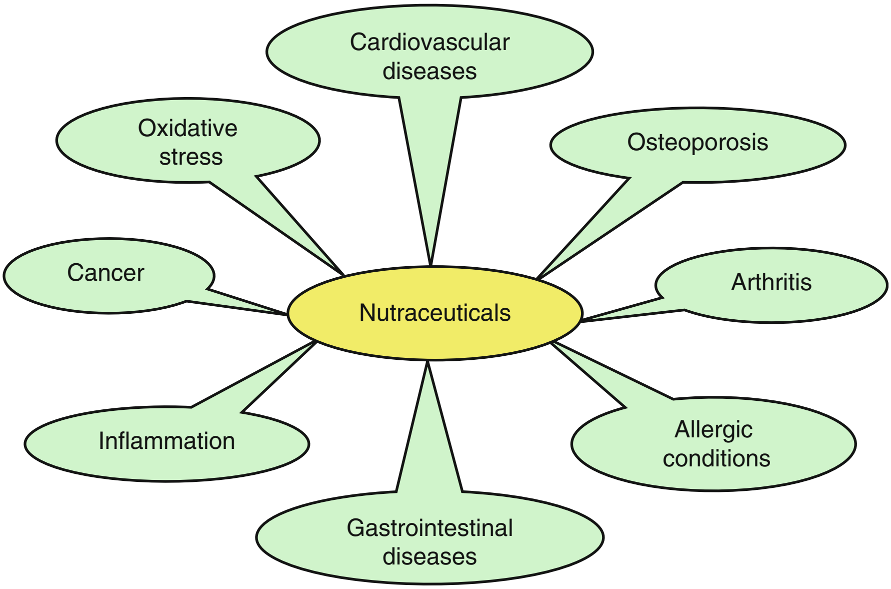
Cardiovascular diseases
Oxidative stress
Osteoporosis
Cancer
Nutraceuticals
Arthritis
Inflammation
Allergic conditions
Gastrointestinal diseases

Fig. 82.2 Role of nutraceuticals in prevention of illness

2 Classification of Nutraceuticals

Nutraceuticals are nonspecific biological therapies used to promote wellness, prevent malignant processes, and control symptoms. It is a broad umbrella term used to describe any product derived from food sources that provides extra health benefits in addition to the basic nutritional value found in foods. The definition of nutraceuticals and related products often depends on the source. Phytochemicals and antioxidants are two specific types of nutraceuticals. It has been proved that phytochemicals found in foods may help to provide protection from diseases such as cancer, diabetes, heart disease, and hypertension, for example, carotenoids found in carrots. Antioxidants may be helpful in avoiding chronic diseases, by preventing oxidative damage in body [8]. There are multiple different types of products that come under the category of nutraceuticals:

1. Dietary supplements
2. Functional foods
3. Medical foods
4. Farmaceuticals

2.1 Dietary Supplements

A dietary supplement, also known as food supplement or nutritional supplement, is a product that contains nutrients derived from food products that are concentrated in liquid or capsule form. The Dietary Supplement Health and Education Act

(DSHEA) of 1994 defined a dietary supplement as “a product taken that contains a ‘dietary ingredient’ intended to supplement the diet and the ‘dietary ingredients’ may include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandular, and metabolites.” Dietary supplements do not have to be approved by the US Food and Drug Administration (FDA) before marketing. Dietary supplements are not intended to treat or cure disease [9] whereas nutraceuticals more emphasize the expected results of these products, such as prevention or treatment of diseases [10].

2.2 Functional Foods

Functional foods are designed to allow consumers to eat enriched foods close to their natural state, rather than by taking dietary supplements manufactured in liquid or capsule form. Functional foods are either enriched or fortified by a process called “nutrification.” This practice restores the nutrient content in a food back to similar levels from before the food was processed. For example, Activia® is a probiotic yogurt marketed to help “regulate your digestive system.” It contains a subspecies strain of *Bifidobacterium animalis*, which is marketed as *Bifidus Regularis*®. Other functional foods include red grapes and cranberry juice (for the oligomeric proanthocyanidins, OPCs) and oat bran (for the fiber content), all with health benefits attributed to “non-nutrient” compounds as classified by standard agreement of the term. Cranberry juice cocktail is now marketed as a functional food with a structure/function claim regarding support of urinary tract health. Several companies are marketing breakfast cereals as functional foods when enriched with inulin (an oligosaccharide) or psyllium. Plasma total cholesterol was reduced by 7.9 % and total triglycerides by 21.2 % in young men consuming a 50-g serving of a breakfast cereal fortified with 18 % inulin for 4 weeks [11]. Similarly, breakfast cereal containing 50 g of soluble fiber as psyllium, oat, and barley showed that it significantly reduced total cholesterol and low-density lipoprotein (LDL) concentrations in the blood as compared to a wheat bran cereal for middle-aged mildly hypercholesterolemic men who were already eating a diet low in saturated fat [12]. Plant sterol-enriched margarines, originally projected as dietary supplements but now marketed in the United States as functional foods, were shown to reduce blood cholesterol levels in mildly hypercholesterolemic subjects in a human trial [13]. Functional foods are an emerging field in food science due to their increasing popularity with health-conscious consumers. Like dietary supplements, functional foods are not required to undergo safety and efficacy testing prior to marketing.

2.3 Medical Foods

Medical foods are considered by the FDA as “formulated to be consumed or administered internally under the supervision of a physician, and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, on the basis of recognized scientific principles, are established by medical evaluation" [14]. Nutraceuticals and supplements are not classified as medical foods as they do not meet these requirements. Medical foods are always designed to meet certain nutritional requirements for people diagnosed with specific illnesses and can be ingested through mouth or tube feeding. They are regulated by the FDA and prescribed/monitored by medical supervision. In order to be considered a medical food, the product must, at a minimum:

1. Be a food for oral ingestion or tube feeding (nasogastric tube)
2. Be labeled for the dietary management of a specific medical disorder, disease, or condition for which there are distinctive nutritional requirements
3. Be intended to be used under medical supervision

Drugs can be found sometimes in foods and can participate in the metabolism. However, these substances are not normally exposed in humans at the dose at which they exert their beneficial effect. Some dietary supplements are drug-like when ingested in amounts that could never be achieved in the diet, even though they are essential nutrients when ingested in smaller quantities, for example, tryptophan is an essential amino acid required for metabolism and incorporation into proteins at low dose in humans. At high dose, it increases 5-hydroxytryptamine levels in brain and thus acts as a drug to treat insomnia.

Unlike dietary supplements, medical foods can be labeled for medical conditions such as Alzheimer disease. Dietary supplements must be labeled for so-called structure and function claims and cannot make claims to treat or prevent disease [9]. For example, ginkgo may be labeled "supports memory function" but not "for treatment of dementia." A drug or medical food could be labeled "for treatment of dementia associated with Alzheimer disease."

2.4 Farmaceuticals

According to a report written for the United States Congress entitled "Agriculture: A Glossary of Terms, Programs, and Laws," farmaceuticals is a melding of the words farm and pharmaceuticals. It refers to medically valuable compounds produced from modified agricultural crops or animals (usually through biotechnology). Proponents believe that using crops and possibly even animals as pharmaceutical factories could be much more cost-effective than conventional methods (i.e., in enclosed manufacturing facilities) and also provide agricultural producers with higher earnings. The term farmaceuticals is more frequently associated, in agricultural circles, with medical applications of genetically engineered crops or animals [15].

3 Phytochemicals as Nutraceuticals

It is well established that plant-derived foods such as wine, fruits, nuts, vegetables, cereals, pulses, and spices exert beneficial effects on human health particularly to conditions related to aging. With increase in average life span, chronic age-related diseases are increasing and correlative with dietary habits such as cardiovascular diseases, neurodegenerative diseases, type II diabetes, and several types of cancer. This has provided a way to look into bioactive molecules present in the diet. These bioactive molecules present in plant-derived foods are not nutrients primarily. These phytochemicals have been shown to exert a wide range of biological activities. Though, they have low potency as compare to a drug molecule, but because of regular intake, they may exert remarkable long-term physiological effects.

Three major classes of secondary metabolites are involved in these phytochemicals, which are alkaloids, terpenes, and phenolics [16]. There are many phytochemicals recognized with health benefits such as sulfur-containing compounds of garlic family, various terpenoids, and polyphenols (anthocyanins, flavones, flavanols, isoflavones, stilbenes, ellagic acid, etc.).

3.1 Alkaloids

Among nitrogen-containing compounds and alkaloids, glucosinolates (present in cruciferous vegetables such as broccoli, cauliflower, and cabbage) and purine alkaloids (present in tea, coffee, etc.) are widely consumed, and their beneficial health-promoting effects are well established [17–19]. Glucosinolates are transformed into isothiocyanates, dithioelthiones, and sulforaphanes. Glucosinolates are released from damaged plant cells and converted by the enzyme myrosinase into isothiocyanates [20]. The current interest in glucosinolates is largely focused on their ability to protect against cancer. There is good evidence from epidemiological studies showing an inverse relationship between consumption of cruciferous vegetables and risk of cancer [21, 22]. These compounds inhibit the neoplastic effects of various carcinogens at a number of organ sites [18].

Caffeine, a purine alkaloid, is mostly ingested in coffee, tea, and cola soft drinks and remains the world’s most widely used pharmacologically active substance. Caffeine is potentially beneficial to human health since it increases extracellular levels of acetylcholine and serotonin (both are neurotransmitter hormones involved in the transmission of nerve impulses) by binding to adenosine receptors in human brain suggesting that caffeine usage can reduce age-related cognitive decline [17].

3.2 Terpenes

Terpenes, present in green foods, soy plants, and cereals, are the largest class of phytonutrients. When terpenes are modified chemically, such as by oxidation or rearrangement of the carbon skeleton, the resulting compounds are generally referred to as terpenoids. Terpenoids are also known as isoprenoids. They are important for plants due to their ability to fix carbon through photosynthetic reactions using photosensitizing pigments like chlorophyll and carotene. These compounds provide protection from certain diseases such as chronic damage and growth dysregulation in animals as these are utilized for hormonal and growth regulatory functions [18]. Tocotrienols and tocopherols are the most studied terpene antioxidants that occur naturally in cereals. Tocotrienols and tocopherols and their derivatives possess antiproliferative and apoptotic effects and help in preventing or reducing the risk of breast cancer in women [23].

Carotenes (tetraterpenes) are a large group of intense red and yellow pigments found in all plants that photosynthesize. Carotenoids (carotenes without oxygen) are fat-soluble pigments widely distributed in plants and animals and possess antioxidant activity. They provide health benefits by decreasing the risk of disease, particularly certain cancers, stroke, heart disease, and eye disease, and strengthen the immune system. Carotene and xanthophylls are two types of molecules that comprise carotenoids. They play an essential nutritional role as they are precursors to vitamin A. Carotenoids from vegetable juices is able to enhance the immune system in those who consume a low-carotenoid diet [24]. The carotenes γ-carotene, lycopene, and lutein protect against uterine, prostate, breast, colorectal, lung, and digestive tract cancers [25, 26]. Limonoids (tetranortriterpenes) are present in the plants belonging to the family Rutaceae (e.g., citrus fruits) and Meliaceae. They possess chemotherapeutic activity [27] and provide protection to lung tissue [28].

Phytosterols are another important terpene subclass. Two sterol molecules that are synthesized by plants are β-sitosterol and its glycoside. In animals, these two molecules exhibit anti-inflammatory, antineoplastic, antipyretic, and immune-modulating activity [29]. Phytosterols were reported to block inflammatory enzymes, for example, by modifying the prostaglandin pathways in a way that protected platelets [30].

3.3 Phenols

In an excellent work, occurrence, structure, and the role of secondary metabolites in human diet have been described [31], which includes phytochemicals present in fruits, vegetables, herbs and spices, nuts, cereals, and beverages [32]. Simple phenols and polyphenolics are widely distributed in the plant kingdom. Phenolics are characterized by having at least one aromatic ring with one or more hydroxyl groups attached [33]. All phenolics can be broadly divided into flavonoids {flavonols (quercetin), flavones (apigenin), flavanols (catechin and its derivatives, proanthocyanidins), anthocyanidins (anthocyanins), flavanones (hesperidin), isoflavones (genistin, diadzin)} and non-flavonoids {stilbenes (resveratrol), phenolic acids (gallic acid, hydroxybenzoic acid, hydroxycinnamic acid)} [31].

Details about structure, biosynthesis, and classification of phenolics are described in the other chapters in this handbook. Plant phenolics have a distinctive ability to form non-covalent, intermolecular complexes with each other and with both large and small molecules. Recognition of the antioxidant activities of many polyphenols has established correlation with the health benefits by such compounds [34]. This leads to the development of commercial products containing free-radical-scavenging phytochemical mixtures, for example, Pycnogenol (procyanidin extracted from *Pinus maritima*). Table 82.2 represents a list of polyphenolic compounds used in nutraceuticals and their biologic effects on human health.

Table 82.2 Polyphenolic compounds used in nutraceuticals and their biologic effects

Active polyphenolic compound	Source	Biologic effect	References
Quercetin	Citrus fruits, apples, onions, parsley, tea, and red wine	Potent antioxidant, anti-inflammatory, antiallergic, anticancer, inhibits mitogen-activated protein (MAP) kinase in human epidermal carcinoma cells, inhibits tumor growth	[35–37]
Ellagic acid	Pomegranate, raspberries, cranberries, *Terminalia chebula* fruit, pecans, and walnuts	Strengthens the immune system, anticancer, induces apoptosis of cancer cells and suppresses angiogenesis, prevents heart disease and liver fibrosis, promotes wound healing, prevents the binding of carcinogens to DNA	[38]
Luteolin	Thyme, peppermint, basil herb, celery, and artichoke	Possesses free-radical scavenging activity, protects human single-cell DNA from oxidative attack	[39]
Curcumin	Turmeric roots	Suppresses the production of cytokines such as IFN-γ, interleukins, and TNF; inhibits the inducible nitric oxide synthase (iNOS)	[40]
Resveratrol	Grapes, raisins, berries, peanuts	Antiaging, anticarcinogenic, anti-inflammatory, and antioxidant properties; improves insulin sensitivity in type 2 diabetic patients	[41]
Apigenin	Parsley, thyme, peppermint, red wine and tomato sauce	Antidepressant, anticancer, antitumor, anti-inflammatory	[42]
Ferulic acid	Rice, wheat, oats, coffee, apple, peanut, orange and pineapple	Provides neuroprotection against oxidative stress-related apoptosis by inhibiting ICAM-1 mRNA expression after cerebral ischemia, hypotension, and hypoglycemia	[43–45]
Caffeic acid	White grapes, white wine, olives, spinach, cabbage, asparagus and, coffee	Exerts antidepressive and anxiolytic-like effects through indirect modulation of the alpha 1A-adrenoceptor system	[46]
Catechins	Tea, chocolate, red wine, apples, and berries	Prevents human plasma oxidation by delaying the consumption of endogenous lipid-soluble antioxidants and inhibiting lipid oxidation	[47]

3.4 Flavonoids

Flavonoids are commonly present in fruits and vegetables, while isoflavones are present in soybean and other pulses. Biological activities of flavonoids include action against free radicals, free-radical-mediated cellular signaling, inflammation, allergies, platelet aggregation, microbes, ulcers, viruses, tumors, and hepatotoxins [18, 21, 48, 49]. Anti-inflammatory and antibacterial activities of flavonoids are well established in case of luteolin and kaempferol, apigenin, and myricetin [18]. Curcumin, a dietary pigmented polyphenol, suppressed tumor promotion in mouse skin possibly through suppression of protein kinase C activity and nuclear oncogene expression [40, 50].

3.5 Catechins and Stilbenes

Major sources of catechins are grapes, berries, cocoa, and green tea. Tea contains considerable amounts of gallic acid esters, such as epicatechin, epicatechin gallate, and epigallocatechin gallate (EGCG). The green tea polyphenol EGCG has beneficial activity in a number of human diseases. It reduces several harmful effects in pathologic conditions (diseases of insulin dysregulation, neurodegenerative diseases, anxiety, memory loss, multiple sclerosis, etc.) in part by altering intracellular signal transduction pathways, by scavenging reactive oxygen and nitrogen species and iron and by affecting cytokine production and expression of neurotransmitters or their receptors [19].

Catechin is one of the major phenolics in grapes and red wines, and it is considered to be responsible for part of the protective effects of red wine against atherosclerotic and cardiovascular diseases [18]. Besides catechin, grapes and red wines contain several other polyphenolics including mainly flavonoids and stilbenes.

Stilbenes constitute large class of compounds, monomers, and oligomers (mainly dimers, trimers, and tetramers), formed by oxidative condensation of the resveratrol monomer. Stilbenes exhibit potent biological activities in vitro on several targets that might be able to influence favorably several physiological and pathological processes, leading to protective effect against cardiovascular diseases and cancer [51]. Epidemiological studies on moderate red wine consumption and reduced risk of cardiovascular diseases are popularly known as “French paradox.”

3.6 Anthocyanins

Anthocyanins impart red-purple color to fruits and flowers due to varying composition of its aglycones such as pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. The color of these pigments is influenced by pH and metal ion complexes [52]. A large number of recent reports suggest that anthocyanins and anthocyanin-rich berries or their extracts exhibit a wide range of protective effects with potential benefits for human and animal health [52, 53]. It has been observed that some of these effects may be related to the chemical characteristics and associated antioxidant activity of these compounds. These compounds may also alter gene expression to modulate health benefits [54, 55]. Though, the available data may not be conclusive for beneficial effects of anthocyanins in a particular disease, the increasing number of evidence suggest beneficial effects of anthocyanin-rich berries or their extracts on decrease in oxidative cell damage, increase in levels of reduced glutathione (it protects from reactive oxygen species (ROS)), protection from DNA damage, increased peripheral blood flow, improvement in muscle fatigue, enhanced endogenous oxidant defense system, decreased blood pressure, cardioprotective effects, neuroprotection and inhibition of lung cancer, metastasis, etc. These beneficial effects were concluded on the basis of modulation of disease-related marker measurements [52, 53, 56, 57].

3.7 Flavanones

The most extensively investigated are the aglycones naringenin and hesperetin (glycosidic forms called naringin and hesperidin) present in grapefruits and oranges, respectively. These compounds exhibit a wide range of biological and pharmacological activities, such as antioxidant, hypocholesterolemic, hypoglycemic, prevention of bone losses, and antitumor. These beneficial effects have consequences in protection against diseases like cardiovascular, diabetes, osteoporosis, and cancer [52].

3.8 Isoflavones

Isoflavones are flavonoids commonly called as phytoestrogens and one of the most extensively investigated polyphenols. These are present in soybeans, soybean products, and other pulses, widely consumed in Asian and Southeast Asian countries. Isoflavones have attracted attention mainly due to their role in the amelioration of postmenopausal symptoms such as hot flushes and osteoporosis [52, 58, 59]. The other beneficial and important biological properties have been established which are related to effects on cardiovascular diseases, cognitive functions, and breast and prostate cancers. These conclusions were based on wide-ranging studies and parameters which showed positive effects, for example, reduction of hot flushes, the excretion of bone resorption markers, increase in bone mineral density, the lower LDL and total cholesterol, reduction in colon cancer, and modulation of immune function [60, 61]. Chemical structures of some important molecules of these phytochemical classes are represented in Fig. 82.3.

Phytochemicals, acting as nutraceuticals, play an important role as hormetic compounds and protect from some chronic diseases. Natural environmental stresses stimulate plants to produce specific endogenous chemicals that help protect the plant against the change in conditions that could otherwise be detrimental. These are regarded as “hormetic compounds” [62]. Under stressful conditions, plants produce these specialized chemicals which are transferred to animals while eating

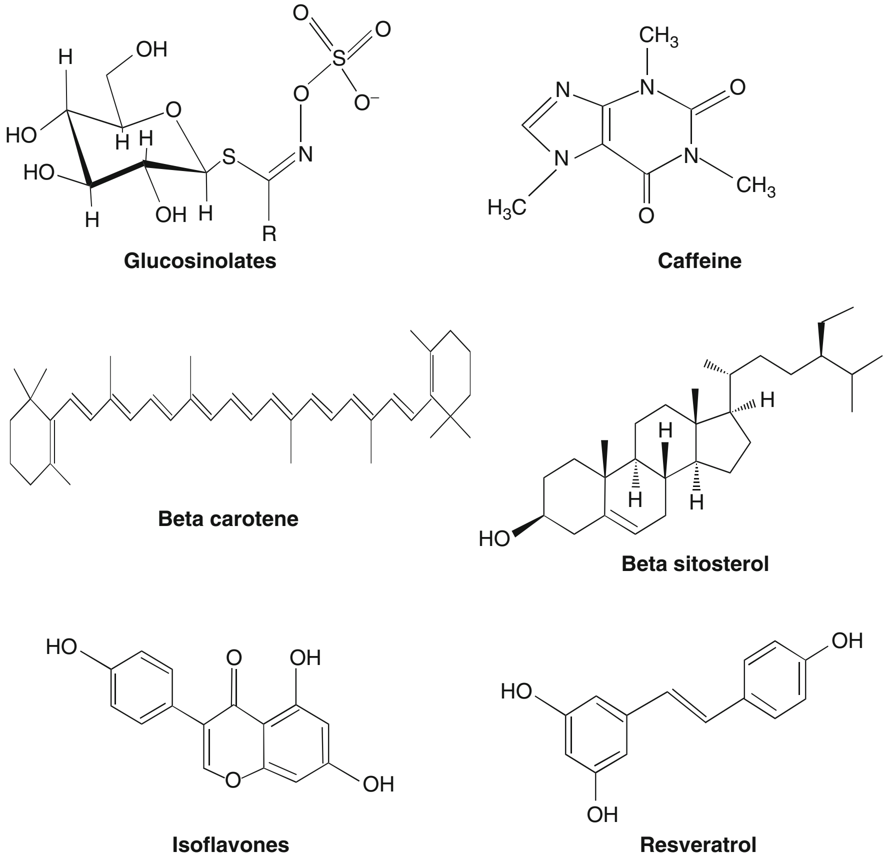

Fig. 82.3 Chemical structures of some important molecules: (**a**) glucosinolates, (**b**) caffeine, (**c**) beta carotene, (**d**) beta sitosterol, (**e**) isoflavones, and (**f**) resveratrol

the plants. Similar protective mechanisms are activated in the cells of the animals as are activated in the cells of the plants. When these plant chemicals act in animals in this way, they are referred as "xenohormetic compounds." The xenohormesis hypothesis [63] proposes that organisms have evolved to respond to stress-signaling molecules (hormetic compounds) produced by dietary plant species where they live, so enabling them to be prepared to resist potentially detrimental effects. Hormetic compounds are present in all vegetables and fruit at different levels. Among the most well known are various berry fruits, broccoli, cocoa, and green tea. One of the most potent hormetic compounds is the polyphenolic compound resveratrol which enhance stress resistance and thus survival pathways in cells via stimulation of sirtuins [64]. The prevention of platelet aggregation by resveratrol involves its selective inactivation of the prostaglandin H2 synthase, cyclooxygenase (Cox)-1, over Cox-2 [65]. Sirtuin or Sir2 proteins are a class of proteins that possess either histone deacetylase or mono-ribosyltransferase activity. Resveratrol, via stimulation of the sirtuin, Sirt1-mediated deacetylation of PGC-1 (peroxisome proliferator-activated receptor-γ coactivator, a crucial regulator of mitochondrial biogenesis and function), improves mitochondrial function and energy balance [66, 67]. Resveratrol also shows dilatation of blood vessels, anti-atherosclerotic effects, lowering of lipid peroxidation, protection of endothelial cells against apoptosis, lowering of blood pressure, oxidative stress, etc. Tea catechins, genistein, quercetin, etc., are other major hormetic compounds.

4 Use of Antioxidants as Nutraceuticals

Antioxidants are substances which retard or prevent the deterioration, damage, or destruction caused by oxidation. Oxidative stress arises by the generation of reactive oxygen species (ROS) and reactive nitrogen species in case of lack of antioxidant defense or by the increase of oxidative processes in the body. Free radicals are a major cause of many degenerative diseases. Many chronic diseases such as cardiovascular diseases, neurodegenerative diseases, and carcinogenic processes are related to free radicals [68]. Antioxidants form an integral part of the nutraceutical market. They are the major part of the most commonly known nutrients. Many compounds possess direct or indirect antioxidant activity. These are quite large in number and diverse in nature and prevent oxidation by neutralizing free radicals at relatively small concentrations. The only official definition of antioxidants is related to "dietary antioxidants" [68]. The definition proposed by the Panel on Dietary Antioxidants and Related Compounds of the Food and Nutrition Board is that "a dietary antioxidant is a substance in food that significantly decreases the adverse effects of ROS, reactive nitrogen species, or both on normal physiological function in human" [69]. Antioxidants prevent the damage at the cellular level by using the following mechanisms: they may reduce the energy of the free radical or suppress radical formation or repair damage and reconstitute membranes. Dietary intake of antioxidants exerts many potential benefits. Ingestion of antioxidants present in fruits and vegetables or administration of synthetic antioxidants decreases certain chronic diseases of aging [8]. For example, high dietary intake of vitamin E may prevent Parkinson's disease [70]. Many vitamins can inhibit low-density lipoproteins (LDL) oxidation, protecting heart against diseases [71]. Terpenes have a unique antioxidant activity as they react with free radicals by partitioning themselves into fatty membranes by virtue their long carbon side chain and help in cure of diseases. Phenols protect plants from oxidative damage. They also act as antioxidant protectant for humans. Phenolic antioxidants such as tocopherols, green tea polyphenols, and phytoestrogens decrease oxidative cell injuries and inflammatory reactions improving brain's health [72, 73]. Scientific focus on the identification of health-protectant components within food and their mechanism(s) of action, such as beta glucan in oat bran, directs attention to the term nutraceuticals rather than to the whole-food concepts implicit in a term such as functional foods [74].

5 Drawbacks of Nutraceuticals

The use of nutraceuticals is increasing due to their easy availability without a prescription from a health-care provider. Natural products are gaining popularity, as in some diseases, standard drugs fail. But, there are some drawbacks in using these products. The lack of quality control is a major area of concern for nutraceuticals [75]. Nutraceuticals are regulated as foods, not as drugs. The plant material quality and manufacturing processes are regulated by food laws, which lack the specificity requirement for botanical drugs. This can have potentially fatal consequences such as contamination with toxins after fungal infection of raw plant material or with other ingredients [76, 77]. Adulterations have been reported in the medical literature because they remain undetected due to a lack of pharmaceutical quality control [78]. Absence of quality control not only increases the risk to the consumer but also results in problems to conduct adequate research that demonstrate the potential health benefit of nutraceuticals or ensures their safety [79].

The purity and dosage requirements of most of the nutraceuticals are not clear. They do not have the scientific basis of dose recommendation [52]. Cost is another main factor in the production of nutraceuticals. The raw materials are often very costly. Most commercially available nutraceuticals contain a mixture of compounds. Often, the non-declared and unhealthy ingredients that are non-herbal in nature and origin are present (e.g., high content of saturated fat in tortilla chips). Thus, an unhealthy food product can be marketed with the implication of a health benefit. Interferences between compounds during uptake are also not known [52, 79].

6 Market Trends of Nutraceuticals

Rapid advances in science and technology, increasing health-care costs, an aging population, and rising interest in attaining wellness through diet are some important factors due to which interest in nutraceuticals is increasing [80]. The 2004 global nutraceuticals market at the retail level is estimated at approximately $106 billion and is poised to grow at a compounded annual growth rate of 6.0 % during 2004–2009 to be around $140 billion in 2009 [80]. Nearly 2/3 of the American population take at least one type of nutraceutical health product. The use of nutraceuticals is increasing as they give desirable therapeutic outcomes with reduced side effects and fit into the today's lifestyle. The nutraceutical industry in the USA is about $ 86 billion. This amount is slightly higher in Europe and in Japan and represents 47 % of the total annual food sales in the Japanese population that consume nutraceuticals [81]. India and China are becoming the fastest-growing markets. One possible explanation for the growth of nutraceuticals in the USA is the aging majority population. As the average age of the citizens continues to rise, the populations increase its focus on health and wellness. Various types of nutraceutical products are currently available in the market which include fortified cereals, vitamins and mineral supplements, energy drinks and tablets, foods to reduce cholesterol levels, and protein powders. [82]. Resveratrol and

Table 82.3 List of some nutraceuticals available in market

S. no.	Brand name	Constituents	Function	Company
1.	Xangold	Lutein esters	Healthy vision	Cognis Nutrition & Health, USA
2.	Cholestaid	Alfalfa saponins extract	Reduce cholesterol	Omni Nutraceuticals, Inc., USA
3.	Green Tea Max™	Active antioxidants such as green tea (leaf) extract, turmeric, red clover, grape seed extract, pine bark extract, ellagic acid	Potent antioxidant; provides nutritional support to heart, brain, and general cellular functions in the body	Nature's Benefit, Inc., USA
4.	Ferradol Food® Powder	Carbohydrate, protein, niacinamide, calcium, iron, zinc, vitamins	Nutritional supplement for children and adults	Pfizer Limited, India
5.	Z Trim	Corn fiber	Zero calorie fat replacer	US Department of Agriculture
6.	IsoExtend-W™	Isoflavones	Women health (menopause)	Physica Pharma, France
7.	Magnum Big C™	Creatine	Increases intracellular energy and endurance; intensifies muscle hardening, strength, and energy	Magnum Nutraceuticals
8.	BeneFlora Probiotic	Lactobacillus, Bifidobacterium and fructooligosaccharides	Improve gastrointestinal health and systematic immunity	Ortis natural herbal supplements, UK
9.	Nutrilite Iron-Folic	Iron and folic acid	Nutritional supplement, energy provider	Amway Corporation, USA
10.	Acai Berry Pure	Antioxidants, vitamins, minerals, amino acids, proteins, and fiber	Reduces free radicals that can cause cancer and other diseases, promotes heart health, and lowers cholesterol	Apex Nutraceuticals, USA

isoflavonoids from soybean are pioneer bioactive molecules put in market in different products ranging from skin improvement products, antioxidant products to antiaging products. Red wine therapy (polyphenolics) includes drinking massage and bath in red wine for the improvement of health [83]. Wine growers associate of Bordeaux, France, not only promote red wine consumption but support research leading to finding out the bioactive molecules involved in French paradox at the Institute of Sciences of Vine and Wine, Villenave-d'Ornon, (at the outskirt of Bordeaux) France [84]. Selected nutraceutical market products are presented in Table 82.3 and Fig. 82.4. The nutraceutical market is becoming more competitive with the entry of pharmaceutical and major food companies into the nutraceutical arena such as Kellogg, Heinz, Quaker Oats, Unilever, Cargill, Hormel, GlaxoSmithKline, Warner-Lambert, Johnson & Johnson, and Wyeth.

Magnum Big C^{TM} Nutrilite Iron-Folic Acai berry pure

Cholestaid Z-trim BeneFlora-Probiotic

Fig. 82.4 Pictures of some commercially available nutraceutical products

Indian nutraceutical market in 2008 is estimated to be US dollar one billion. While the global market has been growing at a compound annual growth rate (CAGR) of 7 %, the Indian market has been growing much faster at a CAGR of 18 % for the last 3 years, driven by functional food and beverages categories. The Indian nutraceutical market is dominated primarily by pharmaceuticals and “fast-moving consumer goods (FMCG)” companies which are very few pure-play nutraceuticals companies. Some major companies marketing nutraceuticals in India are GlaxoSmithKline Consumer Healthcare, Dabur India Ltd., Cadila Healthcare, EID Parry Limited, Zandu Pharmaceuticals, Himalaya Herbal Healthcare, Amway, Sami Labs Limited, Elder Pharmaceuticals Ltd., and Ranbaxy Laboratories Limited.

7 Conclusion

In present system of medicine and healthcare, nutraceuticals play a significant role. These products are extremely active, have profound effect on cell metabolism, and possess little adverse effects. The limitations of nutraceuticals such as lack of quality control and dose recommendation are the major areas of concern. Nutraceutical professionals and regulatory bodies need to play a crucial role for safety maintenance and advances of nutraceuticals. Food fortification is just beginning and has immense scope for the benefit of human population in underdeveloped and developing countries. This can lead to rapid elimination of problems related to malnutrition and associated diseases. Production of such nutraceuticals at industrial level has huge economic consequences and results in development of related technologies.

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Functional Grapes

83

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Contents

1 Introduction 2582
2 Chemical Composition 2583
2.1 Phenolic Compounds 2584
2.2 Other Bioactive Compounds in Grape 2589
3 Biological Activities 2590
3.1 Bioactivity and Bioavailability of Phenolic Compounds 2590
3.2 Antioxidant Activities of Grape and Grape Derivative Products 2594
3.3 Cardioprotective Activity of Grape and Grape Derivative Products 2596
3.4 Anticarcinogenic Activity of Grape and Grape Derivative Products 2597
3.5 Neuroprotective Activity of Grape and Grape Derivative Products 2598
4 Functional Food from Grapes 2600
4.1 Enrichment of Dairy Products 2600
4.2 Enrichment of Meat Products 2601
4.3 Enrichment of Bread 2602
4.4 Enrichment of Seafood 2603
4.5 Enrichment of Grape Derivative Beverages 2604
5 Conclusion and Perspectives 2605
References 2606

Abstract

Grape is one of the earliest cultivated plants all around the world. Health-benefiting grape properties have been widely studied in vitro, ex vivo, and in vivo. These properties are mainly attributed to phenolic composition, which

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is also responsible for many quality properties. Grape contains anthocyanins, flavonols, flavanols, hydroxycinnamic acid derivatives, hydroxybenzoic acids, and stilbenes. All these show bioactivity and, therefore, antioxidant, cardioprotective, anticarcinogenic, neuroprotective, and other activities are nowadays associated with grape consumption. Clinical studies on the intake of grape or grape derivative products report positive results. For this reason, numerous food products are enriched with different types of grape extracts. Grape extracts are added to meat, fish, dairy products, bread, and beverages so as to increase their nutritional value. The functional product market is an emerging market, and this type of products can be expected to increase in the near future. In this sense, further research is required.

Keywords
Anticarcinogenic • antioxidant • bioactive • cardioprotective • functional • grape • neuroprotective • phenolic compounds

1 Introduction

The Vitaceae family consists of almost 1,000 species, grouped into 17 genera. Grapevines are classified in the *Vitis* genus, and the most widely cultivated *Vitis vinifera* is generally accepted to comprise up to 5,000 true cultivars used in wine, table (fresh fruit), and dried grape manufacture around the world. The *Vitis vinifera* grape is one of the earliest cultivated plants and is thought to have originated in the region between the Mediterranean and the Caspian Seas. Vine cultivars are thought to have slowly spread eastward across southern Asia and westward around the Mediterranean Sea. The Germplasm Resources Information Network (www.ars-grin.gov) of the United States Department of Agriculture describes the genera and 43 species, 5 natural hybrids, and 15 varieties of species in *Vitis*. *V. Vinifera* as the most successfully used grape species, with thousands of wine, table, and raisin grape cultivars grown throughout the world's temperate zones.

The numerous uses of the grapevine fruit, especially for wine and beverages, have made it one of the most important plants worldwide. Grapes are grown in more than 90 countries and are the world's largest fruit crop with a total production of 69 million tons. The countries with the greatest acreage are Spain, France, Italy, Turkey, China, and the United States [1].

Grapes have been praised for thousands of years for their medicinal and nutritional values. Since the ancient age of human civilization, grapes have been considered as a fruit with "healing power." Egyptians loved grapes, and ancient Greek and Roman philosophers and physicians heralded their healing powers, particularly as fermented grape juice or wine. This health character of grapes has been supported scientifically in recent years.

Chronic diseases are the most prevalent cause of death in the world, led by cardiovascular diseases and followed by cancer, chronic lung diseases, and diabetes mellitus. Epidemiological studies indicate that high serum cholesterol might have a strong correlation with increased risk of coronary heart disease (CHD). These findings led to the classical diet – heart hypothesis – which postulated the primary role of saturated fat and cholesterol in the development of atherosclerosis and CHD. In addition, initial studies suggested a direct relationship between high dietary fat intake and increased risk of breast and colon cancer. However, large prospective studies have not only addressed the effects of high dietary fat intake but also indicated the prevention of certain chronic conditions with a vegetable/fruit-rich diet. In recent years, the relationship between the consumption of specific foods and/or overall dietary patterns and the risk of CHD has been examined. Accumulating evidence from epidemiological, case control, and cohort studies suggests that a vegetable/fruit-rich diet may offer protection against chronic diseases. A study published in JAMA in 1999 by Joshipura et al. [2] clearly proved the need for higher fruit and vegetable consumption to reduce the risk of certain diseases such as ischemic stroke. However, no apparent further reduction in risk was observed beyond six servings per day. Another study has shown reduced risk of myocardial infarction in women consuming five fruit and vegetable servings [3]. The abovementioned literature suggests that fruits and vegetables may have an important role in keeping a healthy lifestyle. This observation leads to a million dollar question: which components of fruits and vegetables may be responsible for this protective effect? In this sense, many clinical trials have used different bioactive compounds for chronic disease prevention. Polyphenols are thoroughly researched for their human health-benefiting properties at different stages and have reached clinical studies, which confirm the potency of these bioactive compounds.

This chapter deals with grapevine products as functional foods and sources of nutraceuticals. Complex grape polyphenol chemistry is reviewed, and emphasis is laid on health benefits arising from the consumption of grape products. Finally, functional food from grapes is examined.

2 Chemical Composition

Grape contains (per 100 g): Water (80.5 g), carbohydrates (18.1 g), fat (0.16 g), protein (0.72 g), fiber (0.9 g), potassium (191 mg), sodium (2 mg), phosphorous (20 mg), calcium (10 mg), magnesium (7 mg), iron (0.36 mg), zinc (0.07 mg), vitamin C (10.8 mg), vitamin B1 (0.07 mg), vitamin B6 (0.09 mg), vitamin B2 (0.07 mg), vitamin A (66 IU), and vitamin E (0.19 mg). All above provide around 69 Kcal per 100 g of intake (from USDA Nutrient Database for Standard Reference, Release 24; http://ndb.nal.usda.gov/ndb/foods/list).

Moreover, apart from these main components, grapes contain secondary metabolites such as flavonoid and non-flavonoid phenolic compounds, sesquiterpenes, and melatonin. Most of the grape's medicinal value can be attributed to its seed and skin, which researchers have found to be rich in nutritional value due to the presence of polyphenolic antioxidants. At least 500 different types of antioxidants have been found in various parts of this fruit [4].

2.1 Phenolic Compounds

Grapevine quality, as with most plants, mainly depends on its metabolites. Metabolite production is especially sensitive to external conditions. In particular, the chemical diversity of grapevine is mostly affected by secondary metabolites.

These secondary metabolites consist of a wide array of species-specific chemicals and belong to different phytochemical groups such as alkaloids, terpenes, antibiotics, volatile oils, resins, cardiac glycosides, tannins, sterols, saponins, and phenolics, many of which have proven highly valuable for the pharmaceutical, agrochemical, food, and fragrance industries [5]. In general, secondary metabolites are known to play key physiological functions in plants, including their adaptation to the environment [6], acquired resistance to pests and diseases, pollinator attractant capacity, and the building of symbiotic relations with microorganisms [7]. They are also very often crucial in quality determination in food attributes (color, taste, and aroma) and colors and pigments in ornamental plants [8].

We have focused on phenolics because they are a large and complex group of metabolites that particularly contribute to the (sensorial and bioactivity) features of grapes.

There are a large number of phenolic compound structures and, therefore, a large number of properties as well. Grapevine phenolics may either arise from the fruit (mainly skins and seeds) and vine stems or be products of yeast metabolism. Their schematic biosynthesis is shown in Fig. 83.1. Briefly, the phenylpropanoid pathway generates most phenolic compounds found in nature, including flavonoids and stilbenoids. Biosynthesis of phenylpropanoid compounds is not only developmentally activated in specific tissues and cell types but can also be activated in other tissues in response to environmental stresses such as wounding, pathogen infection, or UV irradiation. Phenylalanine is an end product of the shikimate pathway. The structural diversity of phenylpropanoids derived from phenylalanine and the key phenylpropanoid intermediate *p*-coumarate is due to the action of enzymes and enzyme complexes that bring about region-specific condensation, cyclization, aromatization, hydroxylation, glycosylation, acylation, prenylation, sulfation, and methylation reactions [9].

2.1.1 Anthocyanins

Anthocyanins are the most abundant pigments in red grape skins (Fig. 83.2a). These water-soluble pigments are responsible for blue, red, and purple color in red grape skin and red wine. Anthocyanins of *Vitis* are monoglucosides of five anthocyanidins, namely, delphinidin, cyanidin, petunidin, peonidin, and malvidin. Acylated anthocyanins are esters of the glucose moiety of the free anthocyanins with acetic, *p*-coumaric, or caffeic acids (Fig. 83.2a).

Malvidin 3-glucoside is the main anthocyanin in all the varieties of *Vitis vinifera*. The amount of anthocyanins in grapes, as well as in all phenolics, depends on the grapevine variety and is highly influenced by viticultural and environmental factors such as light, temperature, altitude, soil type, water, nutritional status, pathogenesis, and various developmental processes [10]. Temperature has a great

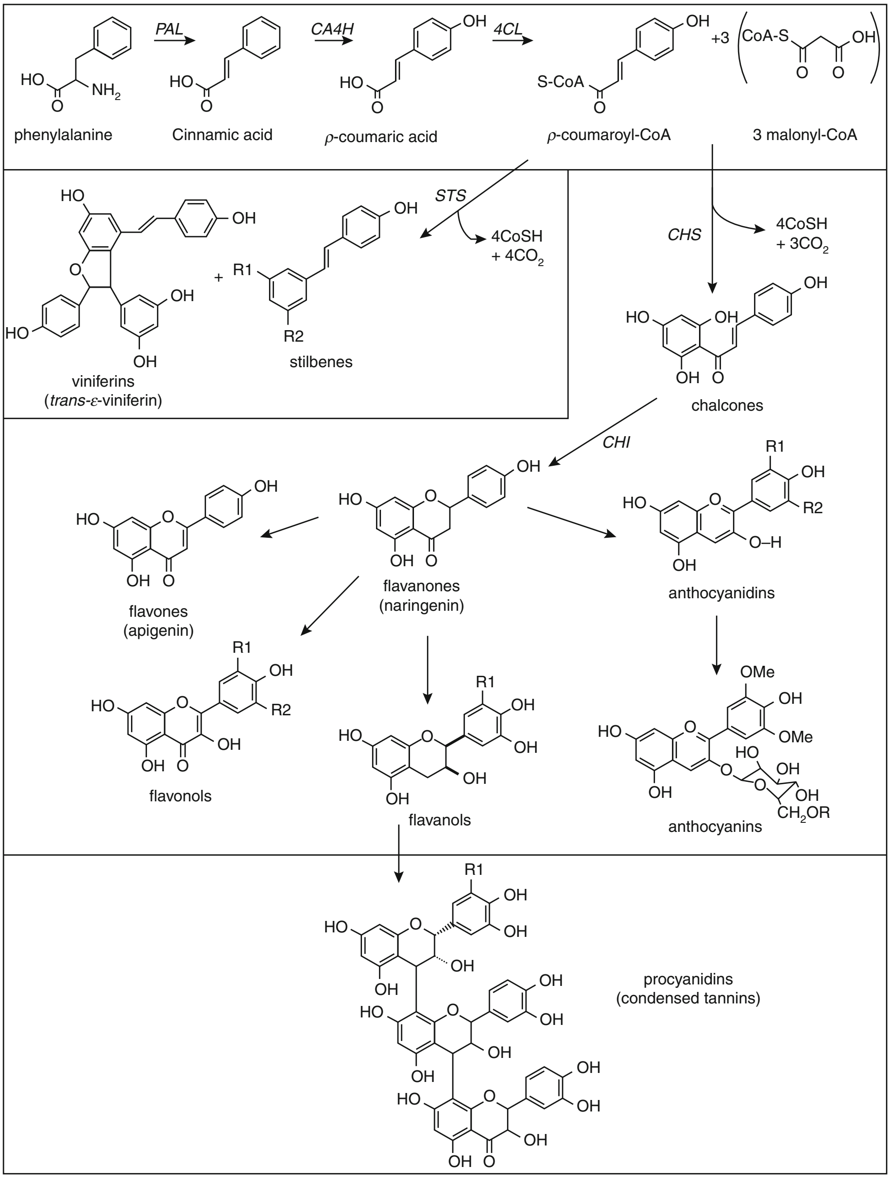
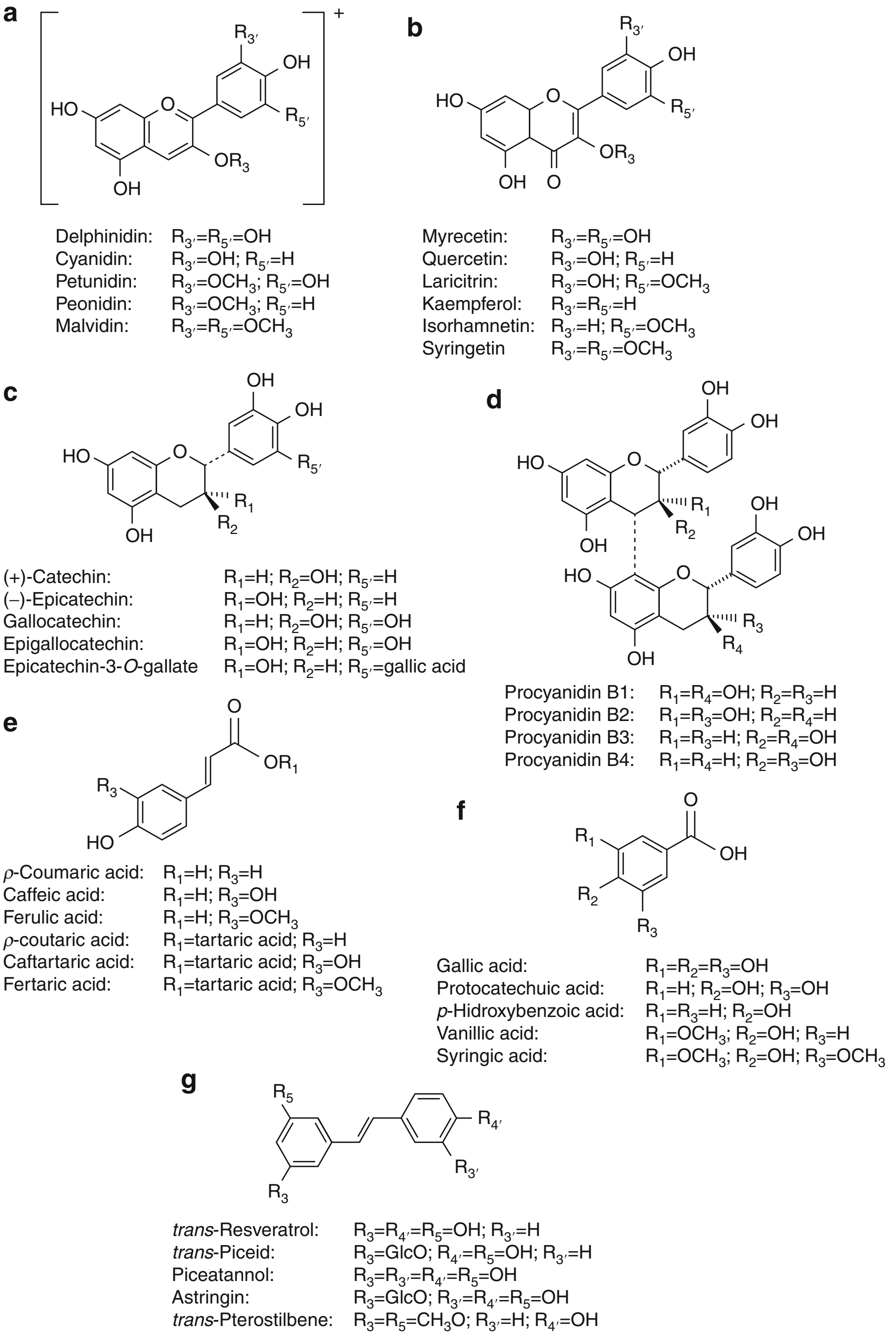

PAL
CA4H
4CL
+3
phenylalanine
Cinnamic acid
ρ-coumaric acid
ρ-coumaroyl-CoA
3 malonyl-CoA
STS
4CoSH
+ 4CO$_2$
CHS
4CoSH
+ 3CO$_2$
viniferins
(*trans*-ε-viniferin)
stilbenes
chalcones
CHI
flavones
(apigenin)
flavanones
(naringenin)
anthocyanidins
flavonols
flavanols
anthocyanins
procyanidins
(condensed tannins)

Fig. 83.1 Biosynthesis pathway of grape phenolics

influence on anthocyanin biosynthesis. Anthocyanin levels in Cabernet Sauvignon grapes are higher when day temperatures remain constant at around 20°C rather than 30°C. Therefore, increased anthocyanin content is associated with grapes grown at higher altitudes. However, this relationship is complicated by the effect of diurnal differences in temperature: lower night temperatures result in greater


a
$R_{3'}$
OH
HO
O
$R_{5'}$
OR_3
OH
+
b
$R_{3'}$
OH
HO
O
$R_{5'}$
OR_3
OH
O
c
OH
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$R_{5'}$
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HO
O
R_3
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OH
OH
e
O
OR_1
R_3
HO
f
O
R_1
OH
R_2
R_3
g
R_5
$R_{4'}$
$R_{3'}$
R_3

Delphinidin:	$R_{3'}=R_{5'}=OH$
Cyanidin:	$R_{3'}=OH$; $R_{5'}=H$
Petunidin:	$R_{3'}=OCH_3$; $R_{5'}=OH$
Peonidin:	$R_{3'}=OCH_3$; $R_{5'}=H$
Malvidin:	$R_{3'}=R_{5'}=OCH_3$

Myrecetin:	$R_{3'}=R_{5'}=OH$
Quercetin:	$R_{3'}=OH$; $R_{5'}=H$
Laricitrin:	$R_{3'}=OH$; $R_{5'}=OCH_3$
Kaempferol:	$R_{3'}=R_{5'}=H$
Isorhamnetin:	$R_{3'}=H$; $R_{5'}=OCH_3$
Syringetin	$R_{3'}=R_{5'}=OCH_3$

(+)-Catechin:	$R_1=H$; $R_2=OH$; $R_{5'}=H$
(−)-Epicatechin:	$R_1=OH$; $R_2=H$; $R_{5'}=H$
Gallocatechin:	$R_1=H$; $R_2=OH$; $R_{5'}=OH$
Epigallocatechin:	$R_1=OH$; $R_2=H$; $R_{5'}=OH$
Epicatechin-3-*O*-gallate	$R_1=OH$; $R_2=H$; $R_{5'}$=gallic acid

Procyanidin B1:	$R_1=R_4=OH$; $R_2=R_3=H$
Procyanidin B2:	$R_1=R_3=OH$; $R_2=R_4=H$
Procyanidin B3:	$R_1=R_3=H$; $R_2=R_4=OH$
Procyanidin B4:	$R_1=R_4=H$; $R_2=R_3=OH$

ρ-Coumaric acid:	$R_1=H$; $R_3=H$
Caffeic acid:	$R_1=H$; $R_3=OH$
Ferulic acid:	$R_1=H$; $R_3=OCH_3$
ρ-coutaric acid:	R_1=tartaric acid; $R_3=H$
Caftartaric acid:	R_1=tartaric acid; $R_3=OH$
Fertaric acid:	R_1=tartaric acid; $R_3=OCH_3$

Gallic acid:	$R_1=R_2=R_3=OH$
Protocatechuic acid:	$R_1=H$; $R_2=OH$; $R_3=OH$
p-Hidroxybenzoic acid:	$R_1=R_3=H$; $R_2=OH$
Vanillic acid:	$R_1=OCH_3$; $R_2=OH$; $R_3=H$
Syringic acid:	$R_1=OCH_3$; $R_2=OH$; $R_3=OCH_3$

trans-Resveratrol:	$R_3=R_{4'}=R_5=OH$; $R_{3'}=H$
trans-Piceid:	$R_3=GlcO$; $R_{4'}=R_5=OH$; $R_{3'}=H$
Piceatannol:	$R_3=R_{3'}=R_{4'}=R_5=OH$
Astringin:	$R_3=GlcO$; $R_{3'}=R_{4'}=R_5=OH$
trans-Pterostilbene:	$R_3=R_5=CH_3O$; $R_{3'}=H$; $R_{4'}=OH$

Fig. 83.2 (**a**–**g**) Chemical structure of the main bioactive compounds in grape

Table 83.1 Phenolic compounds in red and white grapes

Family	Phenolic compound	Red grape (mg/kg fw)	White grape (mg/kg fw)	References
Anthocyanins	Dp-3-glc	500–5,000	–	[13–16]
	Cy-3-glc			
	Pt-3-glc			
	Pn-3-glc			
	Mv-3-glc			
	Acetyl-derivatives			
	p-Coumaroyl-derivatives			
Flavonols	Q-3-glc/glu/gal/rut/glugal/gluxyl	3–300	1–200	[13, 16–19]
	K-3-glc/glu/gal			
	M-3-glc/glu			
	I-3-glc/glu/gal			
	L-3-glc/gal			
	Syr-3-glc			
Total flavan-3-ols	Catechin	2,500–11,000	2,500–11,000	[17, 20, 21]
	Epicatechin			
	Gallocatechin			
	Epigallocatechin			
	Epicatechin-3-*O*-galate Procyanidin B1, B2, B4, C1			
Hydroxycinnamic acid derivatives	Caffeoyl tartaric	1.5–50	4–45	[15–17, 22]
	Coumaroyl tartaric			
	Feruloyl tartaric			
Hydroxybenzoic acids	Gallic acid	2–5	2–5	[22]
	Protocatechuic acid			
	p-Hydroxybenzoic acid			
	Vanillic acid			
	Syringic acid			
Stilbenes	*trans*-/*cis*-Resveratrol	0–22	0–8.5	[23–25]
	trans-/*cis*-Piceid			
	Piceatannol			
	Astringin			
	Pterostilbene			
	ε- and δ-Viniferin			

accumulation of anthocyanins [11]. The accumulation of anthocyanins starts at the veraison stage with occasional decreases toward the end of ripe stage, especially in hot climates [12]. In fact, reduced grape berry color has been observed in very hot seasons. Whether this decrease occurs through degradation of existing anthocyanins or reduced anthocyanin biosynthesis is not known. Hence, the range of concentration varies from 500 to 5,000 mg/kg fw (Table 83.1).

2.1.2 Flavonols

Flavonols are found in grape only as glycosides of myricetin, quercetin, laricitrin, kaempferol, isorhamnetin, and syringetin (Fig. 83.2b). The corresponding aglycones can be found in wine, together with 3-glycosides. Glucose is the common sugar attached to the C-3 position of kaempferol, quercetin, myricetin, and isorhamnetin, but glucuronic acid has also been found as glycosylation of quercetin, kaempferol, myricetin and isorhamnetin, and laricitrin. Furthermore, quercetin has been found in grapes as 3-galactoside, 3-rhamnosylglucoside (also called rutin), 3-glucosylgalactose, and 3-glucosylxyloside. Kaempferol, laricitrin, and isorhamnetin have been described as galactoside derivatives (Table 83.1).

As described for anthocyanin, climate impacts on the amount of flavonol in grape. In contrast to anthocyanin and tannin synthesis, which are scarcely affected by shading treatments, a positive relationship was observed between sunlight exposure and increased flavonol accumulation [26, 27].

Flavonols are exclusively found in red and white grape skins, and its concentration ranges from traces to 300 mg/kg fw (Table 83.1).

2.1.3 Flavan-3-ol Monomers and Procyanidins

Flavan-3-ols are another large family of polyphenolic compounds comprising mainly catechin, epicatechin, epicatechin 3-*O*-gallate, gallocatechin, epigallocatechin, and epigallocatechin 3-*O*-gallate (Fig. 83.2c). Procyanidins, also known as condensed tannins, are both oligomeric and polymeric compounds arising from polyhydroxy flavan-3-ol and flavan-3,4-diol units and their epimers through C4 → C8 or C4 → C6 bonds. In grape seeds and skin, about 20 procyanidin dimers and trimers have been identified [28], B1, B2, B3, and B4 being the main ones (Fig. 83.2d). In *Vitis,* procyanidins are mainly present in grape seed, skin, and stem tissues. In the grape seed, they represent the major fraction of total polyphenols, characterized by a lower polymerization degree than those in grape skin. However, skin procyanidins are more easily extracted during winemaking, thus conferring organoleptic properties on wine such as astringency and bitterness [29]. Moreover, the grape seed contains procyanidin of catechins, epicatechin, and epicatechin gallate units, while grape skin and wine show procyanidins mostly based on epicatechin and epigallocatechin units [30].

Unlike anthocyanins and flavonols, climatic conditions have little effect on flavan-3-ols, as these compounds mainly occur in the seed. In fact, climate seems to have greater effect on composition than on quantity. Low-vigor vines show grapes with higher procyanidin content, increased proportion of epigallocatechin subunits in procyanidins, and increased polymer size, and, therefore, these compounds show decreased astringency [10]. Besides, considerable differences in types and concentrations have been observed among cultivars [31].

Seed flavanol monomers and polymers ranged from 240 to 730 and from 330 to 790 mg/kg fw, respectively [17, 20]. Skin flavonol monomers and polymers ranged from 10 to 40 and from 40 to 100 mg/kg fw, respectively. The total amount of procyanidins is reported to vary from 1.7 to 4.4 g/kg of berries in skin, 1.1–6.4 g/kg in seeds, and 0.2–1 g/kg in pulp [21] (Table 83.1).

2.1.4 Hydroxycinnamic Acid Derivatives

Hydroxycinnamics are the third most abundant group of phenolic compounds in grapes and mainly comprise caffeic, coumaric, ferulic acids, and their corresponding tartaric esters (Fig. 83.2e). The hydroxycinnamic esters are more concentrated (2- to 100-fold) in grape skin than in pulp. Differences in total amount and proportion have been reported according to grape varieties [32].

These compounds are major phenols in white wine, and their concentration in grape ranges from traces to 50 mg/kg fw (Table 83.1). In addition, these compounds are important constituents of acylated anthocyanins.

2.1.5 Hydroxybenzoic Acids

Hydroxybenzoic acids are a minor group of phenolic compounds in grapes (Fig. 83.2f). The commonest ones are gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, and syringic acid, which are mainly found in free form [33, 34]. Hydroxybenzoic acids are mostly represented by gallic acid, which is found both free and acyl substituent in flavan-3-ols. Two forms of gallic acid (i.e., 3-*O*-b-glucopyranoside and 4-*O*-b-glucopyranoside) have been reported in grape [35]. They are found in grape skin, and their total amount ranges from 2 to 5 mg/kg fw (Table 83.1).

2.1.6 Stilbenes

Stilbenes are minor compounds in grapes. However, their importance is due to the fact that grape is the main source of stilbenes in diet. Stilbenes are essentially located in grape skin [36, 37] but have also been reported in grape seeds [38] and grape stem [39] (Fig. 83.2g). The main stilbenes in grape are resveratrol (*trans* and *cis*) and piceid (*trans* and *cis*), but some others have also been described: piceatannol, astringin, pterostilbene, ε-viniferin, and δ-viniferin [40].

As described for the other polyphenols, the amount of resveratrol in grape depends heavily on many factors such as grape variety, geographic region, and agronomic and climatic factors. Furthermore, as resveratrol is a grape-synthesized phytoalexin after grape exposure to biotic or abiotic stress, the presence of resveratrol in grapes depends on the degree of stress exposure.

Total stilbenoid concentration was calculated taking into account just piceids and resveratrol (the major ones) and resulted higher for red than for white grapes [23]. Total stilbenoid concentration can reach 8.5 and 22 mg/kg fw in white and red grapes, respectively (Table 83.1).

2.2 Other Bioactive Compounds in Grape

Sesquiterpenoids are compounds that show woody, spicy, sweet, floral, clove, and fresh-like flavors. More than 40 sesquiterpenoids have been identified in grapes. However, quantification is a difficult task due to the lack of standards. Sesquiterpenoids have been described to contribute to the varietal volatile composition between 56% and 80% [41].

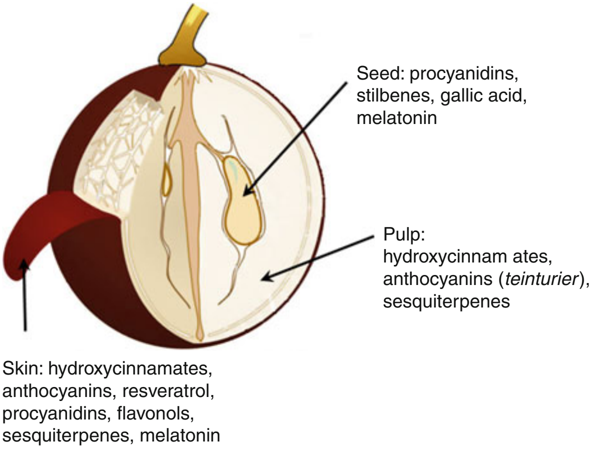
Seed: procyanidins, stilbenes, gallic acid, melatonin
Pulp: hydroxycinnam ates, anthocyanins (*teinturier*), sesquiterpenes
Skin: hydroxycinnamates, anthocyanins, resveratrol, procyanidins, flavonols, sesquiterpenes, melatonin

Fig. 83.3 Tissue localization of phenolic compounds in grape berry

Another bioactive compound described in grape and wine is melatonin. As reported for phenolic compounds, differences in genotypes and agrometeorological conditions contribute to the differing melatonin content reported in grape. Melatonin in grape ranged from traces to 0.9 ng/g fw [42, 43]. Some authors suggest that the synthesis of melatonin in wine is directly related to yeast metabolism [44].

Figure 83.3 shows the distribution/location of all abovementioned compounds in grape seed, skin, and pulp.

3 Biological Activities

From a medical point of view, the bioactive compounds of plant food are not considered pharmaceuticals, though they can improve life quality and expectancy. These compounds are named nutraceuticals, since they act as dietary therapeutic or pharmaconutrients (i.e., compounds that, if regularly included in diet, can prevent, block, or delay the onset of major chronic diseases).

First of all, it is worth mentioning the most remarkable bioactivities and bioavailability by phenolic family compounds separately. Secondly, we shall describe the different bioactivities attributed to the consumption of grape derivatives.

3.1 Bioactivity and Bioavailability of Phenolic Compounds

Anthocyanins have been reported to be strong antioxidants. They inhibit the growth of cancerous cells and inflammation, act as vasoprotectors, and have antiobesity effects [45, 46]. It has been described that delphinidin may preserve endothelium integrity, as endothelium alteration leads to several pathologies, including cardiovascular diseases such as atherosclerosis, and is often associated with cancer [47].

Anthocyanins, as a supplement, have also shown beneficial results on HDL- and LDL-cholesterol concentrations. Increased HDL-cholesterol and decreased LDL-cholesterol were reported in a recent study on dyslipidemic patients who were given 160 mg anthocyanins twice daily throughout a 12-week trial. Furthermore, the anthocyanin supplementation led to decreased mass and activity of plasma cholesteryl ester transfer protein and increased cellular cholesterol efflux to serum [48].

Anthocyanins seem to be absorbed very rapidly but rather inefficiently [49]. However, the bioavailability of anthocyanins may have been underestimated due to either methodological inaccuracy or the different chemical forms that anthocyanins can take depending on pH. This may explain why all anthocyanins' effects are contingent on sufficient bioavailability in terms of exposure at both cell and organism level through the diet [50].

Flavonols comprise some of the most prominent dietary antioxidants. Among the flavonols, quercetin can be singled out, for it presents a wide variety of pharmacological activities that provide protection not only against osteoporosis, certain forms of cancer, and pulmonary and cardiovascular diseases but also against aging [51]. Even more significantly, quercetin plays a pivotal role in reducing blood pressure by reducing oxidative stress in a dose-dependent way [52, 53]. In the 1970s, quercetin was reported to be mutagenic. However, more recent studies in vivo indicate that quercetin is not carcinogenic. In fact, in the USA and Europe, quercetin supplements are commercially available, and their beneficial effects have been reported in clinical trials [54]. With respect to its bioavailability, quercetin is absorbed in humans and can reach high concentrations that are sufficient to increase plasma antioxidant capacity [55]. Moreover, quercetin glucosides, one of the main flavonols present in grape, are among the polyphenols most readily absorbed in humans [49].

Flavan-3-ols with various types of structure act as antioxidants, free radical scavengers, and anticarcinogenic. They have cardiopreventive, antimicrobial, and antiviral properties and may also play a significant role in maintaining neurological health [56]. Relationships have been established between the structure of flavan-3-ols and their strong antioxidant and free radical scavenging properties. Their antioxidant function depends on the ring structure and number of catechol groups [57]. However, some evidence points out that the opposite must also be considered, as flavan-3-ols may behave as antinutrients, procarcinogens, pro-oxidants, hemorrhage inducers, mutagens, or hepatotoxins depending on the source, type, amount, and existence of other dietary factors [58].

Bioavailability of flavan-3-ol monomers is generally good, although it differs markedly among different compounds. Catechin, present in high concentrations in the plasma of Mediterranean diet consumers, is able to reduce the progression of atherosclerosis in vivo [59]. The fact that grape is one of the main sources of catechin in diet supports the finding that grape has anti-atherosclerotic effect [60].

Procyanidins are considered to be among the most effective antioxidants present in grapes. When rabbits were fed with procyanidins from grape seed extract, the compounds were active in preventing lipid oxidation while in the digestive tract [61]. The consumption of proanthocyanidin-rich foods has been shown to (1) increase plasma antioxidant capacity, (2) have positive effects on vascular function, and (3) reduce platelet activity in humans [62]. Unlike flavan-3-ol monomers, procyanidins are less permeable through cell walls and are therefore absorbed less readily. In fact, their polymerization impairs intestinal absorption [49, 56]. However, the health effects of proanthocyanidins may not require efficient absorption through the gut. These compounds may have direct effects on the intestinal mucosa and protect it against oxidative stress and carcinogens. Procyanidins bioactivity is not only due to their activity itself but also to their metabolites bioactivities and also to the modulation of intestinal bacterial population [63].

Hydroxycinnamic acid derivatives have shown antioxidant and anti-inflammatory properties in vivo and in vitro. They contribute to DNA protection and help to prevent Alzheimer's disease [64, 65]. However, data are still too limited for an informed assessment of hydroxycinnamics [49].

Stilbenes in general, and *trans*-resveratrol in particular, have been reported to be responsible for various beneficial effects. Resveratrol's biological properties include antibacterial and antifungal effects, as well as cardioprotective, neuroprotective, and anticancer action [40]. Anticancer resveratrol activity is one of the most promising bioactivities of resveratrol. In 1997, Jang et al. [66] reported the ability of resveratrol to inhibit carcinogenesis at multiple stages (initiation, promotion, and progression). Their finding that topical application of resveratrol reduces the number of skin tumors per mouse by up to 98% triggered research on resveratrol all around the world. Resveratrol could slow down tumor development through multiple complementary mechanisms. It inhibits the enzymatic activity of both forms of cyclooxygenase, which implies a reduction in the risk of developing many cancers. Another mechanism by which resveratrol could combat tumor formation is induction of cell cycle arrest and apoptosis. Its antiproliferative and pro-apoptotic effects in tumor cell lines have been extensively documented in vitro [67] and are supported by down regulation of cell cycle proteins [68] and increased apoptosis [69] in tumor models in vivo. However, in some in vivo experiments, resveratrol failed to impact cancer, which suggests that other factors such as dosage, delivery method, tumor origin, and other diet components could all contribute to the efficacy of resveratrol treatment. Overall, in vivo studies clearly show a promising use of this molecule in cancer treatment. Other remarkable activity of resveratrol is its neuroprotective character. It is able to penetrate the blood-brain barrier and exerts strong neuroprotective effect. Moreover, it has been shown to combat neuronal dysfunction in Huntington's, Alzheimer's, and Parkinson's diseases [70]. Resveratrol also has positive effects on longevity and age-related deterioration [71–73]. Other stilbenes such as piceatannol and viniferins are usually found in grape in lower concentrations than resveratrol, and, as a result, their bioactivity has been studied less than that of resveratrol. Nevertheless, some of their health-benefiting properties have also been researched [40, 74, 75].

Numerous studies on animals and humans have shown resveratrol's low bioavailability. Once it is absorbed, at least 70% of the ingested resveratrol is readily metabolized to form mainly glucuronide and sulfate derivatives. Since the in vivo concentration of individual metabolites from ingested resveratrol can be much higher than that of resveratrol itself, further studies on the activity of its metabolites become necessary.

Resveratrol binds to albumin, and albumin has been suggested a natural polyphenol reservoir in in vivo context, where it might play a pivotal role in the distribution and bioavailability of circulating resveratrol [76]. The accumulation of resveratrol in other organs such as heart, liver, and lung after chronic administration was described for the first time in 1996 [77] and has more recently been confirmed [78, 79] and extended to bile, stomach, and kidneys [80]. It is also worth considering the potential interactions between polyphenols. For example, resveratrol has been shown to synergize with both quercetin and ellagic acid in the induction of apoptosis in human leukemia cells [81], with ethanol in the inhibition of iNOS expression [82], with vitamin E in the prevention of lipid peroxidation [83], with catechin in the protection of PC12 cells from β-amyloid toxicity [84], with nucleoside analogues in the inhibition of HIV1 replication in cultured T lymphocytes [85], and with tyrosol and β-sitoesterol in modulation of LDL oxidative stress and PGE2 synthesis [86].

Sesquiterpenoids have been related with medicinal plants with different health applications, mainly anti-inflammatory [87], anti-HIV [88], antibacterial [89], and antitumor activity [88]. Up to date, no study has covered the biological activity of sesquiterpenoids from *Vitis vinifera L* grapes. Sesquiterpenoids such as farnesol and nerolidol have been reported to have the ability to enhance bacterial permeability and susceptibility to exogenous antimicrobial compounds. These compounds increase the susceptibility of *Staphylococcus aureus* and *Escherichia coli* to antibiotics by disrupting the normal barrier function of the bacterial cell membrane, allowing permeation into the cell of exogenous solutes such as antibiotics [90].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurohormone produced in the pineal gland. Its biological properties have been studied in depth, particularly those concerning the circadian rhythm. Melatonin shows antioxidant properties as a direct free radical scavenger and a stimulator of antioxidant enzymes [91–94]. Melatonin's role in neuroprotection is an important issue. Melatonin has been tested in sleep disorders. It generally reduces sleep latency and improves sleep especially in case of disturbed circadian phasing. In this case, melatonin was found particularly effective in patients with neurodegenerative diseases [95]. The European Food Safety Authority (EFSA) accepted the health claims related to melatonin and alleviation of subjective feelings of jet lag. Melatonin dosage must be within 0.5 and 5 mg per day [96]. Numerous research attempts have been made or are currently being developed to mitigate neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases and amyotrophic lateral sclerosis. Melatonin was shown to inhibit Aβ fibrillogenesis [97, 98]. Melatonin

Table 83.2 Antioxidant compounds from grapes and main bioactivity

Family	Most prominent bioactivity against disease	References
Anthocyanins	Decrease cardiovascular and cancer risk	[45–48]
Flavonols	Provide protection against osteoporosis, cancer, pulmonary and cardiovascular diseases	[51–53]
Total flavan-3-ols	Anticarcinogenic, cardiopreventive, antimicrobial, and antiviral properties	[56, 57]
Hydroxycinnamic acid derivatives	DNA protection and help prevent Alzheimer's disease	[64, 65]
Stilbenes (resveratrol)	Cardioprotective, neuroprotective, antiaging, and anticancer actions	[66, 70, 71]
Sesquiterpenes	Anti-inflammatory and antibacterial actions	[87, 89]
Melatonin	Neuroprotection action	[70]

(200 mg/kg) has recently been shown to reduce edema in impacted striatum versus traumatic brain injury [99].

In addition to plasma melatonin levels, urine-excreted melatonin metabolite 6-sulfatoxymelatonin is easy to determine. Indeed, higher melatonin excretion in the first morning urine was 16% higher in women with higher quartile vegetable intake in comparison to those with lower quartile intake [100]. Moreover, nutrition habits and lifestyle have also been correlated with melatonin [101]. A statistically inverse relation was found between age, smoking, and body mass index, on one hand, and urinary 6-sulfatoxymelatonin [102].

The most important bioactivities of these compounds in diseases are summarized in Table 83.2.

Moreover, many of the described health properties may not be attributed to single polyphenols present in grape but rather to synergism among polyphenols themselves and/or between them and other types of bioactive compounds. For instance, some studies on grape or grape juice are detailed next.

3.2 Antioxidant Activities of Grape and Grape Derivative Products

The most widely researched biological activity of polyphenols is their antioxidant capacity, though they also have a plethora of more or less correlated properties such as antimutagenic, anti-inflammatory, antitumoral, antineurodegenerative, antihypertensive, and cardioprotective activities.

Oxidative stress is broadly defined as a perturbation of cellular homeostasis, so that the production rate of reactive oxygen species (ROS) exceeds their neutralization rate. If homeostasis is not reestablished, oxidative stress may progress toward the onset of apoptotic cell death and tissue degeneration [103]. In order to cope with excessive production of free radicals, human bodies have developed sophisticated mechanisms for maintaining redox homeostasis. These protective mechanisms include ROS scavenging or detoxification, ROS production blockage, and sequestration of transition metals, as well as enzymatic and nonenzymatic antioxidant defenses both endogenous (body-produced) and exogenous (diet-supplied). Among them, dietary polyphenols have been widely studied for their strong antioxidant capacities and other properties that regulate cell functions [104].

Flavonoids act as antioxidants by donating electrons and stopping radical chains. This activity is attributed to phenolic hydroxyls, especially in 3′,4′ positions in the B-ring and to the 2,3-double bond in the C-ring, thus increasing with the number of OH groups in A and B rings [105]. In fact, most epidemiological and intervention studies on flavonoids' beneficial effects have been focused on their antioxidant capacity.

The antioxidant capacity of red grapes was evaluated in HepG2 (human hepatocellular liver carcinoma) cells and positively correlated with the total phenolic content and the oxygen radical absorbance capacity (ORAC) values of grape extracts. Results suggest that increasing fruit consumption is a suitable strategy to counteract oxidative stress [106].

A similar conclusion is drawn in a study developed on oxidative stress markers in 32 healthy subjects. Daily consumption of grape juice (10 mL/kg body weight) for 2 weeks resulted in increased resistance of LDL to ex vivo oxidation, comparable to the value obtained after α-tocopherol. Furthermore, decreased protein-carbonyl concentration was observed at the same time [107]. These results agree with those contributed by other authors, who showed that the daily intake of grape juice (125 mL) for 1 week in a group of six men and six women led to significantly reduced LDL oxidation [108]. In a short-term study, the acute intake of a phenolic-rich juice (400 mL), with grapes as a major ingredient, improved the antioxidant status in healthy subjects according to their plasma thiobarbituric levels [109]. In a group of 27 hemodialysis patients, regular ingestion of concentrated red grape juice (100 ml) for 2 weeks reduced inflammatory biomarkers to a greater extent than vitamin E [110].

High daily intakes of grape juice are not feasible in most population. To overcome this difficulty, new derivative products such as power extracts are proposed. The daily supplementation of lyophilized grape powder (36 g) for 4 weeks reduced urinary F2-isoprostanes (in women), which are biomarkers of oxidative stress, in pre- and postmenopausal women [111]. The administration of grape seed extract (600 mg/day) for 4 weeks was reported to have led to significant improvement in insulin resistance and plasma CRP markers in a group of 32 type 2 diabetic patients [112]. The consumption of black grape (1 g/kg body weight) has also been described to exert similar effects to juice and powder: significantly increased antioxidant potential was observed in healthy volunteers 4 h after ingestion [113].

Furthermore, this antioxidant capacity of grape is also able to protect DNA oxidation and therefore mutagenesis-, carcinogenesis-, and aging-related DNA damage. Daily grape juice supplementation (480 mL) for 8 weeks led to reduced DNA strand breaks in peripheral lymphocytes, apart from decreasing the amount of released ROS [114]. Similarly, treatment of human lymphocytes with grape seed extract reduced the frequency of micronuclei by 40% and the production of malonyldialdehyde, a biomarker of lipid peroxidation, by 30%, while it increased the activity of antioxidant enzymes catalase and glutathione S-transferase by 10% and 15%, respectively [115].

3.3 Cardioprotective Activity of Grape and Grape Derivative Products

Vessel injury and thrombus formation are the cause of most ischemic coronary syndromes, and in this setting, activated platelets stimulate platelet recruitment up to the growing thrombus. Hypertension is one of the major risk factors for cardiovascular disease, with an impact on global health. Multiple studies have suggested that various dietary factors are associated with blood pressure and hypertension. However, the effects of fruit and vegetable consumption on plasma lipid levels, diabetes, and body weight have not yet been thoroughly explored [116]. Evidence suggests an inverse relationship between grape product consumption and cardiovascular disease. In studies conducted with grapes and grape juice, clinical trials demonstrated improved endothelial function, reduced platelet aggregation, and a positive influence on biomarkers such as LDL and HDL. In a study on 15 patients with coronary artery disease, the consumption of 8 mL/kg/day of red grape juice for 2 weeks improved the endothelial function and reduced the susceptibility of LDL-cholesterol to oxidation [117]. In fact, Vison et al. found that red grape juice, in contrast with orange juice, enriched LDL and VLDL and reduced their oxidation susceptibility in vitro, ex vivo, and in vivo [118]. Regarding platelet aggregation, many promising results have been reported. Platelet incubation with red grape juice led to inhibited aggregation, enhanced the release of platelet-derived NO, and reduced superoxide production. Oral consumption of standardized grape extract (100 and 200 mg/kg) provided significant cardioprotection by improving postischemic ventricular recovery and reducing myocardial infarction in rats [119].

In other study, 20 healthy subjects were supplemented with grape juice (7 mL/kg) for 14 days. Significantly decreased platelet aggregation, increased platelet-derived NO release, and decreased superoxide production were observed. The suppression of platelet-mediated thrombosis represents a potential mechanism for the beneficial effects of purple grape products in cardiovascular disease [120]. Similarly, proanthocyanidin-rich extract of grape seed (50 and 100 mg/kg for 3 weeks) had cardioprotective effects against reperfusion-induced injury in isolated rat hearts [121]. A lower dose (36 g of lyophilized power grape/day for 4 weeks) was tested in pre- and postmenopausal women. Lipoprotein metabolism, oxidative stress, and inflammatory markers were achieved, and, therefore, CHD risk factors were reduced [122].

Moreover, numerous studies have been developed on CHD patients. The intake of red grape polyphenol extract – which contains epicatechin, catechin, gallic acid, *trans*-resveratrol, ε-viniferin, rutin, quercetin, p-coumaric, and ferulic acid – improved the endothelial function in CHD patients. Similar results have been found for red grape juice [123]. Hypercholesterolemic patients were asked to consume red grape juice (500 mL/day) and red wine (250 mL/day) for 14 days. Results showed increased brachial artery flow-mediated dilation in both cases. However, increased endothelium-independent vasodilation was observed only in red wine-drinking patients [124]. Similar results and the same conclusions were drawn from a similar study on red grape juice. The daily ingestion of moderate amounts of red grape juice improves endothelial function in patients with atherosclerotic vascular disease, has no adverse affects on lipid and glucose metabolism, and reduces LDL susceptibility to oxidation [117, 125]. The consumption of purple grape juice offered protection against the oxidation of LDL-cholesterol, as shown by an in vivo study with dogs, monkeys, and humans from which the flavonoids of purple grape juice and red wine could be inferred to be able to inhibit the initiation of atherosclerosis [126].

3.4 Anticarcinogenic Activity of Grape and Grape Derivative Products

Cancer is a term commonly used for diseases in which abnormal cells divide uncontrollably and invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. Cancer is not just one disease but many diseases. There are more than 100 different types of cancer, hence, the complexity of cancer treatment. It is the second leading cause of death worldwide after heart disease, and its risk and incidence increase with patient age (www.cancer.gov). In addition to genetic factors, environmental and nutritional factors play a main role in cancer etiology. In westernized countries, breast, prostate, and colon-rectum cancers predominate because diets are usually rich in animal-source foods and refined carbohydrates and deficient in plant foods. Conversely, in developing countries, where diets are largely based on cereal/starchy foods, esophageal, stomach, and liver cancers are more incident [127].

Fruit and vegetable consumption may reduce the risk of oropharynx, esophagus, lung, stomach, and colon-rectum cancers. A recent study has evaluated the potential effect of interventions aimed at increasing the intake of fruits and vegetables up to the recommended level (500 g/day) on future cancer incidence in Europe. Data on cancer incidence and daily intake of fruit and vegetables were collected for France, Germany, the Netherlands, Spain, and Sweden. The results predicted 212,000 fruit- and vegetable-related cancer cases in these countries in 2050, out of which 398 (0.19%) might be prevented if the 500 g/day fruit and vegetable intake was achieved in the aforementioned countries. The largest absolute impact was observed for lung cancer with 257 (out of 136,517) preventable cases if the intervention was successfully implemented. Increasing fruit and vegetable consumption has a small impact on reducing the burden of cancer in Europe. Nevertheless, although health impact is rather limited, it can be used as a tool in chronic disease prevention [128]. In another previous study, quantitative conclusions regarding the contribution of fruit and vegetables intake to the occurrence of oral cancer were drawn from a meta-analysis. Promising results were contributed in this study. The authors conclude that each portion of fruit consumed per day significantly reduces the risk of oral cancer by 49%. Moreover, the multivariable meta-regression showed that the lower risk of oral cancer associated with fruit consumption was significantly influenced by both the type of fruit consumed and the time interval of dietary recall [129].

Many of the studies on the cancer-preventive mechanisms of phenolic compounds have been focused on individual compounds (mainly on resveratrol, procyanidins, and melatonin at too high concentrations to be achieved via dietary consumption). In contrast, few intervention studies have been found in the database. Some of them, related with their antioxidant activity, are cited in Sect. 3.2 in this chapter. Some others are detailed below. The effects of black grape extract, including seeds, on the activity of DNA turnover enzymes in cancerous human colon tissues have been tested. Results showed that the extract inhibited the activity of the enzymes involved in rapid DNA synthesis. Thus, extract intake may have beneficial effects on colon human cancer [130]. In vitro studies on grape seed extract (usually on grape seed extract) also showed promising efficacy against both angiogenesis and metastasis, which are involved in cancer progression in mammary, colon, prostate, and breast carcinoma [131–134]. Skin carcinogenesis in mice was reduced by combining topical and dietary treatment with freeze-dried grape powder [135].

Literature review for this chapter found only one human study that had examined the relationship between grape products and cancer. In an intervention study involving smoking and nonsmoking humans, Park et al. found that supplementation with 480 mL/day of purple grape juice for 8 weeks decreased lymphocyte DNA damage, reduced the release of reactive oxygen species by 15%, and reduced DNA damage to a greater extent in smokers (25% vs. 18% decrease). While these results suggest grape juice's potential anticarcinogenic role, further well-designed studies in humans are needed to corroborate these findings.

3.5 Neuroprotective Activity of Grape and Grape Derivative Products

Neural dysfunction and metabolic imbalances underlie many progressive neurodegenerative conditions such as Alzheimer's, Huntington's, and Parkinson's diseases. As commented before, resveratrol and melatonin can penetrate the blood-brain barrier and exerts strong neuroprotective effects, even at low doses. Several studies support that the onset of the neurodegenerative disease may be delayed or mitigated with the use of dietary chemopreventive agents that provide protection against β-amyloid plate formation and oxidative damage [136, 137].

The effect of grape seed extract, rich in procyanidin, on the stress-induced neuronal cell death model has been studied in vitro using a hippocampal neuron-rich culture. The extract led to increased interleukin-6, which protected neuronal cells from death by oxidative stress [138]. Ono et al. [139] showed that a commercially available grape seed polyphenolic extract (MegaNatural-AZ) significantly attenuated Alzheimer´s disease-type cognitive deterioration and reduced cerebral amyloid deposition. Similarly, Wang et al. [140, 141] found that a naturally derived grape seed polyphenolic extract can significantly inhibit amyloid β-protein aggregation into high-molecular-weight oligomers in vitro.

Regarding in vivo studies, when orally administered to Tg2576 mice, this polyphenolic preparation significantly attenuated Alzheimer's disease-type cognitive deterioration, coincidentally with reduced HMW soluble oligomeric Aβ in the brain. Grape seed-derived polyphenolics were suggested to be useful agents to prevent or treat Alzheimer's disease. Moderate consumption of two unrelated red wines made from different grape species (Cabernet Sauvignon and Muscadine) and characterized by different component composition of polyphenolic compounds significantly attenuated the development of the Alzheimer's disease-type brain pathology and memory deterioration in a transgenic Alzheimer's disease mouse model [142]. Treatment with Cabernet Sauvignon was found to reduce the generation of Alzheimer's disease-type Aβ peptides, while the Muscadine treatment was found to attenuate Aβ neuropathology and Aβ-related cognitive deterioration in Tg2576 mice by interfering with oligomerization of Aβ molecules to soluble HMW Aβ oligomer species, which are responsible for initiating a cascade of cellular events resulting in cognitive decline. These authors suggested the possibility of developing a "combination" of dietary polyphenolic compounds for Alzheimer's disease prevention and/or therapy by modulating multiple Aβ-related mechanisms.

Concord grape juice supplementation has been shown to reduce inflammation, blood pressure, and vascular pathology in patients with cardiovascular disease. Besides, the consumption of such flavonoid-containing foods is associated with reduced risk of dementia. In addition, preliminary animal data have indicated improved memory and motor function with grape juice supplementation, suggesting its potential for cognitive benefits in aging humans. In this initial research on neurocognitive effects, 12 older adults with memory decline but not dementia took part in a randomized, placebo-controlled, double-blind trial with Concord grape juice supplementation for 12 weeks. Significant improvement was observed in verbal learning measurements, as well as nonsignificant enhancement of verbal and spatial memory. The intervention was observed to have no appreciable effect on both depressive symptoms and weight or waist circumference. These preliminary findings suggest that Concord grape juice supplementation may enhance cognitive function in older adults with early memory decline. In addition, they set a basis for further comprehensive research on its potential benefits and assessment of its mechanisms of action.

Prospective cohort studies on flavonoid intake and risk of developing dementia have led to inconsistent results [143–148], since high blood levels of homocysteine may be increased in Alzheimer's disease and hyperhomocysteinemia may contribute to disease pathophysiology by vascular and direct neurotoxic mechanism [149]. The effect of a polyphenol-rich antioxidant beverage on plasma homocysteine levels in Alzheimer patients has been recently evaluated. With this purpose, Morillas-Ruiz et al. performed a multicenter, randomized, double-blind controlled clinical trial with polyphenol supplementation in 100 subjects [150]. Twenty-four patients with initial Alzheimer's disease, 24 patients with moderate AS, and 52 controls were randomly assigned to assume either a polyphenol-rich antioxidant beverage or an identical placebo beverage (200 ml/day) for 8 months. The fasting plasma homocysteine concentration levels measured before and after the ingestion of the beverage showed higher baseline levels in Alzheimer patients than in both mild-Alzheimer and control patients. The antioxidant beverage versus placebo attenuated homocysteine increase in the control and Alzheimer groups, especially in the mild one, yet no other effects were achieved.

4 Functional Food from Grapes

Functional foods were first described by Roberfroid [151] as "Food similar in appearance to conventional food that is intended to be consumed as part of a normal diet, but has been modified to subserve physiological roles beyond the provision of simple nutrient requirements." More simply put, functional foods are foods that may provide health benefits beyond basic nutrition. Functional foods can arise from a desire to provide additional benefits to consumers in the way of enhanced nutrition. They can also be useful in making nutrients more available by providing particular dietary components in foods, thus increasing their usual availability and palatability [152].

Enrichment of foods with phenolics can be a promising strategy to produce functional foods with higher antioxidant activity. A number of products such as "grape seed extract," "grape extract," "red wine powders," "anthocyanin extracts," and even "leaf extracts" are currently being marketed and employed in the formulation of dietary supplements. Among these, grape seed extract (GSE) seems the most widely extended. In addition, plant callus/cell cultures have proven potentially promising for the production of secondary metabolites [153]: mainly anthocyanins and other phenolics in grapes [154–156]. These in vitro cultures involve some advantages over fresh fruit extracts such as the possibility of continuous production of natural compounds, large scale production depending on specific needs, lower cost and opportunity for the manipulation of the direction of the biosynthesis of anthocyanins, or other phenolics [155].

Finally, other grape derivative, phenolic-rich products are by-products: mainly pomace, which is made of solid residues; 80% of grape production is used in winemaking, and therefore millions of tons of grape pomace are produced within a few weeks after harvest. Pomace's phenolic extracts show high antioxidant and antimicrobial properties [157].

The following section contributes a revision of different grape extract-enriched food products.

4.1 Enrichment of Dairy Products

In cheese-making, the curd is generally made by coagulating milk casein with an enzyme, an acid, and either with or without further curd treatments by heat, pressure, salt, and fermentation with selected microorganisms. The retention coefficient is an important parameter in predicting the recuperation rate of value-added functional ingredients such as polyphenols. Indeed, low-molecular-weight soluble compounds are often lost to a great extent in the cheese whey. A high retention coefficient is considered desirable in the cheese-making process, as higher retention coefficients indicate reduced loss of functional ingredients. The retention coefficients of phenolic compounds in cheese are attributable to the interactions between phenolic compounds and proteins. Therefore, there is growing interest in determining the interactions between bioactive ingredients and milk proteins to enhance their recovery in cheese [158]. In this sense, grape extracts including skin, pulp, and seed (among other extracts) were added as functional components to prepared cheese. The nutritional value of cheese product was improved, since the retention coefficient of bioactive compounds and antiradical activity in cheese curd increased 1.5- and 3.5-fold, respectively [158]. However, it affected milk's gel-formatting kinetics, mainly because of pH and curd moisture content, and textural properties were therefore affected too [159]. Further research is needed before applying this technology in the food industry.

In fact, many aspects beyond the functional properties must be considered for the addition of polyphenols to dairy products. Apart from their antioxidant capacity, their sensorial attributes such as bitterness or the astringency of procyanidins can be easily translated into the dairy product [160]. When grape seed extract was added to low-fat ultra-high temperature (UHT) milk, the flavor characteristics of the milk were suppressed. Moreover, astringency and bitterness were also detected in fortified milks. The authors suggest joining this extract to cyclodextrins, thus forming complex polyphenols that result in reduced perception of sensory attributes [160, 161].

Yogurt is another fermented dairy product; beyond its nutritional characteristics and importance for the human diet, yogurt is not currently considered a significant source of phenolic compounds. Therefore, plant-based additives have been used to enhance phenolic content in yogurt [155, 162].

In recent research, high anthocyanin and phenolic levels (as much as 17.7 and 78.46 mg/kg, respectively) were reported in yogurts inoculated with extracts obtained from red grape varieties or grape callus cultures. Thus, high level of free radical scavenging capacity was also observed. Phenolic acids such as gallic acid, caffeic acid, p-coumaric acid, vanillic acid, gentisic acid, vanillin, catechin, epicatechin, *trans*-resveratrol, hesperidin, and quercetin were identified in fortified yogurts. These compounds were concluded the main constituents of the antioxidant power of yogurts enriched with functional ingredients. However, decreased total phenolic content, anthocyanin content, and antioxidant activity were observed in all assayed yogurt samples along time (2 weeks of storage). Finally, the authors state that grape callus/cell cultures can be a valuable alternative for the biomanufacturation of chemopreventive and nutraceutical fortified yogurt [155].

4.2 Enrichment of Meat Products

Grape seed extract (GSE) has proven antioxidant activities both in vivo and in vitro in various meat products [163–167]. In the meat system, GSE proves its antioxidant activity by reducing the amount of primary (e.g., lipid hydroperoxides and hexanal) and secondary (e.g., thiobarbituric acid reactive substances – TBARS) lipid oxidation products [163]. GSE has reduced rancid flavor development and antioxidant activities in various meat products like raw beef, cooked beef, raw and cooked pork patties, turkey, and ground chicken breast and thigh meat [168–173]. The antioxidant activity of GSE is concentration-dependant between 0.02% and 0.1% [168]. Grape seed extract at 0.1% (w/w) is an effective radical scavenger in muscle tissues and has been shown to reduce secondary oxidation products in beef, chicken, and turkey during refrigerated storage [168, 173, 174]. At this level (0.1% w/w), GSE can be used as an effective antioxidant in both raw and cooked meat systems. Addition of GSE ($\geq$1,000 μg/g) results in minor increase ("a" – redness values) in the surface color of raw meat and retention (due to the anthocyanins present in GSE) in cooked meat, which may have a negative impact on consumer preference based on meat product color without affecting meat eating quality [168, 171, 175]. The addition of GSE (6,000 ppm) does not change flavor scores in irradiated and nonirradiated whole chicken breasts [176]. Furthermore, GSE (0.1% w/w) was observed to have no effect on pH, yield, and water activity in ground chicken breast samples [170].

Other authors have researched the usefulness of grape seed flour from grape by-products [177]. Grape seed flour can beat alternative materials used in various food products due to its high antioxidant activity and high dietary fiber content (http://www.vitis-vital.de/). Grape seed flour was incorporated into frankfurters at seven different concentrations (from 0% to 5%), and its effects were observed on the researched products' physical, nutritional, and sensory features. Oxidation in frankfurters was minimized with increasing levels of grape seed flour in their formulation as a result of the strong antioxidant properties of grape seed flour. Moreover, increased levels of grape seed flour led to frankfurters' increased total dietary fiber and water-holding capacity, thus increasing the products' value. However, the products' sensorial properties were significantly modified, and further research therefore becomes necessary to improve product palatability [177].

4.3 Enrichment of Bread

Bakery products, particularly bread, have a significant share in the food guide pyramid for daily food choices recommended by the US Department of Health and Human Services. Therefore, the development of polyphenol-enriched bread is an efficient way to increase polyphenol intake.

In a recent study, different amounts of grape seed extract (300, 600, and 1,000 mg), source of catechin and procyanidin, were added to bread ingredients before the bread-making process. Although thermal processing decreased the antioxidant activity of the extract, fortified bread showed significantly higher antioxidant capacity. Moreover, the antioxidant capacity of the extract contributed to the reduction of N^{ε}-carboxymethyllysine, a potential toxicant in food, in enriched bread. Regarding bread sensory properties, only a favorable change in bread color was observed, with no significant alteration in quality parameters (sweetness, porosity, astringency, and stickiness) [178].

The study evaluated the effect of grape by-products (GP) on the chemical composition, soluble and insoluble dietary fiber, phenolic compounds and antioxidant activity, and organoleptic characteristics of sourdough mixed rye bread. The following samples of sourdough mixed rye bread were prepared: control bread and breads with GP at four different levels, 4%, 6%, 8%, and 10%. The addition of GP significantly improved dietary fraction contents, as bread with 10% added GP accounted for 39% and 37% higher contents of soluble and insoluble dietary fiber than control bread. The assay of radical scavenging activity and reducing ability showed that GP addition greatly enhanced the antioxidant properties of mixed rye breads. The profiles of phenolic compounds of supplemented breads were dominated by procyanidin B1 and B2, catechin, epicatechin, caffeic acid, and myricetin. Increased GP levels led to significantly increased bread hardness and gumminess. Although both control bread and supplemented breads showed common volatile compound profiles, slight differences were observed in the concentration of these components. Sensory evaluation of GP-enhanced breads revealed that a maximum of 6% GP could be added to prepare acceptable products.

4.4 Enrichment of Seafood

Lipid oxidation is still today a problem in the food industry, especially for products that contain marine lipids. The high content of polyunsaturated fatty acids (PUFA), highly beneficial for human health particularly in preventing cardiovascular diseases, in fish oil makes it very attractive to the rising market of functional products. However, at the same time, this high PUFA content makes marine lipids highly susceptible to oxidation, consequently affecting fish oil quality during storage through flavor, odor, color, and texture deterioration and even producing toxic compounds [179].

Antioxidant phenolics are often added to food to inhibit the initiation and propagation of oxidation's radical chain reactions, thus delaying the oxidation process. New antioxidants capable of retarding oxidation in fish oil-enriched foods would be desirable, especially if these new antioxidants had relevant biological properties. In this sense, grape procyanidins and resveratrol derivatives have been examined as inhibitors of oxidation in fresh Atlantic horse mackerel [180, 181]. Procyanidins were added at 50 and 100 ppm (W/W) to minced muscle fish. Procyanidin supplementation stabilized the fish product and maintained its functionality associated with the presence of PUFA and α-tocopherol. Regarding the stability and biological activity of grape procyanidins during chilled storage, their antioxidant activity has been described to remain stable for more than one year at 4 °C [180]. On the other hand, resveratrol, piceid, and some resveratrol derivatives were tested at similar concentration (100 ppm) on minced muscle fish. Resveratrol and piceid showed notable antioxidant activity in fish oil-in-water emulsions.

Neither lipophilization nor glycosylation of resveratrol led to improved antioxidant efficiency [181]. The authors conclude that these biologically relevant phenols could be used as natural antioxidant in this type of food matrix.

4.5 Enrichment of Grape Derivative Beverages

As the fastest growing segment in the food industry, functional beverages represent an attractive alternative to conventional food products for health conscious individuals. Through the incorporation of fortifying agents and antioxidant-eliciting ingredients, ordinary beverages are being transformed into "superfoods" [182, 183]. Functional beverages and plant-based fortified products are especially attractive to consumers [153] and represent new opportunities for beverage producers.

Newly developed foods and beverages have incorporated grape-derived extracts to gain health appeal [184]. A concentrated food ingredient comprising green tea and grape skin and seed extracts has been developed. A mix of green tea extract (3.0 g/L)+grape skin extract (12.0 g/L)+grape seed extract (0.5 g/L)+fungus extract (0.1 g/L)+vitamin C (0.3 g/L) yielded 1,155 mg/L of polyphenol content [185]. Other authors have formulated new grape extracts rich in procyanidins: 15% monomers and 20% of dimers and up to 30% of trimers, tetramers, and pentamers by weight [186]. The product was tested in the treatment of prehypertensive patients and was successful in reducing both systolic and diastolic blood pressure by 8% on a dose of 300 mg/day for 8 weeks.

In 2008, a patent application by Perlman et al. [187] presented an interesting use of the "rich" by-product of the grape juice industry. Based on the fact that grape pomace solids contain at least ten times greater amounts of polyphenols than pressed grape juice, the authors proposed the fortification of such juice with pomace polyphenol extract. The beverage was tested at different extract concentrations for sensorial acceptance and antioxidant activity. Unacceptable astringency was observed together with significantly increased antioxidant activity. As described for other food products, cyclodextrin was proposed for successful reduction of the astringent sensation. Draijer et al. [188] described the invention of a beverage containing 550 mg of red wine polyphenols + 250 mg of red grape polyphenols added to 200 mL of a soy-based drink. Results of daily doses for breakfast suggest a positive impact on blood pressure.

Wine has traditionally been identified as a health-benefiting product due to its effects on coronary heart disease after the so-called French paradox [189] delaying tumor onset [190] and its high antioxidant activity [191]. These benefits have been ascribed to phenolic compounds abundant in red wine. As previously commented, resveratrol is one of the main bioactive compounds found in wine. Wine with higher resveratrol content may therefore be regarded as "functional wine" due to resveratrol's positive health effects. Some research is currently being developed in this sense. Metabolic engineering has been used to obtain transgenic yeasts that enhance resveratrol content in wine [192, 193]. In some cases, the conversion rate was higher than 20-fold [194]. Moreover, as resveratrol is a phytoalexin synthesized by grapes after exposure to biotic or abiotic stress, the presence of resveratrol in grapes depends on the degree of stress exposure. Preharvest chemical treatments such as BTH, chitosan, methyl jasmonate, jasmonic acid, salicylic acid, beta-aminobutyric acid, ozone, aluminum chloride, and UVC can be used to enhance nutraceutical grape properties [40]. Guerrero et al. obtained resveratrol-enriched red wines by using UVC [195], while Gaudette et al. did it by adding resveratrol directly to the wine [183]. Both studies concluded that increased resveratrol content does not alter wine's quality properties. In addition, enriched wines showed significantly higher antioxidant capacity compared to control wines [183].

Market studies suggest that the functional attribute in wine positively and significantly affects the probability of selecting a particular red wine and consumer willingness to pay for this attribute [196]. However, it should be mentioned that the term "functional" cannot be used for beverages with over 1.2% alcohol content in Europe according to the EU Regulation 1924/2007. Remarkably, a winery is already commercializing this type of wines in the Australian market (www.winedoctor.com.au). They extract resveratrol from grape and add it to wine, thus achieving resveratrol wine content up to 100 g/L – an outstanding achievement regarding usual resveratrol content in wine.

5 Conclusion and Perspectives

From an economic viewpoint, grapevine is one of the most important crops in the world and has a deeply rooted significance in human culture. The functional ingredients of grapevine include several flavonoids that have been reported to show different activities including antioxidant, anticancer, and prevention of cardiovascular disease, as well as the treatment of several neurological disorders. Grape seed extract and its active components such as proanthocyanidins, resveratrol, and quercetin seem to be potent antioxidants. The consumption of grapes and grape juice is likely to have positive effects on human health.

In this sense, numerous food products are enriched with grape polyphenols, thus entering the market of functional foods. Functional foods (and nutraceuticals) constitute a promising field to improve health and prevent age-related chronic diseases. There has been a growing interest in researching, developing, and commercializing functional food. Moreover, recent trends have shown consumers' growing interest in many health-promoting food and supplements. The addition of fruit polyphenols to food products is increasing with the currently emerging popularity of functional food (ISI Web of Knowledge; 1,585 entries in the last 11 years). For that reason, further research is needed, since the effectiveness of functional products in vivo is a really complex topic on which there is still too little knowledge. In this way, both synergistic effects among phenolics and the effect of food matrices on bioavailability will be taken into account.

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Potential Neuroprotective Actions of Dietary Flavonoids

84

David Vauzour, Marcus Rattray, Robert J. Williams and Jeremy P. E. Spencer

Contents

1 Introduction 2618
2 Flavonoid Bioavailability and Accessibility to the Brain 2619
3 Flavonoids and Memory, Learning, and Neurocognitive Performance 2620
4 Flavonoids and Neurodegeneration 2621
5 Flavonoids and Neuroinflammation 2624
6 Mechanisms Underpinning the Beneficial Effects of Flavonoids 2626
7 Summary 2629
References 2630

Abstract

Flavonoids exert a multiplicity of neuroprotective actions within the brain, including a potential to protect neurons against injury induced by neurotoxins, an ability to suppress neuroinflammation, and the potential to promote memory,

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learning, and cognitive function. These effects appear to be underpinned by their interaction with critical protein and lipid kinase signaling cascades in the brain leading to an inhibition of apoptosis triggered by neurotoxic species and to a promotion of neuronal survival and synaptic plasticity. Through these mechanisms, the consumption of flavonoid-rich foods throughout life holds the potential to limit neurodegeneration, decrease neuroinflammation, and prevent or reverse age-dependent loses in cognitive performance. The intense interest in the development of drugs capable of enhancing brain function means that flavonoids may represent important precursor molecules in the quest to develop a new generation of brain-enhancing drugs.

Keywords

Flavonoids • memory • neurodegeneration • neuroinflammation • signaling pathways

Abbreviations

AD	Alzheimer’s disease
Arc/Arg3.1	Activity-regulated cytoskeletal-associated protein
ASK1	Apoptosis signal-regulating kinase 1
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CaMKIV	Calcium/calmodulin kinase IV
CREB	Cyclic AMP regulatory-binding protein
EGCG	Epigallocatechin-3-gallate
ERK1/2	Extracellular signal-regulated kinase 1 and 2
GSPE	Grape seed polyphenolic extract
JNK	c-jun N-terminal kinase
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR	The mammalian target of rapamycin
NGF	Nerve growth factor
PD	Parkinson’s disease
PI-3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
ROS	Reactive oxygen species

1 Introduction

Due to significant advances in medical science over the past century, there has been a gradual increase in human life span, with people over the age of 60 expected to double between 2000 and 2050 [1]. Although this is a great achievement, an increase in age-related diseases including neurodegenerative disorders has been observed to parallel the extended life span. This will soon have profound economical and social implications, and it is already becoming a burden for health-care systems. Aging is an important risk factor for neurodegenerative diseases, of which Alzheimer's disease and Parkinson's disease are the most common. Neuronal loss underlies the clinical impairment in these conditions, and this cell death is associated with numerous pathogenic cellular and molecular events [2]. The majority of existing drug treatments for neurodegenerative disorders can afford symptomatic relief but are not disease-modifying, that is, cannot prevent the underlying degeneration of neurons. Therefore, there is an urgent need to develop therapeutic interventions capable of preventing the progressive loss of neurons. Because many of these neurodegenerative diseases have been linked to increases in oxidative stress, strong efforts have been aimed at exploring dietary and therapeutic antioxidant strategies to combat the neuronal damage. Recent attention has focused on the neuroprotective effects of major dietary polyphenols called flavonoids, which have been effective in protecting against both age-related cognitive and motor decline in vivo. While historically research focused on their antioxidant properties [3], recent data support the view that flavonoids, and their in vivo metabolites, do not act as conventional hydrogen-donating antioxidants but may exert modulatory actions in cells through actions at protein kinase and lipid kinase signaling pathways [4]. This chapter will highlight the neuroprotective mechanisms of flavonoids through their ability to interact with neuronal signaling pathways and their potential to modulate neuroinflammation, to counteract neurotoxin-induced neurodegenerative disorders, and to enhance memory, learning, and cognitive performances.

2 Flavonoid Bioavailability and Accessibility to the Brain

Many studies have reported the bioavailability of flavonoids in the systemic circulation [5–8], however, little is known about their uptake within the central nervous system (CNS; brain and spinal cord). In order to understand whether these phenolic compounds affect neurons and glial cells, it is crucial to ascertain their presence within the cerebral tissue. In order for flavonoids to access the brain, they must first cross a tightly regulated, selectively permeable endothelial cell layer which isolates the CNS tissue from the vasculature, the blood-brain barrier (BBB). The BBB is permeable to nutrients and actively excludes many substances from the central nervous system [9]. Using in vitro models, researchers have provided the first information on the capacity of flavonoids to traverse the BBB [10] and demonstrated that less polar *O*-methylated metabolites appear to be capable to greater brain uptake than the more polar flavonoid glucuronides [11]. The degree of entry of flavonoids or their metabolites into the CNS was also observed to depend on their interactions with transporters, such as P-glycoprotein, expressed in the BBB whose function is to export xenobiotics and unwanted metabolites [12].

For example, P-glycoprotein is considered to be responsible for the differences between naringenin and quercetin flux into the brain in situ [10]. Further to in vitro models, animal investigations have also substantiated these findings and indicated that flavanones were able to enter the brain following their intravenous administration [13], while epigallocatechin gallate [14], epicatechin [15], and anthocyanins [16, 17] were found in the brain after their oral administration. Furthermore, several anthocyanins have been identified in different regions of the rat [18, 19] and pig brains [20, 21] of blueberry-fed animals. Altogether, these results indicate that many flavonoids are able to traverse the BBB and localize in the brain, suggesting that they can directly exert neuroprotective and neuromodulatory actions.

3 Flavonoids and Memory, Learning, and Neurocognitive Performance

There is a growing interest in the potential of phytochemicals to improve memory, learning, and general cognitive ability [22, 23]. A recent prospective study aimed at examining flavonoid intake in relation to cognitive function and decline has provided strong evidence that dietary flavonoid intake is associated with better cognitive evolution, that is, the preservation of cognitive performance with aging [24]. In particular, subjects included in the two highest quartiles of flavonoid intake had better cognitive evolution than subjects in the lowest quartile and after 10 years follow-up. Subjects with the lowest flavonoid intake had lost on average 2.1 points on the Mini-Mental State Examination, whereas subjects with the highest quartile had lost 1.2 points. Such data provides a strong indication that regular flavonoid consumption may have a positive effect on neurocognitive performance as we age.

There has been much interest in the neurocognitive effects of soy isoflavones, primarily in postmenopausal women [25, 26]. Isoflavone supplementation has been observed to have a favorable effect on cognitive function [27], particularly verbal memory, in postmenopausal women [28], and a 6- and 12-week supplementation was observed to have a positive effect on frontal lobe function [29]. Furthermore, animal studies have also indicated that isoflavones are capable of improving cognitive function [30, 31]. However, there is still uncertainty regarding their effects as some large intervention trials have reported that isoflavone supplementation does not lead to cognitive improvements [32]. The rationale behind the potential of isoflavones to exert positive effects on cognitive function is believed to lie primarily in their potential to mimic the actions and functions of estrogens in the brain [33]. For example, postmenopausal women who undertake estrogen replacement therapy have a significantly lower risk for the onset of Alzheimer's disease than women who do not [34]. They may also be effective by affecting the synthesis of acetylcholine and neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in hippocampus and frontal cortex [35, 36].

There is also extensive evidence that berries, in particular blueberries, are effective at reversing age-related deficits in motor function and spatial working memory [37–39]. In addition to spatial memory, blueberry supplementation has been shown to improve "object recognition memory" [40] and "inhibitory fear conditioning learning" [41, 42]. Blueberry appears to have a pronounced effect on short-term memory [42] and has also been shown to improve long-term reference memory following 8 weeks of supplementation [38]. Tests using a radial arm maze have supported these findings and have provided further evidence for the efficacy of blueberries [39]. Indeed, these have shown that improvements in spatial memory may emerge within 3 weeks, the equivalent of about 3 years in humans. The beneficial effects of flavonoid-rich foods and beverages on psychomotor activity in older animals have also been reported [37, 43]. In addition to those with berries, animal studies with tea [44] and pomegranate juice [45] or pure flavonols such as quercetin, rutin [46], or fisetin [47] have provided further evidence that dietary flavonoids are beneficial in reversing the course of neuronal and behavioral aging.

The flavonoid-rich plant extract, *Ginkgo biloba*, has also been shown to induce positive effects on memory, learning, and concentration [48, 49]. *Ginkgo biloba* has a prominent effect on brain activity and short-term memory in animals and humans suffering from cognitive impairment [50, 51] and promotes spatial learning in aged rodents [52, 53]. Furthermore, *Ginkgo biloba* promotes inhibitory avoidance conditioning in rats with high-dose intake leading to short-term, but not long-term, passive avoidance learning in senescent mice [54, 55]. However, the pharmacological mechanisms by which *Ginkgo biloba* promotes cognitive effects are unclear, with its ability to elicit a reduction in levels of reactive oxygen species (ROS) [56], to increase cerebral blood flow [57], to modulate membrane fluidity [54], to interact with muscarinic cholinergic receptors [58], and to protect the striatal dopaminergic system [59] all being suggested as possible mechanisms underlying its actions in the CNS.

4 Flavonoids and Neurodegeneration

There are a number of epidemiological studies which suggest that plant-derived flavonoid-rich foods or supplements might delay the initiation and progression of Alzheimer's disease (AD) and related neurodegenerative disorders. With regard to AD, independent prospective cohort studies have associated the consumption of polyphenolic-rich vegetables, fruit juices, and red wine with delayed onset of the disease [60, 61]. This is in accordance with previous studies linking high consumption of flavonoids to improvements in dementia [24, 62], and collectively, these reports lend some support to the underlying hypothesis that dietary intervention with plant-derived flavonoid-rich foods or supplements could impact on the development of AD. Not all prospective studies have reached the same positive conclusions however, and in the Honolulu-Asia aging study, midlife flavonoid intake, as estimated using mean intake of green and black tea, was not associated with altered risk of late-life incident dementia [5]. Despite this, much of the subsequent work in the field has focused on the potential bioactivity of catechins which are abundant in tea. Indeed, the green tea flavanol epigallocatechin-3-gallate (EGCG) is regarded as a lead candidate molecule for use in AD and is part of an ongoing clinical trial where it is being given in combination with donepezil to 50 patients with AD (NCT00951834).

Many of the preclinical studies of the effects of flavonoids in AD have focused on models where there is increased production of beta-amyloid (Aβ). Aβ is a small protein produced by the enzymatic cleavage of amyloid precursor protein (APP). Aβ is aggregation prone and forms oligomeric species which are directly toxic to synapses and can aggregate further to form amyloid plaques, extracellular protein deposits which are a hallmark of Alzheimer's disease pathology [63]. Studies using transgenic mouse models of AD pathology have begun to address the possible mechanisms involved in the apparently beneficial effects of catechin-rich diets. Oral administration of EGCG for 6 months to Tg2576 mice, a strain which overexpresses the Swedish mutation of APP, reduced Aβ pathology and improved cognition [64]. Similarly, long-term green tea catechin administration improved spatial learning and memory in senescence-prone mice [65]. The mechanisms underlying these changes are not clear but might be linked to increased non-amyloidogenic processing of APP, through stimulating the activity of α-secretase, which cleaves APP at a site which prevents the formation Aβ species [66–68], or could be due to disruption of the interaction of amyloid with cAbl/Fe65 which might alter its ability to be processed into toxic species [69]. Alternatively, it is conceivable that EGCG reduces Aβ plaque pathology by inhibiting amyloid aggregation and fibrillization either as a result of metal chelation activity [70–72] or by favoring the formation of nontoxic (off-target) oligomers [73]. Interestingly, in addition to possessing the ability to inhibit the formation of β-sheet rich amyloid fibrils, EGCG also converts large mature Aβ fibrils into smaller nontoxic aggregates [74]. These are significant observations although very serious consideration must be given as to whether dietary EGCG could drive Aβ disaggregation in AD brain as the micromolar concentrations required to exert these effects in vitro will not be easily achievable in vivo. Anti-amyloidogenic activity is not unique to EGCG, and a number of other flavonoids, most notably myricetin, bind to Aβ fibrils and prevent further fibrillization [75–77]. Gallic acid and catechin-rich grape seed polyphenolic extract (GSPE) administered for 5 months to Tg2576 mice also inhibited cognitive deterioration coincident with reduced levels of soluble high molecular weight oligomers of Aβ [78]. Repeated intraperitoneal injection of the polymethoxylated citrus flavone, nobiletin, has similar effects [79]. However, it is worth noting that beneficial effects have been observed with flavonoids in some AD mouse models without obvious alterations in pathology. For example, feeding blueberry to APP + PS1 double transgenic mice prevented deficits in cognitive performance at 12 months but without altering the Aβ burden [80].

Although these are clearly important studies in that they show in principle that chronic exposure to polyphenolics can influence AD pathology and behavior in vivo, it is likely that the optimal flavonoid structures possessing the necessary bioactivity and bioavailability have not yet been identified. Other mechanisms of action are also possible. Interestingly in this regard, certain flavonols and flavones have been reported to inhibit and suppress expression of an enzyme BACE-1, which is required for the production of Aβ from APP [81, 82]. This observation is consistent with some of the observed Aβ lowering effects reported for flavonoid-rich extracts in vivo and in vitro. The identification of those flavonoid structures possessing the greatest potential inhibitory activity at BACE-1 and defining their precise mechanisms of action are needed.

Despite the well-established and compelling link between Aβ and AD, Aβ pathology and cognitive deficits are not well correlated. Consequently, beneficial effects of flavonoids on cognition may be unrelated to changes in Aβ per se but to key downstream changes, for example, in phosphorylation and fibrillization of tau, a protein which, when abnormally phosphorylated, is found in neurofibrillary tangles: another pathological hallmark of AD. Indeed, a number of flavonoids including myricetin and epicatechin 5-gallate have been shown to potently inhibit heparin-induced tau aggregation [83]. Moreover, grape seed polyphenolic extract (GSPE) also inhibits tau fibrillization, promotes the loss of preformed tau aggregates, and disrupts paired helical filaments [84–87]. (−)-Epigallocatechin-3-gallate (EGCG) appears to have broadly similar effects. (−)-Epicatechin and hesperetin hold the potential to inhibit the development of tau pathology through an alternative mechanism relating to their ability to enhance phosphorylation of a key regulatory enzyme, Akt, to inhibit GSK3β-induced hyperphosphorylation of tau [88, 89]. Whatever the mechanisms involved, collectively, this suggests that orally active flavonoids could have utility in AD beyond anti-Aβ actions.

The potential utility of flavonoids in neurodegeneration extends beyond dementia, and there is also considerable interest in their therapeutic potential in Parkinson's disease (PD). The neurodegeneration observed in PD appears to be triggered by multifactorial events including neuroinflammation, glutamatergic excitotoxicity, increases in iron, and/or depletion of endogenous antioxidants. There is a growing body of evidence to suggest that flavonoids may be able to counteract the neuronal injury underlying these disorders and thus slow the progression of the disease [23, 90]. There is good evidence to suggest that the consumption of green tea may have a beneficial effect in reducing the risk of PD [91], as has been extensively reviewed elsewhere [92, 93]. The efficacy of green tea is likely to be mediated by the effects of EGCG, which has been shown to attenuate the selective degeneration of dopamine neurons in animal models of PD induced by toxins including 6-hydroxydopamine [94] and MPTP [95]. The mechanism of protection is not known, but EGCG has been noted to interact with and modulate signaling pathways involved in neuroprotection, notably protein kinase C (PKC) and PI3 kinase, and has been implicated in reducing dopamine neuron damage in the substantia nigra by the chelation of iron: a mechanism which is also relevant to AD pathology. In vitro studies have also indicated that flavonoids might act to prevent PD pathology via their ability to prevent the formation of the endogenous neurotoxin, 5-*S*-cysteinyl-dopamine [96, 97].

EGCG can also reduce hippocampal neuronal injury induced by transient global ischemia [98]. Neuroprotective effects of flavonoids have also been observed in animal models of Huntington's disease, where the flavonol fisetin has been reported to be effective in reducing pathophysiology through its actions on the extracellular signal-regulated kinase (ERK) pathway [99, 100]. Collectively, these studies suggest that flavonoids have the potential to confer benefit in diverse neurodegenerative disorders. Some of the major neuroprotective mechanisms are discussed in more detail below.

On a note of caution, however, there is still insufficient data to support the clinical use of flavonoids in the treatment of neurodegeneration, and there have been a number of disappointing results from human intervention studies for dementia with various dietary polyphenolics and antioxidants such as curcumin and Gingko biloba. The challenge ahead, therefore, is to proceed cautiously until rigorous randomized controlled clinical trials have been undertaken to determine empirically if flavonoids have efficacy in individuals affected by dementia and other neurodegenerative conditions.

5 Flavonoids and Neuroinflammation

Neuroinflammation is an important defense mechanism in the CNS which typically results from cellular damage but may also arise from other stimuli including infection. While it is a beneficial process, sustained neuroinflammatory processes are known to participate in CNS disease states [101]. For example, neuroinflammation contributes to the progressive neuron death observed in Alzheimer's disease [102], Parkinson's disease [103], and also with neuronal cell death and damage associated with cerebral ischemia [104].

Neuroinflammation is a complex process which involves several CNS cell types and is characterized by a strong reaction of glial cells, namely, microglia: cells within the CNS with an immune function, similar to macrophages; and astrocytes: cells which support neuronal function and maintain BBB integrity. During neuroinflammation, proinflammatory chemical mediators can be released from cells resident in the CNS, including neurons themselves, endothelial cells of the vasculature, and glial cells [101]. If the BBB is impaired, the neuroinflammatory stimulus may involve infiltrating T and B lymphocytes and macrophages which interact with cells resident in the CNS (neurons, microglia, astrocytes) through a complex series of interactions which are not completely understood [105]. In either case, the neuroinflammatory state is characterized by a marked activation of microglia and astrocytes. This response is typically associated with a coordinated cellular response which includes activation of intracellular pathways dependent on the proinflammatory transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). During neuroinflammation, activated microglia and astrocytes can release a number of factors which are toxic to neurons; these include inflammatory cytokines such as interleukin-1 beta (IL1β), tumor necrosis factor alpha (TNFα), nitric oxide (NO), and glutamate [101, 106, 107].

The best example of a CNS disease state with a major neuroinflammatory component is multiple sclerosis (MS), a chronic debilitating disease which is characterized by demyelination, progressive irreversible axonal damage, and inflammation [105]. The most effective therapies to date act by reducing inflammation and activation of the immune system [108], showing that suppression of neuroinflammation has a positive effect in the disease. Dietary modification to improve multiple sclerosis symptoms and progression is an attractive proposition. Many patients with MS already use special diets, for example, gluten-free and milk-free or dietary supplementation with polyunsaturated fatty acids, vitamins, antioxidants, and/or herbal supplements (e.g., *Gingko biloba*). However, so far, a truly beneficial supplement or protective factor with a sound scientific base has not been elucidated. In part, this is attributed to poor design of the clinical trials [109] but also reflects the fact that the most potent interventions have not been found or, indeed, searched for systematically. Although to date there is no correlation between dietary intake of fruit and vegetables and incidence of MS [110, 111], flavonoids have the potential to be clinically useful in abrogating MS pathology. The studies to date have not excluded links between fruit and vegetable consumption and lowered MS incidence [112]. Dietary supplementation with flavonoid compounds has not been tested in man.

There is data which show encouraging positive effects of flavonoids in animal and in vitro models relevant to MS. A flavanol, (−)-epigallocatechin-3-*O*-gallate (EGCG), delivered orally reduces symptom severity in the autoimmune encephalomyelitis model of relapsing-remitting MS by reducing inflammation and increasing neuroprotection [113]. The flavonol quercetin has also been reported to be effective in the Experimental Autoimmune Encephalomyelitis (EAE) mouse model and reduces T cell proliferation in vitro at concentrations exceeding 10 μM [114]. Two Dutch groups independently identified a number of promising flavonoids using in vitro assays. Hendriks et al. [115] tested six flavonoids and found that one, luteolin, was the most effective at suppressing myelin phagocytosis by the macrophage cell line RAW 264.7 (IC_{50} of 20 μM). Several other flavonoids (quercetin, fisetin, and apigenin) were also effective in this assay but with potencies an order of magnitude lower. In the rat EAE model, luteolin (but not quercetin) showed clinical protection [116]. A separate group tested six flavonoids for their ability to alter T cell proliferation [117]. They showed that micromolar concentrations of luteolin, apigenin, fisetin, and quercetin (but not morin or hesperetin) suppress the production of the cytokine interferon-gamma (IFNγ) from lymph node-derived T cells but, paradoxically, worsen clinical severity in the EAE model. There is strong evidence that the flavone wogonin and a related compound baicalein can inhibit inflammatory responses in macrophages in vitro and in vivo [118, 119]. Thus, the studies to date show promising proof of concept of beneficial effects of flavonoids in suppressing immune and inflammatory responses in models of MS.

During activation of glial cells in neuroinflammatory states, various transcription factors including NF-κB, activator protein-1 (AP-1), and the signal transducer and activator of transcription-1 (STAT-1) have been shown to be involved in proinflammatory responses in astrocytes and microglia [120–124] which can contribute to neuronal death. Of these transcription factors, the NF-κB system is the most studied system in the context of neuroinflammation. Suppression of this pathway can be neuroprotective [125]. Its activation is seen in a number of neurodegenerative states, for example, in postmortem Alzheimer's disease patients, cells in the vicinity of β-amyloid plaques show increased NF-κB immunoreactivity. Numerous flavonoids have been shown to have the ability to inhibit NF-κB in different cell types. The flavonol quercetin (50 μM) reduces phosphorylation of NF-κB subunits in human peripheral blood mononuclear cells [126] and suppresses NF-κB in a microglial cell line [127]. The flavanone oroxylin A (80 μM) reduces LPS-induced NO production and NF-κB activity in RAW 246.7 macrophages [128]. The flavone apigenin (5–15 μM) blocks LPS stimulation of the NF-κB pathway in RAW 246.7 macrophages and reduces κB-transcriptional activity [129]. The flavanol EGCG (5–15 μM) reduces LPS-induced NFκB-activity in peritoneal macrophages [130] and reduces T cell proliferation accompanied by inhibition of NF-κ B [113]. Catechin (0.13–2 mM) has been reported to increase mouse microglial cell survival following exposure to the oxidative agent *tert*-butyl hydroperoxide (tBHP) by suppressing NF-κB activation [131]. The flavone luteolin (20 μM) reduced LPS-induced NF-κB transcriptional activity in fibroblasts [132]. The flavone wogonin (50 μM) was shown to reduce NF-κB activation in C6 glioma cells and prevent microglial activation [133], and baicalein is reported to inhibit $NO^\bullet$ production and NF-κB activity in microglia [134, 135]. The isoflavone genistein has been shown to reduce expression of iNOS in astrocytes, through inhibition of NF-κB activation [136]. While the data gives proof of principle that NF-κB is a potential target of flavonoids, the concentrations required for positive effects of those particular compounds in vitro are high, in the micromolar range, that is, at concentrations which cannot be obtained through the diet. It is likely that, for most of those studies, the antioxidant effects of the flavonoids used account for the positive effects on suppressing NF-κB activation. We have tested dietary-relevant concentrations of flavonoids and shown them to be bioactive in suppressing certain responses in primary astrocytes mediated by transcription via the antioxidant response element [137]. However, at this concentration range (0.1–1 μM), we find flavonoids of different classes are unable to suppress NF-κB signaling pathways in primary astrocytes [169]. Therefore, while flavonoids may be effective agents at suppressing neuroinflammation in vivo, at this time, we do not regard the NF-κB signaling system as the primary signaling system responsible for the effects of flavonoids in vivo.

6 Mechanisms Underpinning the Beneficial Effects of Flavonoids

Historically, the biological actions of flavonoids have been attributed to their antioxidant properties, either through their reducing capacities per se or through their possible influences on intracellular redox status. However, their classical hydrogen-donating antioxidant activity is unlikely to be the sole explanation for the bioactivity of flavonoids in vivo, as during absorption they are extensively metabolized to glucuronides, sulfates, and *O*-methylated forms which are reduced in their antioxidant potential [4]. Rather, it has become evident that flavonoids are more likely to exert their neuroprotective actions by the modulation of intracellular signaling cascades, of particular interest in this context are those protein kinases which are central to pro-survival or pro-death pathways in neurons.

After ingestion, flavonoids are thought to reach sufficiently high concentrations in the CNS, that is, in the high nanomolar range, to exert pharmacological activity by binding to specific protein targets. Numerous studies now show important effects of flavonoids at sub-micromolar concentrations where antioxidant effects are unlikely to be relevant. The effects of flavonoids on neuronal signaling pathways are highly concentration dependent and are likely to be related to their ability to exert high-affinity receptor agonist-like actions at low concentrations (low to mid nanomolar) and direct enzyme inhibition at higher concentrations (high nanomolar to micromolar) [138, 139].

The precise site for the first point of interaction of flavonoids with neurons is still unclear in most cases. Potential flavonoid-binding sites on neurons include adenosine [140], $\mathrm{GABA_A}$ [141, 142], and testosterone receptors [143], and a specific plasma membrane binding site for polyphenols in CNS tissue has been proposed [144]. Evidence indicates that they are capable of regulating signaling pathways, particularly protein kinases, in a number of ways which include: (1) binding to enzymes or receptors which control kinase activation, (2) by modulating the activity of kinases directly, (3) by affecting the function of important phosphatases, which act in opposition to kinases, and (4) by modulating signaling cascades lying downstream of kinases, that is, transcription factor activation to selectively control gene expression [23, 145]. It is beyond the scope of this chapter to list all the pathways which have been shown to be regulated by flavonoids, so we focus on a few key signaling pathways which are intimately associated with neuron survival and plasticity.

There is much evidence to support the actions of nanomolar concentrations of flavonoids, in particular flavanols and flavanones, on the ERK pathway [89, 146], which are, in general, calcium dependent and mediated by interactions with upstream kinases MEK1 and MEK2 and potentially membrane receptors [147]. ERK activation often leads to the activation of the cAMP response element-binding protein (CREB), a transcription factor. CREB is considered to be critical in the induction of long-lasting changes in synaptic plasticity and memory [148, 149]. CREB activation regulates the expression of a number of important genes, including brain-derived neurotrophic factor (BDNF), thus has a pivotal role in controlling neuronal survival and synaptic function in the adult central nervous system [150, 151]. Regulation of BDNF is of particular interest as it is linked with the control of synaptic plasticity and long-term memory [152]. Decreases in BDNF and pro-BDNF have been reported in Alzheimer's disease [153], and a polymorphism that replaces valine for methionine at position 66 of the prodomain of BDNF is associated with memory defects and abnormal hippocampal function in humans [154].

Recent studies have shown that spatial memory performance in rats supplemented with blueberry correlates well with the activation of (CREB) and with increases of BDNF in the hippocampus [42]. Blueberry flavonoid-induced activation of CREB and BDNF expression has also been shown to lead to the activation of the PI3 kinase/Akt signaling pathway [42], via the binding of BDNF to pre- or postsynaptic TrkB receptors. Fisetin, a flavonoid found in strawberries, has been shown to improve long-term potentiation and to enhance object recognition in mice by a mechanism dependent on the activation of ERK and CREB [155].

In general, in vitro studies show that many flavonoids, at submicromar concentrations, activate ERK, as determined by measuring increased phosphorylation of this enzyme. In cortical neurons, the flavanol (−)-epicatechin (0.1 and 0.3 μM) induces both ERK1/2 and CREB activation [47], while nanomolar concentrations of quercetin are effective at enhancing CREB activation [156]. Other flavonoids have also been found to influence the ERK pathway, with the citrus flavanone, hesperetin, capable to activating ERK1/2 signaling in cortical neurons at nanomolar concentrations [157], and flavanols such as EGCC restoring ERK1/2 activities in 6-hydroxydopamine-treated or serum-deprived neurons [94]. This ability to activate the ERK pathway is not restricted to neurons and has also been observed in fibroblasts exposed to nanomolar concentrations of epicatechin [158].

As well as effecting the ERK/CREB/BDNF axis, flavonoids are known to modulate the activity of an enzyme system associated with neuroprotection, Akt (also known as PKB). One of the major enzymes which controls Akt/PKB activity is the lipid kinase, PI3K. In cortical neurons, flavonoids such as the citrus flavanone hesperetin (0.1 and 0.3 μM) cause the activation of Akt/PKB and the consequent inhibition of proteins associated with cell death such as apoptosis signal-regulating kinase 1 (ASK1), Bad, caspase-9, and caspase-3 [89]. The activation of Akt by flavonoids in hippocampal neurons has been shown to trigger the increased translation of specific mRNA subpopulations [159], including the activity-regulated cytoskeletal-associated protein (Arc/Arg3.1) [42]. Arc is also under the regulatory control of both BDNF [160] and ERK signaling [161]. Increased Arc expression may facilitate changes in synaptic strength and the induction of morphological changes in dendritic spines [162]. In support of this, studies have indicated that changes in neuronal morphology occur in response to flavonoid supplementation [163] and that certain flavonoids can influence neuronal dendrite outgrowth in vitro [164] (Fig. 84.1).

As well as pro-survival effects, some flavonoids may inhibit important protective enzymes. Flavonoids can inhibit PI3K via direct interactions with its ATP binding site [165]. The structure of flavonoids determines whether or not they act as potent inhibitors of PI3K [166]. One of the most selective PI3K inhibitors available, LY294002, was modeled on the structure of quercetin [167, 168]. Quercetin and some of its in vivo metabolites have been shown to be neurotoxic in vitro, by inhibiting pro-survival Akt/PKB signaling pathways by a mechanism of action consistent with quercetin and its metabolites acting at and inhibiting PI3K activity [156]. In addition, some flavonoids may be capable of interacting directly with ERK kinases, such as MEK-1 to cause ERK inhibition: the flavone backbone (2-phenyl-1,4-benzopyrone) has close structural homology to a specific MEK-1 inhibitor, PD98059 (2′-amino-3′-methoxyflavone). This data suggests that flavonoid supplementation must be treated with caution; while many compounds are likely to enhance neuroprotective signaling, others may produce unwanted inhibition of key enzymes important for cell survival.

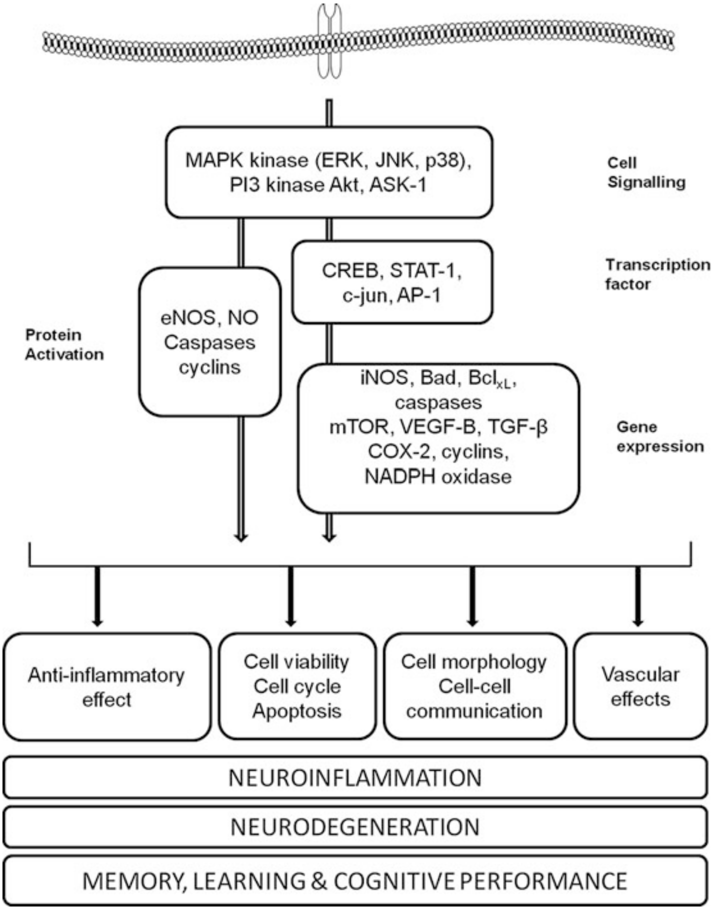
MAPK kinase (ERK, JNK, p38), PI3 kinase Akt, ASK-1
Cell Signalling
CREB, STAT-1, c-jun, AP-1
Transcription factor
eNOS, NO Caspases cyclins
Protein Activation
iNOS, Bad, Bcl$_{xL}$, caspases mTOR, VEGF-B, TGF-β COX-2, cyclins, NADPH oxidase
Gene expression
Anti-inflammatory effect
Cell viability Cell cycle Apoptosis
Cell morphology Cell-cell communication
Vascular effects
NEUROINFLAMMATION
NEURODEGENERATION
MEMORY, LEARNING & COGNITIVE PERFORMANCE

Fig. 84.1 *The interaction of flavonoids with cellular signaling pathways involved in neurodegeneration, neuroinflammation, and learning and memory*. Flavonoid-induced activation and/or inhibition of MAP kinase and PI3 kinase signaling leads to the activation of transcription factors which drive gene expression. For example, activation of ERK/Akt and the downstream transcription factor CREB by flavonoids may promote changes in neuronal viability and synaptic plasticity, which ultimately influence neurodegenerative processes. Flavonoid-induced inhibition of the JNK, ASK1, and p38 pathways leads to an inhibition of both apoptosis in neurons and a reduction of neuroinflammatory reactions in microglia (reduction in iNOS expression and NO$^{\bullet}$ release). Alternatively, their interaction with signaling may lead to direct activation of proteins such as eNOS, which controls nitric oxide release in the vasculature and thus may influence cerebral blood flow

7 Summary

The neuroprotective actions of dietary flavonoids involve a number of effects within the brain, including a potential to protect neurons against injury induced by neurotoxins, an ability to suppress neuroinflammation, and the potential to promote memory, learning, and cognitive function. This multiplicity of effects appears to be underpinned by their capacity to interact with important neuronal signaling cascades in the brain leading to an inhibition of apoptosis triggered by neurotoxic species and to a promotion of neuronal survival and differentiation. Although the consumption of flavonoid-rich foods throughout life may hold a potential to limit neurodegeneration and prevent or reverse age-dependent deteriorations in cognitive performance, at present, the precise temporal nature of the effects of flavonoids on these events is unclear. For example, when one needs to begin consuming flavonoids in order to obtain maximum benefits is not yet known. There are a vast number of flavonoids available, and while many have similar beneficial effects on neuroprotection in animal models, those flavonoids which are the most effective are not yet known. Due to the intense interest in the development of drugs capable of enhancing brain function, flavonoids may represent important precursor molecules in the quest to develop a new generation of brain-enhancing drugs.

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Improvement of Nutraceutical Value of Food by Plant Symbionts

85

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Contents

1 Introduction 2642
2 Health-Promoting Phytochemicals as Affected by Agronomic Management 2643
3 Plant Beneficial Symbionts: Arbuscular Mycorrhizal Fungi 2645
4 Nutraceutical Value of Mycorrhizal Plants 2647
4.1 Case Study 1: Globe Artichoke 2650
4.2 Case Study 2: Tomato 2651
5 Conclusion 2652
References 2653

Abstract

Consumers and producers have recently shown an increasing interest in health-promoting properties of plant fresh foods, which are currently considered "functional foods." They contain phytochemicals playing a key role in promoting human health by reducing oxidative damages, modulating detoxifying enzymes, stimulating the immune system, and showing chemopreventive actions. Recent findings revealed that the content and composition of phytochemicals in fresh fruit and vegetables is greatly affected by plant genotype, harvest season, soil quality, and agronomic and environmental factors, including mycorrhizal symbioses established by arbuscular mycorrhizal fungi (AMF) with most crop plants. AMF promote plant growth and health and reduce the need of chemical fertilizers and pesticides, leading to less environmental damage and to improved

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food quality and health. They also enhance the biosynthesis of plant secondary metabolites with health-promoting activities, such as polyphenols, carotenoids, flavonoids, phytoestrogens, and activity of several antioxidant enzymes. Recent studies reported a higher nutraceutical value in mycorrhizal globe artichoke and tomato, two plant species largely cultivated for human consumption, suggesting that AMF inoculation may represent a suitable biotechnological tool to be implemented in agri-food chains aimed at producing safe and healthy food.

Keywords

Agri-food chains • arbuscular mycorrhizal fungi • beneficial microorganisms • functional food • globe artichoke • health-promoting compounds • mycorrhizal symbiosis • phytochemicals • plant secondary metabolites • tomato

1 Introduction

Consumers and producers have recently shown an increasing interest in health-promoting properties of plant foods, which represent an important societal issue. Fresh fruits and vegetables are currently evaluated not only for their size, weight, appearance, and flavor but also for their nutritional and nutraceutical value, that is, their content in vitamins, mineral nutrients, dietary fibers, and secondary metabolites. Plant fresh foods are considered “functional foods,” “nutraceutical foods,” or “pharmafoods,” since many epidemiological studies have reported that their consumption may play a key role in promoting human health by preventing chronic diseases and decreasing the risk of mortality from cancer and cardiovascular diseases [1–6]. Though, in most cases, it was not investigated whether the biological activity was linked to a specific plant molecule or to additive and synergistic combinations of phytochemicals [7].

Phytochemicals, represented by thousands of secondary metabolites produced by plants belonging to many different families, genera, and species, are dietary plant molecules which, consumed daily, can beneficially modulate human metabolism [8]. In particular, they represent a rich source of natural antioxidant compounds able to reduce or prevent oxidative damages to different biological molecules, such as lipids, proteins, and nucleic acids [9], damages caused by the action of reactive oxygen species (ROS) deriving from cell aerobic respiration [10–12]. Beyond their antioxidant activity, phytochemicals play a beneficial and functional role in human health by modulating detoxifying enzymes [13] and hormone metabolism [14, 15] and by stimulating the immune system [16], showing also antibacterial and antiviral activity [17].

The most important phytochemicals are represented by polyphenols, widespread compounds functioning as scavengers of free radicals and quenchers of single oxygen formation [18]. Polyphenols are widely distributed in plants and are generally considered beneficial, since they affect different processes in mammalian cells, suggesting an anticarcinogenic and antiatherogenic role [19, 20]. Flavonoids, represented by more than 5,000 bioactive compounds, many of which can be found in food and beverages – such as quercetin in tea, kaempferol in cabbages, and myricetin in blackberry and red vine – have been reported to reduce the risk of cardiovascular diseases, to exert a chemoprotective action, and to have a phytoestrogenic activity [21]. Glucosinolates, phytochemical compounds including more than 130 different molecules mainly occurring in Brassicaceae [22], are considered "plant food protection agents" since a clear correlation has been shown between cruciferous plant consumption and cancer risk reduction [6, 23–25]. Other epidemiological data reported that isoflavones have different health-promoting effects, protecting from cardiovascular diseases [26].

Different phytochemical molecules have shown a chemopreventive action [27]: for example, the synthetic oleanane triterpenoid, CDDO-methyl ester, is a potent antiangiogenic agent [28]; epigallocatechin-3-gallate from green tea inhibits tumor angiogenesis and vascular tumor growth [29]; polyphenol curcumin prevents hematogenous breast cancer metastases in immunodeficient mice [30]; polyphenol xanthohumol from hop shows antileukemia effects in Bcr/Abl-transformed cells [31]; hyperforin from *Hypericum perforatum* blocks neutrophil activation of matrix metalloproteinase-9 and restrains inflammation-triggered angiogenesis [32]; lycopene from tomato is active in inflammation and in chemoprevention of prostate cancer [27]; polyphenolic compounds contained in red wine are able to inhibit vascular endothelial growth factor expression in vascular smooth muscle cells [33].

Nevertheless, it is important to note that any health-promoting activity of plant food depends on its bioavailability and bioefficacy, which are often related to individual variables, such as microbiome structure and composition, digestive processes, and absorption in the intestine [34–36].

Some phytochemicals, such as isoflavones and other flavonoids, as well as lignans, coumestans, and stilbenes, display estrogenic (and antiestrogenic) activity and are generally called phytoestrogens, notwithstanding the fact that they do not show any structural similarity to naturally occurring estrogens [37]. For example, lignans contained in seeds, sprouts, fruits, vegetables, and whole grains are active in cancer prevention, similarly to isoflavones [38]. These compounds have been studied for their putative preventive role in osteoporosis, menopausal symptoms, arteriosclerosis, heart disease, and cancer, and some were considered alternative to synthetic compounds for therapeutic purposes in humans [39, 40]. They have a complex mode of action via interaction with the nuclear estrogen receptor isoforms ERa and ERb, exhibiting either estrogen-agonist or estrogen-antagonist effects [41]. In particular, antiestrogenic compounds can antagonize estrogen-dependent processes in their target tissues, counteracting the growth of estrogen-related cancers. Moreover, other polyphenols and lycopene have been considered promising pharmacological agents in cancer prevention, as a result of their antiproliferative effects and their inhibitory action on the human estrogen receptors [42–44].

2 Health-Promoting Phytochemicals as Affected by Agronomic Management

Phytochemicals play a major role in ecological interactions between plants and the surrounding environment, being active against pathogens and viruses, in allelopathic interactions, in insect chemoattraction, and in defense mechanisms against biotic and abiotic stresses. As plant secondary metabolites, they can be constitutively expressed but can also be induced by diverse factors, including attack by fungal and bacterial pathogens and herbivores [45]. Recent findings revealed that the content and composition of phytochemicals in fresh fruit and vegetables (FAVs) is greatly affected by other variables, that is, plant genotype, harvest season, cultivation site and techniques, and soil quality, and by agronomic practices, such as quantity and quality of available nutrients and light, irrigation, use of pesticides and chemical fertilizers, and conventional/organic management [46–48]. Such results increased interest in nutraceutical and functional foods, which stimulated scientists to boost research on beneficial plant secondary metabolites and on the best genetic and agronomic approaches to increase their concentration in FAVs [49, 50].

A key reference paper provided a comprehensive database (collected as part of the USDA National Food and Nutrient Analysis Program) of the total phenolic content and antioxidant capacity in over 100 different foods, including fruits, vegetables, nuts, dried fruits, spices, and cereals [51]. Other investigations reported thorough lists of nutraceutical properties of FAVs, depending on different genetic and agronomic variables. Plant genotype was one of the main factors assessed: the antioxidative activity of 92 plant phenolic extracts was found to vary with plant species, which showed highly variable total phenolic content, calculated as gallic acid equivalents (GAE), ranging from >20 mg g^{-1} GAE in berries to <12.1 mg g^{-1} GAE in apples [11]. Two different cultivars of tomato contained 1.0 and 10 mg kg^{-1} of lycopene, whose content ranged from 10 to 100 mg kg^{-1} in the same tomato variety analyzed at two different ripening stages – turning and red – respectively [52]. Plant genotype affected the production of different metabolites, such as ascorbic acid, whose concentrations ranged from 20 to 300 mg kg^{-1} in apple, from 300 to 500 mg kg^{-1} in orange, and from 290 to 800 mg kg^{-1} in kiwi, depending on the cultivar type [53, 54] and glucosinolates in broccoli, which showed huge variability among 50 screened cultivars [55]. In fruit plants, rootstock type affected the nutritional and nutraceutical quality of peel and flesh of peach fruits, which showed the highest antioxidant capacity associated to a high level of carotenoids and phenols in the rootstocks Mr. S 2/5 and Barrier 1, compared with Ishtara and GF 677 [56, 57].

Several conventional breeding programs in different countries, boosted with the aim of obtaining cultivars with enhanced concentrations of phytochemicals, were successful: for example, improvements were registered in tomato lines with 10–25 times increased concentration in β-carotene, compared with conventional varieties [58, 59] and in new peach and plum genotypes rich in phenolic compounds and antioxidant capacities [60, 61]. Such promising and important results allow us to foresee that in the years to come, new breeding programs will lead to the selection of cultivars with enhanced concentrations in phytochemicals.

Beyond plant genotype, agronomic and environmental factors may play a major role in increasing phytochemical content of FAVs, and it is tempting to speculate on the possibility of selecting the best performing growth conditions and techniques to enhance health-promoting properties of crops.

The production of phytochemicals, such as ascorbic acid, phenolic compounds, carotenoids, and glucosinolates, as a result of plant exposure to low temperatures and high light intensity during the growth period, was much variable in quantity and composition in diverse FAVs. However, high light exposure or intensity generally produced positive effects in the concentration of ascorbate, phenolic compounds, carotenoids, and glucosinolates of FAVs, probably due either to enhanced photo-oxidative stress or to increased photosynthesis [50]. The same variable trend was shown by treatments with specific wavelength irradiation, such as red light, blue light, and UV-B on carotenoids concentration in tomato, suggesting that light influences carotenoid metabolism in a very complex way [62, 63].

Other agronomic factors, such as drought and high salinity, are still under investigation for their putative positive effects on phytochemical concentration in diverse FAVs. Interesting responses to water availability were found in grapevine and field-grown olive trees: deficit irrigation regimes increased the concentration of phenolic compounds, improving the quality of grape and virgin olive oil [64, 65]. Other findings suggested that irrigation with saline water may improve carotenoids content and antioxidant activity of tomatoes [66, 67].

It is important to highlight that the extremely high increases in some phytochemicals obtained through genetic selection, conventional breeding, and metabolic engineering – reaching up to 10–25-fold in carotenoids, 20-fold in glucosinolates, and 36-fold in kaempferol-rutoside – raised concerns on their safety [50], since some compounds, such as polyphenols, generally considered beneficial for their anticarcinogenic and antiatherogenic role [19], have proved to be genotoxic at high concentrations [68, 69]. On the contrary, the increases in phytochemical concentration produced by environmental and agronomic factors – ca. twofold – were claimed to "*represent arguably a good balance between effectiveness and safety*" [50].

Such interesting suggestion gives further support to projects aiming at continuing and extending research on the best agronomic techniques and managements to enhance the production of beneficial phytochemicals in crop plants, cereals, fruits, and vegetables in the years to come. One of the most promising agronomic factors affecting plant secondary metabolic pathway is represented by arbuscular mycorrhizal fungi, belonging to Glomeromycota, an important ecological and economical group of beneficial soil microorganisms that establish mutualistic symbioses with the roots of the vast majority of plant species.

3 Plant Beneficial Symbionts: Arbuscular Mycorrhizal Fungi

Mycorrhizal symbioses are beneficial associations between plant roots and soil-borne fungi, occurring in about 90 % of land plants and involving 240,000 plant species and 6,000 fungal species. Depending on host plants and fungal symbionts, many different mycorrhizal types have been observed in nature; nevertheless, their effects on host plants are similar: a larger growth due to a better nutritional status, a higher tolerance to biotic and abiotic stresses, and a general higher fitness, compared with non-mycorrhizal plants [70]. The most widespread type of mycorrhizal symbiosis is the arbuscular mycorrhiza, which is distributed from arctic to subantarctic regions, in temperate and tropical grassland and forests, scrub and desert ecosystems, from sand dunes to alpine sites [71]. Arbuscular mycorrhizal (AM) symbioses occur within all phyla of land plants, in most plant families, except genera and species belonging to Brassicaceae, Chenopodiaceae, and Cyperaceae, and plants which are exclusively hosts of other mycorrhizal fungi.

The most important agricultural fodder and grain crops form AM symbioses: from cereals, including rice, corn, barley, and wheat, to legumes and fruit trees including citrus, peach, grapevine, and olive and from vegetables like onion, strawberry, tomato, and potato to economically important species, such as sunflower, cassava, cotton, sugarcane, tobacco, coffee, tea, cocoa, rubber, oil palm, and banana [71]. AM fungi (AMF) show low host specificity and are obligate biotrophs, that is, they cannot be cultivated on synthetic media in the absence of the host. Such inability represents the major constraint to their large biotechnological application. Their life cycle starts with spore germination, originating a short-lived asymbiotic mycelium, which is able to recognize host roots and to differentiate infection structures, the appressoria, on the root surface. Hyphae developed from appressoria grow within the root cortex, intercellularly along the longitudinal root axis, and then penetrate within cortex cells, forming haustoria-like branched structures, the arbuscules, originating from the dichotomous branching of intracellular hyphae which progressively reduce their diameter. Arbuscules are the key structure of the mutualistic symbiosis, representing the site where nutrient exchanges between plant and fungus occur. After establishing the symbiosis, plant-derived carbon is transferred to AM symbionts, reaching up to 20 % of total photosynthate, and then transformed into trehalose and other fungal polyols [72]. Such carbon is essential for the extraradical growth of AMF, which produce large mycelial networks exploring the surrounding environment and efficiently absorbing mineral nutrients from the soil, as a result of the high surface-to-volume ratio of the hyphae [73, 74]. Eventually the fungus is able to complete its life cycle by the formation of new spores.

A large body of investigations showed that AMF have important beneficial effects on plant growth, increasing the transfer of soil mineral nutrients, such as phosphorus (P), nitrogen (N), sulfur (S), potassium (K), calcium (Ca), iron (Fe), copper (Cu), and zinc (Zn), and improving plant tolerance to root pathogens and drought [70]. Differential increases in P and N supply to host plants after inoculation of diverse AMF have been ascribed to phenotypic and functional properties of the extraradical mycorrhizal mycelium [75–79], depending also on the occurrence and differential expression of P transporter and N assimilation fungal genes [80–82].

The mechanism involved in the improved P nutrition of mycorrhizal plants is represented by the efficient soil exploration by fungal hyphae extending beyond the depletion zone caused by the fast absorption of P from the soil solution, which cannot be rapidly replenished, given the poor mobility of P in the soil [83]. Electron microscope studies and chemical analyses revealed that hyphal P translocation is operated through the accumulation of polyphosphate granules within hyphal vacuoles, which are transferred from soil-based to root-based hyphae [84–88]. P is then released to the host cells in the arbuscules by means of polyphosphatases and alkaline phosphatases [89–95]. As AMF are coenocytic organisms, that is, they do not possess cross walls, the high flow rates of protoplasm occurring within their hyphae can explain the rapid mobilization and transfer of P and the other soil nutrients.

Indeed, the inflow rates of P in extraradical hyphae may range from 2 to 20 × 10^{-6} mol m^{-2} s^{-1} [96–99], while bidirectional protoplasmic flow rate, measured on the basis of cellular particles movement (presumably vacuoles, nuclei, fat droplets, organelles, granules), ranges from 2.98 to 4.27 μm s^{-1} [100, 101]. However, it is important to underline that the most important factor affecting the transfer of nutrients from soil to root-based hyphae is represented by the flux of mineral nutrients through appressoria, which for P is 3.8 × 10^{-8} mol cm^{-2} s^{-1} [102].

A great diversity has been reported among plant species in the extent to which they depend on AMF. For example, some plant species such as *Citrus* spp. are totally dependent on mycorrhizal inoculation during the seedling stage [103], showing no response to P addition, while other species, such as grasses, show a low mycorrhizal dependency [104]. However, the different degrees of mycorrhizal dependency vary with plant species and cultivars, soil P content, root colonization, and efficiency of the inoculated fungal isolate [105].

Moreover, AMF may represent underground communication ways [106], also activating defense pathways before the pathogen attacks. For example, tomato plants colonized by the same AM symbiont showed increased expression of defense-related genes and higher levels of disease resistance enzymes in healthy "receiver" plants after inoculation of "donor" plants with a pathogen [107].

In conclusion, AMF represent fundamental key factors promoting plant growth and health by processes such as the acquisition of nutrients and water, the modulation of plant hormonal balance, the protection from pathogens, and the abiotic stress protection. Thus, AMF play a major role in organic production of food by reducing the need of chemical fertilizers and pesticides, leading not only to less environmental damage but also to improved food quality and health. Contemporary trends toward low-input, sustainable agriculture consider mycorrhizal inoculation with efficient AM fungal species a promising biofertilization strategy in order to enhance plant growth, yield, and quality.

Recently, AM fungal symbioses have been shown to modify several aspects of host plant metabolism and have been proposed as an environmentally friendly and efficient strategy to enhance plant biosynthesis of secondary metabolites with health-promoting activities.

4 Nutraceutical Value of Mycorrhizal Plants

Many evidences indicate that AM fungal symbiosis may induce changes in primary and secondary metabolism of host plants, for example, increasing the production of secondary compounds – including polyphenols – in the roots of mycorrhizal plants [108–113]. Indeed, the colonization of root cortical cells by AMF causes diverse cytological and metabolic changes: a marked proliferation of plastids, the activation of Krebs cycle, and the modification of plastid biosynthetic pathways during intracellular arbuscule development leading to increased metabolic activity and to higher production of fatty acids, apocarotenoids, and amino acids, such as tyrosine, which, together with phenylalanine, is the main precursor of plant polyphenols in the phenylpropanoid metabolism [114–117]. Interestingly, in *Trifolium repens* plants, accumulation of some flavonoids – quercetin, acacetin, and rhamnetin – was exclusively detected in roots of plants inoculated with *Glomus intraradices* [118].

Several studies investigated also the influence of AM fungal symbiosis on plant shoot metabolic activities. Some authors suggested that accumulation of secondary compounds in the shoots of mycorrhizal plants may represent the main factor conferring resistance to fungal pathogens [119–122]. For example, higher contents of phenolic compounds in mycorrhizal plants were correlated with a reduced severity of diseases caused by *Phytophthora nicotianae* and *Botrytis fabae* in tomato and *Vicia faba*, respectively [123, 124], and with a decrease of *Ralstonia solanacearum* population in tomato [125].

AM fungal inoculation, besides inducing larger plant photosynthetic rates [126, 127], may produce other important biochemical changes, for example, in the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis, correlated with apocarotenoid accumulation [128], and in physiological mechanisms leading to the accumulation of secondary metabolites, such as phenolic acids, carotenoids, and polyphenols, both in roots and in shoots [129–132]. Moreover, AMF induced also alterations in the activity of superoxide dismutase (SOD) in roots and shoots of different plant species [133–137] and of several antioxidant enzymes in roots of *Phaseolus vulgaris* [138] and in shoots of lavender, rice, and three Mediterranean shrubs [127, 139, 140], suggesting that the induction of plant protective mechanisms could alleviate oxidative damages caused by drought, salt, and other environmental stresses, leading to plant increments in shoot biomass. Accordingly, higher levels of enzymes active in the removal of reactive oxygen species (ROS) in mycorrhizal roots suggested that colonized plants may respond to oxidative stresses by the accumulation of antioxidative enzymes and carotenoids [141–144].

The inoculation of the AM fungal species *Glomus mosseae* and *Glomus versiforme* triggered transient enhancement of the levels of transcripts encoding phenylalanine ammonia-lyase in *Oryza sativa* and *Medicago truncatula* roots, respectively [141, 145], while increased chalcone synthase (CHS) transcript accumulation was found in *M. truncatula* roots colonized by *G. versiforme* [141] and *G. intraradices* [146]. Other effects of AM fungal symbiosis on secondary metabolism, such as phytohormone dynamics, were also reported [147–149].

A recent study found that mycorrhizal red clover showed impaired levels of isoflavones with estrogenic activity, such as biochanin A, formononetin, genistein, or daidzein [150], and natural plant metabolites called “phytoestrogens,” which are believed to play an important preventive role in osteoporosis, menopausal symptoms, arteriosclerosis, heart diseases, and cancer [39, 151] and are investigated as alternative to synthetic compounds for therapeutic purpose in humans [37]. In the species *Arnica montana*, which contains several groups of active secondary compounds, AM fungal inoculation induced sesquiterpene lactones accumulation both in roots and in shoots [131].

Several studies investigated AM fungal effects on the production of phytochemicals in medicinal and aromatic plants. Higher accumulation of antioxidant compounds (rosmarinic acid and caffeic acid) and essential oils was reported in shoots of *Ocimum basilicum* (sweet basil) inoculated with different *Glomus* species [130, 152, 153]. Essential oil concentration increased in fruits of *Coriandrum sativum*, *Anethum graveolens*, and *Trachyspermum ammi* (+43 %, +90 %, and +72 %, respectively, compared with controls) inoculated with *Glomus macrocarpum* and *Glomus fasciculatum* [154, 155], with differences between the two species of fungal symbionts. The concentration of essential oil showed about 62.5 % increase in *Foeniculum vulgare* seeds produced by plants inoculated with *G. fasciculatum*, compared with non-mycorrhizal controls. Moreover, chemical characterization of the essential oil extracted from seeds produced by mycorrhizal plants revealed an enrichment in anethol concentration [156].

Interestingly, essential oil accumulation may be partly due to a non-nutritional effect of AMF, since mycorrhizal *Origanum* sp. plants produced higher oil concentrations compared with those obtained from non-mycorrhizal plants showing equivalent P content [157].

Root concentration of the alkaloid forskolin, a potent cardioactive and hypotensive diterpenoid compound, was consistently higher in *Coleus forskohlii* plants inoculated with different AM fungal species, compared with non-mycorrhizal controls, with the largest increase (+147 %) when the symbiont *Glomus bagyarajii* was used [158]. Phytochemicals with therapeutic value produced by *Echinacea purpurea* (pigments, caffeic acid derivatives, alkylamides, and terpenes) increased their concentration up to 30 times after inoculation with the AM fungal species *G. intraradices* and *Gigaspora margarita* and the entomopathogenic endophyte *Beauveria bassiana* [159].

An increase in thymol derivatives production was observed in roots of *Inula ensifolia* plants inoculated with *G. intraradices* and *Glomus clarum*: the highest concentration of all the analyzed compounds was found in roots colonized by *G. clarum* [160]. Enhanced concentrations of anthraquinone derivatives (hypericin and pseudohypericin) were reported in *H. perforatum* shoots after inoculation with *G. intraradices* and with a mixed AM fungal inoculum [161].

Interestingly, a positive correlation between AM fungal root colonization of the medicinal plant *Castanospermum australe* and the content of castanospermine – an alkaloid of the indolizidine type – was found both in seeds and leaves, under field and greenhouse conditions, respectively [162].

So far a few studies investigated the relationship between plants, AM fungal colonization, and phytochemicals concentration in plants used for human nutrition. In the greenhouse, anthocyanins, carotenoids, and, to a lesser extent, phenolics occurred in higher concentration in the leaves of mycorrhizal lettuce plants than in non-mycorrhizal controls [163], while AM fungal inoculation of *Allium cepa* plants in pots significantly enhanced the total antioxidant capacity of bulb biomass [164]. In a greenhouse experiment, fruits of tomato plants, inoculated with a microbial mix of AMF and different bacteria, showed higher glucose, fructose, malate, and nitrate contents, while β-carotene, lycopene, and lutein contents increased when the substrate was amended with the microbial mix and green compost [165].

A 2-year field study and a multidisciplinary research reported higher nutraceutical value in mycorrhizal globe artichoke and tomato, plants largely cultivated for human consumption.

4.1 Case Study 1: Globe Artichoke

Globe artichoke, *Cynara cardunculus* var. *scolymus* (L.) Fiori, is an ancient perennial plant species (Asteraceae) native to the Mediterranean Basin, probably domesticated by Romans and diffused by the Arabs in the Southern Mediterranean area during the Middle Ages [166]. Globe artichoke, cultivated for its immature flower heads and for its high contents of phytochemicals, including polyphenols and inulin [167, 168], is considered a functional food, according to the definition of the European Commission on Functional Food Science in Europe (FuFoSE) [169, 170]. Artichoke leaves, rich in polyphenols, are utilized by the pharmaceutical industry for the production of commercial extracts for their choleretic, hypocholesterolemic, and antioxidant bioactivities, as a result of high contents in chlorogenic acid, cynarine, and luteolin [171–173]. Artichoke flower heads are among the richest sources of dietary phenolic antioxidants [174], whose levels depend on cultivar genetic diversity, harvest time, and climatic conditions during plant growth [175, 176].

A recent study reported, for the first time, large increases in total polyphenolic content (TPC) and antioxidant activity, expressed as antiradical power (ARP) in both leaves and flower heads of mycorrhizal globe artichoke plants assessed in microcosm and in the field [177]. Artichoke plants were inoculated in microcosm with the AM fungal species *G. mosseae* and *G. intraradices* and with a mixture of them, then transplanted and grown for 2 years in the field, where plants treated with the inoculum mixture showed large increases of main flower head fresh weight, not only in the first (92.8 %), but also in second year (70.6 %). Such mycorrhizal inoculation responses, persisting for 2 years after field transplant, stimulated further molecular studies, aimed at assessing the persistence of inoculated AMF in the field. ITS rDNA sequences clustering with those of *G. mosseae* and *G. intraradices* were retrieved only from inoculated plant roots.

Leaves of mycorrhizal artichoke grown in microcosm showed enhanced phenolic complement, depending on the inoculum composition. TPC and ARP were significantly higher (50 % and 33 %, respectively) in plants inoculated with the *Glomus* mixture, compared with control plants (Fig. 85.1). In the field, inoculated plants showed higher phenolics content in the edible parts (flower heads), compared with controls, with the highest values detected in plants inoculated with the

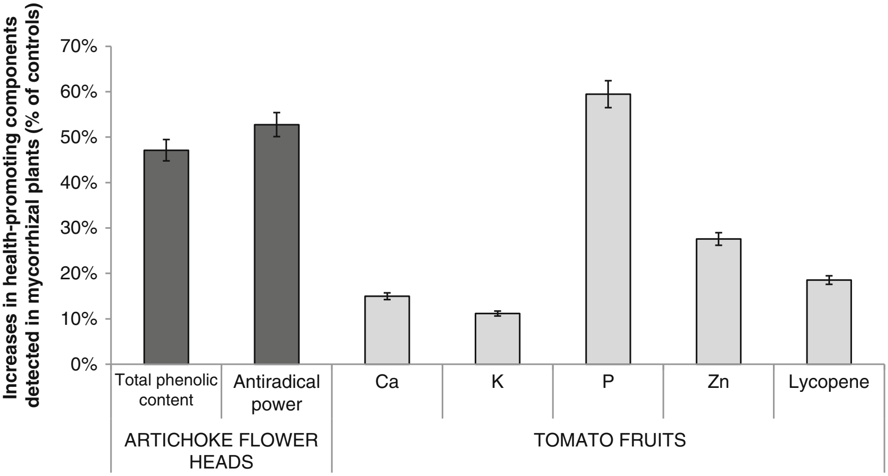

Fig. 85.1 Increases in health-promoting compounds detected in artichoke flower heads and in tomato fruits produced by mycorrhizal plants, compared with non-mycorrhizal controls. Increases are statistically significant ($P< 0.01$)

combination of the two AM fungal species. Higher TPC values were found in secondary flower heads (second year of field cultivation) compared with main flower heads (first year of field cultivation). Antioxidant activity of flower heads followed the same pattern of phenolics accumulation: ARP increases (52 % and 32 % in the first and second year in the field, respectively) were higher in plants inoculated with the mixed AM fungal inoculum. As mycorrhizal inoculation has been proved to induce changes in host plant secondary metabolism, including the phenylpropanoid biosynthesis, further studies should investigate the role of mycorrhizal symbiosis in the activity of two hydroxycinnamoyltransferases, recently isolated and functionally characterized in globe artichoke [178, 179].

4.2 Case Study 2: Tomato

Tomato (*Solanum lycopersicum* L.) is extensively cultivated worldwide, and its fruits have assumed the status of "functional foods" as a result of epidemiological evidence of reduced risks of certain types of cancers and cardiovascular diseases [180, 181]. They are a reservoir of diverse antioxidant molecules, such as lycopene, ascorbic acid, vitamin E, carotenoids, flavonoids, and phenolics, and may provide a significant part of the total intake of beneficial phytochemicals, as a result of their high consumption rates. Among carotenoids, lycopene has a strong antioxidant activity and is able to induce cell-to-cell communications and modulate hormones, immune systems, and other metabolic pathways [182].

The levels of lycopene and other beneficial phytochemicals in fresh tomato fruits may be affected by many agronomic factors, such as cultivars [58], cultural practices and ripening stage [183], and also by cultivation conditions, such as the establishment of mycorrhizal symbioses in plant roots. Recent works reported that mycorrhizal inoculation improved tomato growth and the production of fruits, which contained significantly higher quantities of ascorbic acid and total soluble solids [184].

A recent multidisciplinary work for the first time investigated the nutraceutical value and safety of mycorrhizal tomato fruits produced by mycorrhizal plants inoculated with the AM fungal species *G. intraradices*, by assessing the antioxidant, estrogenic/antiestrogenic, and genotoxic activity of tomato fruits [185]. The data obtained showed that mycorrhizal inoculation positively affected the growth and mineral nutrient content of tomato fruits, with enhanced uptake of soil mineral nutrients, that is, Ca, K, P, and Zn, whose concentrations in tomato fruits produced by inoculated plants were higher than in the controls. Interestingly, fruit P and Zn contents were 60 % and 28 % higher than those of controls, suggesting an important role of the symbiosis in improving the nutritional value of tomatoes, in particular for Zn, which is considered a key human mineral nutrient [186].

Lycopene content of tomato fruits from mycorrhizal plants was 18.5 % higher than that of controls (Fig. 85.1). Such an evidence of plant secondary metabolism modification was not linked with the production of putative unsafe compounds, such as mutagenic ones, since tomato extracts induced no in vitro genotoxic effects. Indeed, the two genotoxicity tests used, that is, the Ames *Salmonella*/microsome mutagenicity assay (the “first line” for detection of gene mutation) and the human lymphocyte MN test (a “first line” for detecting chromosome aberrations), excluded the presence of any putative phytochemical with DNA-damaging activity. Given the chemoprotective/chemopreventive effects of lycopene, it is tempting to suggest that the higher lycopene content detected in mycorrhizal tomato fruits could represent an important factor in neutralizing the DNA-damaging activity of any possible mutagenic compound occurring not only in tomato fruits but also in other foods consumed together with tomatoes.

Moreover, tomato fruit extracts – both hydrophilic and lipophilic fractions, originating from mycorrhizal plants – strongly inhibited 17-β-estradiol-human estrogen receptor binding, showing significantly higher antiestrogenic power, compared with controls. As lycopene represents a promising pharmacological agent in cancer prevention on account of its antiproliferative effects and inhibitory action on the human estrogen receptors [42, 43], the data obtained allow us to suggest that tomato fruits and those produced by mycorrhizal plants at a higher rate could antagonize the estrogen-like activity elicited by several environmental-industrial xenobiotics to which humans are exposed through the food chain [185].

5 Conclusion

Many epidemiological studies have reported that the consumption of FAVs may play a fundamental role in promoting human health by preventing chronic diseases and decreasing the risk of mortality from cancer and cardiovascular diseases. Thus, plant fresh foods are considered “functional foods,” not only because of their nutritional properties but also for their content in phytochemicals, which may vary depending on environmental and agronomic conditions, including plant mycorrhizal status. AM symbionts are ecologically and economically important beneficial fungi playing a major role in sustainable food production systems: they determine higher plant growth rates, increase resistance to biotic and abiotic stresses, and reduce the need of chemical fertilizers and pesticides, allowing a safe production of high-quality food. As they enhance the biosynthesis of many different compounds with nutraceutical value in leaves, roots, and fruits of plants used for human nutrition, the inoculation of selected AM fungal species and isolates may represent a suitable and environmentally friendly biotechnological tool to be implemented in agri-food chains aimed at producing safe and healthy food. Further studies are needed to answer questions as to whether different AM fungal species and isolates may differentially modulate plant secondary metabolism and the production of phytochemicals; whether inoculation time, technique, and protocols may change fungal performance affecting healthy compounds accumulation; and whether agronomic managements and practices may influence the persistence of AMF in the field and their efficacy in inducing favorable changes in the biosynthesis of secondary metabolites with health-promoting activity.

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Part X

Terpenes: General Biology and Biotechnology

Terpenes: Chemistry, Biological Role, and Therapeutic Applications

86

Priyanka P. Brahmkshatriya and Pathik S. Brahmkshatriya

Contents

1 Introduction 2667
2 Terpenes: General Overview 2668
3 Classifications of Terpenes 2669
4 Biosynthesis of Terpenes 2671
5 Terpenes: Therapeutic Applications 2673
5.1 Anticancer Activity 2673
5.2 Antibacterial Activity 2676
5.3 Antiviral Activity 2677
5.4 Activity Against CNS Disorders 2677
5.5 Activity Against CVS Disorders 2678
5.6 Antihyperglycemic Activity 2678
5.7 Anti-inflammatory Activity 2679
5.8 Antiparasitic Activity 2680
5.9 Antioxidant Activity 2680
5.10 Immunomodulatory Activity 2681
5.11 Skin Penetration–Enhancing Activity 2681
5.12 Miscellaneous Activity 2682
5.13 Side-Effects of Terpenes 2683
6 Conclusion 2683
References 2684

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Abstract

Terpenoids are naturally occurring hydrocarbons produced by a wide variety of plants and animals. They are classified based on five-carbon (isoprene) units as their building blocks, numbering more than 55,000 molecules having been discovered till date. Different terpenes include hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), and polyterpenes (>C30). Diverse functional roles of terpenoids have been critically studied and well-accepted now. Some of them include natural flavor additives for food or fragrances in perfumery and in traditional and alternate medicines as aromatherapy. Biosynthetically, terpenoids are formed via two major synthetic pathways: mevalonic acid (MVA) pathway and 2*C*-methyl-D-erythritol-4-phosphate (MEP) pathway. Biologically active terpenoids span various orders of magnitude. Most comprehensively studied of which is the effect of terpenes in prevention and treatment of cancer. Illustratively, Taxol derivative (paclitaxel and docetaxel) are among the widely used drugs in cancer chemotherapy. Other important therapeutic uses of terpenoids include antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory, antioxidants, antiparasitic, immunomodulatory, and as skin permeation enhancer. Since many of these molecules are only found in very low levels in nature, their massive harvesting to obtain sufficient amounts of the drug including synthetic biology and metabolic engineering provides innovative approaches to increase the production of terpenoids.

Keywords

Antibacterial • anticancer • antiparasitic • anti-inflammatory • antiviral • biosynthesis • isoprene unit

Abbreviations

COX	Cyclooxygenase
DMAPP	Dimethylallyl pyrophosphate
DXP	1-Deoxy-D-xylulose-5-phosphate
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
GPP	Geranyl pyrophosphate
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IL	Interleukin
iNOS	Inducible nitric oxide synthetase
IPP	Isopentenyl pyrophosphate
MEP	2*C*-Methyl-D-erythritol-4-phosphate
MIC	Minimum inhibitory concentration
MVA	Mevalonic acid
NF	Nuclear factor

PG	Prostaglandin
PL	Phospholipase
TNF	Tumor necrosis factor
WBC	White blood cells

1 Introduction

Secondary metabolites are small molecule organic compounds that are not essential to the growth and development of the producing organism and are not classified by structure [1]. It is estimated that well over 300,000 secondary metabolites exist, and it's thought that their primary function is to increase the likelihood of an organism's survival by attracting or repelling other organisms. Lack of secondary metabolites does not result in severe complications such as death, but generally, long-term impairment of the organism's survivability or fertility is seen. Natural products often play a key role in plant defense against herbivory and other interspecies defenses [2]. Often, plant secondary metabolites may be referred to as plant natural products, in which case they illicit effects on other organisms.

Most of the secondary metabolites of interest fit into categories which classify them based on their biosynthetic origin. Secondary metabolites are chemicals produced chiefly by plants for which no role has yet been found in photosynthesis, growth, or reproduction. Secondary metabolites are diverse; many thousands have been identified in several major classes. Each plant family, genus, and species produces a characteristic mixture of these secondary metabolites. These chemicals are also used as taxonomic characters in classifying plants. Humans use these natural products as medicines, flavorings, and recreational drugs.

Secondary metabolites can be classified on the basis of their chemical structure (e.g., cyclic and acyclic, containing a sugar moiety), composition (presence or absence of elements, e.g., nitrogen), and aqueous solubility, or based on the pathway by which they are synthesized (e.g., phenylpropanoid, which produces tannins). A simple classification includes three main groups: *terpenes* (made from mevalonic acid, composed almost entirely of carbon and hydrogen), *phenolics* (made from simple sugars, containing benzene rings, oxygen, and hydrogen), and *nitrogen-containing compounds* (extremely diverse). However, there is no strict scheme for classifying natural products: their extreme diversity in structure, function, and biosynthesis allows them to fit neatly into a few simple categories. In practice, however, scientists often speak of five main classes of natural products: terpenoids and steroids, fatty acid–derived substances and polyketides, nonribosomal polypeptides, alkaloids, and enzyme cofactors. Natural products are medicinally of great importance. Most pharmaceuticals are based on plant chemical structures, and secondary metabolites are widely used for recreation and stimulation (the alkaloids nicotine and cocaine, the terpene cannabinol). The study of such plant use is called "*ethnopharmacology*."

2 Terpenes: General Overview

The term “terpene” was given to the compounds isolated from “turpentine” (Latin *balsamum terebinthinae*), a volatile liquid isolated from pine trees. Turpentine contains the “resin acids” and some hydrocarbons, which were referred to as terpenes. The term “terpene” was originally coined to describe a mixture of isomeric plant hydrocarbons of the molecular formula $C_{10}H_{16}$ occurring in the essential oils.

Terpenoids, sometimes called as isoprenoids, are a subclass of the prenyllipids (terpenes, prenylquinones, and sterols). They represent the oldest group of small molecular products synthesized by plants and are one of the most widespread groups of natural products. Terpenoids can be described as modified terpenes, where methyl groups are replaced or removed, oxygen atoms removed or added (hydrogenated/dehydrogenated), or the carbon skeleton is modified by oxidation or rearrangement. Thus, many authors use the term “terpenes” more broadly, to include the terpenoids. Terpenes are the most numerous and structurally diverse secondary metabolites among various natural products (Fig. 86.1) [3].

Historically, terpenes are one of the most ancient natural products having diverse applications. As they are chiefly found in essential oils, they were used in the Ancient Egypt for various religious reasons. Camphor was introduced in Europe from the East by the Arabs in the eleventh century. The process of obtaining plant essential oils by fatty extraction was known by the early Middle Ages. In the twelfth century, Villanosa described distillation of oils from sage and rosemary. He made an *oleum mirabile* from oils of turpentine and rosemary. Analyses of oils of turpentine were made in 1818 by Houston, and it was in 1866 when Dumas proposed the name “terpene,” derived from turpentine, instead of camphor for crystalline oxygenated substances extracted from essential oils. In 1887, Wallach proposed that one isoprenic unit of five carbon atoms (C_5H_8) is always present in the molecule of terpenes [4]. The structures of camphor, pinene, citral, and β-carotene were established by Bredt (1893), Wagner (1894), Tiemann (1895), and Wackenrodder (1837), respectively. It was due to the discovery of chromatographic and spectroscopic techniques that the period since 1945 has seen an extensive explosion in natural product chemistry. Ruzicka (1953) discovered the isoprene unit based on the concept of the “isoprenic rule” [5] which was later completed by Lynen [6] and Bloch [7]. In 1956, mevalonic acid was shown to be a biosynthetic precursor of cholesterol [8], and later, its incorporation into a number of terpenes has been shown.

More than 55,000 terpenes have been isolated, and this number has almost doubled each decade [9, 10]. The diverse functional roles of many terpenoids have been characterized. Eucalyptus, conifer wood, balm trees, cinnamon, cloves, citrus fruits, coriander, ginger, lavender, lemongrass, lilies, carnation, caraway, peppermint species, roses, rosemary, sage, thyme, violet, and many other plants or parts of those (stems, leaves, blossoms, roots, rhizomes, fruits, seed) are well known to smell pleasantly, to taste spicy, or to exhibit specific pharmacological activities which chiefly are attributed to the presence of terpenes. Also, terpenes have shown to be useful as hormones (gibberellins), photosynthetic pigments (phytol, carotenoids),

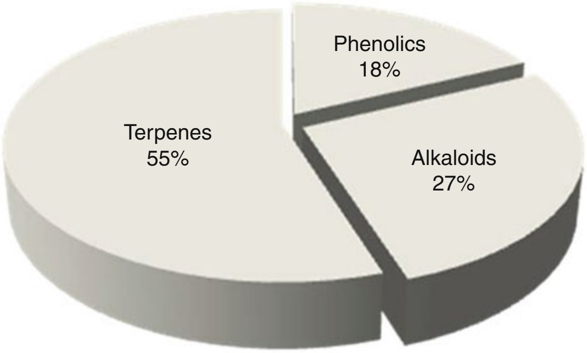
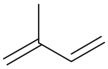


Fig. 86.1 Population of different plant secondary metabolites in nature

electron carriers (ubiquinone, plastoquinone), and mediators of polysaccharide assembly, as well as communication and defense mechanisms [11].

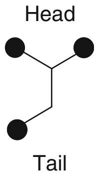
3 Classifications of Terpenes

Thermal decomposition of terpenoids gives isoprene as one of the product. As mentioned previously, Wallach pointed out that terpenoids can be built up of isoprene unit. *Isoprene rule (C5 rule)* states that the terpenoid molecules are constructed from two or more isoprene units.



Isoprene unit

Further, Ingold suggested that isoprene units are joined in the terpenoid via “head-to-tail” fashion. *Special isoprene rule* states that the terpenoid molecules are constructed of two or more isoprene units joined in a “head-to-tail” fashion.



However, exceptions exist to this rule. For example, carotenoids are joined tail to tail at their central, and there are also some terpenoids whose carbon content is not a multiple of 5.

Most natural terpenoid hydrocarbon have the general formula (C_5H_8)n. They can be classified on the basis of value of n or number of carbon atoms present in the structure. As with any class of compounds, not all terpenes contain even numbers of

Table 86.1 Classification of terpenes

Terpenes	Isoprene units	Carbon atoms
Hemiterpenes	1	5
Monoterpenes	2	10
Sesquiterpenes	3	15
Diterpenes	4	20
Sesterpenes	5	25
Triterpenes	6	30
Carotenoids	8	40
Rubber	>100	>500

intact isoprene units, with a few being degraded isoprene moieties with missing carbons such as C_{19} diterpenoids. The simplest of all is hemiterpene, consisting of a single five-carbon isoprene unit (C_5H_8). Their biological significance is not much established.

Mono-, sesqui-, di-, and sesterpenes contain the isoprene units linked in a head-to-tail fashion. The triterpenes and carotenoids (tetraterpenes) contain two C_{15} and C_{20} units, respectively, linked head to head. Many terpenes are hydrocarbons, but oxygen-containing compounds such as alcohols, aldehydes, or ketones are also found to fall in the class of terpenes. These derivatives are more frequently named as terpenoids.

A rational classification of the terpenes has been established based upon the number of isoprene (or isopentane) units incorporated in the basic molecular skeleton (Table 86.1). Some illustrative examples are depicted in Fig. 86.2.

Monoterpenoids are composed of two isoprene units ($C_{10}H_{16}$) that can be found in various states of oxidation in acyclic, monocyclic, and bicyclic forms. Monoterpenoids can be further classified based on their cyclic carbon systems, with a number of them being pharmacologically active. Sesquiterpenoids compounds ($C_{15}H_{24}$) contain three isoprene units, occurring in simple to complex mono- and polycyclic rings. Triterpenoids are C_{30} compounds ($C_{30}H_{48}$) which have wide distribution from more than 40 different carbon skeletons. Carotenes are examples of tetraterpenoids ($C_{40}H_{64}$). Natural rubber is an example of polyterpenoid which contains many cis-isoprene units.

Mono- and sesquiterpenes are the main constituents of the essential oils, while the other terpenes are constituents of balsams, resins, waxes, and rubber. Oleoresin is a roughly equal mixture of turpentine (85 % C_{10}-monoterpenes and 15 % C_{15}- sesquiterpenes) and rosin (C_{20}-diterpene) that acts in many conifer species as a toxic material to invading insects and their pathogenic fungi [12]. A number of angiosperm species have inducible terpenoid defensive compounds (phytoalexins) [13]. These include both sesquiterpenoid and diterpenoid types. Isoprenoid units are also found within the framework of other natural molecules. Thus, indole alkaloids, several quinones (vitamin K), alcohols (vitamin E, vitamin A formed from β-carotene), phenols, and isoprenoid alcohols (also known as terpenols or polyprenols) also contain terpenoid fragments. The biogenesis,

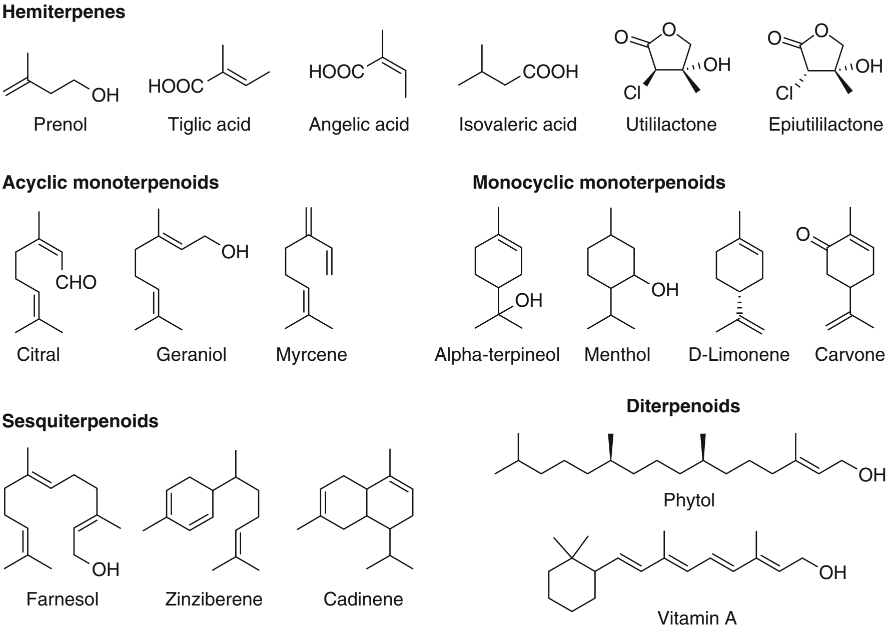

Hemiterpenes
OH
Prenol
HOOC
Tiglic acid
HOOC
Angelic acid
COOH
Isovaleric acid
O
O
OH
Cl
Utililactone
O
O
OH
Cl
Epiutililactone
Acyclic monoterpenoids
CHO
Citral
OH
Geraniol
Myrcene
Monocyclic monoterpenoids
OH
Alpha-terpineol
OH
Menthol
D-Limonene
O
Carvone
Sesquiterpenoids
OH
Farnesol
Zinziberene
Cadinene
Diterpenoids
OH
Phytol
OH
Vitamin A

Fig. 86.2 Chemical structures of some representative terpenes

molecular regulation, and function of plant terpenoids have been the subject of a review [14]. According to literature, there are more than 1,000 monoterpenes, 7,000 sesquiterpenes, and 3,000 diterpenes, and the number is still increasing [15].

4 Biosynthesis of Terpenes

Terpenes are found in each living organism which is derived from the branched C5 carbon skeleton of isoprene. Each isoprenoid is constructed using a different number of isoprene unit repeats, cyclizations, rearrangements, and further oxidation of the carbon skeletons [16, 17]. Biosynthetically, the majority of terpenoids are formed by the well-known mevalonic acid (MVA) pathway (Fig. 86.3), but they may also be formed through triose phosphate utilizing nonmevalonate pathway [18, 19]. The nonmevalonate pathway is also called the 2*C*-methyl-D-erythritol-4-phosphate (MEP) or the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway. In all eukaryotes (all mammals, the cytosol and mitochondria of plants, and fungi), terpenes are synthesized by the mevalonate pathway [20]. The nonmevalonate pathway is present mainly in eubacteria and cyanobacteria, green algae, apicomplexan parasites, and in higher plants [18]. Details on the progress of the elucidation of the biosynthetic pathways have been the subject of many reviews [21–24].

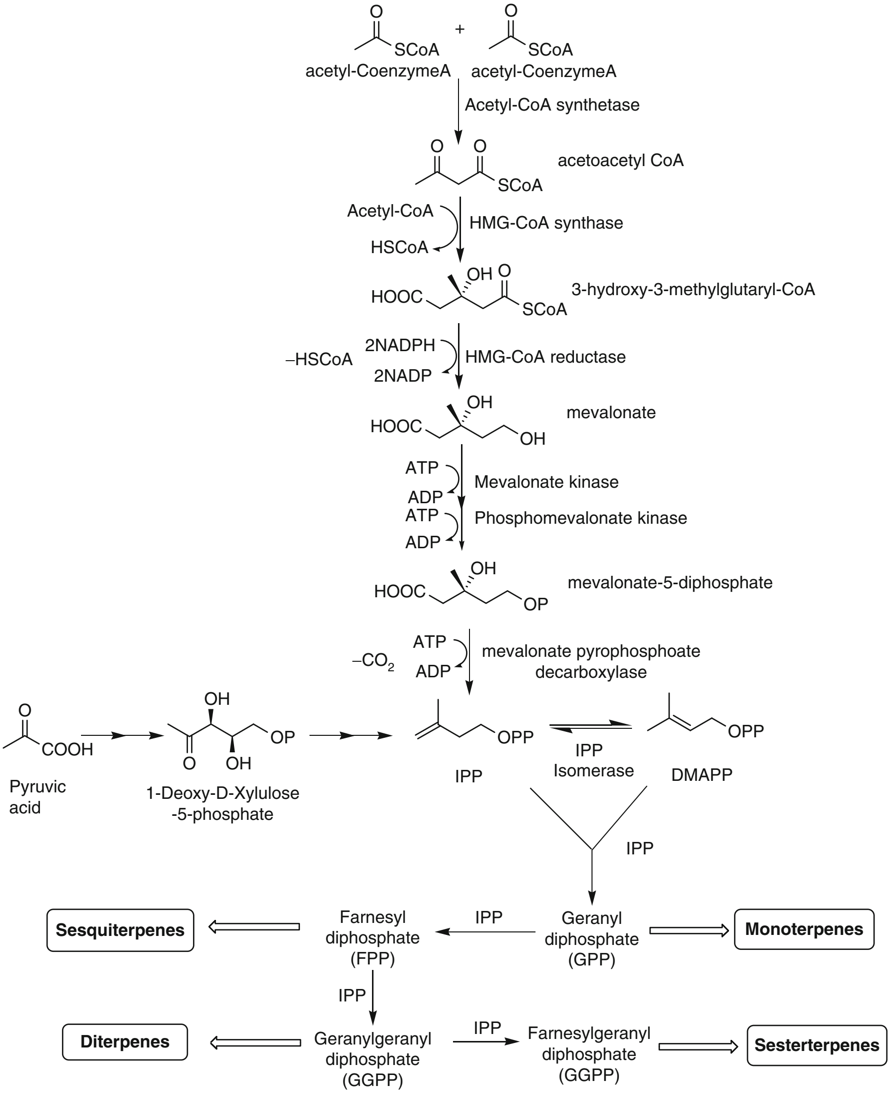

SCoA
acetyl-CoenzymeA
acetyl-CoenzymeA
Acetyl-CoA synthetase
acetoacetyl CoA
Acetyl-CoA
HSCoA
HMG-CoA synthase
HOOC
3-hydroxy-3-methylglutaryl-CoA
–HSCoA
2NADPH
2NADP
HMG-CoA reductase
mevalonate
ATP
ADP
Mevalonate kinase
Phosphomevalonate kinase
mevalonate-5-diphosphate
–CO2
mevalonate pyrophosphoate decarboxylase
Pyruvic acid
1-Deoxy-D-Xylulose -5-phosphate
IPP
IPP Isomerase
DMAPP
Sesquiterpenes
Farnesyl diphosphate (FPP)
Geranyl diphosphate (GPP)
Monoterpenes
Diterpenes
Geranylgeranyl diphosphate (GGPP)
Farnesylgeranyl diphosphate (GGPP)
Sesterterpenes

Fig. 86.3 Illustrative biosynthetic pathway of terpenes in eukaryotes (mevalonate pathway)

Although, terpenoids show enormous chemical and structural diversity, their backbones are synthesized from only two universal precursors: isopentenyl pyrophosphate (IPP) and its highly electrophilic allylic isomer dimethylallyl pyrophosphate (DMAPP) [25]. IPP is isomerized to DMAPP by the enzyme isopentenyl pyrophosphate isomerase. The mevalonate pathway for the biosynthesis of terpenoids has been illustrated in Fig. 86.3. To summarize, the active isoprene unit (IPP) is repetitively added to DMAPP or a prenyl diphosphate in sequential head-to-tail condensations catalyzed by the prenyl transferases. In the biosynthesis of mono- and higher terpenoids, the starting molecule is DMAPP, which condenses with an IPP unit to for geranyl pyrophosphate (GPP), which leads to synthesis of monoterpenoids. Sequential condensation of IPP with GPP leads to formation of farnesyl pyrophosphate (FPP) which is the precursor of sesquiterpenoids. Condensation of IPP with FPP forms geranylgeranyl pyrophosphate (GGPP), the precursor of diterpenoids. GGPP and IPP condense to form squalene, which is the precursor of triterpenes, lipids, and steroids. Finally, condensation of appropriate intermediate precursor and/or IPP units leads to synthesis of tetra- and polyterpenoids. Terpenoid synthases or cyclases are the group of enzymes which catalyze the reactions where the primary terpene skeletons are formed from the earlier substrates. The parent skeletal type of mono-, sesqui-, and diterpenes is usually further modified by the activity of an array of different catalyzing enzymes (e.g., hydroxylases, dehydrogenases, reductases and glycosyl, methyl and acyl transferases) which together generate thousands of different terpenoid structures [26].

5 Terpenes: Therapeutic Applications

Of the approximately 55,000 terpene structures known, very few have been investigated from the perspective therapeutic applications. For much of the last century, terpenes were depicted in as products of detoxification or overflow metabolism. However, starting in the late 1960s, various terpenes were shown to be toxins, repellents, or attractants to other organisms, which led to the belief that they have a potential role as prevention and cure of various ailments [11]. Thus, it has been proven time and again that aside from their immense commercial value, terpene products have important biological functions in plants. In the following section, we have described most of the important pharmacological actions of plant terpenes and their plausible role in diverse therapeutic categories.

5.1 Anticancer Activity

Spectrum of activity of terpenoids as anticancer agents is doubtlessly the most explored area since last few decades. Many studies have shown that many dietary monoterpenes are helpful in the prevention and therapy of different cancers [27–30]. Some representative terpenes which possess anticancer activity are shown in Fig. 86.4.

Epidemiological studies demonstrate that dietary monoterpenes may be effective in the prevention and treatment of cancer [31]. Monocyclic monoterpenes D-limonene (Fig. 86.2) and perillyl alcohol (Fig. 86.4) are shown to inhibit the development of liver, skin, mammary, colon, lung, prostate, forestomach, and pancreatic carcinomas in a dose-dependent manner [29, 31–35]. Moreover, the metabolites of D-limonene such as perillic acid, dihydroperillic acid,

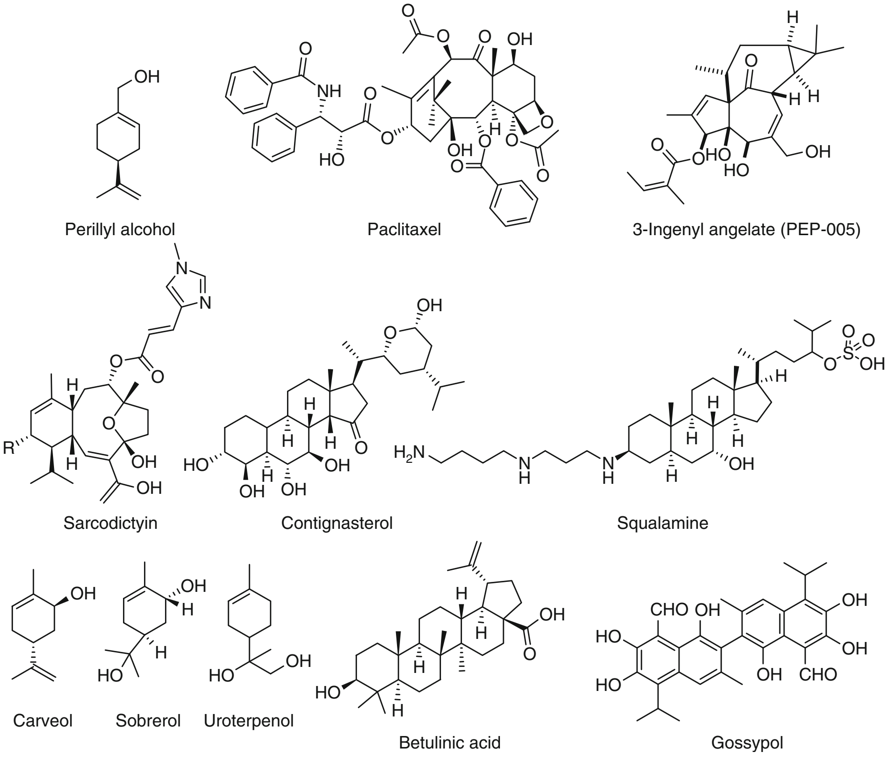

Perillyl alcohol
Paclitaxel
3-Ingenyl angelate (PEP-005)
Sarcodictyin
Contignasterol
Squalamine
Carveol
Sobrerol
Uroterpenol
Betulinic acid
Gossypol

Fig. 86.4 Chemical structures of anticancer terpenes

limonene-1,2-diol, and the oxygenated molecule of D-limonene, carvone (Fig. 86.2), have also been shown to have potent antitumor activities [28, 32–37].

Paclitaxel (Fig. 86.4), originally known as Taxol, a complex diterpenoid from the bark of specific yew tree, is a potent and one of the most widely used anticancer agents. Paclitaxel has shown excellent activity against breast and ovarian cancers [38], as well as AIDS-related Kaposi's sarcoma [39]. Docetaxel, also used clinically, is a semisynthetic analogue of paclitaxel. Taxanes enhance stability of microtubules, preventing the separation of chromosomes during anaphase [40]. 3-Ingenyl angelate (referred to as PEP005, Fig. 86.4), a hydrophobic diterpene ester isolated from the plant *Euphorbia peplus*, is another anticancer agent used principally for the treatment of skin cancer. 3-Ingenyl angelate is a modest inhibitor of protein kinase C family and causes rapid mitochondrial disruption and cell death by primary necrosis, resulting in a favorable cosmetic gloss of the skin [41]. Efficacy and toxicity of PEP005 has been the subject of an interesting review [42]. Sarcodictyin (sarcodictyin A, R = CH_3; sarcodictyin B, R = C_2H_5; Fig. 86.4) and contignasterol (Fig. 86.4) derivatives belong to the class of marine terpenoids and are demonstrated to be active against cancer in preclinical or clinical studies [43]. Squalamine (Fig. 86.4) is another terpenoid currently evaluated in phase II trials for the treatment of lung and ovarian cancer [44, 45].

Farnesol and geraniol (Fig. 86.2) have also been demonstrated to have anticancer activities against pancreatic cancers [46, 47]. Monoterpenes such as carveol, uroterpenol, and sobrerol (Fig. 86.4) have shown activity against human mammary carcinomas. Carvone (Fig. 86.2) has also been shown to reduce pulmonary adenoma and forestomach tumor formation [48, 49]. In an in vitro antitumor study, betulinic acid (Fig. 86.4) has been shown to induce apoptosis of several human tumor cells, whereas ursolic acid and oleanolic acid reduced leukemia cell growth and inhibited the proliferation of several transplantable tumors in animals [50]. In a recent study, a new class of monoterpenes designated as miliusanes was isolated from *Miliusa sinensis*. A number of the miliusanes such a smiliusol, miliusate, and miliusane-1 demonstrated potent cytotoxic activity against a panel of cancer cell lines [51]. In animal models and at cellular level, a number of dietary monoterpenes prevent the initiation and progression of cancer and along with regression of existing malignant tumors. Monoterpenes administered as a pure chemical or in orange peel oil inhibited the development of chemically induced rodent mammary, kidney, skin, lung, and forestomach cancers in different animal models [31, 33, 52–56]. Many sesquiterpene lactones have also been reported as potential anticancer agents [57]. Retinoids, which belong to the class of dipterenoids, have been shown to play an important role in regulating the growth and differentiation of different cell types, viz., normal, premalignant, and malignant [58]. In animal models, a significant suppression of development of different tumor types (skin, breast, oral cavity, lung, prostate, bladder, liver, and pancreas) was observed [59, 60]. Certain retinoids have also shown efficacy in inhibiting the development of primary cancers of the skin, cell lung cancer, and breast cancer [61, 62]. Several saponins have been reported as potent cytotoxic agents [63–65]. The biologically active constituents in *Eleutherococcus senticosis* are a complex mixture of triterpene saponins known as ginsenosides. In a cell-based assay, anti-proliferative effect of ginsenosides on human prostate cancer LNCaP cell lines has been reported [66]. Epidemiological and intervention studies indicate that carotenoids are potential agents for the chemical prevention of carcinogenesis [67, 68]. It is believed that the effects of carotenoids on colon and prostate cancers are mainly attributed to lutein and lycopene, respectively [69, 70]. Halomin, a halogenated acyclic monoterpene derivative isolated from the red alga *Portieria hornemnnii* is an effective anticancer agent against renal, brain, colon, and non–small cell lung cancer cell lines [71].

Gossypol (Fig. 86.4) is a sesquiterpene dimer found in cotton. Gossypol occurs as a mixture of two enantiomers because of restricted rotation around the central binaphthyl bond. The ratio of (+)- to (−)-gossypol varies widely among cotton cultivars, and each enantiomer has different biological activities. (−)-Gossypol inhibits the growth of cancer cells more than the (+) enantiomer [72] and inhibits male fertility in humans [73]. In China, gossypol has been used to treat infertility in males, and studies have shown an increase in sperm production.

A recent review describes the role of terpenoids present in cruciferous vegetables in the prevention and inhibition of cancers of different types. The review also illustrates different mechanisms by which these terpenoids act as anticancer agents [74]. Two new pentacyclic triterpene esters, uncarinic acids A and B, were discovered by cytotoxic activity of *Uncaria rhynchophylla* which was determined by bioactivity-guided fractionations. These compounds showed inhibition of phospholipase Cγ1 (PLCγ1) (IC_{50} = 35.66 and 44.55 μM, respectively), an enzyme which induces proliferation in human cancer cells [75]. Thus, terpenes represent a promising new class of agents which may be approved as supplementary drugs in contemporary oncology. Chemical and structural diversity in some anticancer terpenoids is exemplified in Fig. 86.4.

5.2 Antibacterial Activity

Squalamine, apart from its known anticancer activity [45], has also been shown to be a potent antibacterial, antifungal, and antiprotozoic agent [44]. Various diterpenes extracted from *Salvia* species exhibited antibacterial activities against a variety of organisms such as *E. faecalis, B. subtilis, S. aureus, S. epidermis, E. coli*,and *P. mirabilis* [76]. Mixtures of different monoterpenes like terpinen-4-ol, *R*-terpineol, 1,8-cineole, and linalool have been shown to possess antibacterial activity against Gram-positive and Gram-negative bacteria isolated from the oral cavity, skin, and respiratory tract [77]. Various studies have reported antibacterial activity of the monoterpene (+)-menthol including those against *S. aureus* and *E. coli* [78, 79]. Diterpenoid ferruginol, a few sesquiterpenes, and their lactone derivatives have also shown potent antimycobacterial activity [80]. Terpenes also display antifungal activity. One illustrative example is the optical isomers of carvone, which are found to be active toward many pathogenic fungi [37]. Cinnamon oil has shown broad-spectrum activity against *Pseudomonas aeruginosa* [81]. John et al. found plant oils from *Neolitsea foliosa*, which also showed some antibacterial properties and included sesquiterpenes such as β-caryophyllene but lacked monoterpenes [82]. Sesquiterpene drimanes have shown potent antibacterial and antifungal activity [83, 84]. The acyclic monoterpenes citronellol, geraniol, and nerol have also been shown to be active against *M. tuberculosis* [85]. Sesquiterpene lactones of the germacranolide, guaianolide, and eudesmanolide types have been shown to be active against *M. tuberculosis* with minimum inhibitory concentration (MIC) values as low as 2 μg/mL [86]. Axisonitrile-3, a marine sesquiterpene isolated from the sponge *Acanthella klethra*, demonstrated potent antimycobacterial activity with MIC of 1.56 μg/mL (50). Puuphenone, a sesquiterpene derivative isolated also from a marine sponge, also showed remarkable 99 % inhibition of *M. tuberculosis* (H3Rv) growth with MIC value of 12.6 μg/mL [87]. (E)-phytol, a diterpene isolated from *Lucas volkensii*, exhibited significant antituberculosis activity with MIC of 2 μg/mL [85]. Carvone and perillaldehyde inhibited the transformation of *Candida albicans* from the coccal to the filamentous form, which is responsible for the pathogenicity of the fungus [37].

5.3 Antiviral Activity

The unusual kaurane-type diterpene neotripterifordin was isolated by Lee and coworkers from the roots of *Tripterygium wilfordii* [88]. This natural product was found to be a potent inhibitor of human immunodeficiency virus (HIV) replication in H9 human lymphocyte cells with an EC50 of 25 nM. Glycyrrhizin, a well-known triterpenoid glycoside isolated from *Glycyrrhiza glabra*, was tested in vitro against RNA viruses like the chandripura virus, measles virus, polio vaccine viruses, polio wild-type viruses, and DNA viruses like the herpes type 1 and 2 viruses. Glycyrrhizin inhibited the DNA virus plaque formation at lower concentrations (0.608 mM), while the RNA viruses were inhibited at higher concentrations (1.216 mM) [89]. Betulinic acid, a triterpenoid isolated from *Syzygium claviflorum*, has been found to contain anti–HIV activity in lymphocytes [90]. Avarol and avarone, a marine sesquiterpenoids isolated from the red sea sponge *Dysidea cinerea*, showed inhibition of HIV reverse transcriptase with respect to its natural substrate (dNTP) [91]. Ilimaquinone, another sesquiterpene derivative isolated from the red sea sponge *Smenospongia* sp., also inhibited HIV reverse transcriptase [92]. Isoborneol demonstrated potent anti–herpes simplex virus-1 (anti-HSV-1) activity by inhibition of virus replication and the glycosylation of viral proteins. As a result, HSV-1 loses its infectivity [36]. Monoterpene derivatives cineol and borneol have also been shown to be potent anti-HSV-1 agents. It has been shown that putranjivain A, isolated from *Euphorbia jolkini* (*Euphorbiaceae*), may affect late stages of HSV-2 replication by inhibiting viral attachment and cell penetration [93]. The terpenoid constituents of *Ganoderma pfeifferi* oil lucialdehyde D and ganoderon A and C were also shown to be potent inhibitors of HSV [94]. Spongiadiol, a tetracyclic furanoditerpene isolated from *Spongia* sp., showed modest inhibition of inhibit HSV1 [95]. Solenolide A, a diterpene lactone isolated from *Solenopodium* sp., also inhibited of rhinovirus ($IC_{50} = 0.39$ μg/mL) along with inhibition of poliovirus III and herpes virus [96].

5.4 Activity Against CNS Disorders

Gb 761, a standardized extract of the leaves of *Ginkgo biloba*, contains 5–7 % ginkgolides and bilobalide (BB) collectively called terpene trilactones [97]. Among various effects shown by EGb 761 in the central nervous system, those related to AD [98], dementia, and memory [99–101] have received most of the attention. EGb 761 is among the most prescribed medications in Germany and France for treatment of dementia [99]. In one of several studies, the water-ethanol extract from roots of *Rhaponticum carthamoides* (which contain several sesquiterpene lactones) injected as a saline solution at 500 mg/kg doses increased the locomotor activity, along with improvement in the learning and memory indices in rats [102]. Also, the root ethanol extract showed a significant improvement in learning and memory ability. Using the maze-training method of active avoidance with punitive reinforcement in experiments in rats, it eliminated the scopolamine-induced memory impairment completely [103]. One study suggests that aqueous extract of *Cecropia glazioui*, which chiefly contains terpenes and flavonoids as its active constituents, promotes an anxiolytic-like effect in mice [104].

5.5 Activity Against CVS Disorders

Petkov et al. demonstrated that the dried residue of ethanol (40%) extract of *Rhaponticum carthamoides* in chloralose-anesthetized male cats at a dose of 200 mg/kg exerted a significant decrease in blood pressure (25–30%) as compared to control group of animals [102]. In a study by Hall et al., some naturally occurring pseudoguaianolides and germacranolides as well as synthetic related compounds were evaluated for their antihyperlipidemic potential where the compounds at a dosage of 20 mg/kg/day resulted in lowering of serum cholesterol by ~30% and of serum triglycerides by ~25% [105]. A recent study reported the first evidence that ginkgolide B protects against cerebral ischemic injury by inhibiting excitotoxicity by modulating the imbalance of excitatory amino acids versus inhibitory amino acids, which may support the traditional use of *Ginkgo biloba* leaves for the treatment of stroke [106]. In a study, administration of ethanolic extract of seeds of *Eugenia jambolana* (which contains a mixture of terpenoids and flavonoids) to alloxan-induced diabetic rats caused a significant hypolipidemic effect with positive alternation in all lipid levels and a decrease in the activity of HMG-CoA reductase [107].

5.6 Antihyperglycemic Activity

Stevioside, a diterpene steviol glycoside extracted from leaves of the plant *Stevia* rebaudiana, has been shown to possess insulinotropic, glucagonostatic, and antihyperglycemic effects. Stevioside and the aglucon steviol have also been shown to potentiate insulin secretion from isolated mouse islets in a dose- and glucose-dependent way [108]. Rebaudioside A, another diterpene glycoside found in *Stevia* leaves, possesses insulinotropic effects, but unlike stevioside, it does not stimulate insulin release at near-normal glucose levels [109]. The four new and four known sesquiterpenoid derivatives were isolated from the roots of *Ferula mongolica* as α-glucosidase inhibitors at the IC_{50} range of 10–100 μM [110]. Centellasapogenol A (an olean-13-ene-type triterpene) and its oligoglycoside, centellasaponin A, were isolated from the methanolic extract of *Centella asiatica* as rat lens aldose reductase inhibitors where methanolic extract of the plant inhibited aldose reductase with an IC_{50} value of 0.80 μg/mL [111–113]. Corosolic acid, isolated from the leaves of *Lagerstroemia speciosa*, displayed a significant glucose transport-stimulating activity at a concentration of 1 μM [114].

5.7 Anti-inflammatory Activity

There is a pool of terpenoids which have demonstrated good anti-inflammatory pharmacological profile [115]. Monoterpenes like linalyl acetate, 1,8-cineole, (−)-linalool, and its esters have shown good anti-inflammatory activity [116, 117]. In particular, 1,8-cineole was found to be useful in bronchitis, sinusitis and steroid-dependent asthma, or in the prevention of returning respiratory infections [118]. Several seco-pseudopterosins and newer pseudopterosins (diterpene glycosides originally isolated from *Pseudopterogorgia elisabethae* [119]) have been demonstrated to possess anti-inflammatory activity and are used for promoting wound healing [115, 120]. Methopterosin (OAS100), a semisynthetic analog of pseudopterosin, also has been reported as potent anti-inflammatory agent [121]. Several plant-derived triterpenoids such as lupane, oleane, ursane, and their natural and synthetic derivatives have also shown good anti-inflammatory profile [122]. *R*-amarin (a pentacyclic terpene) is another example of clinically proven anti-inflammatory terpenoid [123]. Studies have shown that the methanol extract of *Nelumbo nucifera* rhizome as well as its constituent betulinic acid (a steroidal triterpenoid) possessed significant anti-inflammatory activity which was found to be comparable to that of phenylbutazone and dexamethasone [124]. Manolide is the first representative class of marine sesterterpene phospholipase-2 (PLA2) inhibitors possessing potent anti-inflammatory and analgesic activities. However, it was discontinued from phase I trials from further development [121]. Scalaranes, sesterterpenes isolated from *Cacospongia mollior*, are other illustrative examples of PLA2 inhibitors [125, 126]. The sesquiterpene-monoterpene lactone artemisolide, isolated from *Artemisia asiatica*, has been shown to be a potent anti-inflammatory agent acting through inhibition of nuclear factor (NF)-κB. In an in vitro study, artemisolide decreased lipopolysaccharide-induced production of PGE2 and NO in macrophages [127]. Similarly, these and other pro-inflammatory substances (PGE2, TNF-α, and IL1β) were also suppressed by terpinen-4-ol, the main component of the oil of *Melaleuca alternifolia*, and cineole derivatives [128, 129]. Cucurbitacin R has been shown to reduce inducible nitric oxide synthetase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF)-α, and PGE2 in paw homogenates of rats with adjuvant-induced arthritis [130]. Naturally occurring diterpene pepluanone, isolated from *Euphorbia peplus*, has also been shown to reduce NO, PGE2, and TNF-α production by inhibiting COX-2 and iNOS activity and downregulating NF-κB [131]. A recent study showed that *Ganoderma applanatum* terpenes significantly inhibited inflammation by pressing the expression of interleukin (IL)-1β and COX-2 and inhibiting NF-κB translocation in the liver of BaP-treated mice which indicates applicability of these terpenes in renewing the activities of antioxidant enzymes and suppressing inflammatory response [132]. In a phytochemical study of *Cryptomeria japonica* wood, hexane extract purified by bioactivity-guided fractionation exhibited significant activities on inhibition of nitric oxide production and inducible nitric oxide synthase expression. It also showed upregulation of heme oxygenase-1 expression in lipopolysaccharide-stimulated macrophages along with inhibition of cyclooxygenase-2 enzymatic activity ($IC_{50} = 5$ μg/mL) [133].

5.8 Antiparasitic Activity

Many terpenoids have been shown to act as antiparasitic agents in the literature with high efficacy and selectivity [134, 135]. The block-buster drug artemisinin, which is the most widely used parasitic drug in the world, is a sesquiterpene lactone derivative. Biological source of artemisinin is *Artemisia annua*, an herb, which is native to China [136]. It has been used for malaria therapy in China for over 1,000 years for antimalarial chemotherapy. Many other sesquiterpene peroxides, such as yingzhaosu A and yingzhaosu C, also have shown antiparasitic activity, especially against *Plasmodium berghei* [137]. Diterpenes and their lactones, e.g., dehydroabietinol isolated from *Hyptis suaveolens*, demonstrated potent antimalarial activity [138]. Moreover, diterpenes with a nor-abietane skeleton were found to possess leishmanicidal and antiplasmodial action [139]. Espintanol and piquerol A (which belong to the class of monoterpenes) have been reported as antiprotozoal agents. Similarly, monoterpene phenol derivative of cymene, thymol, and their structural derivatives also have been found to have an antileishmanial activity [140]. Menthol derivatives have also been described to possess trypanocidal activity [141]. The well-known steroidal triterpenoic betulinic acid has also been described to have antimalarial activity [142]. Axisonitrile-3, an unusual and irregular terpene with an isonitrile group, isolated from marine sponge *A. klethra*, was found to have antiplasmodial activity [143]. Diisocyanoadociane is an example of another marine sponge diterpene, which has similar antiplasmodial activity to that of axisonitrile-3. Diisocyanoadociane, a tetracyclic diterpene, isolated from the sponge *Cymbastela hooperi*, demonstrated potent in vitro antimalarial activity against *P. falciparum* [144]. Pyrethrins, a group of six closely related monoterpene esters isolated from pyrethrum flowers, have been shown to act as antiparasitic agents against skin parasites such as head lice by blocking sodium channel repolarization of the arthropod neuron, leading to paralysis and death of the parasite [86]. Halorosellinic acid, a sesterterpene derivative isolated from the marine fungus *Halorosellinia oceanica*, has been shown to be a potent antimalarial agent ($IC_{50} = 13$ μg/mL) [145].

5.9 Antioxidant Activity

Many diterpenes are shown to act as primary antioxidants. Inside the body, some diterpenoids act as primary or synergistic antioxidants [146]. Carotenoids are well known as antioxidant agents and act along two different main pathways: physical and chemical radical quenching [147, 148]. Ertas et al. evaluated the antioxidant activity of petroleum ether, methanolic and acetone extracts, and some isolated ent-kaurane diterpenoids from the aerial parts of *Sideritis arguta* by β-carotene bleaching, free-radical scavenging, and superoxide-anion scavenging activity methods [149]. While both methanol and acetone extracts exhibited similar antioxidant activity in all assays, the active diterpenoid, 7-epi-candicandiol, inhibited lipid peroxidation at IC_{50} of 43.1 μg/mL. The extracts of various parts of *Piper umbellatum* (which contains several mono- and sesquiterpenoids) and several isolated compounds have shown interesting antioxidant activities [150].

5.10 Immunomodulatory Activity

There are also large group of monoterpenes, which possess immunomodulatory properties. It was shown that monoterpenes from *Plantago* species increased the activity of human lymphocyte proliferation and secretion of interferon gamma [151]. Carvone, limonene, and perillic acid were found to increase the total white blood cells (WBC) count in mice [49]. Administration of terpenoids to mice increased the total antibody production, antibody producing cells in spleen, bone marrow cellularity, and alpha-esterase positive cells significantly compared to the normal animals. There has been substantial interest in carotenoids as immunomodulatory agents [152–154]. β-Carotene was able to enhance the cell-mediated immune responses, especially in the elderly. In particular, supplementation leads to enhanced activity of natural killer cells and antigen-presenting monocytes. Especially in patients with HIV infection, the plasma status of β-carotene is low, thus increasing the free-radical-induced peroxidation in these patients. The immunomodulatory effect of carotenoids extracted from carrot was also assessed in rats by the analysis of immune parameters in the blood [155]. Significant increases in monocyte neutrophils, percentage lymphocytes, and platelet count were detected in the group that received carotenoid complex in comparison with the control group, which suggests that carotenoids have clear immunomodulatory effects.

5.11 Skin Penetration–Enhancing Activity

In addition to the above-mentioned therapeutic roles, terpenoids are also useful as skin penetration–enhancing agents (for improving transdermal drug delivery). The activity of terpenes as transdermal enhancers is due to a reversible disturbance of the lipid arrangement in the intercellular region of the stratum corneum, which is the thin, outermost layer of the skin, which generally limits the amount of different agents [156]. Reports suggest that terpenes may increase the permeability of the stratum corneum through intercellular lipid disruption, interaction with proteins, or improved access of agents into the stratum corneum [157]. Thus, such enhancement by terpenes facilitates the diffusion of drugs through the skin, thereby increasing their therapeutic value. Apart from these, advantages of terpenes remain that along with their good penetration-enhancing abilities, they have low skin irritation effects and low systemic toxicity [80]. The best penetration improvement activity has been accounted for monoterpenoid cyclic ether 1,8-cineol, as compared to hydrocarbon or even alcohol or ketone functionalized terpenes [158]. Hydrocarbon terpenes like D-limonene have already been approved as an active enhancer for drugs like steroids. Terpenes have also showed their effectiveness to enhance skin permeability for both the lipophilic molecules like indomethacin and for hydrophilic diazepam and propranolol [159]. Moreover, linalool, carvone, and thymol have been shown to enhance the permeability of model drugs such as 5-fluorouracil through skin and mucous membranes. Nerolidol (a hydrophilic sesquiterpene) and 1,8-cineole (monoterpenoid cyclic ether) improved permeability 20-fold and 95-fold over D-limonene, respectively [160]. A study on the effect of four different terpenes (fenchone, thymol, D-limonene, and nerolidol) on the increase of percutaneous permeation of different drugs (nicardipine, hydrocortisone, carbamazepine, and tamoxifen) was reported. Results showed significant increase in the flux of evaluated model drugs. The flux of nicardipine hydrochloride increased by approximately 135-fold, hydrocortisone by 33-fold, carbamazepine 8-fold, and tamoxifen 2-fold [161].

5.12 Miscellaneous Activity

Since centuries, terpenoids are used as flavors and fragrances in foods and cosmetics (e.g., menthol, nootkatone, linalool, and sclareol) [9]. One more upcoming and promising industrial use of monoterpenes is as substitutes for ozone-depleting chlorofluorocarbons [162]. Similarly, terpenes have also been suggested as substitutes for chlorinated solvents in applications such as cleaning electronic components and cables, degreasing metal, and cleaning aircraft parts [163]. One of the best known and most widely used monoterpenes is menthol, which has been used as a topical antipruritic, a counterirritant in external analgesic preparations and as antiseptics. Arbortristoside A and C, derived from the alcoholic extract of the seeds of *Nyctanthus arbortristis* [164]; two diterpenes, andrographolide and neoandrographolide, isolated from *Andrographis paniculata* [165]; and himachalol, a sesquiterpene alcohol, derived from the hexane soluble extract of the wood of *Cedrus deodara* [166], possess significant antiallergic activity comparable to disodium cromoglycate when tested in the experimental models. In a recent study, it was found that limonene directly binds to the adenosine A2A receptor, which may induce sedative effects. Results from an in vitro radioligand binding assay showed that limonene exhibits selective affinity to A2A receptors. Limonene also increased cytosolic calcium concentration, which can be achieved by the activation of adenosine A2A receptors. These findings suggest that limonene can act as a ligand and an agonist for adenosine A2A receptors [167]. It is shown in a recent study that *Phyllanthus emblica* fruit extract (which contains various terpenes and sesquiterpenoids) possesses antidiarrheal and spasmolytic activities, mediated possibly through dual blockade of muscarinic receptors and Ca^{2+} channels, thus explaining its medicinal use in diarrhea [168]. Uchiyama et al. isolated several new and known terpenoids from *Dracocephalum komarovi*. Among the isolated terpenes, komaroviquinone showed the most potent in vitro trypanocidal activity against epimastigotes of *Trypanosoma cruzi*, the causative agent of Chagas' disease, with minimum lethal concentration of 0.4 mM [169].

5.13 Side-Effects of Terpenes

Most of the therapeutically used terpenes are local irritants and, thus, are capable of causing GI signs and symptoms. CNS manifestations are rare but may range from an altered mental status to seizures to coma. Terpenes are well absorbed from the oral cavity as evidenced by the early onset of toxicity in significant ingestions. Terpenes are metabolized by the family of cytochrome P450 enzymes and are excreted as glucuronide conjugates by the kidney. According to the 2009 Annual Report of the American Association of Poison Control National Poison Data System, 3,362 single exposures to disinfectants containing pine oil, 10,714 single exposures to camphor, and 422 single exposures to turpentine were reported [170]. Exposure to pine oil resulted in two deaths, and exposure to turpentine resulted in one death. No deaths resulted from exposure to camphor [170]. As seen from the numbers, despite the some mild toxicity of these agents, morbidity is extremely low which indicates that terpenes are relatively safe compounds.

6 Conclusion

All living organisms produce terpenes for some essential physiological functions including some defense mechanisms for many plants. The enormous number and diversity of structures of terpenoids exist which is attributed to the fact that there are many ways in which the basic C5 units can be combined together and there are different pathways which the organism follows. Although history of terpenes spans since the Egyptians, it was until last few decades that terpene synthetic pathways are being understood in enough detail. Moreover, the use of bioinformatics and molecular databases has helped greatly in analyzing the chemical and molecular mechanisms behind terpene synthesis. Also, recent developments in analytical chemistry have helped in functional studies by providing a much more detailed view of the spectrum of terpenes present in and around different living organisms than was previously available. In the field of medicine and drug discovery, natural product–based therapeutics have seen a resurgence in interest and discovery of interesting compounds, and not surprisingly, terpenoids also has been explored in great detail for their vast variety of pharmacological actions and therapeutic potential. The wide array of structures and functionalities that have been evolved in natural terpenoids provide an excellent pool of molecules for use in human therapeutics. Terpenoids are successfully used for the treatment of cancer, inflammation, and various infectious diseases of microbial and parasitic origin, and many terpenoids are in the discovery pipeline in various other categories. Apart from these, they can also be used as supplementary agents in topical dermal preparations, cosmetics, and toiletries, which further broaden the applications of terpenes in other areas of human healthcare and medicine. Finally, the better understanding of the functions of genes in terpene production could lead to discovering novel pathways of terpene production or even new terpene compounds, which might explore new door for future therapeutic intervention employing terpenoids.

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Biosynthesis of Terpenoids

87

Nelson L. Brock and Jeroen S. Dickschat

Contents

1 Introduction 2695
2 Biosynthesis of the Isoprenoid Monomers IPP and DMAPP 2695
2.1 Terpenes from Acid: The Mevalonate Pathway 2697
2.2 Terpenes from Sugar: The Deoxyxylulose Phosphate Pathway 2698
3 Isopentenyl Diphosphate-Dimethylallyl Diphosphate Isomerases (IDI) 2705
3.1 Type I IDI 2705
3.2 Type II IDI 2707
4 Biosynthesis of Linear Polyisoprenoids 2708
4.1 Homomeric (*E*)-Selective Prenyltransferases 2709
4.2 Heteromeric (*E*)-Selective Prenyltransferases 2711
4.3 The (*Z*)-Selective ζ-Type Prenyltransferases 2713
4.4 Biosynthesis of Squalene and Phytoene 2715
5 Terpene Cyclases 2717
5.1 Type I Terpene Synthases 2719
5.2 Type II Terpene Cyclases 2724
5.3 Bifunctional Type I and Type II Terpene Cyclases 2725
6 Conclusions 2726
References 2726

Abstract

Terpenes form the largest and structurally one of the most intriguing classes of natural products. When looking at their structures, it becomes clear that the evolution of terpene biosynthesis must have been one of the biggest challenges

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for Mother Nature, but her solutions are – as often – surprisingly simple. As will be discussed in this chapter, all terpenes are made up from only two five-carbon monomers that have an intrinsic reactivity for the formation of oligomeric linear chains. Virtually the same reactivity is used in cyclization reactions that convert the linear precursors into (poly)cyclic products, not only with introduction of usually several stereogenic centers but also via cationic intermediates in an aqueous environment. How this works – you will find it here.

Keywords

Deoxyxylulosephosphate pathway • dimethylallyl diphosphate • isopentenyl diphosphate • mevalonate pathway • prenyltransferases • terpene cyclases

Abbreviations

CDP-ME	4-Diphosphocytidyl-2-*C*-methyl-D-erythritol
CDP-MEP	4-Diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate
CMP	Cytidine monophosphate
CPP	Copalyl diphosphate
CTP	Cytidine triphosphate
DecPP	Decaprenyl diphosphate
DMAPP	Dimethylallyl diphosphate
DMASPP	Dimethylallyl thiodiphosphate
DXP	1-Deoxy-D-xylulose 5-phosphate
FMN	Flavin mononucleotide
FPP	Farnesyl diphosphate
GGPP	Geranylgeranyl diphosphate
GPP	Geranyl diphosphate
HexPP	Hexaprenyl diphosphate
HMBPP	(*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
IDI	Isopentenyl diphosphate-dimethylallyl diphosphate isomerase
IPP	Isopentenyl diphosphate
LPP	Linalyl diphosphate
MEcPP	2-*C*-Methyl-D-erythritol 2,4-cyclodiphosphate
MEP	2-*C*-Methyl-D-erythritol 4-phosphate
MVA	(*R*)-Mevalonic acid
NPP	Nerolidyl diphosphate
OctPP	Octaprenyl diphosphate
OSC	Oxidosqualene cyclase (Lanosterol synthase)
SHC	Squalene/hopene cyclase
SPP	Solanyl diphosphate (Nonaprenyl diphosphate)
TIM	Triosephosphate isomerase
TPP	Thiamine diphosphate
UPP	Undecaprenyl diphosphate

1 Introduction

Today, more than 55,000 terpenoids are known, making up the structurally most complex and largest class of natural products [1]. The members of this class can be isolated from almost every single organism, and they play important roles in all processes of life (Fig. 87.1). This is exemplified by the ubiquinones (**1**), ubiquitously found terpenoid molecules that serve in the mitochondrial ATP synthesis as electron carriers in the electron transport chain [2]. The same function in the photosynthetic electron transport is fulfilled by the structurally related plastoquinones (**2**) in the chloroplasts of plants and other photosynthetic organisms. Also of terpenoid origin are the vitamins E (**3**), a group of lipophilic compounds known as tocopherols that are exclusively synthesized by photosynthetically active organisms and must be taken up with their plant diet by all other species [3]. As antioxidant membrane constituents, they preserve oxidative membrane destruction. The retinoids, also termed class A vitamins (**4**), arise from carotenoids by oxidative cleavage and fulfill important roles in the process of vision [4]. Other highly active terpenoid molecules are the steroids that exhibit various regulatory functions. One example is the vitamin D family (**5**) that is involved in processes such as immune suppression, hormone secretion, and intestinal calcium absorption [5]. Finally, the chlorophylls (**6**) are green pigments for the absorption of light energy in photosynthetic organisms. Their porphyrin core structures are decorated with terpenoid side chains.

The structural diversity in terpene secondary metabolites is even higher, and they not only make up the larger portion of the known terpenoid molecules, but they also show a diverse array of bioactivities. In their function as aroma constituents, antibiotics, pheromones, or toxins, they confer a benefit on the producing organism in its particular ecological context. Most importantly, humans can adopt these molecular traits for their own purposes, e.g., in the treatment of infectious diseases or cancer as is exemplified by the clinical use of the antimalaria agent artemisinin (**8**) from *Artemisia annua* or the cytostatic taxol (**9**) from *Taxus brevifolia* that are two of the most promising terpenoid drugs [6, 7].

To understand and control the biosynthesis of terpenes is one of the biggest challenges of natural products chemists. The accumulated knowledge and today’s level of understanding the complex biosynthetic transformations toward terpenes are not only of academic interest but also provide the fundament for the industrial production of pharmacologically useful terpenes by biotechnological processes. The state of the art including the chemistry of the transformed metabolites, the enzymology, and aspects of structural biology in terpene biosynthesis will be presented in this chapter.

2 Biosynthesis of the Isoprenoid Monomers IPP and DMAPP

As was already manifested by the groundbreaking work of Otto Wallach and Leopold Ruzicka who were both awarded the Nobel Prize in chemistry

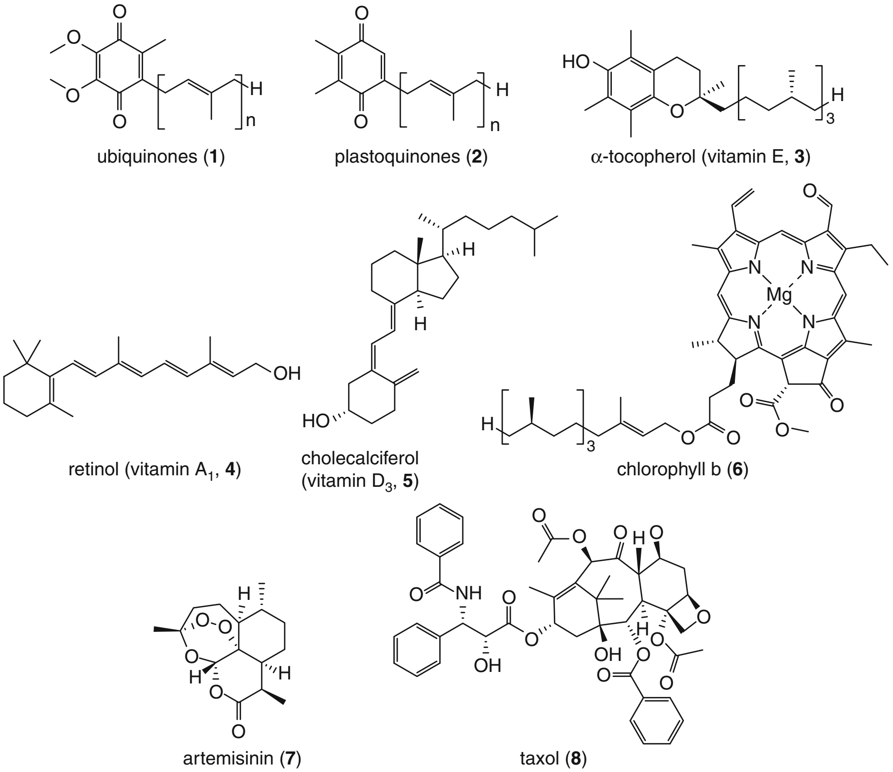


Fig. 87.1 Terpenes with important biological activities

(Wallach 1910; Ruzicka 1939), the usual terpene hydrocarbons are formally oligomers of isoprene [8]. This finding later resulted in Ruzicka's rule [9, 10] that generalized the structures of regular terpenes, exemplified by the cadalanes or cedranes, as constitutional head-to-tail fusions of the isoprene monomers. Structural motifs formally resembling head-to-head or tail-to-tail connections as present, e.g., in the abietanes, were regarded as being irregular (Fig. 87.2, however, such molecules can arise from head-to-tail fused linear polyisoprenoid diphosphates by rearrangements during terpene cyclization, cf. Sect. 5). The biosynthesis of terpenes does not proceed via isoprene itself but relies on two activated molecules that structurally exhibit the same carbon backbone as isoprene: the isoprenoid monomers isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These functional entities serve as precursors to all terpenoid structures and are made via two different pathways. The mevalonate pathway was established by Lynen, Bloch, and Cornforth in the 1950s and is known to take place in animals, fungi, the cytosol of plants, archaea, and a few bacteria, while the deoxyxylulose phosphate pathway has been discovered in the late 1990s and early 2000s with main contributions of Lichtenthaler, Rohmer, Arigoni, and Seto and exclusively occurs in the plastids of plants and green algae and in most bacteria. It is intriguing to see that

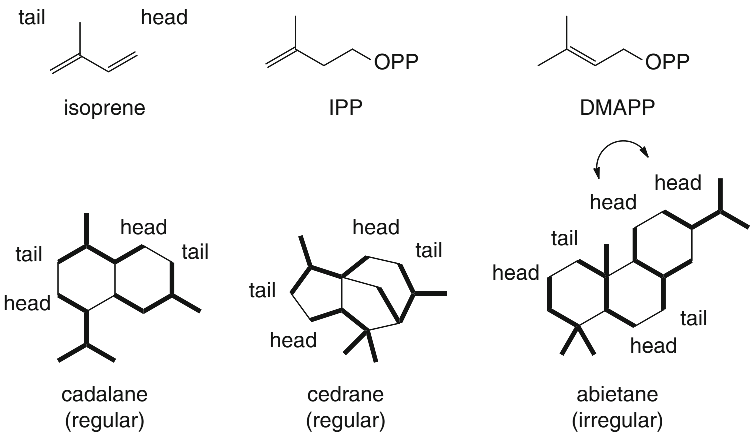
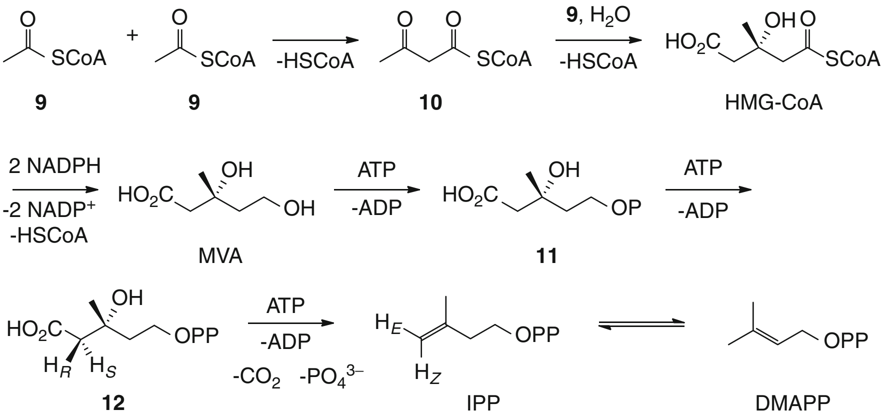


Fig. 87.2 Ruzicka's isoprene rule: regular and irregular terpene skeletons. The irregular head-to-head fusion in the abietanes is highlighted by the *double-headed arrow*

both pathways deliver the same activated isoprenes, IPP and DMAPP, in multiple steps starting from diverse primary metabolites and via completely different intermediates.

2.1 Terpenes from Acid: The Mevalonate Pathway

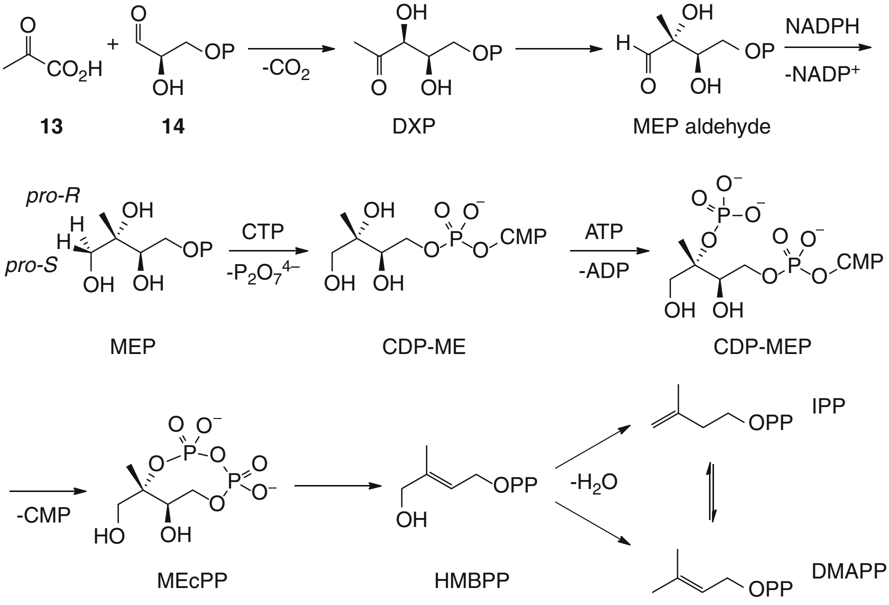
The well-known mevalonate pathway starts with two molecules acetyl-CoA (**9**), the activated form of acetic acid (Scheme 87.1) [11]. In a first step, acetoacetyl-CoA (**10**) is formed via a biological Claisen condensation mediated by the acetoacetyl-CoA acetyltransferase. A biological aldol reaction with a third equivalent of **9** affords (*S*)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) catalyzed by the HMG-CoA synthase [12–14]. During this step, the coenzyme A thioester of the newly introduced acetyl-CoA is hydrolyzed to the corresponding free acid. The subsequent action of the HMG-CoA reductase requires two equivalents of NADPH to reduce HMG-CoA to the eponymous intermediate (*R*)-mevalonic acid (MVA) [15–17]. Twofold phosphorylation with ATP gives first 5-phosphomevalonic acid (**11**) by the mevalonate kinase and then 5-diphosphomevalonic acid (**12**) by the 5-phosphomevalonate kinase [18–21]. In the last step, IPP is furnished by the concomittant dehydration-decarboxylation of **12** in a reaction that is mediated by the 5-diphosphomevalonate decarboxylase under consumption of one equivalent ATP [20, 22, 23]. Stereochemically, in this *anti* elimination, the 4-*pro*-*R* proton of **12** is turned into the (4*Z*)-proton of IPP, and the 4-*pro*-*S* proton converts into the (4*E*)-proton, respectively [24, 25]. As will be discussed in more detail in Sect. 3, the primary product IPP can be transformed into its isomer DMAPP by an isopentenyl diphosphate isomerase (IDI), a reaction that is essential in organisms that solely rely on the mevalonate pathway.



Scheme 87.1 The mevalonate pathway

2.2 Terpenes from Sugar: The Deoxyxylulose Phosphate Pathway

After the discovery and full elucidation of the mevalonate pathway, it was initially believed that this pathway is the only one for the biosynthesis of all isoprenoids. From today's point of view, seemingly slavish adherence to this misleading prospect on terpene biogenesis is sometimes referred to as the mevalonate dogma. However, during the years of further research on terpene biosynthesis, more and more data accumulated, mainly from feeding experiments with isotopically labeled glucose [26–28], that were in disagreement with the mevalonate pathway and raised the question whether the mevalonate dogma holds true. Finally, the ambiguities were refined into a newly discovered pathway starting from a phosphorylated deoxysugar, 1-deoxy-D-xylulose 5-phosphate (DXP) [29]. This second pathway for the biogenesis of IPP and DMAPP is known as the deoxyxylulose phosphate pathway (also referred to as non-mevalonate or methylerythritol pathway). The synthesis of DXP itself requires the two glycolysis products pyruvate (**13**) and glyceraldehyde 3-phosphate (**14**) (Scheme 87.2) [30]. Pyruvate is activated by the thiamine diphosphate (TPP) cofactor and after decarboxylation condensed with **14** to form DXP by the DXP synthase [31–34]. The isomerization to 2-*C*-methyl-D-erythrose 4-phosphate (MEP aldehyde) and subsequent reduction with NADPH to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) are catalyzed by a single enzyme termed DXP reductoisomerase (IspC) [35–37]. During this conversion, the C3 proton of DXP is transferred via the aldehyde position in MEP aldehyde to become the 1-*pro-S* proton in MEP, whereas the 1-*pro-R* proton originates from NADPH [38, 39]. The IspD-mediated activation with cytidine triphosphate (CTP) to 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME) and subsequent phosphorylation with ATP by IspE affords 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate (CDP-MEP) [40–43]. The enzyme IspF cyclizes CDP-MEP to 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) via elimination of cytidine



Scheme 87.2 The deoxyxylulose phosphate pathway

monophosphate (CMP) after which (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) is furnished by reduction with IspG [44–47]. In the terminal step HMBPP is transformed to the isoprene equivalents IPP or DMAPP in a fix ratio of about 5:1 by the oxidoreductase IspH [48, 49]. For fine-tuning of the actual building block requirements for terpene biosynthesis, an isomerase can mediate between IPP and DMAPP, but since both products are made simultaneously, an IDI activity is not essential for organisms that use the DXP pathway [50].

Three enzymes of the DXP pathway are mechanistically particularly interesting, and their enzyme mechanisms have been investigated in various approaches. These are the enzymes IspC, IspG, and IspH that will be discussed in more detail in the following sections.

As outlined above, the reductoisomerase IspC has isomerase and dehydrogenase (reductase) activity. Dehydrogenases can be divided into two distinct classes, types A and B, depending on whether they transfer the hydride ion that is abstracted from their substrate to the *Re* face (type A) or the *Si* face (type B) of the NAD(P)$^+$ cofactor. In case of the reverse reaction, class A enzymes reduce their substrates by transfer of the *pro*-*R*-hydrogen, while class B enzymes transfer the *pro*-*S*-hydrogen of NAD(P)H [51]. The DXP reductoisomerase IspC is a class B dehydrogenase and catalyzes the reductive isomerization of DXP to MEP [39, 39]. The first crystal structure was obtained from the *Escherichia coli* enzyme that crystallizes in a homodimeric fashion (Fig. 87.3a) [53, 54]. Each monomer subunit has a V-shaped topology (Fig. 87.3b) and contains the N-terminal NADPH-binding domain, a C-terminal four-helix bundle, and a middle domain

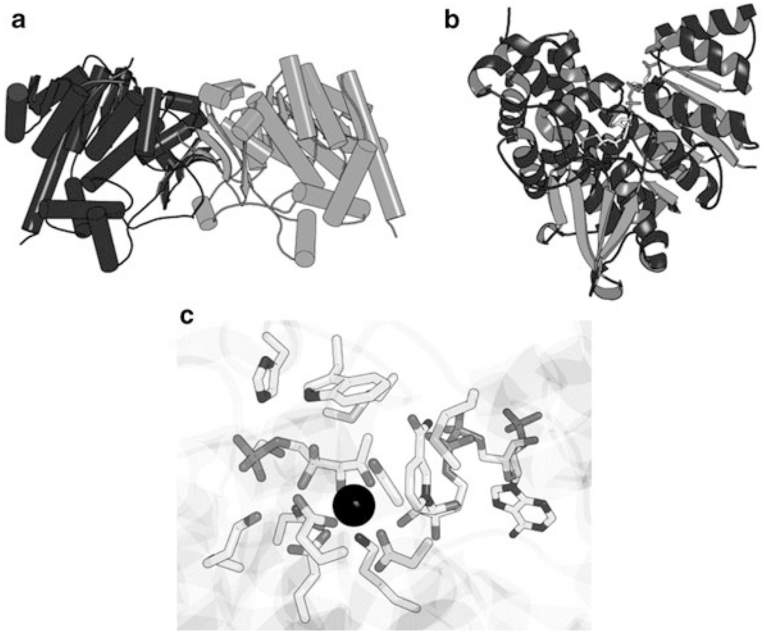
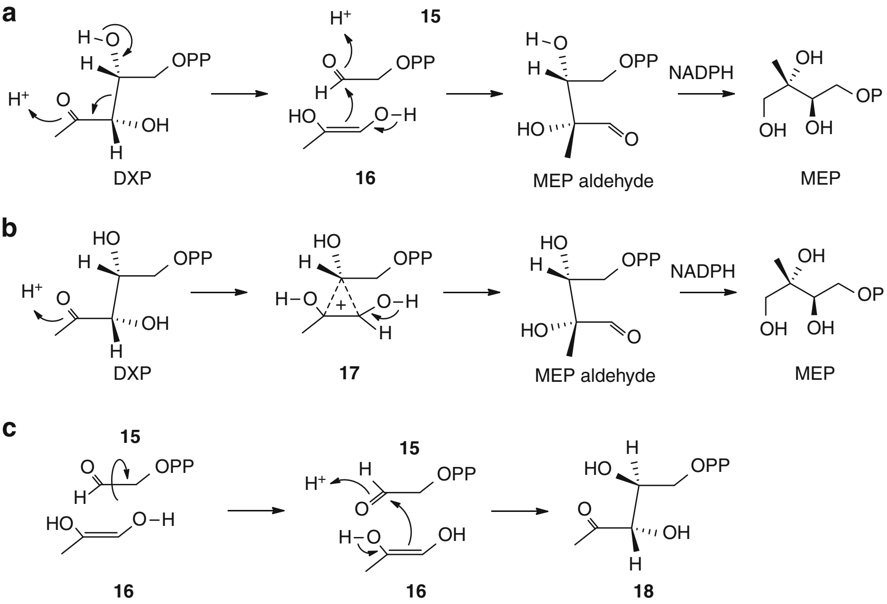


Fig. 87.3 IspC from *E. coli* (1Q0Q) [52]. (**a**) Dimeric structure, (**b**) enzyme complex with NADPH and DXP, and (**c**) close-up on the active site (Mn^{2+} is superimposed from the IspC-Mn^{2+} complex from *Mycobacterium tuberculosis* (4AIC))

that facilitates dimerization. Herein located are conserved aspartate, glutamate, and lysine amino acid residues that bind a divalent metal ion (Mn^{2+}) for catalytic activity (Fig. 87.3c) [53–55].

Although MEP aldehyde has never been observed directly, its intermediacy en route from DXP to MEP seems plausible, as recombinant IspC catalyzes the NADPH-dependent reduction of MEP aldehyde to MEP [56]. For its enzymatic formation from DXP, two possible mechanisms, a retroaldol-aldol mechanism or an α-ketol rearrangement, have been suggested (Scheme 87.3) [56]. The retroaldol-aldol mechanism was supported by kinetic studies and proceeds via the intermediates glycoaldehyde phosphate (**15**) and the (*Z*)-configured enzyme-stabilized enol of hydroxyacetone (**16**) [57–59]. However, no fragment exchange of the putative intermediates could be observed in NMR studies of incubation experiments with IspC and different ^{13}C-labeled substrate isotopologues. This result better fits to the α-ketol-rearrangement mechanism via the transition state **17** or at least points to a very strict fragment containment during the retroaldol-aldol conversion [60]. In contrast, the observed partial epimerization of DXP to 1-deoxy-L-ribulose 5-phosphate (**18**) in the ternary complex of IspC, NADPH, and the substrate was implicated as an evidence for a stepwise mechanism that would allow for rotation of fragment **15** followed by its reaction with **16** to the epimer of DXP, **18** (Scheme 87.3c) [61, 62].


a
H−O
H
OPP
H+
O
OH
H
DXP
H+
15
O
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H
HO
O−H
16
H−O
H
OPP
HO
O
MEP aldehyde
NADPH
OH
OP
OH
OH
MEP
b
HO
H
OPP
H+
O
OH
H
DXP
HO
H
OPP
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O−H
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17
HO
H
OPP
HO
O
MEP aldehyde
NADPH
OH
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O−H
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15
H+
H
OPP
O
H−O
OH
16
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HO
OPP
O
OH
H
18

Scheme 87.3 Suggested mechanisms for the IspC reaction. (**a**) Stepwise retroaldol-aldol mechanism, (**b**) concerted α-ketol-rearrangement mechanism, and (**c**) postulated mechanism for the enzymatic formation of the DXP epimer **18**

The enzyme IspG is mechanistically possibly the biggest challenge of all enzymes within the DXP pathway. The homodimeric IspG proteins from *Aquifex aeolicus* and *Thermus thermophilus* have been structurally characterized [63, 64] and show an N-terminal TIM barrel-shaped domain (Fig. 87.4). TIM barrels are named after the triosephosphate isomerase in which this structural motif was first identified and are protein folds made up from eight pairs of alternating α-helices and parallel β-sheets. A smaller C-terminal domain contains a 4Fe-4S cluster [63, 64]. In the first step of the IspG reaction, MEcPP is bound to the TIM barrel and ring-opened to the cationic intermediate **19** (Scheme 87.4). This may cause a switch in the overall enzyme structure into a closed conformation in which the C-terminal domain of one monomer subunit moves toward the TIM barrel of the other subunit and traps the substrate in between [63, 64]. Thereby, the subsequent reduction with involvement of the 4Fe-4S cluster is enabled, and the product HMBPP is released.

Different mechanistic proposals are discussed for the IspG-catalyzed reduction of MEcPP to HMBPP. As an alternative to the initial ring opening of the substrate's cyclodiphosphate to **19**, the formation of an HMBPP epoxide intermediate was proposed (Scheme 87.4) [48, 65, 66]. In support of this intermediate is the finding that HMBPP epoxide is accepted by IspG and reduced to HMBPP [67, 68]. However, semiempirical model calculations disfavor this intermediate that has also never experimentally been observed. A possible explanation is that HMBPP

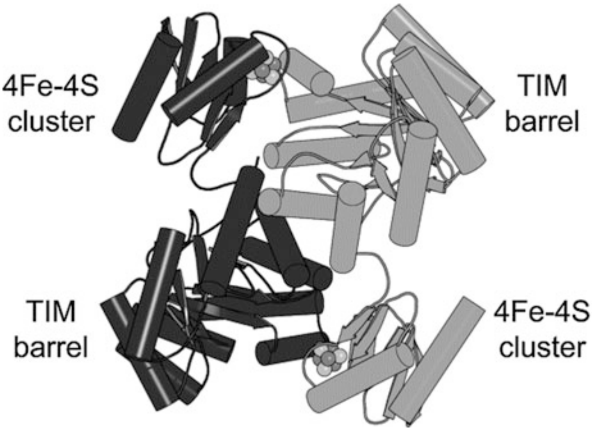
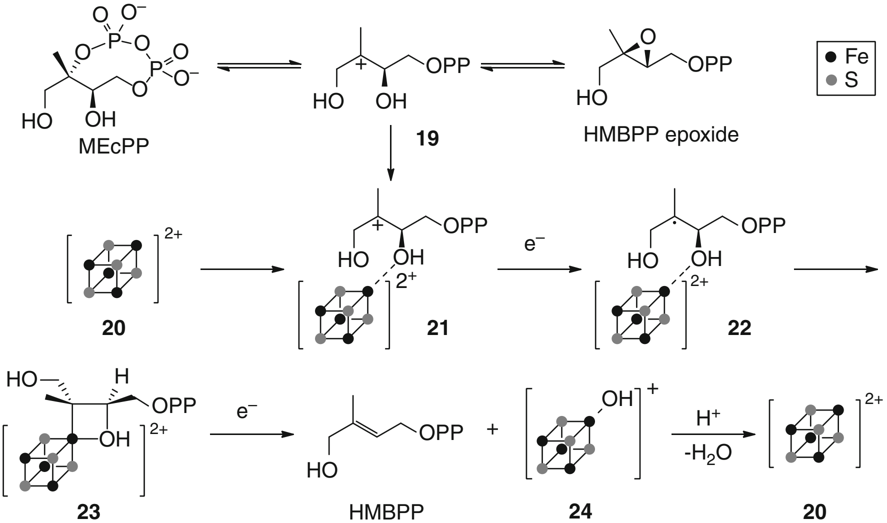


Fig. 87.4 IspG from *A. aeolicus* (3NOY). The enzyme has a homodimeric structure, and each subunit is composed of an N-terminal TIM barrel domain and a C-terminal domain containing the 4Fe-4S cluster



Scheme 87.4 Hypothetical mechanism of the IspG reaction

epoxide, like the native substrate HMBPP, is transformed into cation **19** followed by its reduction. This idea is corroborated by the conversion of HMBPP epoxide into MEcPP by IspG under nonreductive conditions [69]. The reversibility of the C–O bond cleavage at C2 of MEcPP was also demonstrated by incubation of recombinant IspG with ^{18}O-labeled MEcPP [70]. For the reduction of **19** by the 4Fe-4S cluster (**20**), a cascade of substrate binding to **21** followed by one-electron transfer to the radical **22**, protonation and elimination of water to a radical cation (not shown), and a final one-electron transfer to HMBPP were suggested [65, 66]. Semiempirical calculations were in favor of a reverse order of the events, i.e., first

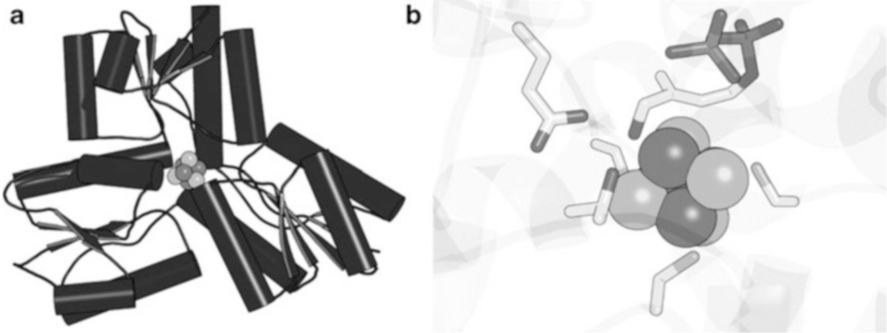
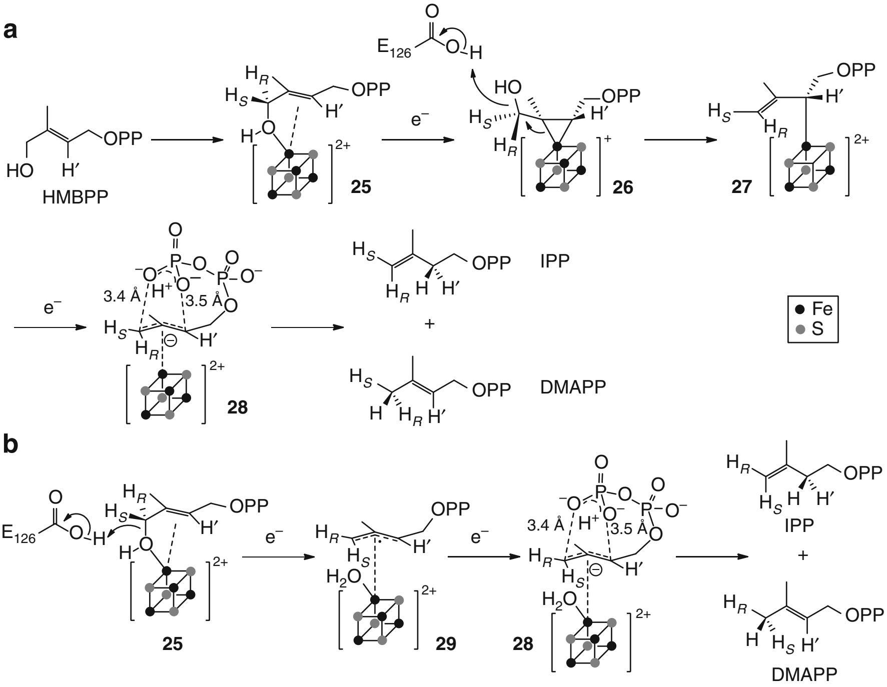


Fig. 87.5 (**a**) IspH from *E. coli* in closed conformation (3KE8) and (**b**) close-up on the active site

a reduction of **22** to an intermediate tertiary anion species (not shown) followed by protonation and elimination of water to yield HMBPP [67]. Recently, upon incubation of reduced IspG with MEcPP or HMBPP epoxide, a paramagnetic species was detected by different EPR techniques. This finding resulted in the suggestion of a ferraoxetane (**23**) intermediate that could directly be formed from **22** [71–73]. Subsequent reduction and *syn* elimination would give HMBPP and the hydroxy complex **24** from which the 4Fe-4S cluster is restored by protonation and hydrolysis [72].

The mechanism of the IspH reaction was also subject to debate for several years. The first IspH crystal structures were reported from *A. aeolicus* and *E. coli* with a 3Fe-4S cluster surrounded by three structurally homologous domains, albeit without any distinct sequence similarity [74, 75]. This cloverleaf structure can therefore be described as pseudo C_3 symmetric (Fig. 87.5a). Three highly conserved cysteine residues, one from each of the surrounding domains, participate in binding of the central 3Fe-4S cluster [74, 75]. In contrast to these findings, EPR measurements indicated the presence of a 4Fe-4S cluster [76, 77]. Since the fourth Fe is not stabilized by a cysteine residue in the active site, an explanation for the initial detection of the 3Fe-4S cluster is given by the eventual loss of the fourth labile iron center. Instead of a cysteine, a conserved threonine residue was found in close proximity to this Fe, and Mössbauer spectroscopy indicated its involvement in binding of the labile Fe atom [78]. Recently, the fourth Fe atom was also crystallographically observed (Fig. 87.5b) [79].

In the first step of the IspH reaction, an alkoxide complex (**25**) is formed between HMBPP and the fourth labile iron atom of the 4Fe-4S cluster (Scheme 87.5) [74, 75, 79]. Two different mechanisms were proposed for the follow-up steps. The mechanism shown in Scheme 87.5a involves the one-electron reduction of **25** to the metallacyclopropane species **26** that was tentatively identified by EPR spectroscopic methods in the unreactive E126A mutant [80]. Alternatively, **26** could be described as a π-complex. Its protonation/dehydration is likely accomplished by the conserved glutamate E126 and may proceed with preliminary rotation of the hydroxymethyl group into a conformation with the hydroxyl group pointing away



Scheme 87.5 Hypothetical mechanisms of the IspH reaction. (**a**) Metallacyclopropane mechanism and (**b**) Birch reduction mechanism

from the 4Fe-4S cluster. This reaction results in the η^1-allyl complex **27** from which the η^3-allyl anion **28** is generated by a second one-electron transfer. The mechanism shown in Scheme 87.5b is essentially a Birch reduction that starts with a protonation/dehydration of **25** and subsequent one-electron reduction to yield the allyl radical **29**. A modification of this mechanims merges these first two steps to convert **25** directly into the allyl radical **29**. A second one-electron transfer leads to the η^3-allyl anion **28** [48, 81–83]. The last step in both mechanisms A and B includes the protonation of **28** from the *Si* face at C3 to yield IPP [84]. The alternative protonation at C1 to DMAPP proceeds most likely from the same side of the molecule, in other words from the *Re* face. Due to its close proximity within a distance of 3.4–3.5 Å to C1 and C3, respectively, the involvement of the substrate's terminal phosphate group in this last step is discussed and may control the observed ~1:5 product ratio of DMAPP and IPP [79, 85].

The two suggested mechanisms could recently be distinguished due to their different stereochemical implications. Mechanism B implies a stereochemical course in which the 1-*pro-S* hydrogen (H_S) of HMBPP transforms into H_Z of IPP, whereas the 1-*pro-R* hydrogen (H_R) must end up as H_E in IPP. However, feeding experiments with deuterated 1-deoxy-D-xylulose isotopologues showed the contrary result. This finding can only be harmonized with the metallacyclopropane mechanism if the conversion of **26** into **27** indeed proceeds out of the conformation of **26** with the rotated hydroxymethyl group [86]. Further support for this idea was given by quantum chemical calculations [80] and recently reported crystallographic data obtained with IspH mutants for which this rotation of the hydroxymethyl group could directly be observed [87].

3 Isopentenyl Diphosphate-Dimethylallyl Diphosphate Isomerases (IDI)

A central role in the biosynthesis of isoprenoids is filled by the isopentenyl diphosphate-dimethylallyl diphosphate isomerase (IDI) that catalyzes the interconversion of IPP and DMAPP. The necessity for such an enzyme was suggested in the 1950s when only IPP was known as a monomeric isoprenoid precursor, but an allylic diphosphate such as DMAPP was assumed to have the higher intrinsic reactivity for polyisoprenoid synthesis [22, 88, 89]. The first enzymatic isomerization of IPP to DMAPP was observed in 1959 from a cell-free extract of baker's yeast [90, 91]. Two types of IDI with essentially no amino acid sequence or structural similarities are able to catalyze this interconversion by completely different enzyme mechanisms. The well-known IDI-I have been identified in animals, plants, fungi, and bacteria, whereas the IDI-II can be found mainly in archaea but also in some bacteria [92, 93].

3.1 Type I IDI

Type I IDI (IDI-I) are Zn^{2+}-dependent metalloproteins which require a second divalent metal ion (Mg^{2+}) for IPP binding via its diphosphate moiety (Fig. 87.6b, c) [94, 95]. The crystal structure of human IDI-I revealed a homodimeric structure (Fig. 87.6a) with a six-coordinate metal ion binding pocket comprised of three histidine and two glutamate residues. These were initially suggested to bind Mn^{2+} [96, 97], but later on, the metal binding pocket was reassigned to have a much higher affinity for Zn^{2+}. Other divalent metal ions (Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}) are suitable substitutes and can, in some cases, even increase IDI-I activity [98, 99]. A participation of the metal cation in the enzymatic reaction was delineated from X-ray analyses of enzyme-inhibitor complexes [94].

The mechanism of the IPP isomerization is a stereoselective antarafacial 1,3-allylic rearrangement in which a protonation at C4 of IPP from the *Re* side is followed by a stereospecific removal of the 2-*pro-R* proton (Scheme 87.6) [24, 100–102]. In the reverse reaction, DMAPP is protonated at C2 from the *Re* side, while a proton of its (*E*)-methyl group is removed. For yeast IDI-I, a slight lack of fidelity was observed resulting in the partial equalization of the (*E*)- and (*Z*)-methyl groups with a rate of about 2% and a removal of the 2-*pro-S* hydrogen of IPP with a rate of 0.5% [103]. The first of these findings supports a stepwise mechanism rather than a concerted process since the tertiary cation intermediate **30** allows for an exchange

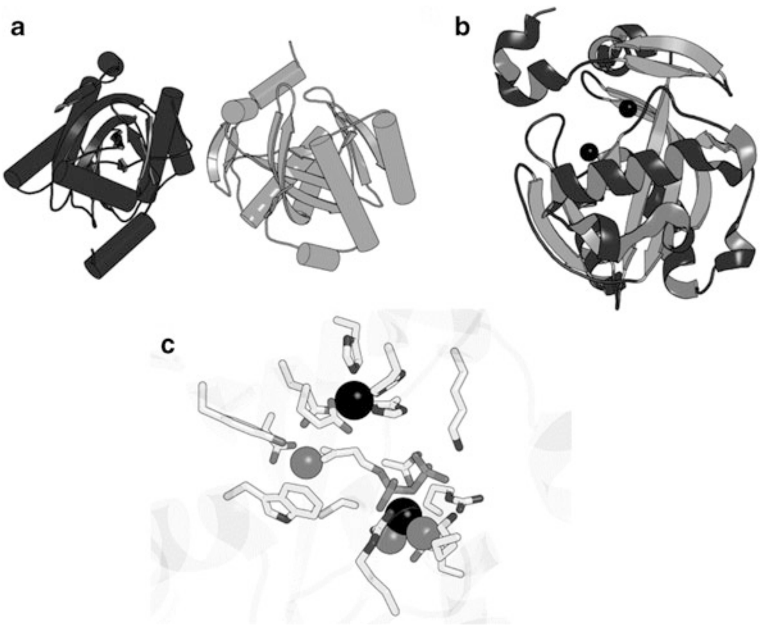
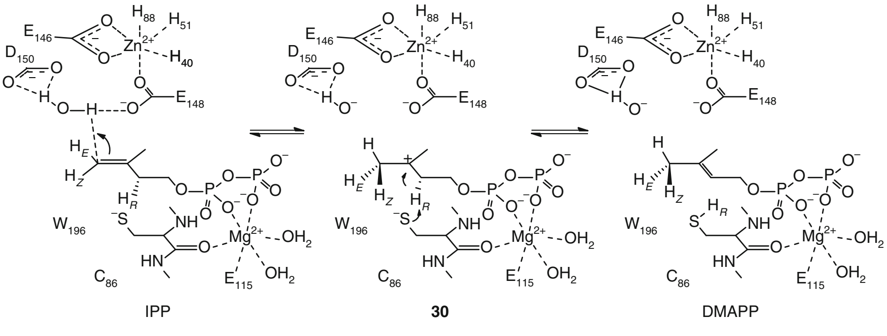


Fig. 87.6 Structure of human IDI-I (2I6K). (**a**) Dimeric structure, (**b**) monomer with Mg^{2+} and Mn^{2+} cofactors, and (**c**) close-up on the active site (DMAPP superimposed from 2ICK)



Scheme 87.6 The IDI-I reaction

of the (*E*)- and (*Z*)-methyl groups by rotation around the C2–C3 single bond. This intermediate **30** is likely stabilized by cation-π-interactions with a tryptophane residue in the active site [96].

The amino acid residues E148 and Y136 have been discussed as proton sources for the initial protonation of IPP. However, E148 is likely unprotonated under physiological conditions due to its coordination to Zn^{2+}. Furthermore, site-directed mutagenesis of the conserved tyrosine in *E. coli* IDI-I results in a 1% residual activity, indicating a structural rather than a direct functional role [104, 105].

Water could act as proton donor, but the crystal structure of *E. coli* IDI-I does not contain a water molecule in the active center. In contrast, the crystal structure of human IDI-I exhibits a water molecule that is stabilized by D150 within the active site [95, 106]. This aspartate residue is conserved in eukaryotic IDI-I which coincides with the ca. 20-fold higher activity of the eukaryotic enzyme as compared to *E. coli* IDI-I [50]. A cystein residue has been suggested as proton acceptor for the deprotonation of **30** [91, 107, 108]. Site-directed mutagenesis of this cystein in yeast IDI-I confirmed its critical role for catalytic activity [109].

3.2 Type II IDI

Type II IDI are flavoenzymes that require a reduced flavin mononucleotide (FMN) and a divalent metal ion (Mg^{2+} or Mn^{2+}) for their action [110–114]. The first enzyme of this class has been discovered in 2001 in *Streptomyces* sp. CL190 [111]. The crystal of IDI-II from the archaeon *Sulfolobus shibatae* shows a tetrameric quarternary structure (Fig. 87.7), whereas the *Bacillus subtilis* enzyme is an octamer [115, 116]. The monomers exhibit a TIM barrel domain as it is typical for flavoproteins. The enzyme's requirement of a redox cofactor is surprising since the overall reaction catalyzed by IDI-II is a simple olefinic double bond isomerization without a net change of the redox state of its substrate. Site-directed mutagenesis experiments demonstrated that the reduced flavin cofactor is only acting as a general acid-base catalyst without being involved in any redox chemistry. Herein, a non-redox functionality of a flavin cofactor was established for the first time [115].

For the IDI-II mechanism, a stepwise proton addition/elimination rather than a concerted process was suggested, as olefinic hydrogen exchange without isomerization was observed for an IPP analogue [117]. In the first step, IPP is protonated at C4 to give the intermediate tertiary cation **30** followed by stereoselective removal of the 2-*pro*-*R*-proton [84, 118]. The reverse reaction follows essentially the same stereochemical course in which the protonation of DMAPP at C2 from the *Re* side is succeeded by a selective deprotonation of the (*E*)-methyl group [84, 119]. In theory, the intermediate **30** would allow for a rotation around the C2–C3-bond, but the reaction has a high fidelity with no observable equalization of the (*E*)- and (*Z*)-methyl groups pointing to a tight binding of the substrate to the FMN cofactor [115]. The suprafacial proton transfer catalyzed by IDI-II was suggested from crystallographic observations in an enzyme-substrate complex, experimentally demonstrated in isotopic labeling experiments, and stands in clear contrast to the established antarafacial process in the IDI-I reaction [115, 120]. The structural data together with the observed stereochemistry of the IDI-II reaction were refined into two alternative mechanisms. Either the N5 and N1 nitrogens of FMN in cooperation (Scheme 87.7a) or the N5 nitrogen alone was implicated to mediate the proton transfer (Scheme 87.7b), whereas a participation of conserved amino acid residues as general acid-base catalysts was experimentally ruled out [115, 120]. In mechanism A, the FMN cofactor has a different protonation state after one

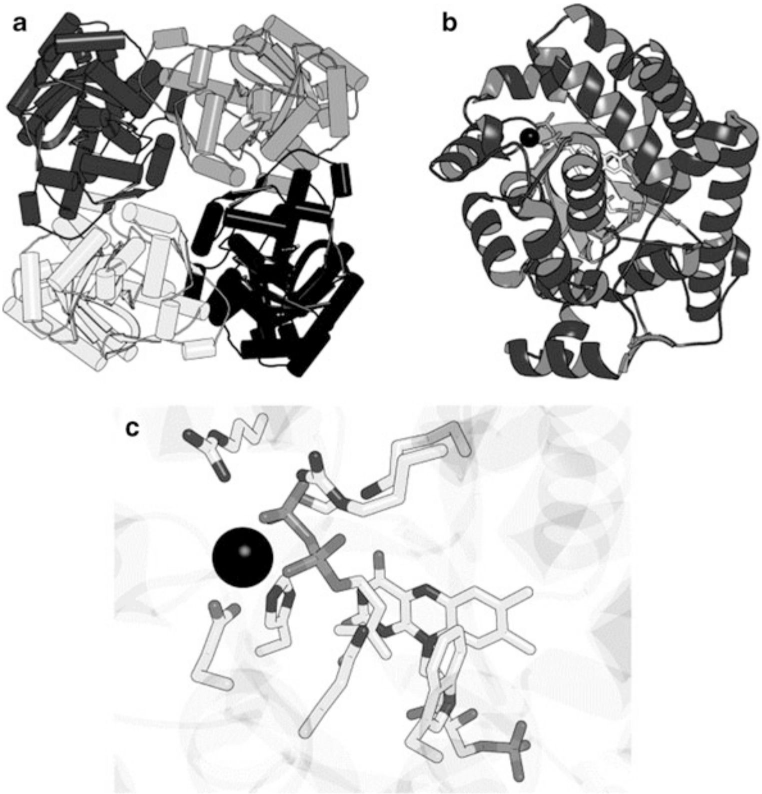
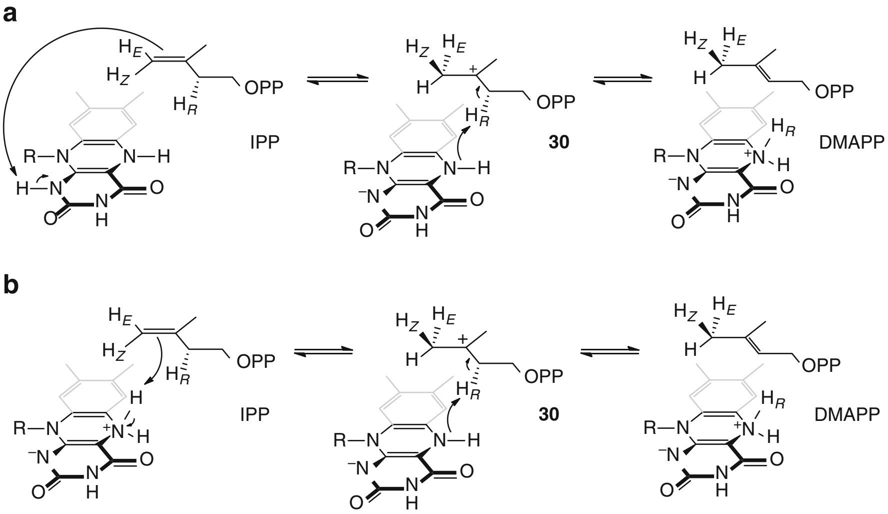


Fig. 87.7 IDI-II from *S. shibatae* (2ZRW). (**a**) Tetrameric structure, (**b**) monomer with FMN cofactor, and (**c**) cocrystallization with Mg^{2+}, FMN, and IPP

turnover and must be restored by a deprotonation/reprotonation event likely with participation of appropriate amino acid residues prior to its next usage. No such net change of the cofactor occurs in mechanism B during one catalytic turnover.

4 Biosynthesis of Linear Polyisoprenoids

The biosynthesis of linear polyprenyl diphosphates is carried out by prenyltransferases. DMAPP and IPP can be fused under catalysis of the geranyl diphosphate synthase to geranyl diphosphate (GPP). Farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are constructed analogously by repeated addition of IPP under catalysis of the FPP synthase and the GGPP synthase. Only a few examples for the biosynthesis of farnesylgeranyl diphosphate are known from archaea [121]. According to Ogura and Koyama, prenyltransferases can be classified into four groups including the homomeric short-chain prenyl diphosphate synthases (class I, C_{10}–C_{25}), the heteromeric medium-chain prenyl diphosphate



Scheme 87.7 The IDI-II reaction. (**a**) Suggested mechanism with involvement of N5 and N1 of the reduced FMN cofactor and (**b**) mechanism with participation only of N5 of FMN

synthases (class II, C_{30}–C_{35}), the homomeric long-chain prenyl diphosphate synthases (class III, C_{40}–C_{50}), and the (*Z*)-polyprenyl diphosphate synthases (class IV) [122].

Recently, a very useful structural classification for proteins involved in terpene biosynthesis has been proposed by Oldfield and Lin [123]. Following this classification, six enzyme domain structures (α, β, γ, δ, ε, and ζ) can be distinguished that occur in different combinations in enzymes for terpene biosynthesis to fulfill various functions. While terpene cyclases are constructed from α-, β-, and γ-domains (Sect. 5) [124], prenyltransferases are made up from α-, δ-, ε-, and ζ-modules. The (*E*)-selective prenyltransferases fall into two classes that either have a homomeric (αα-type or $(\alpha\alpha)_3$-type) [125] or heterotetrameric ($\alpha_2\delta_2$-type) structure, whereas the (*Z*)-selective prenyltransferases constitute a third distinct group with a completely different enzymology (ζ-type) [126]. In contrast to these head-to-tail connecting prenyltransferases, the specialized ε-type prenyltransferases generate the linear precursors squalene and phytoene by a head-to-head linkage of FPP and GGPP, respectively.

4.1 Homomeric (*E*)-Selective Prenyltransferases

The (*E*)-selective homomeric prenyltransferases are responsible for the formation of polyprenyl diphosphates with product chain lengths from GPP (C_{10}) up to decaprenyl diphosphate (DecPP, C_{50}). At least one (*E*)-selective prenyltransferase is essential for every organism as short-chain (*all-E*)-configured polyisoprenoids are the precursors for the pivotal steroids and carotenoids. These α-helical enzymes

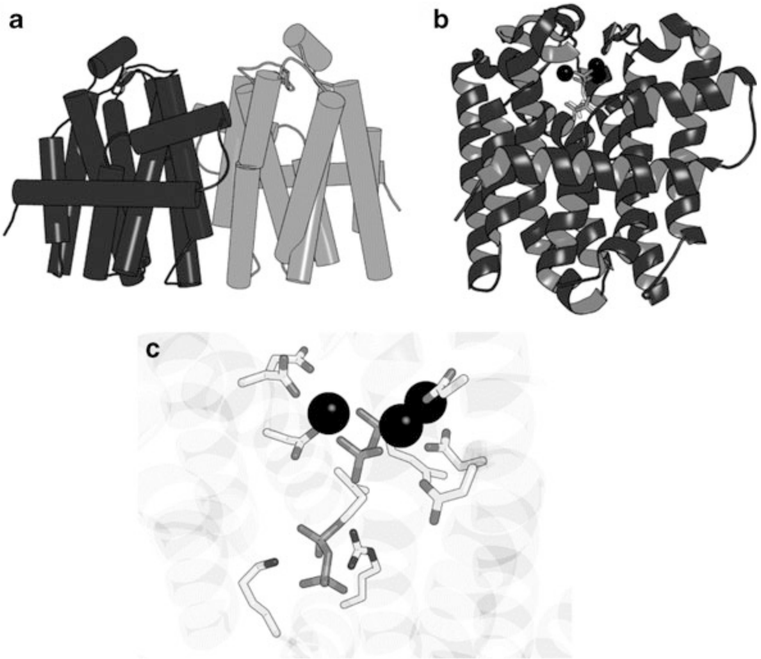
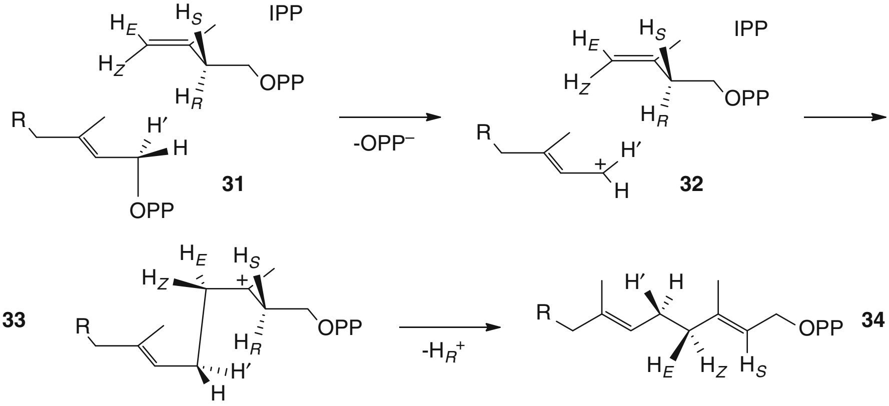


Fig. 87.8 *E. coli* FPP synthase (1RQI). (**a**) Homodimeric structure (αα-type), (**b**) monomer with trinuclear Mg^{2+} cluster, and (**c**) close-up on the active site in complex with Mg^{2+}, IPP, and DMASPP

can appear as homodimers (αα-type) exemplified by *E. coli* FPP synthase (Fig. 87.8a) [127] or as hexameric assemblies ($(\alpha\alpha)_3$-type) like in the human GGPP synthase or the solanyldiphosphate (SPP) synthase from *Arabidopsis thaliana* [128, 129]. The αα-type prenyltransferases require divalent metal ions (usually Mg^{2+} or in some cases Mn^{2+}) as a cofactor. Each α-subunit contains two highly conserved aspartate-rich motifs on opposite sides of the active site with a consensus sequence of DDXXD (Fig. 87.8b). These aspartate residues are responsible for the binding of three divalent metal ions via salt bridges (Fig. 87.8c). The resulting trinuclear magnesium cluster coordinates to the diphosphate oxygen atoms of the allylic diphosphate. The elongation unit IPP is bound with its diphosphate moiety to conserved cationic residues in the homoallylic binding site via electrostatic interactions [127].

In the first step of the chain elongation reaction, diphosphate abstraction from DMAPP is initiated by the Lewis-acidic trinuclear magnesium cluster to generate the allyl cation **32**. This enables attack of IPP at C1 of DMAPP with inversion of the configuration resulting in the tertiary cation **33** [100]. Stereoselective removal of the IPP 2-*pro*-*R* proton finishes the assembly of GPP [100, 130]. As the attack of IPP proceeds at its C4 from the *Si* side [24], H_E of IPP ends up as the 4-*pro*-*S*-hydrogen and H_Z as the 4-*pro*-*R*-hydrogen of the newly formed



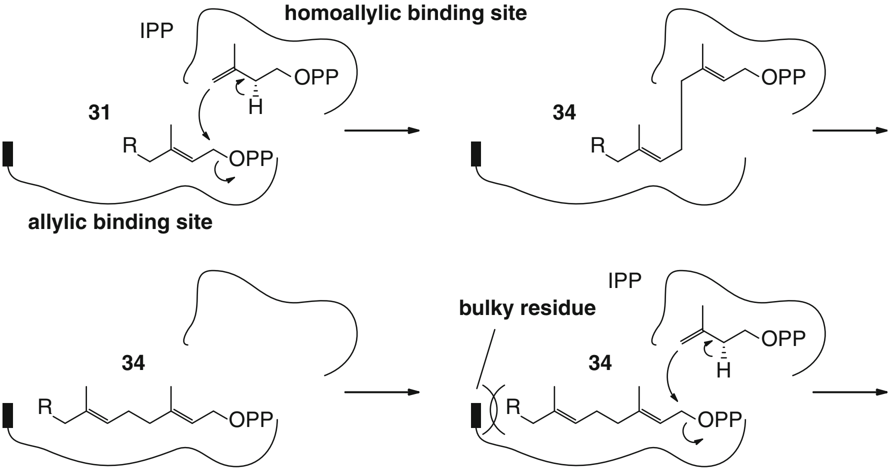
Scheme 87.8 Stereochemical course and intermediates of the polyisoprenoid biosynthesis by (*E*)-selective prenyltransferases

polyisoprenoid **34** (Scheme 87.8). Higher homologues are accessed by ionization of GPP and (*E*,*E*)-FPP that are elongated by IPP addition to yield (*E*,*E*)-FPP and (*all*-*E*)-GGPP, respectively. The stepwise rather than concerted process of the prenyltransferase reaction via the intermediates **32** and **33** was established by incubation of FPP and a less reactive IPP analogue (3-bromobut-3-enyl diphosphate) with the octaprenyl diphosphate (OctPP) synthase resulting in the capture of the farnesyl cation as farnesol [131].

The crystal structures of αα-homodimeric prenyltransferases exhibit a homoallylic binding site for IPP and an allylic binding site for the allylic diphosphate substrate that together constitute a hydrophobic pocket (Scheme 87.9). The individual product chain length of a prenyltransferase is determined by the size of this hydrophobic cleft, i.e., bulky substituents limit the size of the allylic diphosphate substrate and thereby control the maximum chain length of the polyisoprenoid product. Important insights into this control mechanism were obtained by site-directed mutagenesis experiments with avian FPPS, in which such bulky substituents were replaced by smaller ones, thus shifting the product scope to higher homologues of the native FPP product [132]. This idea of chain length control is also supported by a good correlation between the sizes of the hydrophobic clefts and the maximum product chain lengths as revealed by a structural comparison of several enzymes [129, 133, 134].

4.2 Heteromeric (*E*)-Selective Prenyltransferases

The αδ-type prenyltransferases are comprised of two proteins, each of these being inactive on its own, that form an active $\alpha_2\delta_2$-heterotetramer. The first prenyltransferase of this class was described from *Micrococcus luteus* and biochemically characterized as hexaprenyl diphosphate (HexPP) synthase [135].



Scheme 87.9 Product chain length is controlled by the size of the hydrophobic clefts in prenyltransferases

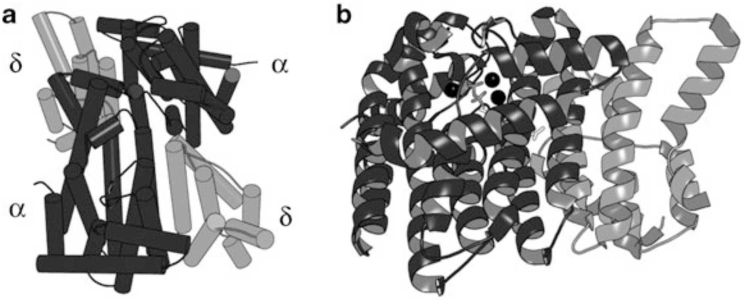


Fig. 87.9 Structure of HexPP synthase from *M. luteus* (3AQC). (**a**) Heterotetramer and (**b**) heterodimer in complex with Mg^{2+} and a less reactive FPP analogue (7,11-dimethyl-2,6,10-dodecatrien-1-yl diphosphate)

GPP synthase from *Mentha* × *piperita* constitutes the first example for which an X-ray structure was obtained [136], followed 1 year later by the crystal structure of HexPP synthase from *M. luteus* [137]. The heterotetramers are made up from two large α- and two small δ-subunits (Fig. 87.9). The α-subunits contain the conserved DDXXD motifs and are structurally related to the α-domains of homomeric prenyltransferases, while the δ-subunits lack the DDXXD motif for catalysis but determine chain length specificity and stabilize the enzyme structure by hydrophobic interactions [137]. The structural similarities between the δ-subunits and the α-domains suggest a close common evolutionary origin. Interestingly, the prenyltransferase from *M.* × *piperita* produces not only GPP but also GGPP without significant amounts of FPP upon prolonged incubation [136].

4.3 The (*Z*)-Selective ζ-Type Prenyltransferases

The (*Z*)-selective prenyltransferases are structurally different to all other prenyltransferases and are made up from so-called ζ-domains. They catalyze the head-to-tail addition of IPP to a growing isoprenoid chain with introduction of a (*Z*)-configured olefinic double bond. Their product chain lengths vary from neryl diphosphate (C_{10}) to undecaprenyl diphosphate (UPP, C_{55}), the latter one being a particularly important molecule for bacterial cell wall biosynthesis [138]. Typically, the long-chain ζ-prenyltransferases including the UPP synthases from *M. luteus* and *E. coli* use (*E*,*E*)-FPP as starter [139–141], while the short-chain enzymes such as the neryl diphosphate synthase from *Solanum lycopersium* and the (*Z*,*Z*)-FPP synthase from *Solanum habrochaites* start the elongation process from DMAPP [142, 143]. An interesting interaction of two ζ-prenyltransferases is known from *M. tuberculosis* where the product of the (*E*,*Z*)-FPP synthase is used by another ζ-prenyltransferase for its further (*Z*)-selective elongation to DecPP [144]. Very long chains up to C_{120} are built by the dehydrodolichyl diphosphate synthase, and polymers composed of more than 10,000 isoprene units are made by the rubber synthases [145–147].

The UPP synthase crystallizes as homodimer, and each monomer exhibits six parallel β-sheets that are surrounded by seven α-helices (Fig. 87.10a) [126, 148, 149]. In agreement with their unique structures, the ζ-prenyltransferases show no sequence homology to (*E*)-selective prenyltransferases and also lack the typical DDXXD motif [139, 140]. However, like the (*E*)-selective enzymes, they require a divalent metal ion for activity [150]. Two highly conserved motifs DGN(G,R)R and, ca. 40 amino acid residues downstream, FSXENXXR are present, and site-directed mutagenesis revealed that the aspartate residue in the first motif is crucial for enzyme activity [151]. Structural analysis confirmed its involvement in Mg^{2+} binding (Fig. 87.10b) that in turn binds the IPP elongation unit [152], while FPP binding proceeds through electrostatic interactions with several cationic residues (Fig. 87.10c) [152–155]. A hydrophobic cavity for binding of the polyprenyl side chain is located adjacent to the active site [148].

The stereochemistry of the (*Z*)-selective prenyl transfer was first investigated for the rubber biosynthesis by the Brazilian rubber tree (*Hevea brasiliensis*) in 1966 by Cornforth and coworkers. Feeding experiments with tritium-labeled MVA were consistent with the specific loss of the 2-*pro*-*S* hydrogen of IPP during chain elongation [156]. Cornforth et al. furthermore observed the specific removal of the 2-*pro*-*R* hydrogen from IPP in the biosynthesis of (*E*,*E*)-FPP (Sect. 4.1) [100]. These and similar observations [157, 158] resulted in the postulate of a strict correlation of the (*E*)-configuration in the product with a specific removal of the 2-*pro*-*R* hydrogen from IPP and vice versa. Later experiments on the (*Z*)-configured malloprenols demonstrated the abstraction of the 2-*pro*-*R* hydrogen from IPP during polyisoprenoid assembly [159–161], a result that contradicted the previously framed rule.

More recently, the complete stereochemical course of rubber biosynthesis in *H. brasiliensis* and *Parthenium argentatum* was investigated in detail. Diphosphate

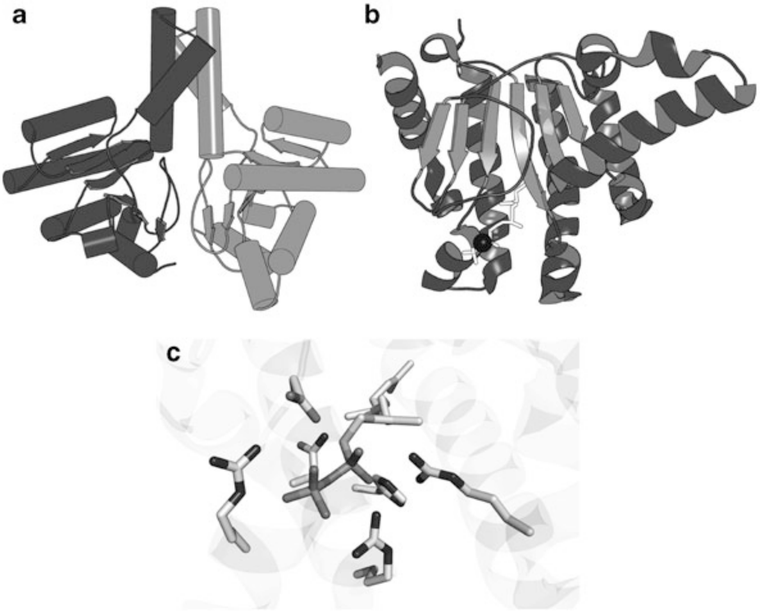
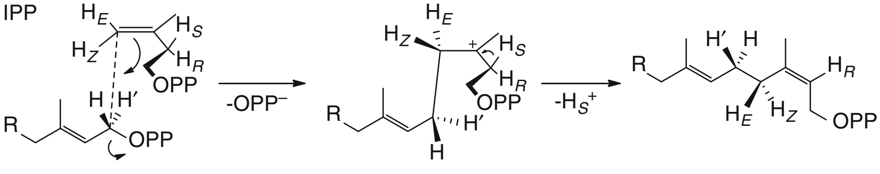


Fig. 87.10 Structures of (*Z*)-selective prenyltransferases (1X06). (**a**) Homodimeric structure of *E. coli* UPP synthase, (**b**) monomer in complex with Mg^{2+} and an unreactive FPP analogue, and (**c**) close-up on the allylic binding site



31 **33** **34**

Scheme 87.10 Stereochemical course of rubber biosynthesis

displacement at the growing chain occurs with inversion of the configuration at C1, IPP attacks the allylic diphosphate **31** from the *Si* face, and the 2-*pro*-*S* proton from IPP is removed (Scheme 87.10) [162]. This stereochemical course is in agreement with the reaction of UPP synthase that also proceeds with attack of IPP from the *Si* face and removal of the 2-*pro*-*S* proton [157, 163]. In contrast to the experiment with (*E*)-selective OctPP synthase (Sect. 4.1), incubation of FPP and 3-bromobut-3-enyl diphosphate with UPP synthase did not result in the capture of farnesol, suggesting that the first step of the reaction catalyzed by (*Z*)-selective prenyltransferases is a concerted DMAPP diphosphate cleavage/IPP attack to **33** without generation of an intermediate allyl cation [131].

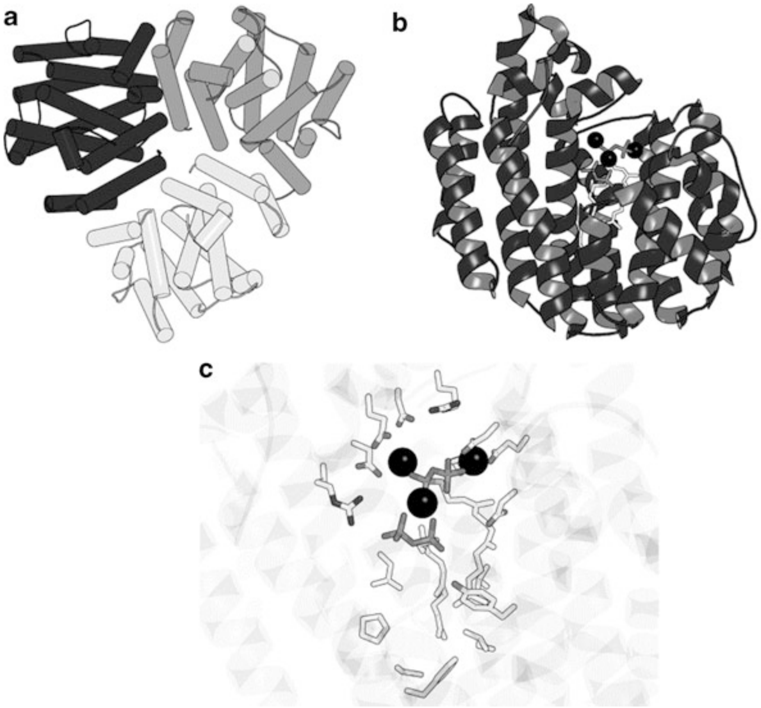
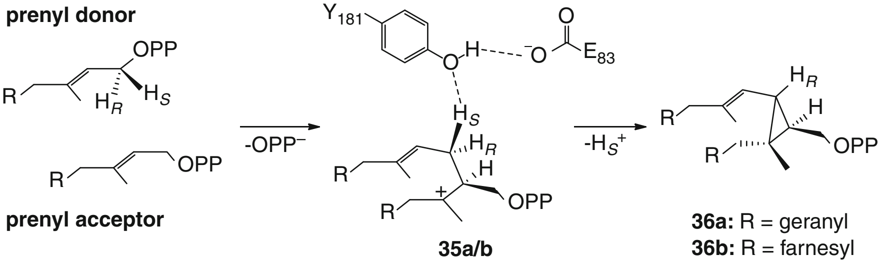


Fig. 87.11 Human squalene synthase (1EZF). (**a**) Homotrimeric structure, (**b**) monomer (Mg^{2+} and FPP superimposed from 2ZCP), and (**c**) close-up on the active site

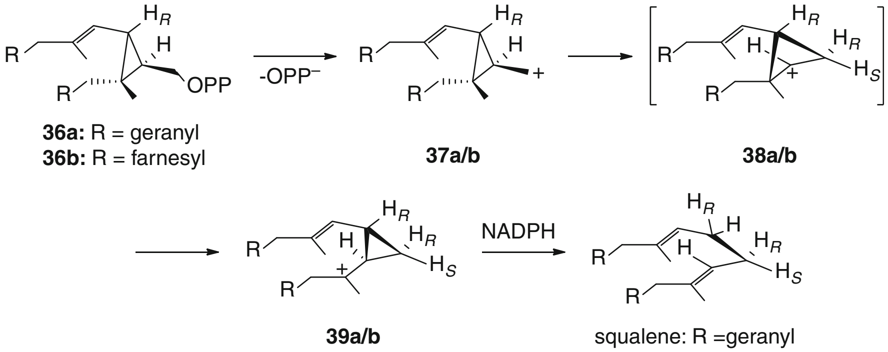
4.4 Biosynthesis of Squalene and Phytoene

In contrast to the head-to-tail connected polyisoprenoids, squalene (C_{30}) and phytoene (C_{40}) arise from a head-to-head condensation of two molecules FPP or GGPP, respectively. The squalene synthase requires divalent metal ions (Mg^{2+}, Mn^{2+}) as well as an NADPH cofactor. Superimposition of the crystal structure of human squalene synthase with avian FPP synthase reveals a close structural homology, although the sequence identity in the superimposed parts is low [164]. Due to their distinct functionality, the highly α-helical squalene synthases (Fig. 87.11) have been designated an own type of head-to-head prenyltransferases termed ε-class. The highly conserved DDXXD motifs in α-prenyltransferases are altered to DTLED and DYLED motifs in the human squalene synthase.

Two molecules of FPP, one acting as prenyl donor and one as prenyl acceptor, are the direct precursors for squalene [165]. The squalene synthase catalyzes the reductive dimerization of FPP under consumption of one equivalent NADPH via the stable intermediate presqualene diphosphate (**36a**, Scheme 87.11) [166–168]. Its relative and absolute configurations were established by degradation studies and total synthesis [169–172]. The formation of presqualene diphosphate (**36a**) proceeds with inversion of configuration at C1 of the prenyl donor and with attack of


prenyl donor
OPP
R
H_R
H_S
R
OPP
prenyl acceptor
-OPP⁻
Y181
O
H
O
E83
H_S
H_R
H
R
+
R
OPP
35a/b
-H_S⁺
H_R
H
R
R
OPP
36a: R = geranyl
36b: R = farnesyl

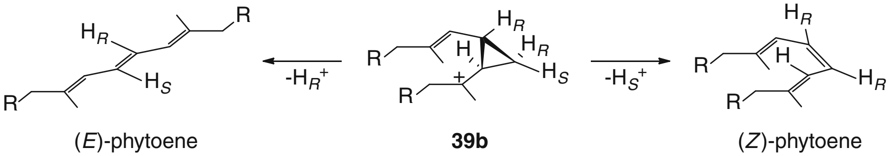
Scheme 87.11 Biosynthesis of presqualene diphosphate (**36a**) and prephytoene diphosphate (**36b**)


H_R
H
R
R
OPP
36a: R = geranyl
36b: R = farnesyl
-OPP⁻
H_R
H
R
+
R
37a/b
H_R
H
H_R
H_S
R
+
R
38a/b
H_R
H
H_R
H_S
R
+
R
39a/b
NADPH
H_R
H
H
H_R
H_S
R
R
squalene: R =geranyl

Scheme 87.12 Rearrangement of presqualene diphosphate to squalene

the olefinic double bond of the prenyl acceptor from the *Re* face to form the tertiary cationic intermediate **35a** [173]. The original 1-*pro-S* proton of the prenyl donor is subsequently removed in order to build the cyclopropyl ring of **36a** [174]. An active site for this first half-reaction was tentatively identified in the crystal structure of the human squalene synthase [164]. The prenyl acceptor and the prenyl donor bind each with two conserved aspartate residues of the DDXXD analogous motifs, in both cases via magnesium salt bridges. The prenyl chains of both FPP molecules fit into an adjacent hydrophobic cavity. A neighboring tyrosine together with a glutamate residue presumably acts as a general base, and two arginine residues located next to the binding site of the prenyl donor could stabilize the diphosphate leaving group [164]. The critical role of several of these residues was confirmed by site-directed mutagenesis [175].

The second step in the squalene biosynthesis is the reductive rearrangement of presqualene diphosphate (**36a**) to squalene. Initial diphosphate abstraction formally gives a primary cyclopropylcarbinyl cation **37a** that rearranges via a secondary cyclobutylium intermediate **38a** to the tertiary cyclopropylcarbinyl cation **39a** (Scheme 87.12) [176–178]. Hydride attack from NADPH at C3 terminates the squalene biosynthesis. Evidence for the existence of the tertiary



Scheme 87.13 Formation of (*E*)-phytoene and (*Z*)-phytoene (R = farnesyl)

cyclopropylcarbinyl cation intermediate was provided by isolation of its water adduct rillingol from incubation experiments of recombinant squalene synthase with FPP and NADPH [169, 179]. No such products derived by water attack to **37a** or **38a** have ever been detected, suggesting that the cyclopropylcarbinyl-cyclopropylcarbinyl rearrangement from presqualene diphosphate (**36**) to the tertiary cation **39a** is a rapid and concerted process rather than a stepwise mechanism [179]. This is also corroborated by the strict inversion of configuration at C1 of the original prenyl acceptor en route to squalene [100, 180]. The mechanism of enzyme catalysis in the second half-reaction is not well understood. A common TSRSF motif and additional highly conserved amino acids build a hydrophobic pocket that shields the intermediate carbocations effectively from capture with water [164]. In particular, the phenylalanine within this motif is crucial for activity by stabilization of the intermediate cationic species via cation-π-interactions [175].

The closely related phytoene synthase, also an ε-type enzyme, catalyzes the head-to-head condensation of two units GGPP to phytoene [181]. Its activity depends on divalent metal ions (Mg^{2+} or Mn^{2+}) and is in vitro strongly stimulated by addition of detergents that remove the water-insoluble product from the active site [181, 182]. Similar to the mechanism of squalene formation, the biosynthesis of phytoene proceeds via the intermediate prephytoene diphosphate (**36b**, Scheme 87.11) [173, 183]. Ionization by diphosphate abstraction to **37b** is followed by cyclopropylcarbinyl-cyclopropylcarbinyl rearrangement to the tertiary cationic intermediate **39b**. Its deprotonation can either proceed with specific abstraction of the 1-*pro-R* proton from the original prenyl acceptor to generate (*E*)-phytoene as in *Mycobacterium* sp., or with abstraction of the corresponding 1-*pro-S* hydrogen to establish (*Z*)-phytoene as in *Phycomyces blakesleeanus* (Scheme 87.13) [184].

5 Terpene Cyclases

Terpene synthases constitute a large class of enzymes that are able to convert just a handful of linear isoprenoid precursors, made from IPP and DMAPP by prenyltransferases, into a large diversity of thousands of terpenoids. As most of the enzyme products are of (poly)cyclic nature, these enzymes are often called terpene cyclases. Two types of terpene synthases can be mechanistically distinguished by their formation of a reactive carbocationic species. Class I terpene synthases contain a highly conserved DDXXD motif as also found in α-type prenyltransferases and perform substrate ionization via metal-triggered abstraction

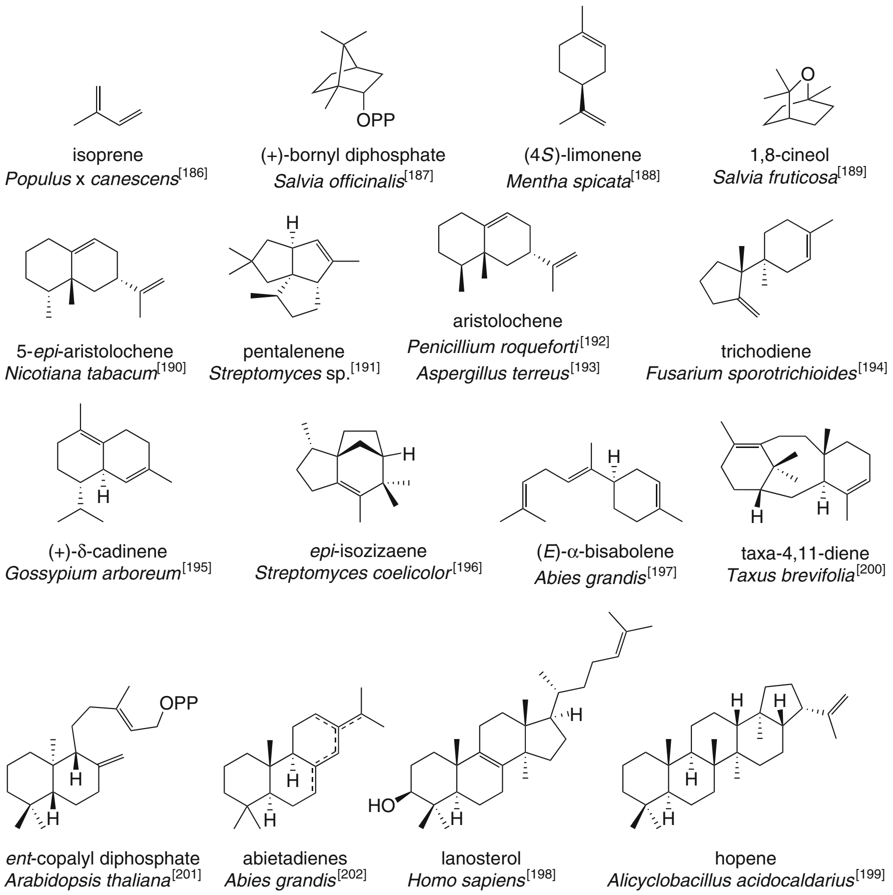

isoprene
Populus x canescens[186]
OPP
(+)-bornyl diphosphate
Salvia officinalis[187]
(4S)-limonene
Mentha spicata[188]
O
1,8-cineol
Salvia fruticosa[189]
5-epi-aristolochene
Nicotiana tabacum[190]
H
pentalenene
Streptomyces sp.[191]
aristolochene
Penicillium roqueforti[192]
Aspergillus terreus[193]
trichodiene
Fusarium sporotrichioides[194]
H
(+)-δ-cadinene
Gossypium arboreum[195]
H
epi-isozizaene
Streptomyces coelicolor[196]
H
(E)-α-bisabolene
Abies grandis[197]
H
H
taxa-4,11-diene
Taxus brevifolia[200]
OPP
H
H
ent-copalyl diphosphate
Arabidopsis thaliana[201]
H
H
abietadienes
Abies grandis[202]
H
HO
H
lanosterol
Homo sapiens[198]
H
H
H
H
hopene
Alicyclobacillus acidocaldarius[199]

Fig. 87.12 Products of structurally characterized terpene synthases

of a diphosphate group, whereas class II enzymes exhibit a highly conserved DXDD motif that induces reactivity by protonation of a carbon-carbon double bond or an epoxide function. The class I enzymes are typically involved in the biosynthesis of hemi-, mono-, and sesquiterpenes. In case of the linear triterpene precursors squalene or squalene epoxide that lack a diphosphate moiety, initiation of cyclization strictly requires a protonation step by a type II terpene cyclase. Both mechanisms can merge in bifunctional diterpene cyclases such as the abietadiene synthase from *Abies grandis* [185]. Several monofunctional type I terpene cyclases from all kinds of organisms [186–197] and a few type II enzymes such as the human lanosterol synthase (oxidosqualene cyclase, OSC) [198] and squalene/hopene cyclase (SHC) from *Alicyclobacillus acidocaldarius* [199] have been structurally characterized (Fig. 87.12), but the diterpene cyclases have eluded from structural characterization for a long time. Only recently the X-ray structures of the taxadiene synthase from *T. brevifolia* (type I) [200], the *ent*-copalyl diphosphate synthase

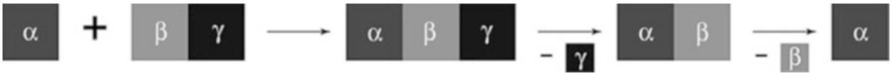


Fig. 87.13 Evolutionary model for terpene cyclases

from *A. thaliana* (type II) [201], and the abietadiene synthase from *A. grandis* [202] have been obtained. These structural information gave important insights into the mechanisms and evolutionary origin of the different classes of terpene synthases.

The structural data together with functional aspects investigated by site-directed mutageneses revealed that terpene cyclases are generally made up from three types of domains in various combinations. This includes α-domains as present in (*E*)-selective prenyltransferases as well as β- and γ-domains. Based on these findings, an evolutionary model has been proposed suggesting that the ancestral βγ-domain proteins such as SHC may have fused to an α-domain prenyltransferase to result in the early αβγ-proteins today seen in the plant diterpene synthases (Fig. 87.13) [124]. Loss of the γ-domain may have resulted in the αβ-domain species as found in plant hemi-, mono-, and sesquiterpene cyclases. The corresponding bacterial and fungal terpene cyclases only contain an α-domain and likely arose by β-domain loss from the plant enzymes.

5.1 Type I Terpene Synthases

Class I terpene synthases are highly α-helical proteins containing conserved aspartate-rich motifs. These motifs bind divalent metal ions (Mg^{2+}) via salt bridges to form a trinuclear metal cluster that complexes the polyisoprenoid for diphosphate abstraction. Like (*E*)-selective prenyltransferases, type I terpene synthases exhibit one conserved aspartate-rich DDXX(D,E) motif, but instead of a second DDXXD on the opposite side of the active center, a consensus sequence of (N,D)D(L,I,V)X (S,T)XXXE (also termed NSE/DTE triad) is found here. One exception is the (+)-δ-cadinene synthase from *Gossypium arboreum* that like prenyltransferases contains two DDXXE motifs [195].

This α-domain is common to all class I terpene synthases and is the only domain in bacterial and fungal class I enzymes such as the pentalenene synthase from *Streptomyces exfoliatus* or the trichodiene synthase from *Fusarium sporotrichioides* (Fig. 87.14a) [191, 194]. Plant hemi-, mono-, and sesquiterpene synthases exhibit a second helical β-domain that resembles a barrel structure. An example of this class is given by the (+)-δ-cadinene synthase from *G. arboreum* (Fig. 87.14b) [195]. In plant diterpene cyclases and in the exceptional case of the (*E*)-α-bisabolene synthase from *A. grandis* (Fig. 87.14c), a third helical γ-domain with a barrel-like structure is present [197, 200, 202]. The β- and γ-domains in the plant enzymes are nonfunctional but are required for correct enzyme folding.

The initial carbocation formation bears the potential for various reactions including intramolecular attack of an olefinic double bond, hydride migrations, or Wagner-Meerwein rearrangements. The transformation of a linear polyisoprenoid

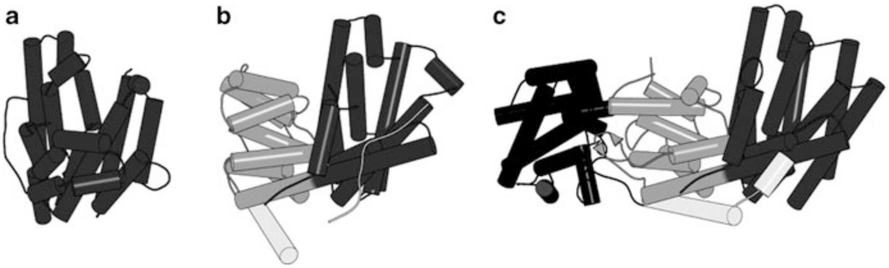
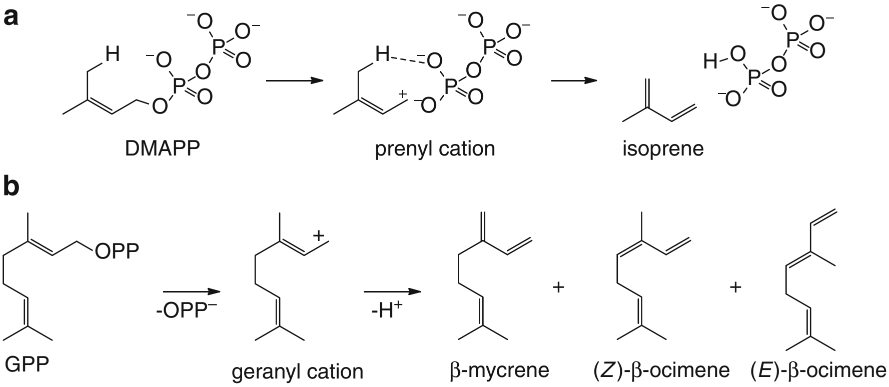


Fig. 87.14 Three different structure types for class I terpene synthases. (**a**) The α-type pentalenene synthase from *S. exfoliatus* (1PS1), (**b**) αβ-type (+)-δ-cadinene synthase from *G. arboreum* (3G4D), and (**c**) αβγ-type (*E*)-α-bisabolene synthase from *A. grandis* (3SAE)

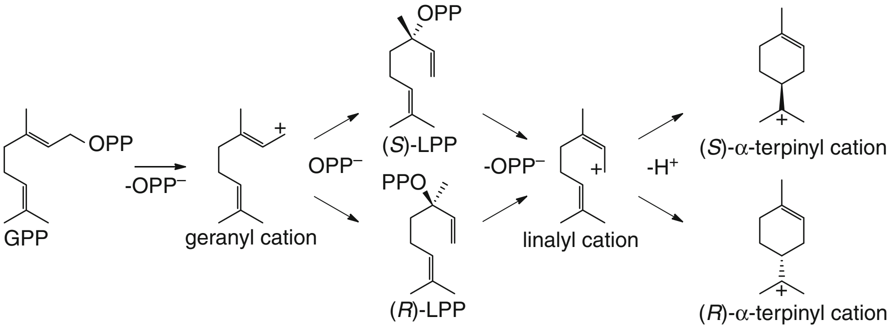
precursor by a terpene cyclase usually involves a multistep cascade of such elementary reactions to assemble a distinct terpenoid. The shape and electrostatic environment within the enzyme's active site determine a certain constitutional fold of the prenyl diphosphate substrate in which the structure of the cyclic product is prearranged [203]. Functionally important in terpene cyclizations are aromatic residues that stabilize the cationic intermediates through cation-π-interactions [204, 205]. Generally, the active site is constructed by hydrophobic rests to exclude water that otherwise may capture the intermediate cations en route to the target terpene. Termination mechanisms include deprotonations or the attack of nucleophiles, in most cases water.

Only about 25 naturally occurring hemiterpenoids are known reflecting the low structural variability within only one isoprenoid unit. Nevertheless, it is estimated that isoprene is released by plants with an annual rate of 500–750 Tg, making it one of the most abundant natural products [206]. The biosynthesis of isoprene is possibly the simplest biosynthetic transformation of any terpene synthase and performed just by ionization of DMAPP to the prenyl cation and proton elimination (Scheme 87.14a). The structure of the isoprene synthase from *Populus* × *canescens* in complex with a DMAPP analogue implicates a substrate-assisted deprotonation of the prenyl cation by the initially removed diphosphate moiety [186]. On the contrary, over 900 monoterpenoids have been described as the structure of GPP gives a lot more opportunities for product diversification. This is already reflected by the larger diversity of linear hydrocarbon products similarly arising by ionization of GPP to the geranyl cation followed by loss of a proton. These two steps potentially yield three alternative hydrocarbons: β-myrcene, (*E*)-β-ocimene, or (*Z*)-β-ocimene (Scheme 87.14b).

Besides a simple ionization-deprotonation process to the acyclic monoterpenes, GPP can also undergo cyclization reactions. The direct Markovnikov-type 1,6-cyclization of the (*E*)-configured geranyl cation by attack of the C6–C7 double bond at the cationic center is impossible since this would give a hypothetical (*E*)-cyclohexene product. Therefore, the isomerization of GPP via the geranyl cation to linalyl diphosphate (LPP) prior to cyclization is required (Scheme 87.15). The possible free rotation of the vinyl group into a *cisoid* conformation privileges


a
DMAPP
prenyl cation
isoprene
b
GPP
-OPP⁻
geranyl cation
-H⁺
β-mycrene
(Z)-β-ocimene
(E)-β-ocimene

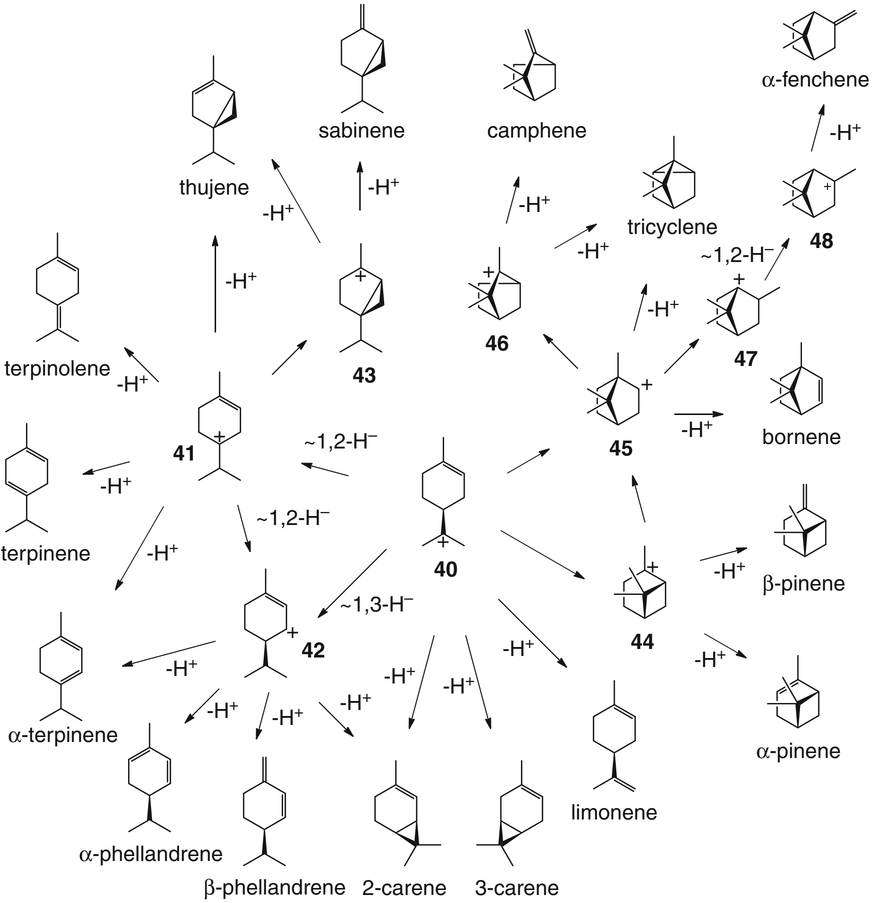
Scheme 87.14 Linear hemi- and monoterpenes


GPP
-OPP⁻
geranyl cation
OPP⁻
(S)-LPP
(R)-LPP
-OPP⁻
linalyl cation
-H⁺
(S)-α-terpinyl cation
(R)-α-terpinyl cation

Scheme 87.15 Cyclization of GPP

LPP to undergo the 1,6-ring closure to the (*S*)- or (*R*)-α-terpinyl cation (**40**). A hypothetic *anti*-Markovnikov 1,7-cyclization has never been reported as it would imply the formation of a less stable secondary cycloheptenyl cation.

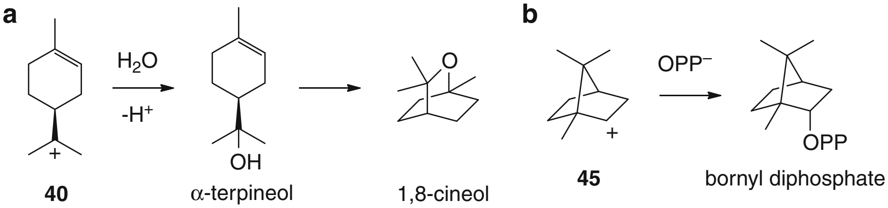
These cyclization reactions open up the possibility for the biosynthesis of a large diversity of (poly)cyclic terpenes. The α-terpinyl cation (**40**) is the key intermediate to a large variety of monoterpenes (Scheme 87.16). Simple deprotonation yields limonene, while 2- and 3-carene can arise by deprotonation with simultaneous construction of a cyclopropyl ring. A 1,2-hydride shift gives the homoterpinyl cation (**41**) as precursor for thujene, terpinolene, α-terpinene, and γ-terpinene. A second 1,2-hydride migration affords the allylic phellandryl cation (**42**), also directly accessable by a 1,3-hydride shift from **40**, that can be deprotonated to α-terpinene, 2-carene, α- and β-phellandrene. The 6,4-ring closure of **41** gives **43**, the precursor for thujene and sabinene, while 7,2-cyclization of **40** to the pinyl cation (**44**) results in the formation of α- and β-pinene after loss of a proton. The secondary bornyl cation (**45**) can be formed by a 7,3-ring closure of **40** or by Wagner-Meerwein rearrangement of **44** and is the precursor for bornene and


α-fenchene
sabinene
camphene
thujene
-H+
tricyclene
48
~1,2-H−
terpinolene
46
47
43
41
~1,2-H−
45
bornene
γ-terpinene
40
~1,3-H−
44
β-pinene
42
α-pinene
α-terpinene
limonene
α-phellandrene
β-phellandrene
2-carene
3-carene

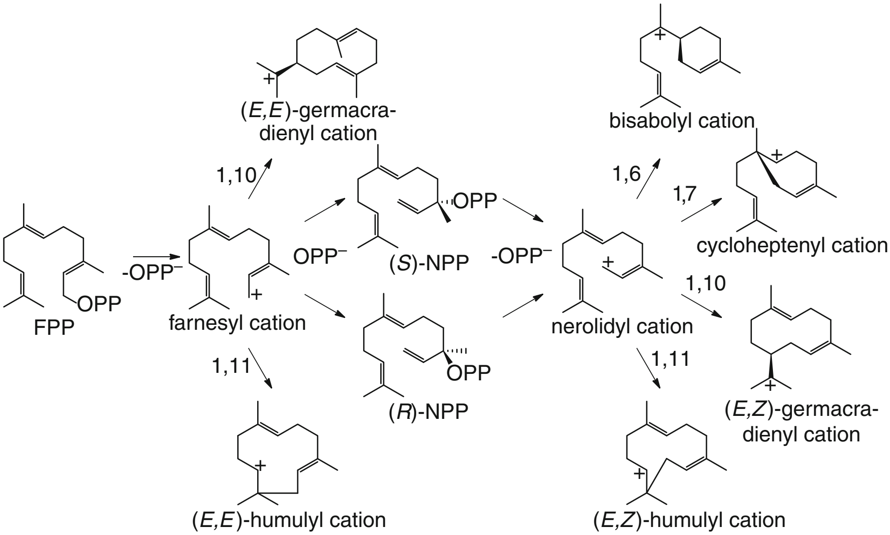
Scheme 87.16 Diversification of monoterpene hydrocarbon skeletons

tricyclene. Its rearrangement to the camphyl cation (**46**) yields camphene or tricyclene by deprotonation. α-Fenchene can arise from **45** by sequential 1,2-methyl migration to **47**, 1,2-hydride shift to **48**, and deprotonation. All reactions are shown starting from the (*S*)-enantiomer of **40** but are also possible from its optical antipode (*R*)-**40** to yield the respective enantiomeric terpene hydrocarbons. Only terpinolene, α-, and γ-terpinene are achiral and may arise from either of the two enantiomers of **40**.

In the last step of terpene biosynthesis, the carbocationic intermediates are in most cases deprotonated to form a terpene hydrocarbon. Alternative termination mechanisms include the attack of nucleophiles to yield a neutral product. The attack of water results in terpenoid alcohols as is exemplified by the reaction of **40** to α-terpineol (Scheme 87.17). Its subsequent reprotonation to a tertiary cation and intramolecular attack of the hydroxy group facilitates the formation of the achiral


a
H_2O
-H^+
+
40
OH
α-terpineol
O
1,8-cineol
b
+
45
OPP^-
OPP
bornyl diphosphate

Scheme 87.17 Alternative termination modes by (**a**) capture with water or (**b**) reattachment of diphosphate


FPP
OPP
-OPP^-
farnesyl cation
+
1,10
(E,E)-germacra-dienyl cation
1,11
(E,E)-humulyl cation
OPP⁻
(S)-NPP
OPP
(R)-NPP
OPP
-OPP⁻
nerolidyl cation
1,6
bisabolyl cation
1,7
cycloheptenyl cation
1,10
(E,Z)-germacra-dienyl cation
1,11
(E,Z)-humulyl cation

Scheme 87.18 Ring closing mechanisms of sesquiterpene biosynthesis

monoterpene 1,8-cineol that is the main product of the cineol synthase from *Salvia fruticosa* [189]. Reattachment of the initially abstracted diphosphate moiety to **45** is a known termination mechanism of the bornyl diphosphate synthase from *Salvia officinalis* [207].

Cyclization modes are even more diverse for the sesquiterpenes than for the discussed hemi- and monoterpenes (Scheme 87.18). *Transoid* sesquiterpene synthases catalyze the ionization of FPP to the farnesyl cation that can cyclize by attack of the C10–C11 double bond. A 1,10-ring closure affords the tertiary (*E*,*E*)-germacradienyl cation in a Markovnikov fashion. Alternatively, the less stable secondary (*E*,*E*)-humulyl cation can be furnished via an *anti*-Markovnikov 1,11-ring closure that is, in contrast to the 1,7-cyclization of the linalyl cation, a well-known reaction. *Cisoid* synthases encourage the reattack of the diphosphate at C3 of the farnesyl cation to give (*R*)- or (*S*)-nerolidyl diphosphate (NPP). As in LPP this allows for rotation of the newly formed vinyl group into a *cisoid* conformation.

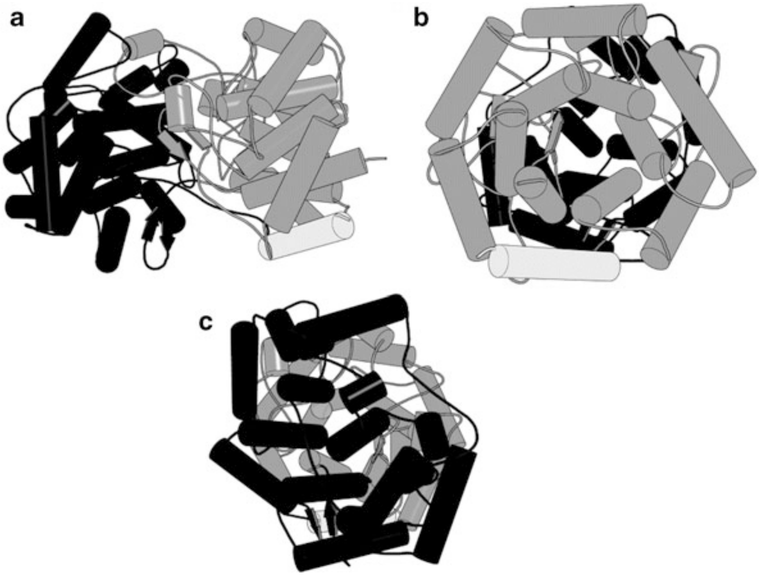
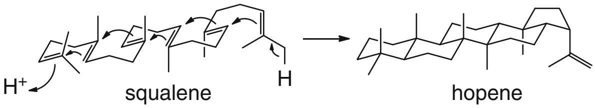


Fig. 87.15 Squalene/hopene cyclase from *A. acidocaldarius* (3SQC). (**a**) βγ-Modular structure, (**b**) top view on the β-domain showing its highly ordered α_6-α_6 barrel structure, and (**c**) top view on the irregular barrel structure of the γ-domain

Subsequent ionization yields the nerolidyl cation that can undergo a 1,6-ring closure to the bisabolyl cation or a 1,10-cyclization to the (*E*,*Z*)-germacradienyl cation, both in a Markovnikov fashion. *Anti*-Markovnikov additions may construct the cycloheptenyl cation via 1,7-cyclization or the (*E*,*Z*)-humulyl cation in a 1,11-ring closure. The 1,11-cyclizations do not generate a chiral center, whereas in all other processes, either of two enantiomeric cations may be formed. Similar as described for the monoterpene cyclases, but with much higher structural variability of the preceding steps, these primary cyclizations can be followed by further ring closures, Wagner-Meerwein rearrangements, and hydride migrations. The last cationic intermediate finally collapses to the neutral terpene product by deprotonation or attack of a nucleophile.

5.2 Type II Terpene Cyclases

Class II terpene cyclases are known for the cyclization of di-, sester-, and triterpenoids. Prominent examples of triterpene cyclases are the human lanosterol synthase (oxidosqualene cyclase, OSC) and the squalene/hopene cyclase (SHC) from *A. acidocaldarius* [1]. Both enzymes have a βγ-domain architecture (Fig. 87.15a). The β-domain exhibits a highly regular α_6-α_6 barrel structure with six inner and six outer helices surrounding a central cavity (Fig. 87.15b) [198, 199, 208]. The γ-domains may have originated from the β-domains by gene duplication



Scheme 87.19 Reaction of the squalene/hopene cyclase

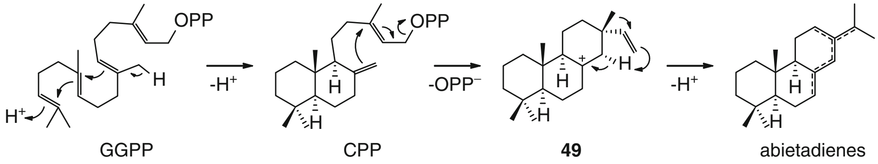
events as the γ-domain fold is similar to a β-domain structure, albeit less regular (Fig. 87.15c) [209]. This ancestral βγ-type of class II terpene cyclases shows multiple characteristic QW motifs with a consensus sequence of (K,R)(G,A)X_{2-3}(F,Y,W)(L,I,V)X_3QX_{2-5}GXW [210, 211]. The glutamine (Q) and tryptophane (W) residues within these motifs facilitate a stacked conformation at the intersection between two α-helices [212] and are implicated to stabilize the overall protein fold against thermal denaturation which is necessary because of the highly exergonic cyclization reactions (~200 kJ/mol) [199, 208]. Site-directed exchange of the tryptophane residues confirmed the stabilizing role for three of the eight QW motifs present in SHC [213].

Monofunctional type II diterpene synthases from plants such as the *ent*-copalyl diphosphate synthase from *A. thaliana* exhibit an αβγ-modular structure [201]. In this type of enzymes, the β-domain contains the conserved DXDD motif, but no DDXXD motif is present within the α-domain which coincides with the lack of type I activity.

The cyclization mechanism of type II terpene cyclases is exemplified by the reaction of the SHC (Scheme 87.19). Important insights into the reaction mechanisms have been obtained from structural data [199, 208]. One of the inner helices of the β-domain of SHC contains a conserved DxD(D,E) motif that is located in the central cavity at the interface between the β- and γ-domains. Its central aspartate residue D376 is polarized via hydrogen bonding to an adjacent histidine residue and protonates the double bond of the squalene substrate to initiate the reaction cascade. Conserved aromatic residues stabilize the intermediate cations by cation-π-interactions. A water molecule is a candidate to act as catalytic base, and this water may also account for the formation of the by-product hopanol. Another bridging water molecule connects D376 to a tyrosine residue and can restore the active site after catalysis by reprotonation of D376.

5.3 Bifunctional Type I and Type II Terpene Cyclases

The bifunctional terpene cyclases with type I and type II activity are restricted to fungal and plant diterpene cyclases. These enzymes like the monofunctional type I or type II plant enzymes exhibit the αβγ-domain architecture, but in contrast to the monofunctional terpene cyclases, both the α-domain and the β-domain contain the highly conserved DDXXD and DXDD motifs, respectively, thus establishing the catalytic activities for type I and type II cyclizations within one enzyme. A model system is the recently structurally characterized abietadiene synthase from *A. grandis* [202] that catalyzes first the class II conversion of



Scheme 87.20 Reaction of the abietadiene synthase

GGPP to copalyl diphosphate (CPP), followed by its transformation into abietadiene via **49** in a class I reaction (Scheme 87.20). Notably, the bifunctional plant terpene synthases are generally involved in the biosynthesis of a large superfamily of terpenes, the labdane-related diterpenoids [214].

6 Conclusions

As presented in this chapter, today, much is known about the process of terpene biosynthesis. The accumulated knowledge includes a detailed picture about the biosynthesis of the terpenoid monomers IPP and DMAPP either via the mevalonate or the DXP route and their interconversion by isomerases. Also, the stereochemical courses and enzyme mechanisms of all transformations have been largely elucidated. Especially the recently obtained structural data of prenyltransferases and various kinds of terpene synthases resulted in an evolutionary model that involves six domains (α, β, γ, δ, ε, and ζ) for the biosynthesis of linear polyisoprenoids from IPP and DMAPP and their subsequent transformation into (poly)cyclic terpenes. All these insights may open up new chances in controlling terpene biosynthesis, e.g., by directed evolution of terpene cyclases or domain swaps in multi-domain enzymes for the production of new terpenes, reconstitution of terpene biosynthetic pathways in heterologous hosts for production optimization, or targeted inhibition of pathways in pathogens for disease control.

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Biotransformation of Terpenoids and Steroids

88

Wang-Yu Tong

Contents

1 Introduction 2734
2 Properties, Classes and Functions of Terpenoids 2735
3 Properties, Classes and Functions of Steroids 2737
4 Terpenoid Metabolic Pathway 2740
4.1 IPP Biosynthesis 2741
4.2 IPP and Terpenoidogenesis 2744
4.3 Paclitaxel Biosynthesis 2744
5 Steroidal Metabolic Pathway 2746
5.1 Lanosterol and Cycloartenol Biosynthesis 2746
5.2 Steroidogenesis 2748
5.3 Steroid Hormone Biosynthesis 2748
5.4 Steroid Microbial Transformation and Degradation 2751
6 Conclusions and Perspectives 2757
References 2758

Abstract

Terpenoids, the largest family of natural products with more than 40,000 structures, refer to a large class of oxygen-containing terpene analogues that can be found in all classes of living things. Similar to terpenes, they are all derived from five-carbon isoprene units assembled and modified in different ways. Steroids, derived from terpenoid building block isopentenyl pyrophosphate, are a subclass of terpenoids that contain a characteristic arrangement of four cycloalkane rings joined to each other. The terpenoids in plant are widely recognized, conceptually and/or empirically, for their aromatic qualities

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and important roles in traditional herbal remedies, also in biomaterials and biofuels. The steroid hormones in animals are often drugs that not only increase protein synthesis in vivo but also control androgenic and virilizing properties. In this chapter, we will describe their properties and classes and functions, biosynthesis and biotransformation and degradation, as well as corresponding enzymes and genes.

Keywords

Terpenoid • terpenoid biosynthesis • terpenoidogenesis • steroid • steroidogenesis • steroid biotransformation and degradation

Abbreviations

CYP11A	Cytochrome P450, family 11, subfamily A
DHEA	Dehydroepiandrosterone
DMAPP	Dimethylallyl pyrophosphate
DOXP	1-Deoxy-D-xylulose 5-phosphate
EDCs	Endocrine-disrupting compounds
FPP	Farnesyl diphosphate
GGPP	Geranylgeranlyl diphosphate
GPP	Geranyl diphosphate
HMBDP	1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate
IPP	Isopentenyl pyrophosphate
MAD	Mevalonate-dependent
MEP	2-C-Methyl-D-erythritol-4-phosphate
MVA	Mevalonate
OCs	Oral contraceptives
RI	Refractive index
SREBP	Sterol regulatory element-binding proteins 1 and 2

1 Introduction

Over 40,000 different identified structures of terpenoids, or isoprenoids, constitute the largest family of natural products [1]. Terpeneoids can be widely found in plants, which are responsible for the scents, flavors, and even colors in many plants [2]. Well-known terpenoids include camphor, menthol, citral, cannabinoids, and so on.

Chemically speaking, terpenoids refer to oxygen-containing terpene analogues. Similar to terpenes, they are derived biosynthetically from isoprene units, $(C_5H_8)_n$. For the terpenoid building block synthesis, there are two biosynthetic pathways, the mevalonate pathway and the non-mevalonate pathway (the MEP/DOXP pathway). The non-mevalonate in the plastids gives rise to the formation of essential oil monoterpenes and linalyl acetate, some sesquiterpenes, diterpenes, and carotenoids and phytol. The mevalonate pathway in the cytosol is responsible for the formation of diverse terpenes, including triterpenes (steroids) and most sesquiterpenes. The biosynthesis of paclitaxel is one of the most important terpenoid synthetical pathways.

Steroids are a class of organic compounds that contain a characteristic arrangement of four cycloalkane rings joined to each other. All steroids are synthesized inside cells either from the sterols lanosterol (in animals and microorganisms) or from cycloartenol (in plants). Both lanosterol and cycloartenol are derived from the cyclization of the triterpene squalene (six isoprene units, 30C) [3–5]. In other words, squalene is the biochemical precursor to the whole family of steroids. Among all of the steroid biosynthesis, steroid hormone biosynthesis is the most concerned.

Hundreds of distinct steroids have been found in plants, animals, and microorganisms, respectively, for example, phytosterols and brassinosteroids in plant, ecdysteroids in insect, cholesterol, sex steroids, corticosteroids and anabolic steroids in vertebrate, and ergosterols in microorganism. Up to now, more than 40 steroid drugs have been marketed since the research and development to steroid drugs was triggered in 1950s [6, 7].

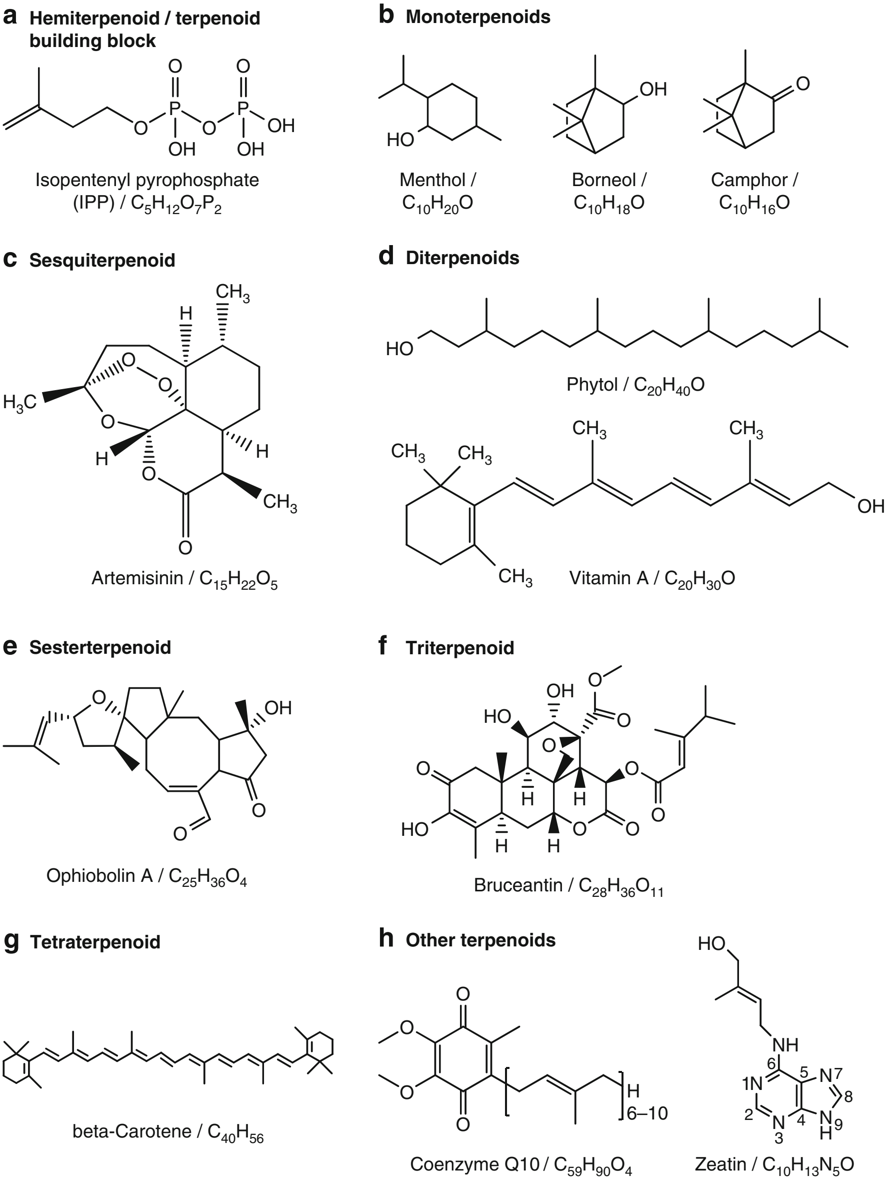
2 Properties, Classes and Functions of Terpenoids

Terpenes are hydrocarbon compounds through the condensation of several isoprene units. Terpenoids, in fact, are modified terpenes, wherein some methyl groups have been removed or replaced, often oxygen atoms added. Most of them are multicyclic structures which differ from one another not only in their basic carbon skeletons but also in functional groups. The structural unit of terpenoid building blocks is isopentenyl pyrophosphate (IPP).

Most terpenoids are optically active and have a bigger refractive index (RI) because of asymmetric carbon atoms in their molecules. Terpenoids commonly are hydrophobic, but their solubility can be improved through the addition of polar groups.

According to the number of isoprene units involved, terpenoids can be classified into [8] (1) hemiterpenoids, one isoprene unit (five carbon atoms) (Fig. 88.1a); (2) monoterpenoids, two isoprene units (10C) (Fig. 88.1b); (3) sesquiterpenoids, three isoprene units (15C) (Fig. 88.1c); (4) diterpenoids, four isoprene units (20C) (Fig. 88.1d); (5) sesterterpenoids, five isoprene units (25C) (Fig. 88.1e); (6) triterpenoids, six isoprene units (30C) (Fig. 88.1f); (7) tetraterpenoids, eight isoprene units (40C) (Fig. 88.1g); and (8) other terpenoids (5nC), isoprene unit condensation compounds (Fig. 88.1h).

Monoterpenoids (derived from geranyl diphosphate, GPP) are a subgroup of terpenoids consisting of two isoprene units (10C). Monoterpenoids may be acyclic or contain rings in their structures. Monoterpenoids usually exist as an oily liquid with distinctive aromas and flavors, such as essential oils, turpentine, and oleoresins of coniferous plants. Important examples of this class include menthol used as topical pain reliever, borneol as disinfectant or deodorant, and camphor as counterirritant, anesthetic, expectorant, antipruritic, etc.



a Hemiterpenoid / terpenoid building block

Isopentenyl pyrophosphate (IPP) / $C_5H_{12}O_7P_2$

b Monoterpenoids

Menthol / $C_{10}H_{20}O$

Borneol / $C_{10}H_{18}O$

Camphor / $C_{10}H_{16}O$

c Sesquiterpenoid

Artemisinin / $C_{15}H_{22}O_5$

d Diterpenoids

Phytol / $C_{20}H_{40}O$

Vitamin A / $C_{20}H_{30}O$

e Sesterterpenoid

Ophiobolin A / $C_{25}H_{36}O_4$

f Triterpenoid

Bruceantin / $C_{28}H_{36}O_{11}$

g Tetraterpenoid

beta-Carotene / $C_{40}H_{56}$

h Other terpenoids

Coenzyme Q10 / $C_{59}H_{90}O_4$

Zeatin / $C_{10}H_{13}N_5O$

Fig. 88.1 The chemical structure of representative terpenoids

Sesquiterpenoids (derived from farnesyl diphosphate, FPP) are a subclass of terpenoids consisting of three isoprene units (15C). Sesquiterpenes are often found in an oily liquid in plants, fungi, marine organisms, and *Streptomyces* species. Sesquiterpenoids usually have antiallergen and anti-inflammatory and allow plants to cope with various types of environmental stress. Artemisinin, isolated from traditional Chinese herb *Artemisia annua*, is the most known sesquiterpenoid for its strong antimalarial activity.

Diterpenoids refer to those compounds having a two consecutive terpenoid structure (20C), usually in crystal state. They can be classified into two subgroups: acyclic and cyclic diterpenoids, for example, phytol (a naturally linear diterpenoid used in preparation of vitamin E and K1) and vitamin A (monocyclo-diterpenoid rich in fish oil). The most important diterpenoids in terms of bioactivities are dicyclo- and tricycle-diterpenoids, such as paclitaxel (Taxol) (details please see in Sect. 4.3 of this chapter below) and its analog docetaxel (Taxotere) for various cancer treatment, and ginkgolides against the aggregation of platelet.

Sesterterpenoids refers to those terpenoids having 25C skeleton compounds. Up to now, only about 40 species are isolated and indentified from several species of plants, marine organisms, and insects. Phytotoxin ophiobolin A, a natural product inhibitor of calmodulin, is an example of sesterterpenoids.

Triterpenoids are essentially derived from the coupling of two sesquiterpene precursors (30C) and known as cancer chemopreventive, antiulcer, and antidiabetic agents; inhibitors of angiogenesis and eukaryotic DNA polymerase, and so on. For example, bruceantin is used as an antineoplastic agent.

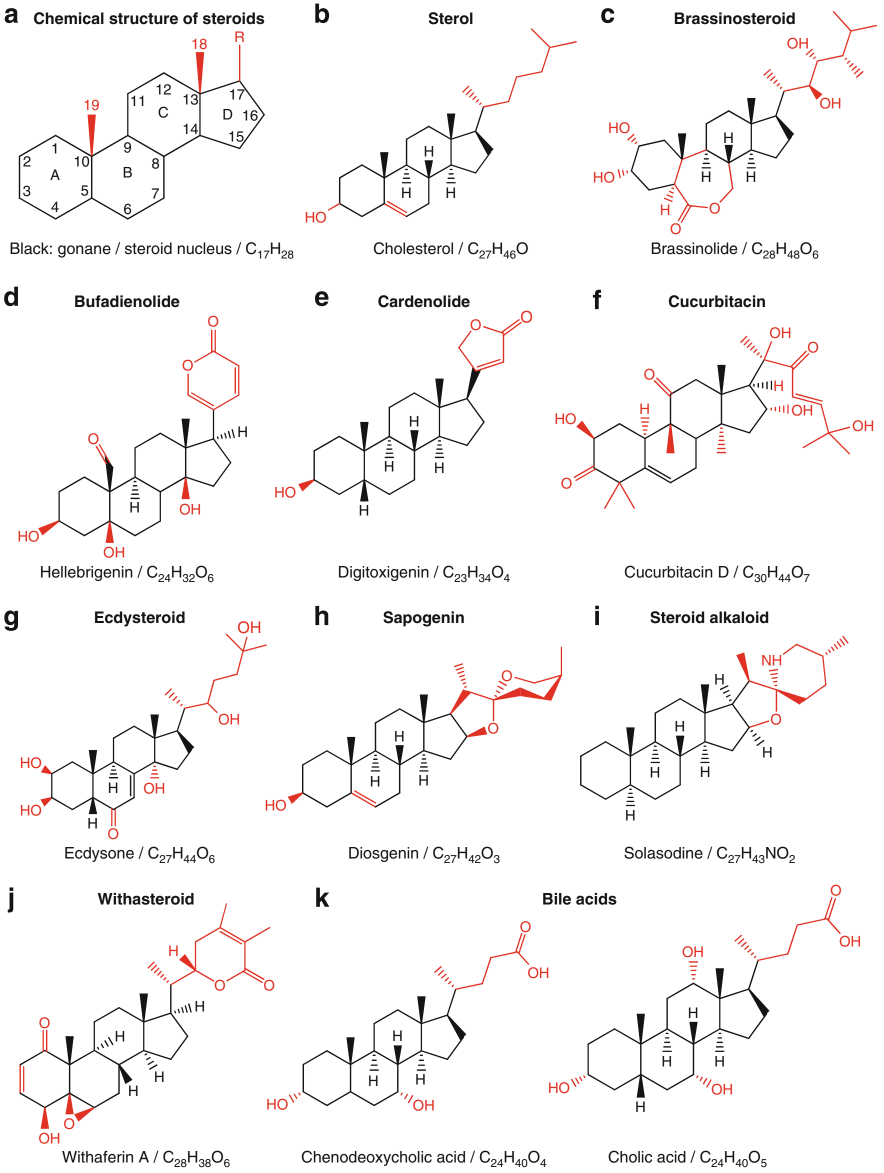
Tetraterpenoids contain 40 carbon atoms, derived essentially from the coupling of two diterpene precursors. Tetraterpenoids easily dissolve in nonpolar organic solvents. Often, they are called as tetraterpenoid pigments because of their conjugated polyene chromophores. The well-known tetraterpenoids are carotenoids (α-, β-, γ-carotene) that are naturally occurring in the chloroplasts and chromoplasts of plants as well as some other photosynthetic organisms like algae and some types of bacteria and fungi. Usually, animals cannot manufacture carotenoids, but can obtain carotenoids through their diets and employ them in various ways in metabolism.

Ubiquinones (UQ), often called coenzyme Q_{10}, are electron carriers in oxidative phosphorylation and photosynthesis, respectively. Ubiquinones consist of quinoid nucleus (derived from the shikimate pathway), 4-hydroxybenzoate (derived from chorismate or tyrosine), and terpenoid moiety. Zeatin, a phytohormone, is a member of the cytokinin family involved in various processes of growth and development in plants. Most cytokinins are adenine-type, where the hydrogen of amino group at C_6 position of adenine is replaced with an isoprenoid.

Note that meroterpenes refer to any organic compounds that have a partial terpenoid structure, such as neoaustin and preaustinoid A produced by *Penicillium* sp.

3 Properties, Classes and Functions of Steroids

Structurally speaking, steroids (note that gonane or steroid nucleus is the simplest steroid without any alkyl side chains) are terpenoid lipids characterized by a distinct carbon skeleton with four basal rings (A, B, C, and D), commonly arranged in



a Chemical structure of steroids

Black: gonane / steroid nucleus / $C_{17}H_{28}$

b Sterol

Cholesterol / $C_{27}H_{46}O$

c Brassinosteroid

Brassinolide / $C_{28}H_{48}O_6$

d Bufadienolide

Hellebrigenin / $C_{24}H_{32}O_6$

e Cardenolide

Digitoxigenin / $C_{23}H_{34}O_4$

f Cucurbitacin

Cucurbitacin D / $C_{30}H_{44}O_7$

g Ecdysteroid

Ecdysone / $C_{27}H_{44}O_6$

h Sapogenin

Diosgenin / $C_{27}H_{42}O_3$

i Steroid alkaloid

Solasodine / $C_{27}H_{43}NO_2$

j Withasteroid

Withaferin A / $C_{28}H_{38}O_6$

k Bile acids

Chenodeoxycholic acid / $C_{24}H_{40}O_4$

Cholic acid / $C_{24}H_{40}O_5$

Fig. 88.2 The chemical structure of representative steroids (*red colors* indicating the distinctive sites)

a 6-6-6-5 fashion, a methyl group(s) at the carbons C_{10} and C_{13}, and an alkyl side chain at carbon C_{17} (Fig. 88.2a). Further, they are individualized by changing the number of carbon atom, the configuration of the side chain and the functional groups attached to the rings.

Although many conventional names (e.g., cholesterol, cortisol, testosterone, etc.) are used in steroid molecules, the use of systematic names (based on IUPAC rules) is recommended. According to the nomenclature, a complete description of a steroid molecule should include the name of their parent compound (e.g., pregnane-21C, androstane-19C, or estrane-18C series) and the name, number, position, and orientation (α or β) of all functional groups.

According to their chemical structure and origin, the wide array of steroids may be classified into the following groups: (1) sterols (Fig. 88.2b), (2) brassinosteroids (Fig. 88.2c), (3) bufadienolides (Fig. 88.2d), (4) cardenolides (Fig. 88.2e), (5) cucurbitacins (Fig. 88.2f), (6) ecdysteroids (Fig. 88.2g), (7) sapogenins (Fig. 88.2h), (8) steroid alkaloids (Fig. 88.2i), (9) withasteroids (Fig. 88.2j), and (10) bile acids (Fig. 88.2k) [9].

Steroids are derived from the same squalene precursor and have an oxygen-dependent biosynthetic pathway beginning with the formation of the first intermediate, 2,3-oxidosqualene (for details, see Sect. 5.1). Sterols, also known as steroid alcohols, are a subclass of steroids and may be found either as free sterols, acylated, alkylated, sulfated, or linked to a glycoside moiety which can be itself acylated. Sterol biosynthesis is nearly ubiquitous among eukaryotes but almost completely absent in prokaryotes. As a result, the presence of diverse steranes (saturated four-cycle skeleton) in ancient rocks has been considered as evidence for over 2.7 billion years of eukaryotic evolution. Cholesterol is the most well-known sterol found in animal cell membranes (for establishing proper membrane permeability and fluidity) and red blood cells.

Brassinosteroids (derivatives of cholestane) are a subgroup of steroids with two vicinal diols (C_2, C_3 and C_{22}, C_{23}) and a six-keto group. Many of them may be considered as sterols. They are plant growth regulators with structural similarity to animal steroid hormones. Up to now, the highest biological activity of the known brassinosteroids is still brassinolide (a plant hormone promoting stem elongation and cell division) that was first isolated from *Brassica napus* pollen [10].

Bufadienolides are polyhydroxy 24C steroids with a pentadienolide ring at C_{17}. More than 250 compounds have been isolated and identified from plants and animals mostly in glycosides. They are important for their cardiotonic activity, insecticidal, and antimicrobial properties. Hellebrigenin is a cardiac steroid first isolated from the European toad in 1955.

Cardenolide structure is closely related to bufadienolides, but these 23C steroids possess a butenolide ring located at C_{17}. As potent cardiotonics, they are widely distributed in plants mostly as glycosides. Digitoxigenin is a typical example of cardenolides. Besides, they are either toxins or insect deterrents.

Cucurbitacins are the most oxygenated 30C triterpenoids with a dimethyl group at C_4 and three methyl groups at C_9, C_{13}, and C_{14}, respectively. About 50 species have been isolated and identified mainly from *Cucurbitaceae* species commonly in glycosides. Some of them are the most bitter substances known. Unlike common steroids, they are not methylated at C-10. Cucurbitacin D, a typical example of cucurbitacins, is a potential new therapeutic drug for treatment of T-cell leukemia.

Ecdysteroids have a common 7-en-6-one chromophore, sometimes a methyl group at C_{24} and several hydroxyl groups increasing their polarity. About 400 ecdysteroids have been isolated and identified from animals (arthropods) and plants. The most common ecdysteroid in plants is ecdysone that was isolated as an insect-molting hormone in 1954.

Sapogenins are oxygenated 27C steroids with a hydroxyl group in C_3. They are widely distributed in plants (occurring in over 90 plant families). They are important in that they can mimic or regulate steroid hormones. Diosgenin (for details, see Sect. 5.4) is the most important sapogenin that has been widely used as precursor to produce diverse steroid drugs.

Steroid alkaloids are a large group of molecules in which a nitrogen atom is integrated into a ring or as a substituent. Steroid alkaloids are only distributed in *Solanaceae*. Solasodine, the most common species in *Solanum*, is a poisonous glycoalkaloid chemical compound; however, their toxic properties can disappear through structural transformation during ripening. Solasodine, as a precursor, can be used for the production of contraceptive pills.

Withasteroids are 28C ergostane-type steroids with a 22,26-lactone and have a number of oxygenated functional groups (hydroxyl, ketone, especially 1-oxo-group, etc.). Over 200 species are isolated and identified predominantly from *Solanaceae*, some of them as glycosides. Withanolides have important pharmacological properties, such as antitumor, immunosuppressive, antimicrobial, ecdysteroid receptor antagonist functions, etc. Withaferin A is the first member of the withanolides first isolated from winter cherry (*Withania somnifera*), which has significant anticancer activity.

Bile acids, synthesized in the liver in mammals, are 24C steroids with a carboxyl group at C_{24} and up to three hydroxyl groups on the steroid nucleus, one being at C_3. Bile acids are important since they are essential in dietary lipid absorption and cholesterol catabolism. The most abundant bile acids in human bile are chenodeoxycholic acid (45%) and cholic acid (31%).

According to the origin of them, steroids can also be divided into (1) animal steroids, (2) plant steroids, and (3) microbial steroids.

The well-known group, animal steroids/vertebrate hormonal steroids, can further be divided into three subgroups, based on their physiological function or their tissue origin: (1) sexual hormones (andostenedione, 17β-estradiol, progesterone, testosterone, etc.), (2) corticosteroids (cortisol, aldosterone, etc.), and (3) neurosteroids (pregnenolone, allopregnanolone, dehydroepiandrosterone/DHEA, etc.).

Steroids, considered as modified triterpenes, are hydrophobic, or lipophilic, readily dissolved by liposoluble solvents. However, the solubility of steroids can be improved through the addition of polar groups.

Note that in popular language, the term "steroids" commonly refers to anabolic steroids that interact with androgen receptors to increase muscle and bone synthesis.

4 Terpenoid Metabolic Pathway

Terpenoid metabolism includes terpenoid biosynthesis (from simpler precursor acetyl-CoA to IPP), terpenoidogenesis (the biosynthesis of terpenoids from IPP and the metabolic interconversion among diverse terpenoids), and terpenoid degradation (β-oxidation pathway).

4.1 IPP Biosynthesis

There are two biosynthetic pathways, (1) the mevalonate pathway and (2) the MEP/DOXP pathway, responsible for the production of the terpenoid building blocks: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which serve as the basis for the biosynthesis of molecules used in processes as diverse as terpenoid/steroid/hormone synthesis, protein prenylation, protein anchoring, cell membrane maintenance, and N-glycosylation.

4.1.1 Mevalonate Pathway

The mevalonate (MVA) pathway or mevalonic acid pathway, sometimes called HMG-CoA reductase pathway or mevalonate-dependent (MAD) route or isoprenoid pathway, is an important cellular metabolic pathway occurring in the cytosol in all higher eukaryotes and many bacteria.

In this pathway (Fig. 88.3), acetyl-CoA (from TCA cycle) first undergoes condensation with another acetyl-CoA subunit via acetyl-CoA C-acetyltransferase to form acetoacetyl-CoA. Acetoacetyl-CoA then condenses with another acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA, which is reduced to mevalonate by NADPH in the cytosol (the rate-limiting step in cholesterol synthesis and a target of statins). Successively, mevalonate is converted to 5-phosphomevalonate, 5-diphosphomevalonate by kinases, and finally to IPP by pyrophosphomevalonate decarboxylase [11].

Several key enzymes in this pathway can be activated through DNA transcriptional regulation of SREBP (sterol regulatory element-binding proteins 1 and 2) [12]. Also, the regulation of this pathway can be achieved by controlling the rate of translation of the mRNA, as well as the level of reductase and kinase (degradation of reductase and phosphorylation).

The mevalonate pathway in cytosol is responsible for the formation of diverse terpenes, also including triterpenes, sterols, and most sesquiterpenes. A number of drugs target the mevalonate pathway, for example, statins used to decrease cholesterol levels and bisphosphonates used to treat various bone-degenerative diseases.

4.1.2 MEP/DOXP Pathway

The MEP/DOXP pathway or non-mevalonate pathway of isoprenoid biosynthesis operating in the plastids is an alternative metabolic pathway that leads to the formation of IPP and its isomer dimethylallyl pyrophosphate (DMAPP) [13]. Unlike the classical mevalonate pathway that is present in higher animals and fungi, the non-mevalonate pathway is often found in plants (note that in green plants, the MEP/DOXP and mevalonate pathways coexist in separate cellular compartments), apicomplexan protozoa and most bacteria.

In this pathway (Fig. 88.4), D-glyceraldehyde-3-phosphate initially condenses with pyruvate to yield 1-deoxy-D-xylulose-5-phosphate (DOXP) through

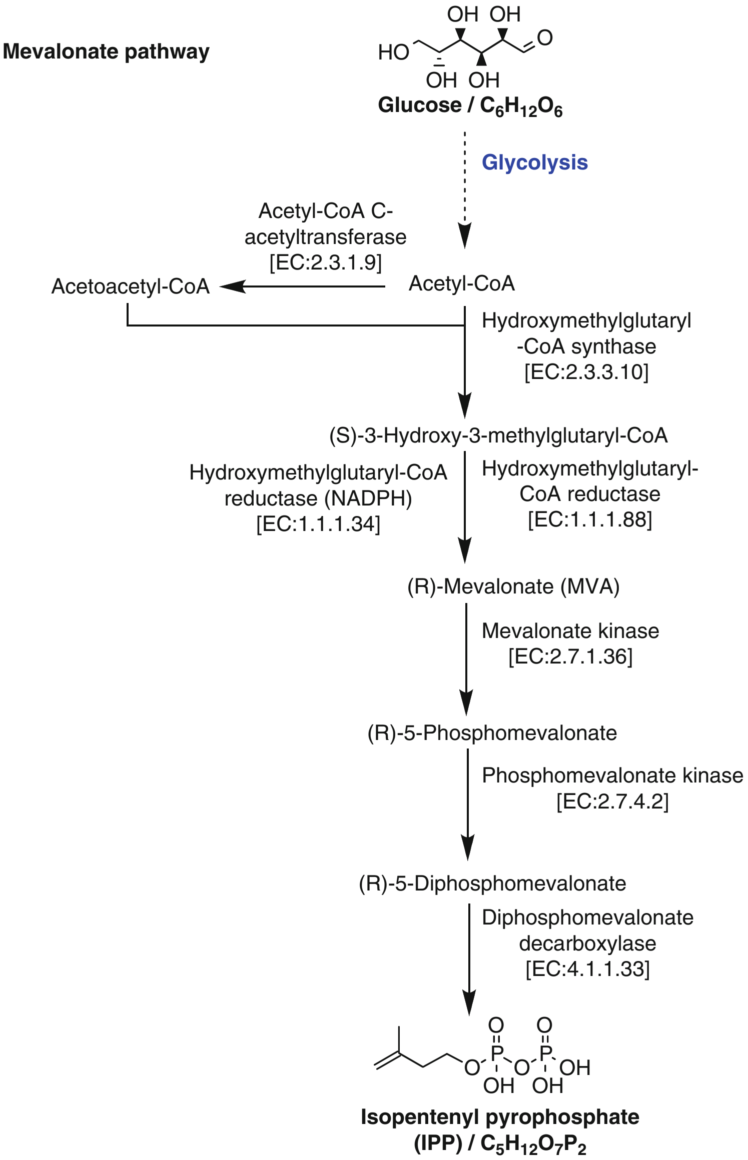

Mevalonate pathway
HO
OH OH
OH OH
O
Glucose / $C_6H_{12}O_6$
Glycolysis
Acetyl-CoA C-acetyltransferase [EC:2.3.1.9]
Acetoacetyl-CoA
Acetyl-CoA
Hydroxymethylglutaryl-CoA synthase [EC:2.3.3.10]
(S)-3-Hydroxy-3-methylglutaryl-CoA
Hydroxymethylglutaryl-CoA reductase (NADPH) [EC:1.1.1.34]
Hydroxymethylglutaryl-CoA reductase [EC:1.1.1.88]
(R)-Mevalonate (MVA)
Mevalonate kinase [EC:2.7.1.36]
(R)-5-Phosphomevalonate
Phosphomevalonate kinase [EC:2.7.4.2]
(R)-5-Diphosphomevalonate
Diphosphomevalonate decarboxylase [EC:4.1.1.33]
O O
O P O P OH
OH OH
Isopentenyl pyrophosphate (IPP) / $C_5H_{12}O_7P_2$

Fig. 88.3 The mevalonate pathway (*solid line arrows* indicating one-step reaction; *dash line arrows* indicating multiple-step reaction)

1-deoxy-D-xylulose-5-phosphate synthase. DOXP is then rearranged and reduced to form 2-C-methyl-D-erythritol-4-phosphate (MEP) by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (two rather different enzymes that share no sequence homology are able to catalyze this reaction) [14]. Successively, MEP is converted

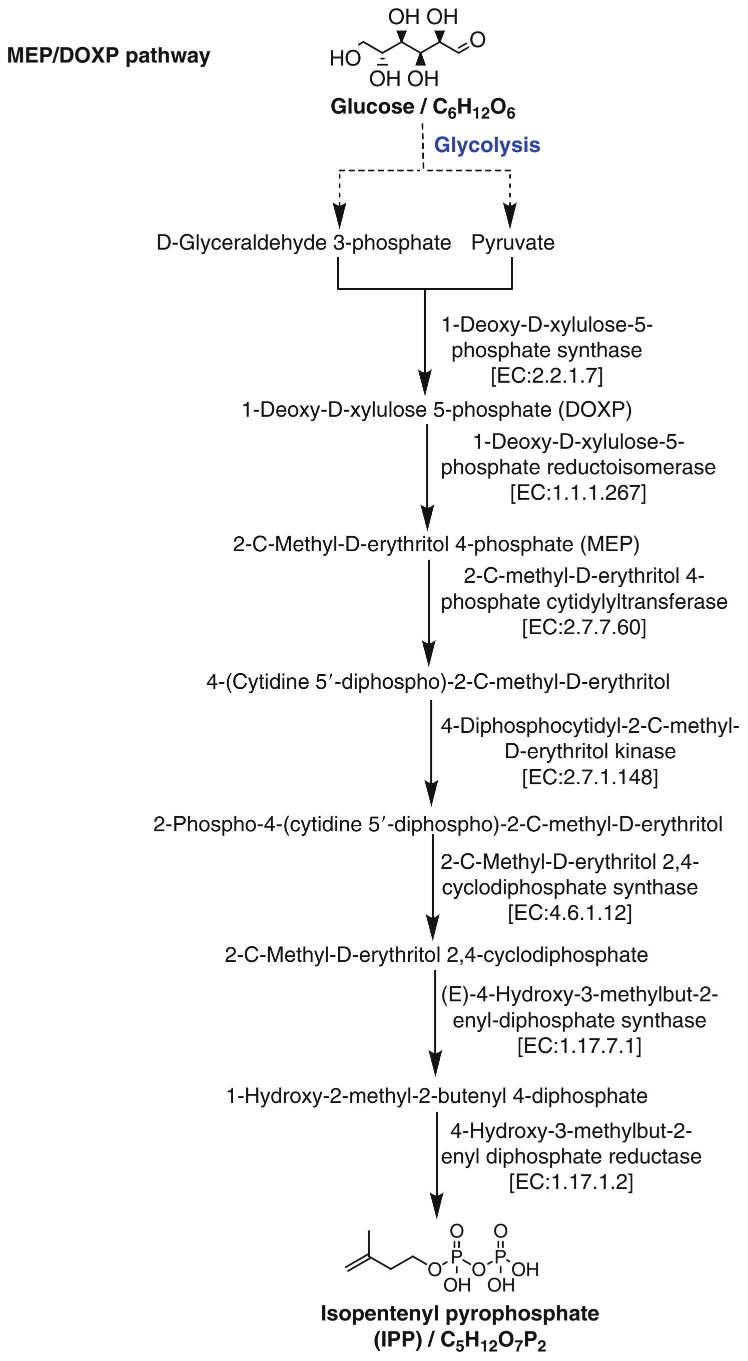

MEP/DOXP pathway
OH OH
HO
O
OH OH
Glucose / $C_6H_{12}O_6$
Glycolysis
D-Glyceraldehyde 3-phosphate
Pyruvate
1-Deoxy-D-xylulose-5-phosphate synthase [EC:2.2.1.7]
1-Deoxy-D-xylulose 5-phosphate (DOXP)
1-Deoxy-D-xylulose-5-phosphate reductoisomerase [EC:1.1.1.267]
2-C-Methyl-D-erythritol 4-phosphate (MEP)
2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase [EC:2.7.7.60]
4-(Cytidine 5′-diphospho)-2-C-methyl-D-erythritol
4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase [EC:2.7.1.148]
2-Phospho-4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol
2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase [EC:4.6.1.12]
2-C-Methyl-D-erythritol 2,4-cyclodiphosphate
(E)-4-Hydroxy-3-methylbut-2-enyl-diphosphate synthase [EC:1.17.7.1]
1-Hydroxy-2-methyl-2-butenyl 4-diphosphate
4-Hydroxy-3-methylbut-2-enyl diphosphate reductase [EC:1.17.1.2]
O O
O P O P OH
OH OH
Isopentenyl pyrophosphate (IPP) / $C_5H_{12}O_7P_2$

Fig. 88.4 The DEP/DOXP pathway

into 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol, 2-phospho-4-(cytidine5'-diphospho)-2-C-methyl-D-erythritol, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate, and 1-hydroxy-2-methyl-2-butenyl-4-diphosphate by corresponding enzymes. Finally, 1-hydroxy-2-methyl-2-butenyl-4-diphosphate is converted by 4-hydroxy-3-methylbut-2-enyl diphosphate reductase into a 5–6:1 ratio of IPP and DMAPP, and further adjusted to 7:3 by isopentenyl diphosphate isomerase for polyisoprenoid biosynthesis.

Usually, the MEP/DOXP pathway in plastids is responsible for the production of essential oil monoterpenes and linalyl acetate, some sesquiterpenes, diterpenes, and carotenoids and phytol. A number of drugs target the mevalonate pathway, for example, fosmidomycin specifically inhibits DOXP reductoisomerase, a committed enzyme in the non-mevalonate pathway. In addition, the intermediate 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP) is the natural activator for human $V\gamma9/V\delta2$ T cells, the major $\gamma\delta$ T-cell population in peripheral blood.

4.2 IPP and Terpenoidogenesis

IPP is an important product in both the mevalonate pathway and the DEP/DOXP pathway and the core precursor inside living organisms for the biosynthesis of diverse terpenoids and other products (Fig. 88.5). IPP can be converted further into DMAPP by the enzyme isopentenyl pyrophosphate *delta*-isomerase, GPP by farnesyl diphosphate synthase, geranylgeranlyl diphosphate (GGPP) by geranylgeranlyl diphosphate synthase, and FPP by farnesyl diphosphate synthase. From them, living organisms can generate diverse terpenoids including monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, carotenoids (C40), zeatins (C10), and other compounds.

For more details about the monoterpenoid, sesquiterpenoid, diterpenoid, triterpenoid, tetraterpenoid, and ubiquinone, zeatin, and other terpenoid-quinone biosynthesis, please log in at http://www.genome.jp/kegg/pathway/map/map00902.html, http://www.genome.jp/kegg/pathway/map/map00909.html, http://www.genome.jp/kegg/pathway/map/map00904.html, http://www.genome.jp/kegg/pathway/map/map00906.html, and http://www.genome.jp/kegg/pathway/map/map00900.html, respectively.

Almost all of terpenoids can be easily assimilated or digested by organisms via β-oxidation pathway similar to those found in fatty acid metabolism after ring cleavage of their structures, if any.

4.3 Paclitaxel Biosynthesis

Paclitaxel, commonly known as Taxol (the registered trademark), is a powerful diterpenoid anticancer drug with an annual market value of several billion dollars [15]. Taxol was first isolated and identified from the bark of Pacific yew, a slow-growing tree that is adapted to a forest shade environment in the Pacific Northwest of North America. Pathway of the biosynthesis of Taxol in *Taxus* has been primarily deciphered as in Fig. 88.6.

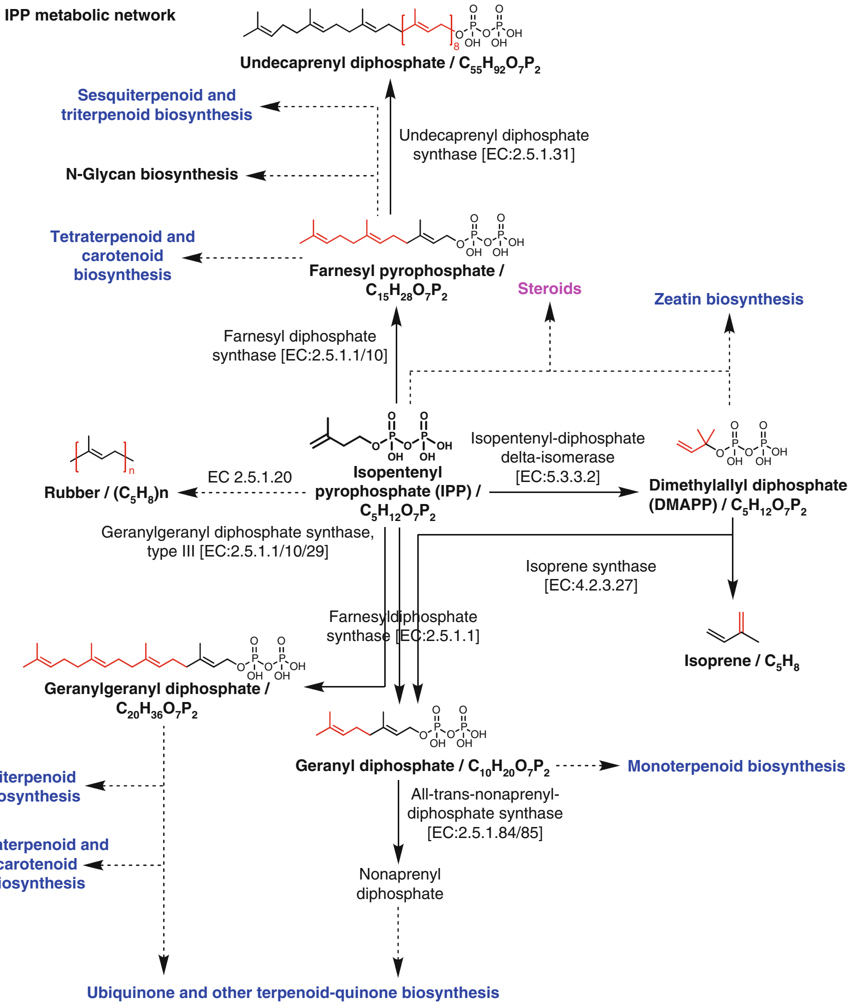

IPP metabolic network
8
O O
P P
O O OH
OH OH
Undecaprenyl diphosphate / $C_{55}H_{92}O_7P_2$
Sesquiterpenoid and triterpenoid biosynthesis
Undecaprenyl diphosphate synthase [EC:2.5.1.31]
N-Glycan biosynthesis
Tetraterpenoid and carotenoid biosynthesis
Farnesyl pyrophosphate / $C_{15}H_{28}O_7P_2$
Steroids
Zeatin biosynthesis
Farnesyl diphosphate synthase [EC:2.5.1.1/10]
n
Rubber / $(C_5H_8)n$
EC 2.5.1.20
Isopentenyl pyrophosphate (IPP) / $C_5H_{12}O_7P_2$
Isopentenyl-diphosphate delta-isomerase [EC:5.3.3.2]
Dimethylallyl diphosphate (DMAPP) / $C_5H_{12}O_7P_2$
Geranylgeranyl diphosphate synthase, type III [EC:2.5.1.1/10/29]
Isoprene synthase [EC:4.2.3.27]
Farnesyldiphosphate synthase [EC:2.5.1.1]
Isoprene / C_5H_8
Geranylgeranyl diphosphate / $C_{20}H_{36}O_7P_2$
Geranyl diphosphate / $C_{10}H_{20}O_7P_2$
Monoterpenoid biosynthesis
Diterpenoid biosynthesis
All-trans-nonaprenyl-diphosphate synthase [EC:2.5.1.84/85]
Tetraterpenoid and carotenoid biosynthesis
Nonaprenyl diphosphate
Ubiquinone and other terpenoid-quinone biosynthesis

Fig. 88.5 IPP and terpenoidogenesis

The biosynthesis of Taxol starts with GGPP as precursor, followed by a series of 19 predicted pathway-specific enzymatic steps. Formation of the first pathway-specific intermediate taxa-4(5),11(12)-diene is catalyzed by a diterpene taxa-4(5),11(12)-diene synthase (EC 4.2.3.17). Then taxa-4(5),11(12)-diene is hydroxylated by several P450 enzymes and further functionalized by a group of acyl and aroyl transferases to yield a putative intermediate with seven alcohol or ester groups, followed by oxidation to introduce a ketone group and form the characteristic oxetane ring of baccatin III. Phenylalanine aminomutase, baccatin III C13-phenylpropanoyl CoA-transferase, and N-benzoyl transferase are involved in the following steps to form the aromatic side chain of Taxol.

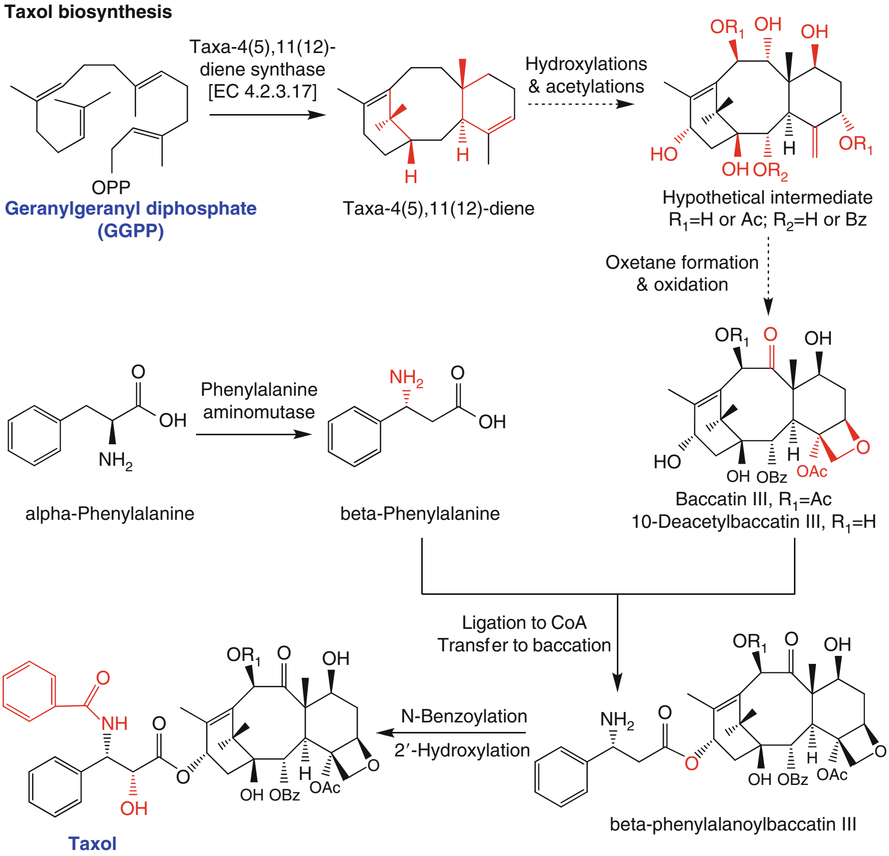

Taxol biosynthesis
Taxa-4(5),11(12)-diene synthase [EC 4.2.3.17]
OPP
Geranylgeranyl diphosphate (GGPP)
H
H
Taxa-4(5),11(12)-diene
Hydroxylations & acetylations
OR1 OH OH
HO
OH OR2
H
OR1
Hypothetical intermediate
R1=H or Ac; R2=H or Bz
Oxetane formation & oxidation
OR1 O OH
HO
OH OBz
H
OAc
O
Baccatin III, R1=Ac
10-Deacetylbaccatin III, R1=H
O
OH
NH2
alpha-Phenylalanine
Phenylalanine aminomutase
NH2
O
OH
beta-Phenylalanine
Ligation to CoA
Transfer to baccation
NH2
O
O
OR1 O OH
OH OBz
H
OAc
O
beta-phenylalanoylbaccatin III
N-Benzoylation
2′-Hydroxylation
O
NH
O
OH
O
OR1 O OH
OH OBz
H
OAc
O
Taxol

Fig. 88.6 Pathway of Taxol biosynthesis in *Taxus*

5 Steroidal Metabolic Pathway

Despite their relative simplicity in chemical structure, steroid metabolism is important for the regulation of their cellular and physiological actions. Steroid metabolism involves in three main levels: (1) steroid biosynthesis (from simpler precursors to steroids lanosterol and cycloartenol), (2) steroidogenesis (the metabolic interconversion among diverse steroids), and (3) steroid degradation.

5.1 Lanosterol and Cycloartenol Biosynthesis

Lanosterol and cycloartenol are two tetracyclic triterpenoids found in animals and plants, respectively, from which all steroids are derived. Triterpenoid squalene is the first precursor in the biosynthesis of phytostanols and phytosterols in plants and

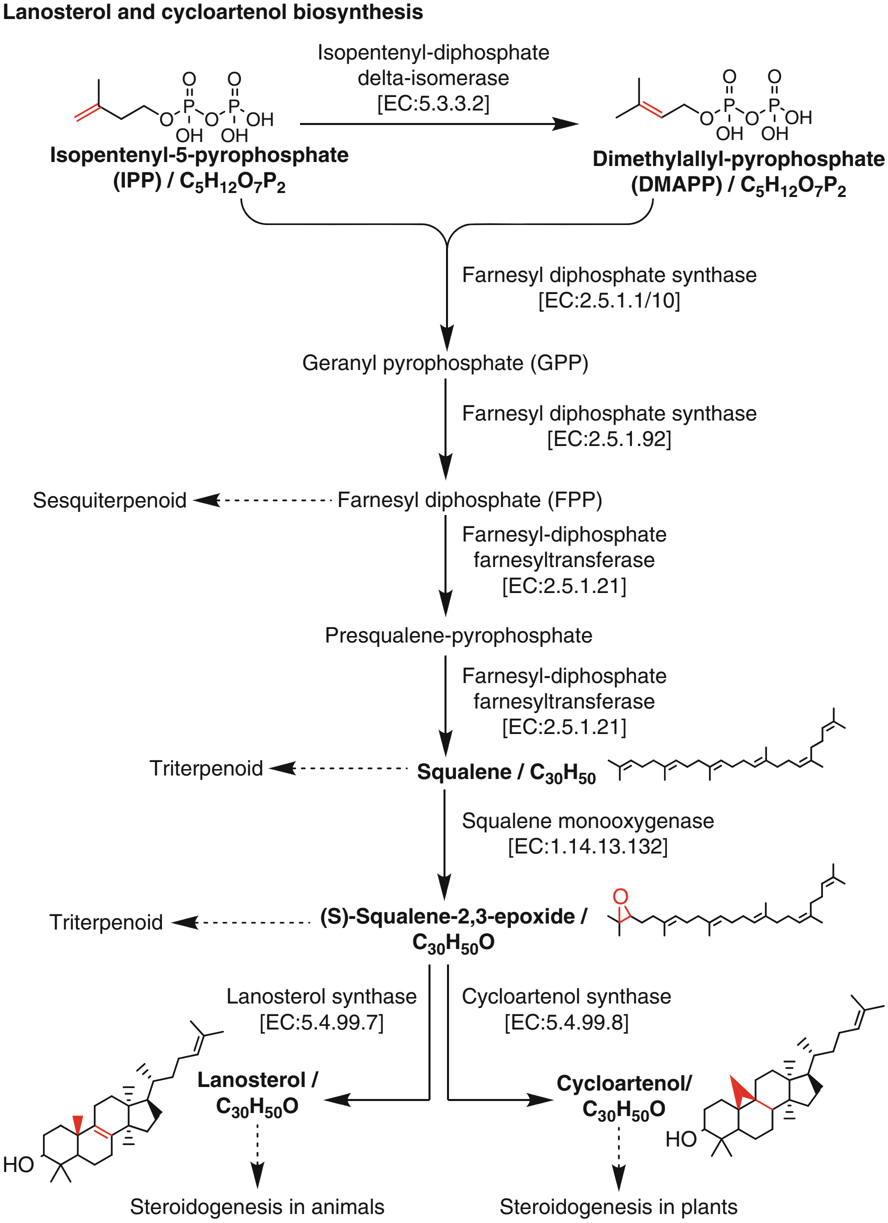

Lanosterol and cycloartenol biosynthesis
Isopentenyl-diphosphate delta-isomerase [EC:5.3.3.2]
O O P P O O OH OH OH
Isopentenyl-5-pyrophosphate (IPP) / $C_5H_{12}O_7P_2$
Dimethylallyl-pyrophosphate (DMAPP) / $C_5H_{12}O_7P_2$
Farnesyl diphosphate synthase [EC:2.5.1.1/10]
Geranyl pyrophosphate (GPP)
Farnesyl diphosphate synthase [EC:2.5.1.92]
Sesquiterpenoid
Farnesyl diphosphate (FPP)
Farnesyl-diphosphate farnesyltransferase [EC:2.5.1.21]
Presqualene-pyrophosphate
Farnesyl-diphosphate farnesyltransferase [EC:2.5.1.21]
Triterpenoid
Squalene / $C_{30}H_{50}$
Squalene monooxygenase [EC:1.14.13.132]
Triterpenoid
(S)-Squalene-2,3-epoxide / $C_{30}H_{50}O$
O
Lanosterol synthase [EC:5.4.99.7]
Cycloartenol synthase [EC:5.4.99.8]
Lanosterol / $C_{30}H_{50}O$
Cycloartenol/ $C_{30}H_{50}O$
HO
HO
Steroidogenesis in animals
Steroidogenesis in plants

Fig. 88.7 The process of steroid biosynthesis

photosynthetic organisms. The identity and distribution of phytostanols and phytosterols is characteristic of a plant species. The biosynthesis of steroids lanosterol and cycloartenol is represented in Fig. 88.7.

In the biosynthesis of steroids lanosterol and cycloartenol, the building blocks DMAPP and IPP are first joined through farnesyl diphosphate synthase to form geranyl pyrophosphate (GPP) and then to form FPP, presqualene pyrophosphate, squalene, and (S)-squalene-2,3-epoxide by corresponding enzymes, sequentially. The product (S)-squalene-2,3-epoxide is used to synthesize the sterols lanosterol (in animals) and cycloartenol (in plants) by lanosterol synthase and cycloartenol synthase, respectively [3]. Further, lanosterol and cycloartenol are converted into other steroids through steroidogenesis.

5.2 Steroidogenesis

Steroidogenesis refers to the biological process by which steroids are generated from one steroid to another, often from cholesterol in animals. The pathways of steroidogenesis are often different in different species. The representative pathways of steroidogenesis in animals and plants are shown in Fig. 88.8.

For more details about the bile acid (sterol), brassinosteroid, and steroid hormone biosynthesis, please log in at http://www.genome.jp/kegg-bin/show_pathway?map00120, http://www.genome.jp/kegg-bin/show_pathway?map00905 and http://www.genome.jp/kegg/pathway/map/map00140.html, respectively.

5.3 Steroid Hormone Biosynthesis

Steroid hormones refer to are a class of biologically active compounds that are commonly derived from cholesterol in vertebrates. In this biosynthesis, the cholesterol side-chain cleavage enzyme cytochrome P450, family 11, subfamily A (CYP11A) catalyzes oxidation of cholesterol (27C) to form the first 21C steroid pregnenolone, which is then converted by a bifunctional enzyme complex 3*beta*-hydroxy-*delta*5-steroid dehydrogenase/steroid *delta*-isomerase [EC:1.1.1.145/5.3.3.1] to the gestagen hormone, progesterone. Pregnenolone and progesterone are the starting compounds for the three groups of steroid hormones: 21C steroids of glucocorticoids and mineralocorticoids, 19C steroids of androgens, and 18C steroids of estrogens.

As shown in Fig. 88.9, (1) progesterone can be converted by hydroxylations at C_{21} and C_{11} to corticosterone, which is further modified by hydroxylation and redox reaction at C_{18} to produce aldosterone (a mineralocorticoid). Cortisol, the main glucocorticoid, is produced from pregnenolone with 17*alpha*-hydroxypregnenolone, 17*alpha*-hydroxyprogesterone, and 11-deoxycortisol as intermediates. (2) Male hormone testosterone can be produced from 17*alpha*-hydroxypregnenolone with dehydroepiandrosterone and androstenedione as intermediates. (3) Female hormones estradiol can be produced from testosterone by oxidative removal of the C_{19} methyl group and subsequent aromatization of ring A.

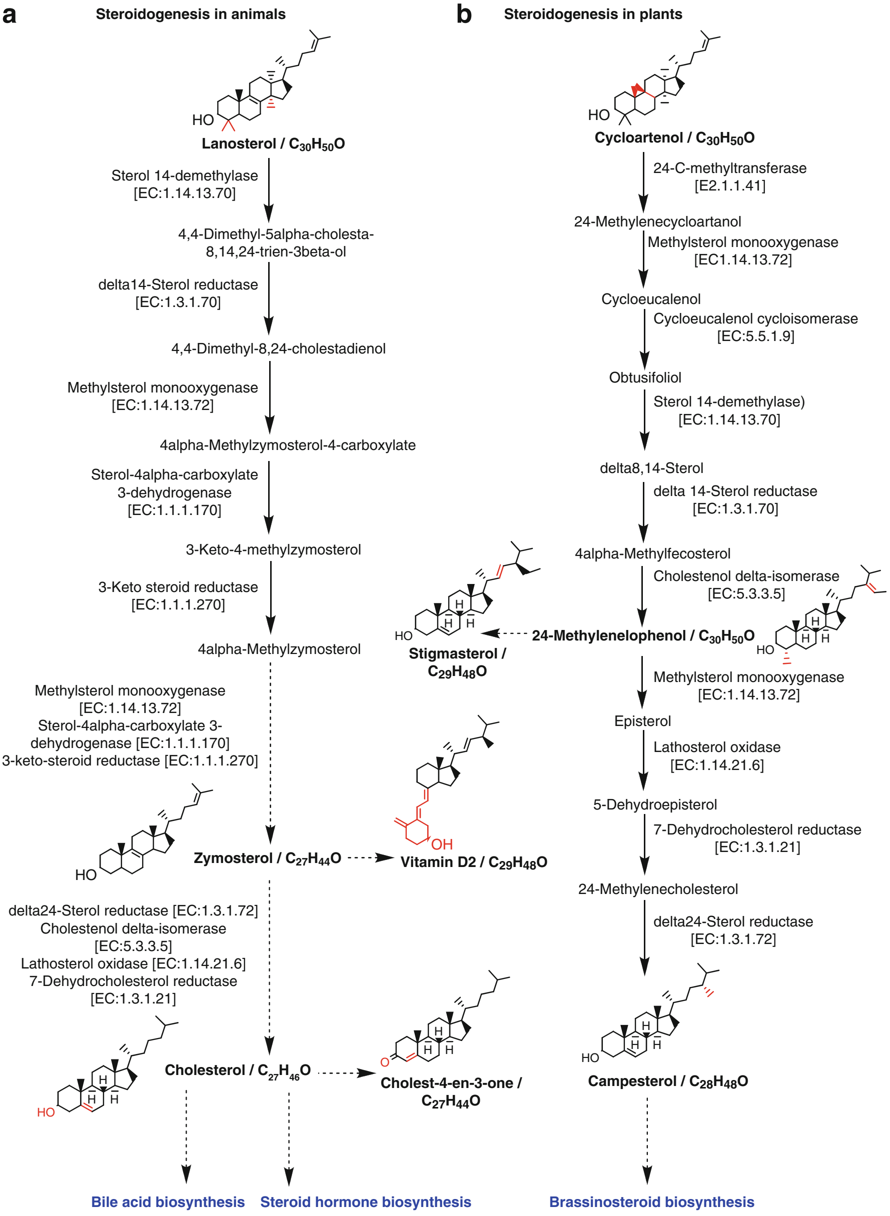

a
Steroidogenesis in animals
HO
Lanosterol / $C_{30}H_{50}O$
Sterol 14-demethylase [EC:1.14.13.70]
4,4-Dimethyl-5alpha-cholesta-8,14,24-trien-3beta-ol
delta14-Sterol reductase [EC:1.3.1.70]
4,4-Dimethyl-8,24-cholestadienol
Methylsterol monooxygenase [EC:1.14.13.72]
4alpha-Methylzymosterol-4-carboxylate
Sterol-4alpha-carboxylate 3-dehydrogenase [EC:1.1.1.170]
3-Keto-4-methylzymosterol
3-Keto steroid reductase [EC:1.1.1.270]
4alpha-Methylzymosterol
Methylsterol monooxygenase [EC:1.14.13.72] Sterol-4alpha-carboxylate 3-dehydrogenase [EC:1.1.1.170] 3-keto-steroid reductase [EC:1.1.1.270]
HO
Zymosterol / $C_{27}H_{44}O$
OH
Vitamin D2 / $C_{29}H_{48}O$
delta24-Sterol reductase [EC:1.3.1.72] Cholestenol delta-isomerase [EC:5.3.3.5] Lathosterol oxidase [EC:1.14.21.6] 7-Dehydrocholesterol reductase [EC:1.3.1.21]
H
H
H
HO
Cholesterol / $C_{27}H_{46}O$
O
H
H
H
Cholest-4-en-3-one / $C_{27}H_{44}O$
Bile acid biosynthesis
Steroid hormone biosynthesis
b
Steroidogenesis in plants
HO
Cycloartenol / $C_{30}H_{50}O$
24-C-methyltransferase [E2.1.1.41]
24-Methylenecycloartanol
Methylsterol monooxygenase [EC1.14.13.72]
Cycloeucalenol
Cycloeucalenol cycloisomerase [EC:5.5.1.9]
Obtusifoliol
Sterol 14-demethylase) [EC:1.14.13.70]
delta8,14-Sterol
delta 14-Sterol reductase [EC:1.3.1.70]
4alpha-Methylfecosterol
Cholestenol delta-isomerase [EC:5.3.3.5]
H
H
H
HO
Stigmasterol / $C_{29}H_{48}O$
24-Methylenelophenol / $C_{30}H_{50}O$
H
H
H
HO
Methylsterol monooxygenase [EC:1.14.13.72]
Episterol
Lathosterol oxidase [EC:1.14.21.6]
5-Dehydroepisterol
7-Dehydrocholesterol reductase [EC:1.3.1.21]
24-Methylenecholesterol
delta24-Sterol reductase [EC:1.3.1.72]
H
H
H
HO
Campesterol / $C_{28}H_{48}O$
Brassinosteroid biosynthesis

Fig. 88.8 The representative pathways of steroidogenesis in animals and plants

Steroid hormones can also be grouped into five groups by the receptors to which they bind: glucocorticoids, mineralocorticoids, androgens, estrogens, and progestogens.

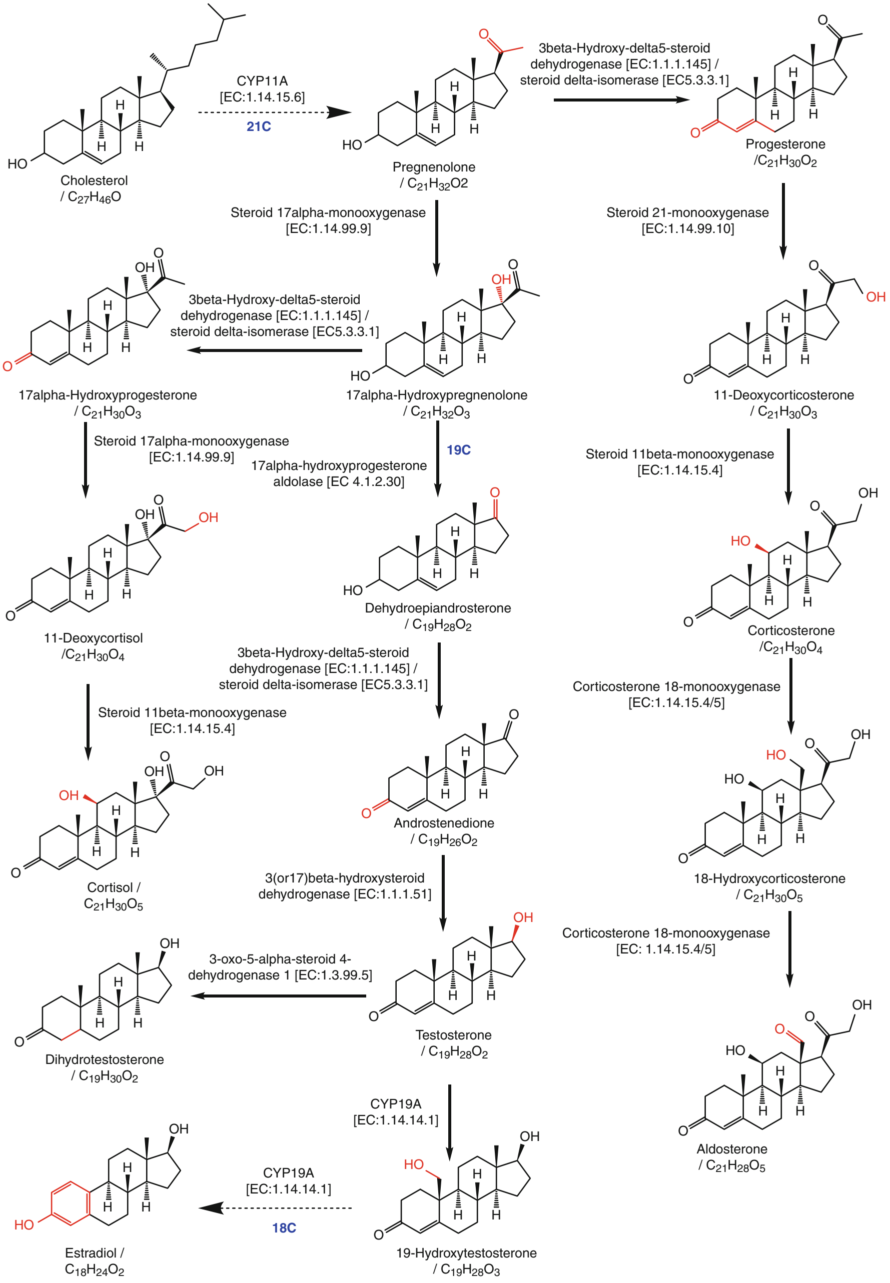

Cholesterol
/ $C_{27}H_{46}O$
CYP11A
[EC:1.14.15.6]
21C
Pregnenolone
/ $C_{21}H_{32}O2$
3beta-Hydroxy-delta5-steroid
dehydrogenase [EC:1.1.1.145] /
steroid delta-isomerase [EC5.3.3.1]
Progesterone
/$C_{21}H_{30}O_2$
Steroid 17alpha-monooxygenase
[EC:1.14.99.9]
Steroid 21-monooxygenase
[EC:1.14.99.10]
3beta-Hydroxy-delta5-steroid
dehydrogenase [EC:1.1.1.145] /
steroid delta-isomerase [EC5.3.3.1]
17alpha-Hydroxyprogesterone
/ $C_{21}H_{30}O_3$
17alpha-Hydroxypregnenolone
/ $C_{21}H_{32}O_3$
11-Deoxycorticosterone
/ $C_{21}H_{30}O_3$
Steroid 17alpha-monooxygenase
[EC:1.14.99.9]
17alpha-hydroxyprogesterone
aldolase [EC 4.1.2.30]
19C
Steroid 11beta-monooxygenase
[EC:1.14.15.4]
11-Deoxycortisol
/$C_{21}H_{30}O_4$
Dehydroepiandrosterone
/ $C_{19}H_{28}O_2$
Corticosterone
/$C_{21}H_{30}O_4$
3beta-Hydroxy-delta5-steroid
dehydrogenase [EC:1.1.1.145] /
steroid delta-isomerase [EC5.3.3.1]
Corticosterone 18-monooxygenase
[EC:1.14.15.4/5]
Steroid 11beta-monooxygenase
[EC:1.14.15.4]
Androstenedione
/ $C_{19}H_{26}O_2$
Cortisol /
$C_{21}H_{30}O_5$
18-Hydroxycorticosterone
/ $C_{21}H_{30}O_5$
3(or17)beta-hydroxysteroid
dehydrogenase [EC:1.1.1.51]
Corticosterone 18-monooxygenase
[EC: 1.14.15.4/5]
3-oxo-5-alpha-steroid 4-
dehydrogenase 1 [EC:1.3.99.5]
Dihydrotestosterone
/ $C_{19}H_{30}O_2$
Testosterone
/ $C_{19}H_{28}O_2$
Aldosterone
/ $C_{21}H_{28}O_5$
CYP19A
[EC:1.14.14.1]
CYP19A
[EC:1.14.14.1]
18C
Estradiol /
$C_{18}H_{24}O_2$
19-Hydroxytestosterone
/ $C_{19}H_{28}O_3$

Fig. 88.9 Steroid hormone biosynthesis

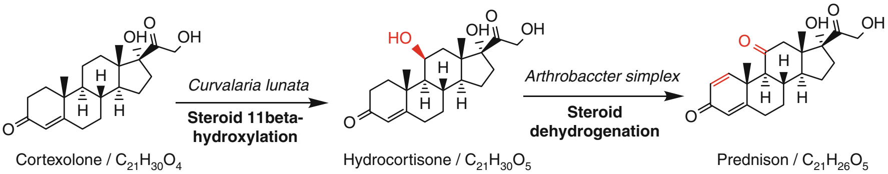

Curvalaria lunata
Steroid 11beta-hydroxylation
Arthrobaccter simplex
Steroid dehydrogenation
Cortexolone / $C_{21}H_{30}O_4$
Hydrocortisone / $C_{21}H_{30}O_5$
Prednison / $C_{21}H_{26}O_5$

Fig. 88.10 Steroid hydroxylation and dehydrogenation in steroidal drug production

5.4 Steroid Microbial Transformation and Degradation

Diosgenin is the primary precursor for the commercial production of cortisone, oral contraceptives (OCs), and many other steroid drugs. Unluckily, both natural and synthetic steroid drugs are powerful endocrine-disrupting compounds (EDCs) which can cause reproductive toxicity and affect cellular development in mammals and are generally regarded as a serious contributor to water pollution. Hence, research interests of many scientists focus on the biotransformation and degradation of steroid compounds.

5.4.1 Steroid Microbial Transformation

Many microbial transformations of steroids have been reported since 1950s, including steroid dehydrogenation/reduction, hydroxylation, esterification, halogenation, methoxylation, isomerization, and acylation at all carbon atoms of the steroid nucleus but C_{10} and C_{13} [6]. The microbial hydroxylation and dehydrogenation have become the most successful examples of large-scale industrial processes with such advantages as higher specificity, higher conversion rate, more moderate reaction conditions, and lower chemical pollution over the chemical synthesis in the production of steroid drugs (Fig. 88.10) [16, 17].

To further find new steroid microbial reactions for steroid drug production, *Streptomyces virginiae* IBL-14, a powerful degradation strain for steroid compounds including estrone, cholesterol, progesterone, isotestosterone, dihydrotestosterone, and hydrocortisone, was isolated by utilizing diosgenin as the sole carbon source [18]. Two new microbial transformations of diosgenin (6-methoxylation and 25-hydroxylation) were found. Also, nine new compounds, including isonuatigenone, 1-dehydroisonuatigenone, nuatigenone, 1-dehydronuatigenone, 26-acetyl-nuatigenone, 6-methoxy-6-dehydrodiosgenone, 6-methoxy-6-dehydroisonuatigenone, 6-methoxy-6-dehydronuatigenone, and 6-dimethoxy-7α-hydroxyldiosgenone were isolated and identified (Fig. 88.11).

5.4.2 Steroid Microbial Degradation

The degradation of steroidal compounds was first presented by Talalay in 1952 [19]. And the best-studied pathway is the metacleavage pathway from the A-ring of testosterone by *Comamonas testosteroni* TA441, a common bacterial degradation

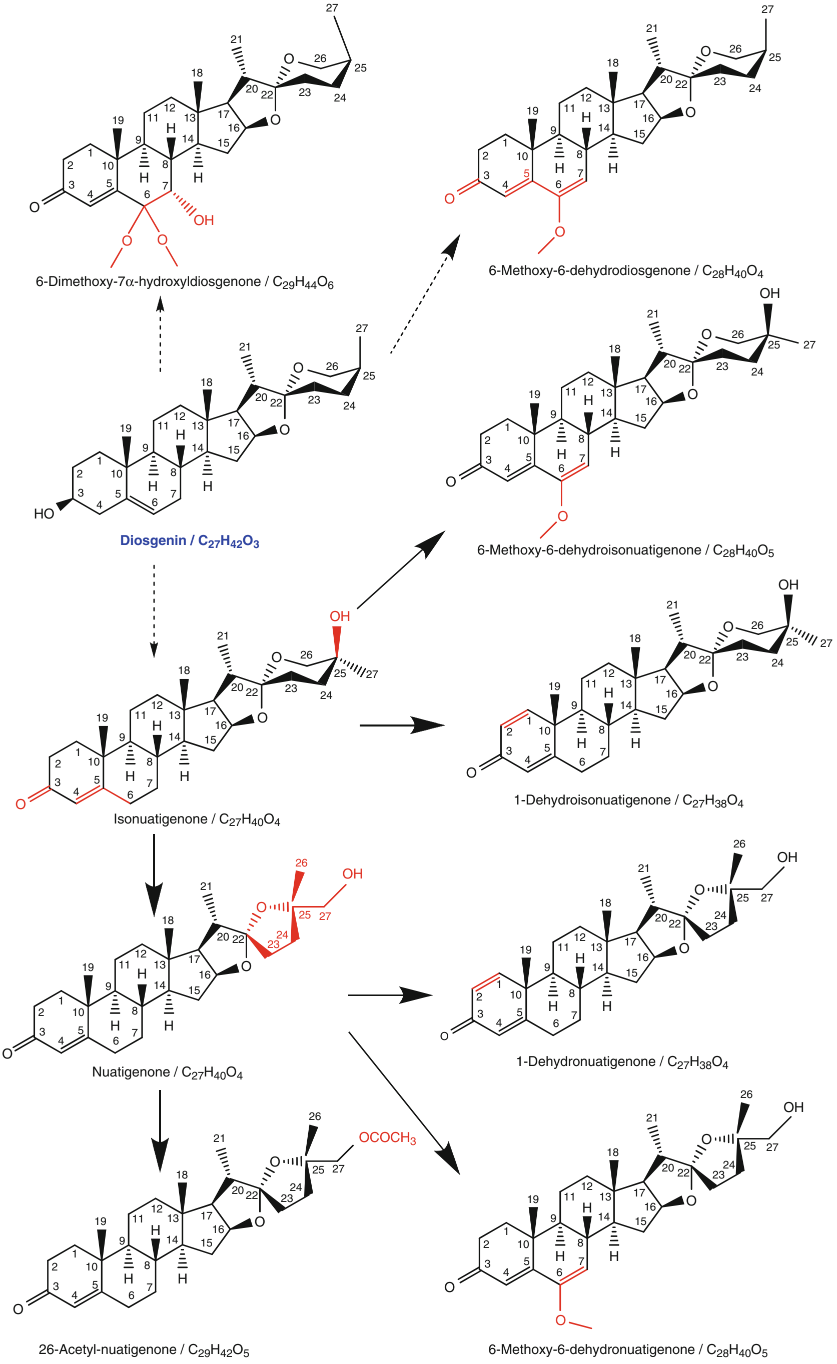

6-Dimethoxy-7α-hydroxyldiosgenone / $C_{29}H_{44}O_6$
6-Methoxy-6-dehydrodiosgenone / $C_{28}H_{40}O_4$
Diosgenin / $C_{27}H_{42}O_3$
6-Methoxy-6-dehydroisonuatigenone / $C_{28}H_{40}O_5$
Isonuatigenone / $C_{27}H_{40}O_4$
1-Dehydroisonuatigenone / $C_{27}H_{38}O_4$
Nuatigenone / $C_{27}H_{40}O_4$
1-Dehydronuatigenone / $C_{27}H_{38}O_4$
26-Acetyl-nuatigenone / $C_{29}H_{42}O_5$
6-Methoxy-6-dehydronuatigenone / $C_{28}H_{40}O_5$
OCOCH$_3$

Fig. 88.11 New metabolic products from diosgenin by *Streptomyces virginiae* IBL-14

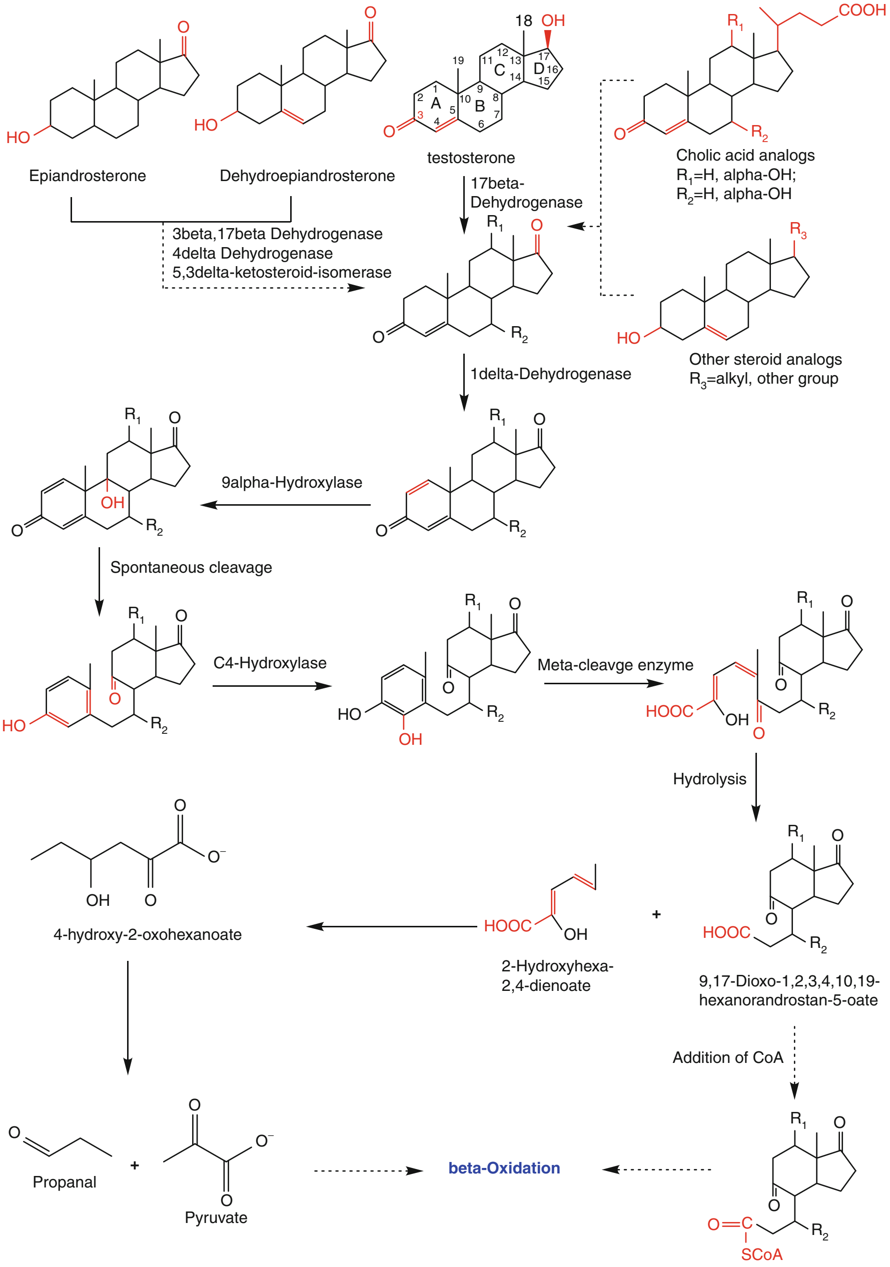

Epiandrosterone
Dehydroepiandrosterone
testosterone
18 OH
19
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
A B C D
Cholic acid analogs
R_1=H, alpha-OH;
R_2=H, alpha-OH
COOH
17beta-Dehydrogenase
3beta,17beta Dehydrogenase
4delta Dehydrogenase
5,3delta-ketosteroid-isomerase
Other steroid analogs
R_3=alkyl, other group
1delta-Dehydrogenase
9alpha-Hydroxylase
Spontaneous cleavage
C4-Hydroxylase
Meta-cleavge enzyme
HOOC
Hydrolysis
4-hydroxy-2-oxohexanoate
2-Hydroxyhexa-2,4-dienoate
9,17-Dioxo-1,2,3,4,10,19-hexanorandrostan-5-oate
Addition of CoA
SCoA
Propanal
Pyruvate
beta-Oxidation

Fig. 88.12 A sketch map of the metacleavage pathway of steroids

Table 88.1 Steroid degradation enzymes and genes in *Comamonas testosteroni*

No.	ATCC11996 Gene or accession no.	TA441 Gene/ ORF	Enzyme/putative product	DNA position (complementary)
			Enzyme/putative product	
1	–		Transcriptional regulator, GntR family	(1432546-1433037)
2	–		Methylcitrate synthase/citrate synthase II	(1433047-1434144)
3	–		Transcriptional regulator, XRE family	1434429-1435901
4	–	*Ste*A	Dehydrogenase for α-oriented hydroxyl group at C-12	1436135-1436866
5	–	*Ste*B	Hydrogenase for ketone group at C-12 to β-oriented hydroxyl group	1436879-1437646
6	–	ORF 7	Hypothetical protein	1437675-1438310
7	–	ORF 6	NADH:flavin oxidoreductase	1438320-1439402
8	AAM77244	*Tes*B	Metacleavage enzyme for aromatized A-ring	1439744-1440598
9	–	ORF1	CoA-transferase alpha subunit	1440615-1441493
10	–	ORF2	CoA-transferase beta subunit	1441506-1442276
11	–	ORF3	Enoyl-CoA hydratase/isomerase family	1442278-1443180
12	–	ORF4	Enoyl-ACP reductase	1443177-1444265
13	–	ORF5	Enoyl-CoA hydratase/isomerase family	1444288-1445046
14	–	ORF21	Acyl-CoA dehydrogenase	1445075-1446229
15	–	ORF22	Acyl-CoA dehydrogenase	1446247-1447386
16	–	ORF23	Thiolase	1447383-1448537
17	–	ORF25	6-Aminohexanoate-cyclic-dimer hydrolase	1448638-1450203
18	–	ORF26	6-Aaminohexanoate-cyclic-dimer hydrolase	1450204-1451793
19	–	ORF27	Short-chain dehydrogenase/reductase family	1451853-1452728
20	–	ORF28	Acyl-CoA dehydrogenase	1452749-1453936
21	–	ORF30	Acyl-CoA dehydrogenase	1453933-1455111
22	ACI39936**	ORF31	Short-chain dehydrogenase/reductase family	1455327-1456112
23	AAV40816**	ORF32	MaoC domain protein	1456115-1456600
24	AAV40815**	ORF33	Acyl-CoA thiolase, acetyl-CoA acetyltransferase	1456668-1457939
25	*tei*R	*Tes*R	LuxR-type transcription factor	1457981-1459228
26	–		Diguanylate cyclase	1459749-1461293
27	–	–	Carnitine dehydratase/bile acid-inducible protein	(1461503-1462636)
28	–	–	(Short-chain dehydrogenase/reductase SDR)	1462821-1463564
29	–	–	(Acetyl-CoA acetyltransferase)	1463926-1465059
30	–	–	(3-Hydroxyacyl-CoA dehydrogenase)	1465077-1467236
31	–		(Glucose-methanol-choline oxidoreductase)	1467325-1469031
32	–		Acyl-CoA dehydrogenase domain protein	1469060-1471471
33	–		Major facilitator superfamily MFS_1	1471575-1472933
34	–		AMP-dependent acyl-CoA synthetase and ligase	1473197-1474822
35	–	(ND)	(Hypothetical protein)	(1474924-1475322)
36	–		Amidohydrolase 3	(1475610-1477325)

(*continued*)

Table 88.1 (continued)

No.	ATCC11996 Gene or accession no.	TA441 Gene/ ORF	Enzyme/putative product	DNA position (complementary)
37	–		Major facilitator superfamily MFS_1	1477575-1478918
38	–		Hypothetical protein	1478915-1479643
39	–		Rieske (2Fe-2S) domain	(1479819-1480928)
40	–		Short-chain dehydrogenase/reductase SDR	(1481087-1481923)
41	–		Major facilitator superfamily MFS_1	(1481945-1483279)
42	–		Coniferyl aldehyde dehydrogenase	1483404-1484828
43	–		Short-chain dehydrogenase/reductase SDR	1485097-1485867
44	–		Transcriptional regulator, LysR family	(1486107-1487042)
45	–		DSBA oxidoreductase	1487110-1487760
46	–	(ND)	(Twin-arginine translocation pathway signal)	1487811-1488710
47	–		Ketosteroid isomerase-like protein	1488719-1489120
48	–		Hypothetical protein	1489139-1489399
49	–		Streptomycin-3-kinase	(1489452-1490153)
50	–		Conserved hypothetical protein	(1490567-1491058)
51	–		GCN5-related N-acetyltransferase	(1491306-1491782)
52	–		Protein of unknown function DUF925	(1491906-1492490)
53	–		NADH:flavin oxidoreductase/NADH oxidase	(1492713-1494839)
54			2,4-Dienoyl-CoA reductase	(1494856-1496871)
55	3α-*hsd*/*hsd*A	3α-*hsd*	3-Hydroxysteroid 3α-Dehydrogenase	1497027-1497800
56	*ksi*	*ksi*	3-Ketosteroid Δ4(5)-isomerase	1497971-1498330
57	*rep*A	*rep*A	Repressor of 3-hydroxysteroid 3α-Dehydrogenase	(1496985-1497803)
57	*rep*B	ORF64	GreA/GreB family elongation factor	(1498493-1499410)
59	–	(ND)	(GCN5-related N-acetyltransferase)	(1499815-1500255)
60	–		Transcriptional regulator, LysR family	(1500300-1501211)
61	–		Short-chain dehydrogenase/reductase SDR	1501320-1502087
62	–		N-Acyl-D-amino-acid deacylase	
63	–		Glutathione-dependent formaldehyde-activating	1502236-1502637
64	–		Enoyl-CoA hydratase/isomerase	(1502710-1503522)
65	–		Short-chain dehydrogenase/reductase SDR	1503622-1504422
66	–		Protein of unknown function DUF925	1504441-1505385
67	–		CaiA, acyl-CoA dehydrogenase-like protein	1505401-1506504
68	–		MaoC-like dehydratase	1506517-1506933
69	–		Lipid-transfer protein	1506957-1508138
70	–		CaiA, acyl-CoA dehydrogenase-like protein	1508156-1509337
71	–		Acyl-CoA synthetase	1509380-1511191
72	–		*Beta*-lactamase domain protein	1511184-1512251
73	–		Acyl-CoA dehydrogenase-like protein	1512264-1513460
74	–		Acyl-CoA dehydrogenase-like protein	1513593-1514720
75	–		Long-chain fatty acid-CoA ligase	1514722-1516428

(*continued*)

Table 88.1 (continued)

No.	ATCC11996 Gene or accession no.	TA441 Gene/ ORF	Enzyme/putative product	DNA position (complementary)
76	–		Putative sigma E regulatory protein, MucB, DUF1329	1516711-1518090
77	–		Protein of unknown function DUF1302	1518126-1519637
78	–		Dienelactone hydrolase	1519751-1520812
79	–		RND efflux transporter	(1520980-1521438)
80	–	(ND)		(1521438-1523921)
81	–		BNR repeat containing glycosyl hydrolase	(1523918-1524925)
82	–		Nonspecific lipid-transfer protein	1525136-1526350
83	–	–	Acyl dehydrogenase protein of unknown function DUF35	1526523-1527299
84	–		(Ferric malleobactin transporter)	1527577-1527855
85	–		TonB-dependent siderophore receptor, transporter	1528207-1530306
86	–	*Tes*G	4-Hydroxy-2-oxohexanoate aldolase	(1530465-1531505)
87	–	*Tes*F	Propionaldehyde dehydrogenase	(1531520-1532443)
88	–	*Tes*E	2-Hydroxyhexa-2,4-dienoic acid hydratase	(1532440-1533255)
89	–	*Tes*D	Hydratase for aromatized A-ring of steroids	(1533260-1534099)
90	–	*Tes*A1	Hydroxylase at C-4 of aromatized A-ring	(1534179-1535156)
91	–	*Tes*A2	Hydroxylase at C-4 of aromatized A-ring	1535436-1536632
92	–	*Tes*H	3-Ketosteroid Δ1-Dehydrogenase	1536737-1538467
93	–	*Tes*I	3-Ketosteroid Δ4-Dehydrogenase	1538483-1540219
94	–	ORF17	Reductase component of C-9 hydroxylase	1540303-1541370
95	–	ORF18	CoA-transferase at C-5 of cleaved B-ring	1541374-1542996

"*ND*" not detected in TA441, "–" not sequenced or sequence incomplete

pathway for aromatic compounds (note that a ketone group at C_3 position and double bonds at Δ1 and Δ4 are indispensable for the aromatization of the A-ring) [20].

The proposed metacleavage pathway (Fig. 88.12) begins with aromatization of the A-ring, followed by a metacleavage accompanying with the production of 2-hydroxyhexa-2,4-dienoate and 9, 17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oate. The former is further converted to 4-hydroxy-2-oxohexanoate, which is then broken down to pyruvate and propanal. Further, all metabolites may be broken down via *beta*-oxidation pathway.

Note that the investigation of the details of the degradation pathway of C-, D-rings of 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oate and other side chains of steroid compounds (E-, F-rings of diosgenin) are still in progress [18].

5.4.3 Steroid Degradation Enzymes and Genes in *Comamonas testosteroni*

Investigation of steroid degradation enzymes and genes in *C. testosteroni* started with the discovery of testosterone degradation by *C. testosteroni* in 1950s [19].

In the metacleavage pathway, the enzymes and genes involved in the A-ring degradation show significant homology to the corresponding genes in bacterial aromatic compound degradation. The enzymes for the degradation reactions are exclusively induced only when bacteria are incubated with steroid compounds [20, 21].

Genes of *C. testosteroni* for the degradation of steroidal backbone structure constitute two main steroid degradation gene clusters, one (named *tes*G-ORF18 in strain TA441) mainly involved in aromatization and cleavage of the A-ring and the other (named *ste*A to *tes*R in strain TA441) involved in β-oxidation of the B-, C-, and D-rings. Further study demonstrates that the two degradation gene clusters are on the same DNA strand several dozens of kilo bases apart, and the 3*alpha*-dehydrogenase gene and the adjacent 5,3*delta*-ketosteroid isomerase gene are located in the DNA region between these clusters (Table 88.1) [20].

6 Conclusions and Perspectives

Current knowledge about terpenoids is primarily based on research in specialized plant chemistry and biochemistry. Modern "omics" techniques are accelerating the discovery of genes and enzymes for biotransformation and regulation of terpenoids. The number of terpenoid-biosynthesis genes found in the plant species that their whole genome sequencing projects have been completed suggests a much wider range of structural diversity and distribution of terpenoids than previously expected. The results of comparative and functional genomics study have also provided new insights into the evolutionary events of repeated gene duplication and subsequent neo-functionalization, as well as the role of allelic variations in new terpenoid biosynthesis [15]. The goal to understand the biotransformation of terpenoids is mainly to fully capture their economic value via metabolic engineering of plant and microbial systems, especially exploring terpenoids as a renewable resource for the production of specialized chemicals and possibly biofuels, as well as for the improving flavor and color of foodstuffs.

Research on steroid chemical and biochemistry has resulted in the discovery of large numbers of steroid compounds; however, efforts are continuously being made for the further applications of steroid compounds, especially in the steroid drug development and their biotransformation and degradation. Genetic manipulation associated with synthetic biology can lead to produce constitutive biocatalysts with high activity and specificity, rather than inducible ones, further to create a large number of new steroid compounds. The exploration of the genetic reservoir of the total microbiota found in nature (metagenome), and the rational designs of enzymes, fermentation, and bioconversion media may lead to the implementation of robust, highly effective biotransformation processes. New biological techniques, particularly highly efficient solid-phase library approaches and high-throughput screening (HTS), associated with combinatorial chemistry, will allow faster and wider screening of new steroidal compounds, to ultimately generate libraries of therapeutically relevant compounds [6, 22].

Besides, the advancements in integrated biotechnology based on integrative strategy, rather than conventional reductionistic approach, will be expected to exert a greater impact on terpenoid and steroid development and utilization.

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Production and Genetic Engineering of Terpenoids Production in Plant Cell and Organ Cultures

89

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Contents

1 Introduction 2763
2 Plant Cell and Organ Cultures for the Production of Secondary Metabolites 2765
2.1 Plant Cell Cultures 2768
2.2 Organ Cultures 2774
2.3 Scaling up to Bioreactor Level 2776
3 Biotechnological Production of Monoterpenes, Sesquiterpenes, Diterpenes, Triterpenes, and Tetraterpenes 2780
3.1 Monoterpenes 2780
3.2 Sesquiterpenes 2782
3.3 Diterpenes 2782
3.4 Triterpenes 2784
3.5 Tetraterpenes 2787

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4 Metabolic Engineering of Terpenes in Plant Cells and Roots 2788
4.1 Artemisinin Production in Engineered Hairy Roots 2788
4.2 Taxol and Taxane Production in Engineered Plant Cells 2789
4.3 Withanolide Production in Engineered Hairy Roots 2791
5 Conclusion 2791
References 2792

Abstract

Among the three main groups of secondary metabolites that can be found in plants, terpenes are by far the largest and most diverse. They play an important role in plants in both primary and secondary metabolism and constitute a source of phytochemicals for human health. Plant biotechnology, based on in vitro culture and genetic engineering techniques, has proved to be a promising tool to increase the production of these bioactive compounds. This chapter describes the plant cell and organ culture techniques used in plant biotechnology, the biotechnological production of the different groups of terpenes using empirical approaches and some examples of genetic and metabolic engineering techniques applied to improve the production of these valuable plant secondary metabolites in plant cell and organ cultures.

Keywords

Biotechnological production • cell cultures • metabolic engineering • roots • terpenes

Abbreviations

CaMV	Cauliflower mosaic virus 35S promoter
CM	Culture medium
DMAPP	Dimethylallyl diphosphate
DXR	1-Deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	1-Deoxy-D-xylulose-5-phosphate synthase
DW	Dry weight
FPP	Farnesyl diphosphate
GGPP	Geranylgeranyl diphosphate
GPP	Geranyl diphosphate
HMGR	3-Hydroxy-3-methyl-glutaryl-CoA reductase
IAA	Indole-3-acetic acid
IPP	Isopentenyl diphosphate
MEP	2-C-methyl-D-erythritol 4-phosphate
MS	Murashige and Skoog medium
OPP	Diphosphate
PGR	Plant Growth Regulators
SQS	Squalene synthase
TXS	Taxadiene synthase

1 Introduction

Terpenes or isoprenoids are the largest and most diverse group of natural compounds. Although all living organisms are able to synthesize terpenes, it is in the plant kingdom where these compounds display the greatest diversity of chemical structures. Terpenes are derived from the five-carbon precursor isopentenyl diphosphate (IPP), produced via the classical mevalonate pathway, or in plants also by the novel MEP (non-mevalonate or Rohmer) pathway [1]. Plant terpenes play important roles in the organism, for example, phytols, the acyclic side chain of chlorophyll; carotenoids, also involved in photosynthetic process; and phytosterols with functions at the membrane level. Plant growth regulators such as gibberellins, abscisic acid, and brassinosteroids also have terpene structures. All these compounds have primary functions in plants and are widely distributed in the plant kingdom, but most terpenes have functions related with plant defense situations or are involved in the relation of the plant with other organisms. These terpenes have a wide distribution in plants and are considered to be secondary compounds [2].

Terpenes are made up of units of five carbons, C5, C10, C15, C20, C25, C30, C40, and so on (Fig. 89.1), and although generally free, they may be modified or derivatized as esters and glycosides or attached to proteins. Mono- and sesquiterpenes are derived from geranyl diphosphate and farnesyl diphosphate, respectively, and are widespread compounds in plants [3]. These lipophilic and volatile compounds normally accumulate in specialized plant structures called essential oil glands (Fig. 89.2). Mono- and sesquiterpenes include aliphatic acyclic, monocyclic, and polycyclic compounds with isolated double bonds, which are substituted with oxygen groups such as aldehydes, ketones or acids (Fig. 89.2), and abscisic acid, a growth regulator involved in plant stress signals. Diterpenes derived from geranylgeranyl OPP are usually components of plant resins. The most commonly encountered diterpenes are polycyclic and nonvolatile acids from conifers and legumes [2], but this group also includes the plant hormone gibberellins. The progenitor of C30 compounds is squalene arising from dimerization of two molecules of farnesyl OPP (Fig. 89.1). Triterpenes constitute a significant portion of the lipid substances of all plants and are precursors to steroids in both plants and animals. Steroids are components of membranes in plants, and brassinosteroids have hormonal functions. Both triterpenes and steroids occur free, as glycosides or in combined forms. Carotenoids derived from the C40 compound phytoene are probably the most widely distributed of all natural pigments, and they are involved in fundamental processes such as photosynthesis. Chemically, carotenoids are polyunsaturated lipid acyclics or have chains that terminate with one or two six-membered rings. These polyolefinic metabolites serve also as vegetative, floral, and fruit pigments (Fig. 89.2).

In all groups of terpenes, it is possible to find compounds with bioactive functions, and for this reason, they have been used since ancient times for their pharmacological properties. Terpenes also give plants their characteristic tastes and flavors and have been used as food additives or cosmeceuticals, and more recently, properties such as the antioxidant activity of carotenoids have given these

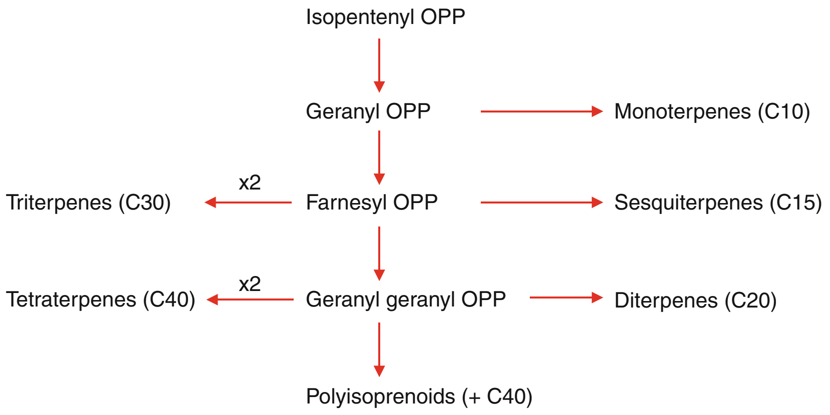

Fig. 89.1 Schematic classification of terpenes

compounds the role of nutraceuticals (Fig. 89.2). The biological activities of some important terpenes are discussed in more detail in other sections, but essential oil components have been used for their antibiotic, expectorant, rubefacient, and carminative properties. In particular, the sesquiterpene artemisinin is widely considered to be the best treatment for uncomplicated malaria when used as a combination therapy [1]. Resins rich in diterpenes are used as feedstocks for products such as insecticides, incense, varnishes, rosin, and adhesives, and as components of drugs and polishes. Triterpenes and steroids also have a wide variety of biological properties. For example, *Panax* saponins have been used as adaptogens and dietary supplements; *Centella* saponins are applied in the cosmetic industry; cardenolides function as cardiovascular drugs; and glycyrrhizin, a triterpenoid glucuronide, is used as a sweetener. However, the most important application of steroidal saponins, such as diosgenin, is as a source of human hormones. Carotenoids have often been used for coloring food products, and their antioxidant properties confer carotenoid-rich nutraceutical properties [4].

Many plants containing high-value terpenes are difficult to cultivate or are becoming endangered because of over-harvesting. Furthermore, the chemical synthesis of some of these compounds, such as the diterpene alkaloid Taxol or steroidal nucleus, is often economically unfeasible due to their highly complex structures and their specific stereochemical requirements [5]. The biotechnological production of high added value terpenes in plant cell or organ cultures is an attractive alternative to the extraction of whole plant material. However, the use of plant cell or organ cultures has had only limited commercial success so far. In this chapter, we describe the main biotechnological systems developed for producing plant secondary metabolism, giving some specific examples of the production of valuable terpenes in plant in vitro cultures. Finally, we discuss the use of a new biotechnological set of tools for improving terpene production in plant cell and organ cultures.

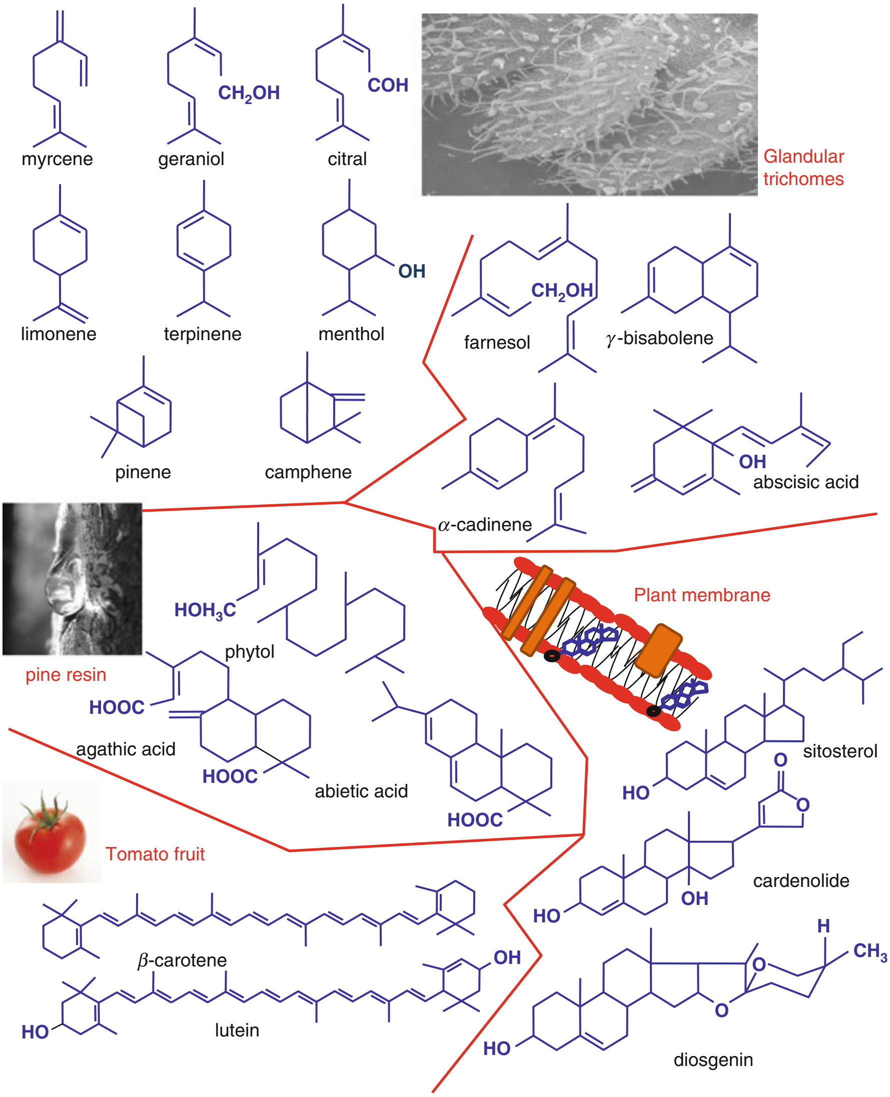
myrcene
geraniol
CH$_2$OH
citral
COH
Glandular trichomes
limonene
terpinene
menthol
OH
farnesol
CH$_2$OH
γ-bisabolene
pinene
camphene
α-cadinene
OH
abscisic acid
pine resin
HOH$_3$C
phytol
Plant membrane
HOOC
agathic acid
HOOC
abietic acid
HOOC
sitosterol
HO
O
O
cardenolide
HO
OH
Tomato fruit
β-carotene
OH
lutein
HO
H
CH$_3$
O
O
HO
diosgenin

Fig. 89.2 Chemical structures of the five main groups of terpenes

2 Plant Cell and Organ Cultures for the Production of Secondary Metabolites

Plants have been used since ancient times as medicines in all cultures that have existed on our planet and still represent an inexhaustible source of new drugs. Table 89.1 gives a short list of some plant products currently used as drugs, most of

Table 89.1 Plant secondary compounds with biological activity

Plant compounds widely used in medicine		
Acetyldigoxine	Scopolamine	Quinine
Ajmalicine	**Centellosides**	Quinidine
Alantoine[a]	**Ginsenosides**	Reserpine
Artemisinin	Espartein	Shikonin
Hyoscyamine	Physostigmine	Theobromine[a]
Bromelain	L-DOPA[a]	Theophylline[a]
Caffeine[a]	**Lanatoside**	**Tetrahydrocannabinol**
Colchicine	Morphine	Podophyllotoxin
Dantron[a]	**Taxol**[a]	**Xanthotoxin**
Deserpine	**Vincristine**	Ouabaine
Digoxin	**Vinblastine**	Codeine
Digitoxin	**Withanolides**	Camptothecin
Ephedrine[a]	Papaverine[a]	Silymarin
Emetine	Pilocarpine	**Diosgenin**

[a] Produced by chemical synthesis

In bold: terpenoid compounds

them plant secondary metabolites, which includes several terpenoid compounds. Although some of them can be synthesized chemically, most are extracted from the plant due to their highly complex structures [4].

This is the case of the diterpene alkaloid Taxol, an important compound for the pharmaceutical industry for its anticancer activity. This compound, like many others synthesized by plants, is found in very small quantities. Extracted from the inner bark of *Taxus* spp., it accumulates at a very low concentration (about 0.02% of dry weight) and its extraction cost is extremely high. Since it is necessary to take 10,000 kg of *Taxus* bark or 3,000 yew trees to produce only 1 kg of the drug, a cancer patient needs approximately 2.5–3 g of Taxol [6]. When the accumulation of the target compound in plants is so low and it is synthesized by an endangered species, it is necessary to look for alternative production techniques, such as processes of chemical synthesis or cultivation. In the case of Taxol, chemical synthesis is not cost-effective, and to produce it in conventional crops, it is necessary to wait many years before obtaining a harvest [7]. In such cases, biotechnological systems based on cell and organ cultures are recommended for the production of the target compounds (Fig. 89.3). In this context, shikonin, berberine, triterpene saponins of *Panax ginseng*, taxanes, and polysaccharides of *Polianthes tuberosa* are all produced commercially by biotechnological systems based on in vitro plant cultures [8].

Although biotechnological processes are expensive and these systems can only be used when the product has a high price in the market, if the specific process is more productive than the plant, it is easy to recover the product from the system, the production is stable, and alternative processes of chemical synthesis do not exist.

In general, not all plant cells are able to develop secondary biosynthetic pathways. These processes are related to cell differentiation and the development of specific tissues and organs [9]. Secondary metabolism depends on precursors

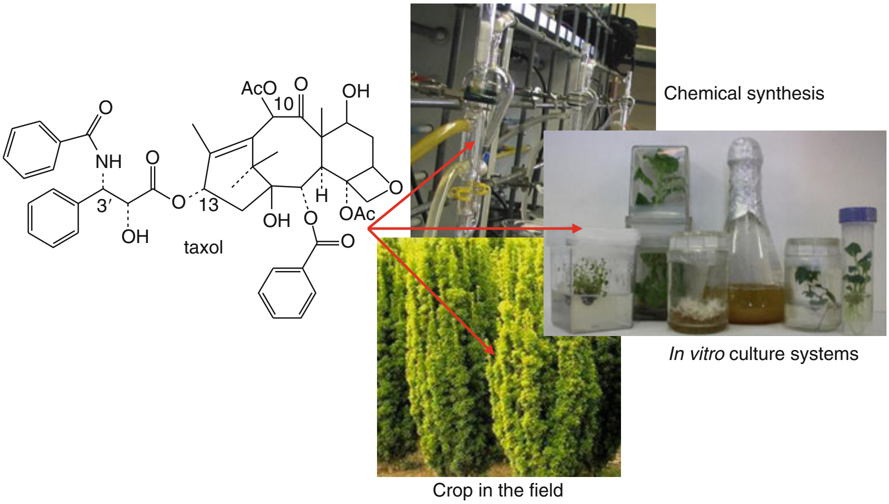
AcO
O
10
OH
O
NH
O
3′
O
13
H
OH
O
OAc
O
OH
O
taxol
Chemical synthesis
In vitro culture systems
Crop in the field

Fig. 89.3 Alternative sources of plant secondary terpenes, including Taxol, for industrial production

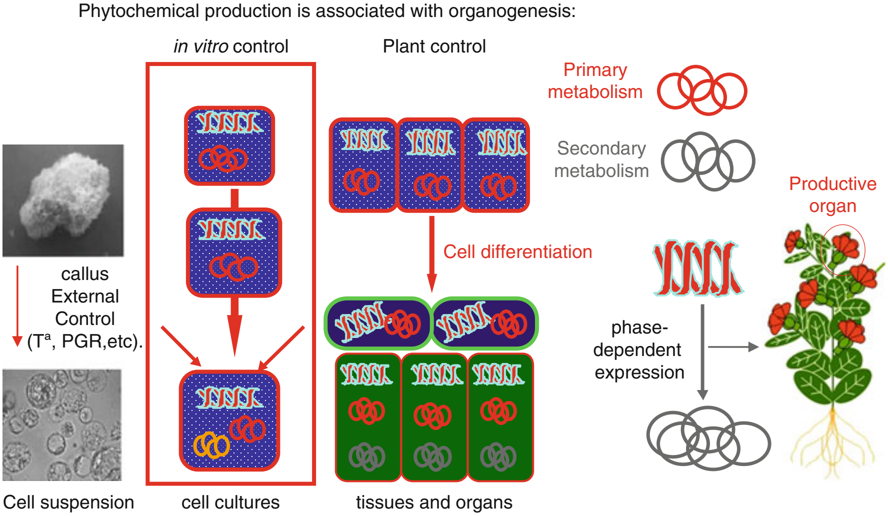
Phytochemical production is associated with organogenesis:
in vitro control
Plant control
Primary metabolism
Secondary metabolism
Productive organ
callus
External Control
(Tª, PGR,etc).
Cell differentiation
phase-dependent expression
Cell suspension
cell cultures
tissues and organs

Fig. 89.4 Schematic representation of the relation between primary and secondary metabolism in plant and cell cultures

provided by primary metabolism, and the biosynthetic routes do not function throughout the life of the plant (Fig. 89.4). They depend on the plant's development and are restricted to certain moments of the plant ontogeny. However, all plant cells with a functional nucleus are totipotent, which means it is possible to control their gene expression and develop plant secondary metabolism even in undifferentiated cells by modifying external factors, such as plant growth regulators, nutritional requirements, light, temperature, etc. [10] (Fig. 89.4). This opens the way to the use of biotechnological systems based on undifferentiated plant cultures, such as calli and cell suspensions, for the production of plant secondary metabolites with biological activities. The most important biotechnological systems based on plant cell and organ cultures are cell suspensions or immobilized cells and the culture of roots and shoots.

2.1 Plant Cell Cultures

2.1.1 Obtaining Cell Cultures

A preliminary step in obtaining plant cell suspensions is to develop callus cultures. Calli are naturally formed from wounds by the infection of microorganisms due to stimulation by endogenous growth hormones, the auxins and cytokinins. It is possible to develop calli artificially by using tissue culture techniques. Auxins are added to the culture medium for callus induction, their type and quantity depending on the nature and source of the explant and its genotype, in addition to other factors. Callus cultures can be maintained for prolonged periods by repeated sub-culturing. The calli need to be friable, and the cell suspension is prepared by transferring a fragment of callus to the liquid medium where it is aseptically shaken to free the cells (Fig. 89.5). Fine cell suspensions are obtained by filtering through a filter of more than 100 μm pore size in order to eliminate the clusters and obtain the isolated cells. They are then cultured by batch or continuous cultures. As the medium is liquid in nature, the callus pieces remain submerged, which creates anaerobic conditions. To overcome this problem, the suspension cultures are shaken by a rotary shaker (100 rpm), which disperses the cells and exposes them to air (Fig. 89.5).

The advantages of cell suspensions over callus culture are the following: (a) they are less heterogeneous, and cell differentiation is less pronounced; (b) they can be cultured in volumes of more than 1,500 L; (c) they can be subjected to more stringent environmental controls; and (d) manipulations for the production of natural products by feeding precursors are possible [12].

Batch cultures are initiated as single cells in 100–250 mL flasks and are propagated by regularly transferring small aliquots of suspension to a fresh medium (Fig. 89.5). Finally, they are scaled up to bioreactor conditions for the production of phytochemicals. Continuous cultures are maintained in a steady state for a long period by draining out the used medium and adding fresh medium. Cell suspensions are also used for (a) induction of somatic embryos/shoots, (b) in vitro mutagenesis and mutant selection, and (c) genetic transformation.

The first compound to be produced commercially in a biotechnological system was shikonin by Mitsui Petrochemical Industries. Shikonin is a red dye used in cosmetics. Currently, several pharmaceutical companies around the world produce plant secondary terpenoids in biotechnological systems, one of the most

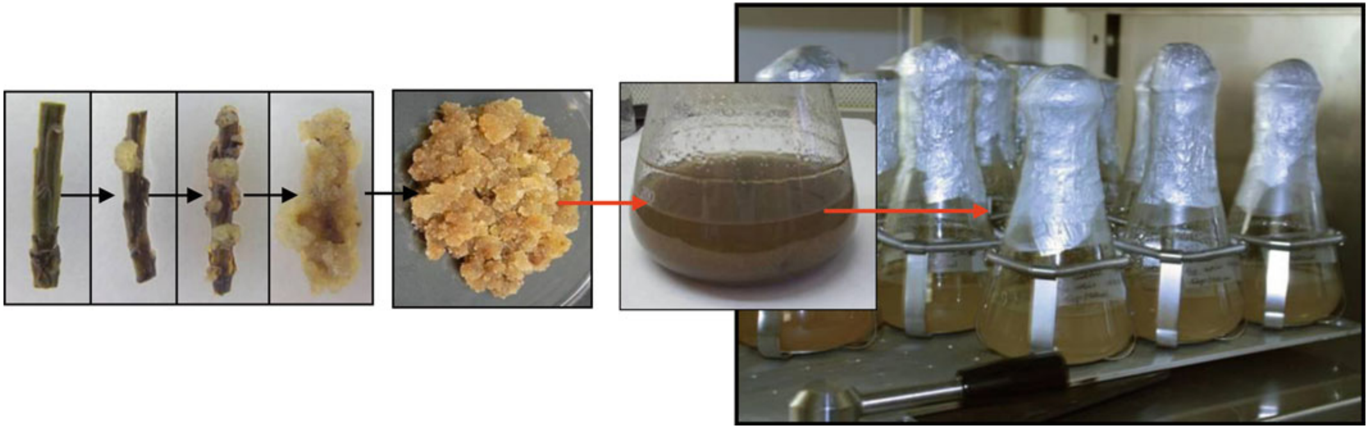

Fig. 89.5 Obtaining a cell suspension culture of *Taxus baccata* for the production of the diterpene alkaloid Taxol. Calli are induced from sterilized segments of the young branches of *T. baccata* trees. When developed from branch segments, callus pieces are isolated and cultured in solid MS (Murashige and Skoog medium). Friable calli are transferred to liquid medium and cultured in a shaker at 110 rpm in the dark at 25 °C. For details of experimental conditions, see the reference [11]

important being Phyton Biotech, which produces the diterpene alkaloid Taxol and other taxanes in *Taxus baccata* cell cultures, or Nitto Denko Co., which manufactures *Panax ginseng* cell mass as a source of triterpene saponins [13]. According to Ramachandra Rao and Ravishankar [10], plant cell cultures have the following advantages over crop plants in the field for producing phytochemicals:

(a) The desired product can be harvested anywhere in the world with strict control of production and quality.
(b) Harmful herbicides and pesticides are avoided.
(c) Climate or ecological problems are not factors.
(d) Growth cycles are of weeks rather than years as in the whole plant.

Nevertheless, not all plant secondary compounds with biological activity can be produced by biotechnological systems. As mentioned above, it is necessary that the production cost is lower than in the whole plant, the product has a high added value in the market (more than 500$ kg^{-1}), and the yield of the process should be over 1–2 mg L^{-1}day^{-1}. To achieve this kind of production, the biotechnological process normally requires optimization.

2.1.2 Optimization of the Production Conditions

Due to the low production of phytochemicals by plant cell cultures, it is frequently necessary to optimize the conditions for producing plant secondary compounds in order to perform a cost-effective bioprocess. The most important steps in production optimization are:

Selection of highly productive cell lines. In vitro techniques promote somaclonal variation, and after callus induction and disaggregation of the callus to obtain cell suspensions, not all the cell lines have the same capacity for the production of phytochemicals [12]. It is therefore necessary to select the most productive cell lines.

Optimization of the growth and production medium. In most cases, phytochemical production is not related with biomass formation, and some culture media and external conditions that promote the latter inhibit the biosynthesis of plant secondary compounds. For this reason, it is necessary to find the best conditions for biomass production as well as for the production of the target compounds.

Addition of biosynthetic precursors. In some biotechnological processes, when the biosynthetic pathway of the bioactive compounds is known, it is possible to add precursors to the culture medium in order to increase the productivity of the system. However, for this, it is necessary that the precursors have a lower market price than the final product of the biosynthetic pathway.

Addition of elicitors. Elicitors are physical or chemical agents that activate secondary metabolism and increase the productivity of the bioprocess. Organic compounds such as methyl jasmonate and salicylic acid are currently used with this aim. Elicitors are used in most biotechnological processes.

Stimulating the release of the target compound to the culture medium. Cell cultures frequently accumulate the bioactive compounds inside the cells or in vacuoles, so it is necessary to harvest the biomass of the culture in order to extract

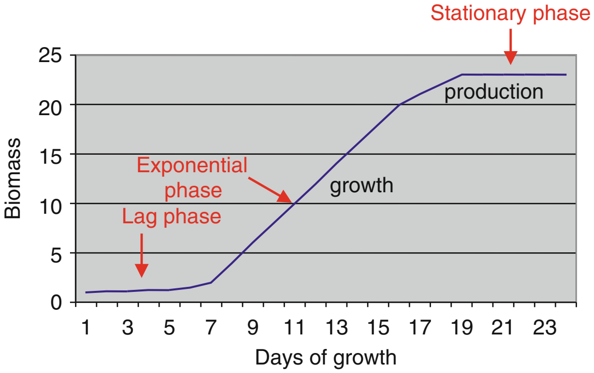

Fig. 89.6 Typical time course of biomass production in a plant cell suspension

the bioactive compounds. Extraction from dry plant matter is a difficult process due to its high quantity of waxes and pigments. Permeabilizing agents can release the phytochemicals caught in the cells to the culture medium, thus facilitating the downstream processing.

The capacity of a plant cell suspension to produce biomass during the culture period shows a sigmoid course with three different phases (Fig. 89.6).

In the first lag phase, the cells do not increase the biomass, while inside the cells, some biochemical processes begin to develop, such as DNA duplication, RNA and protein synthesis, etc. During this phase, cells are preparing all the machinery to initiate the processes of cell division that will start in the second exponential growth phase. During the phase of exponential growth, cells are dividing continuously by mitosis, involving DNA duplication and RNA and protein synthesis [14]. All these processes are included in primary metabolism and use up all the energy and reduced carbon from the cells. In these conditions, secondary metabolism leading to the biosynthesis of plant secondary compounds is inhibited. Finally, in the stationary phase, the biomass of the system remains constant, and the number of cells that divide by mitosis is almost equal to the number of dying cells. It is in these conditions that the cells can accumulate precursors for the biosynthesis of phytochemicals and the machinery of plant secondary metabolism is ready to work.

In most cases, primary and secondary metabolism are antagonistic because they share the same precursors (Fig. 89.7), and, as mentioned, to develop a highly productive bioprocess, it is first necessary to optimize the conditions for biomass production, and when enough biomass has developed, the culture has to be transferred to other conditions in order to boost the secondary metabolism of the cells and achieve the production of phytochemicals.

Based on these facts, the two-stage cultures have been established (Fig. 89.8). In the first stage, all the culture parameters, such as nutrients, PGRs, light, temperature, etc., have to be optimized to produce the maximum quantity of biomass, and then the culture is transferred to other conditions to improve the production of the

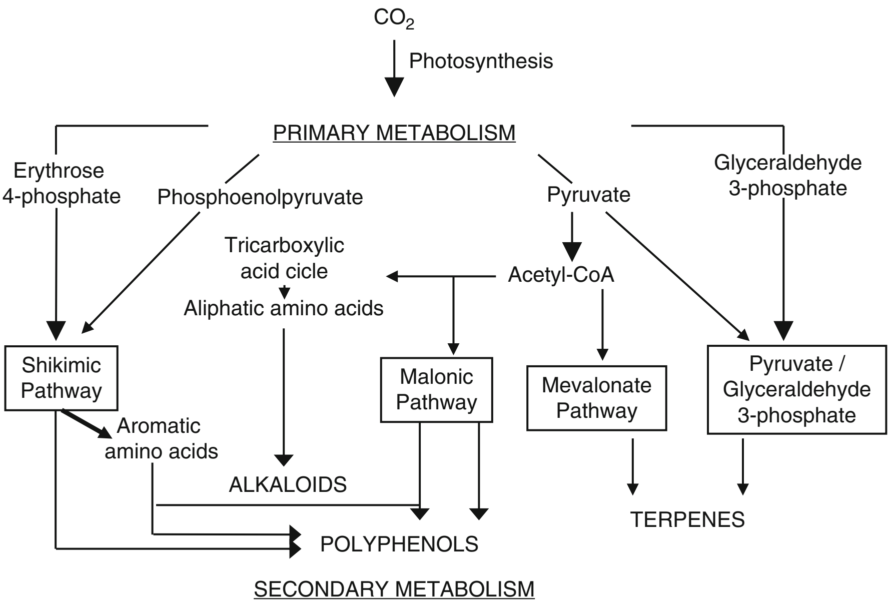
CO2
Photosynthesis
PRIMARY METABOLISM
Erythrose 4-phosphate
Phosphoenolpyruvate
Pyruvate
Glyceraldehyde 3-phosphate
Tricarboxylic acid cicle
Aliphatic amino acids
Acetyl-CoA
Shikimic Pathway
Malonic Pathway
Mevalonate Pathway
Pyruvate / Glyceraldehyde 3-phosphate
Aromatic amino acids
ALKALOIDS
POLYPHENOLS
TERPENES
SECONDARY METABOLISM

Fig. 89.7 Primary and secondary metabolism connections

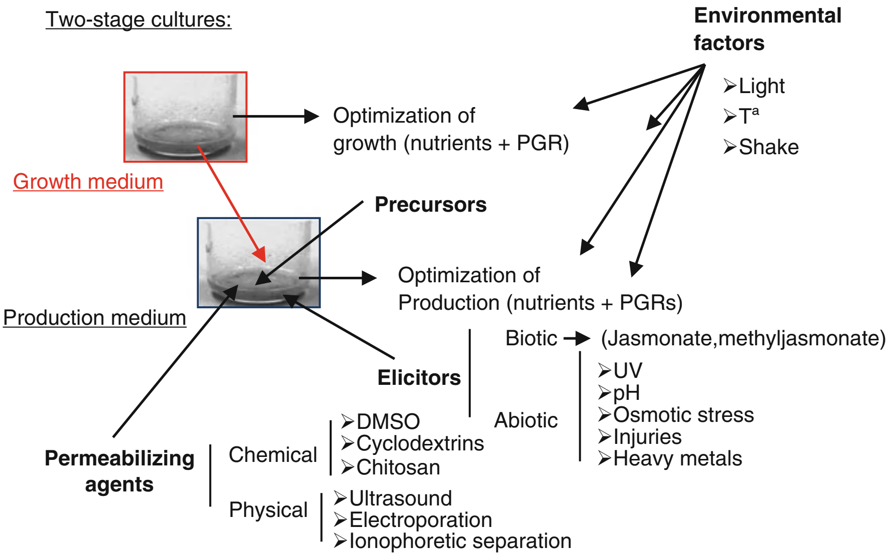
Two-stage cultures:
Environmental factors
➢Light
➢Tª
➢Shake
Optimization of growth (nutrients + PGR)
Growth medium
Precursors
Optimization of Production (nutrients + PGRs)
Production medium
Biotic
(Jasmonate,methyljasmonate)
Elicitors
Abiotic
➢UV
➢pH
➢Osmotic stress
➢Injuries
➢Heavy metals
Permeabilizing agents
Chemical
➢DMSO
➢Cyclodextrins
➢Chitosan
Physical
➢Ultrasound
➢Electroporation
➢Ionophoretic separation

Fig. 89.8 Factors affecting biomass and phytochemical production in a two-stage culture

target compounds. This requires the optimization of nutritional and environmental conditions, but other factors also play an important role, including feeding with precursors and elicitor treatments. Among the biotic elicitors, the most used are jasmonates, for example, methyl jasmonate, which are plant compounds involved in the response mechanism of the plants to stress conditions. Other elicitors like salicylic acid, chitosan, or coronatine are also successfully applied. Physical factors can also be used, for example, UV treatment, high pH, osmotic stress, injuries like shear stress, or heavy metals are commonly applied to increase the production of biotechnological systems. Similarly, permeabilizing agents such as organic solvents and cyclodextrins, and physical agents such as ultrasounds or electrical treatment, not only provoke the release of the bioactive compounds to the culture medium, simplifying the extraction process, but can also increase phytochemical production.

2.1.3 Immobilization

Plant cells are very sensitive to shaking stress, and changing the growth medium for production medium is difficult to perform in cell suspensions [12]. In order to facilitate the changes of the culture medium and to protect cells from shear stress, cell immobilization protocols have been developed. Plant cells can be easily immobilized in by microencapsulation in sugar polymers or by adhesion to inert matrices [15]. The most utilized inner matrices are polyurethane foam or hollow glass fiber, and alginate has been commonly used as a gelling agent. Alginate (1–2%) forms beads in the presence of ion calcium (0.2–0.3 M), entrapping the plant cells inside (Fig. 89.9).

Compared to cell suspensions, immobilized cells have several advantages: the process can develop continuously, while the product is released from the cells and separated from the culture medium; the culture medium can be changed, allowing several compounds to be added and removed easily and quickly; the biomass can be rejuvenated in situ by perfusion of the growth medium to the cells at different time intervals; and relatively low amounts of biomass can be used efficiently [16, 17].

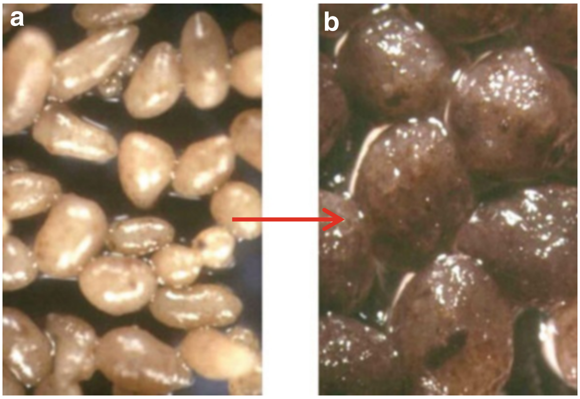

Fig. 89.9 Microencapsulation in alginate beads of Taxol-producing cell cultures of *Taxus baccata*. (**a**) After 1 week of growth. (**b**) After 4 weeks of growth

Table 89.2 Plant secondary metabolites with biological activity synthesized in plant roots

Compounds synthesized in the roots	Genus	Pharmacological activity
Ajmalicine, serpentine	*Catharanthus*	Antihypertensive
Berberine	*Hydrastis*	Hemostatic, antitumor
Bornyl esters	*Valeriana*	Sedative
Ginsenosides	*Panax*	Adaptogen
Hyoscyamine, scopolamine	*Hyoscyamus, Datura, Duboisia*	Anticholinergic
Quinine, quinidine	*Cinchona*	Antimalarial, bitter principle
Reserpine	*Rauwolfia*	Antihypertensive
Rotenone	*Derris*	Insecticide
Shikonin	*Lithospermum*	Bactericide, dye
Thiophenes	*Bidens, Coreopsis, Tagetes*	Solvents

2.2 Organ Cultures

2.2.1 Hairy Root Cultures

Plant roots have a very active metabolism and can biosynthesize a high number of secondary compounds in an exclusive way (Table 89.2). Some of these compounds, including shikonin or triterpenoid saponins of *Panax ginseng* (ginsenosides), can also be biosynthesized in undifferentiated systems such as cell cultures, but in some cases, cell differentiation and organ formation is necessary for producing the bioactive compounds, which is the case of tropane alkaloids, bornyl esters, ajmalicine, and other compounds.

Thus, in the middle of the last century, plant biotechnologists began to work in root cultures. The problem with this type of culture was the low growth rate, and in some cases, adding IAA to improve growth caused a significant decrease in the production of bioactive compounds. Then several years ago, a plant syndrome was discovered: the hairy root syndrome. This condition is provoked by a pathogenic bacterium of plants, *Agrobacterium rhizogenes*, and is characterized by an abundant profusion of roots in the sites of bacterial infection.

A. rhizogenes, as well as *A. tumefaciens*, is able to transfer a section of its plasmid pRi to the plant genome, and the transformed cells begin a process of cell division and cell differentiation to form the so-called hairy roots (Fig. 89.10).

There are several reports indicating that the T-DNA (the DNA fragment from *Agrobacterium* transferred into the plant genome) of *A. rhizogenes* carrying the *rol* genes is responsible for root development. Among the *rol* genes, the most important are the *rol*A gene, which among other actions probably increases the sensitivity of the transformed cells to auxin; the *rol*B gene, which hydrolyzes the inactive conjugates of auxin (auxin + sugar); and *rol*C gene, which hydrolyzes the conjugates of cytokinins. However, most of the effects of these *rol* genes remain unknown [18].

When excised from the plant, hairy roots show a high capacity to grow in culture media without plant growth regulators (Fig. 89.11) and can biosynthesize the same plant secondary compounds as the root of the mother plant. Hairy roots are

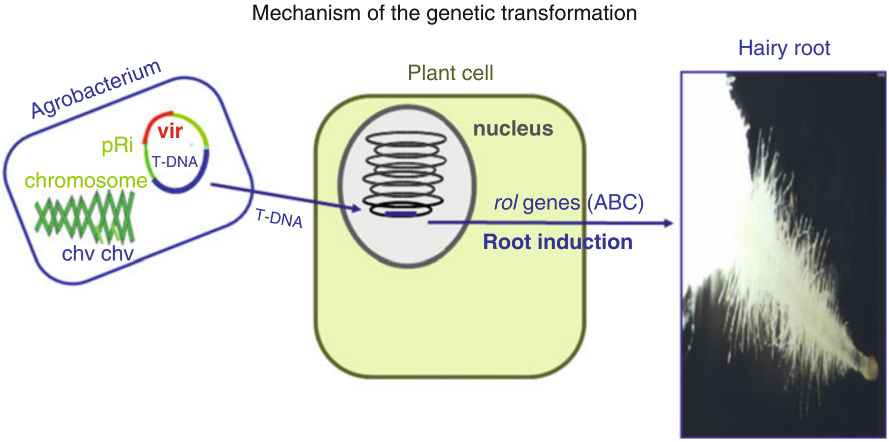
Mechanism of the genetic transformation
Agrobacterium
pRi
vir
T-DNA
chromosome
chv chv
T-DNA
Plant cell
nucleus
rol genes (ABC)
Root induction
Hairy root

Fig. 89.10 Schematic representation of the genetic transformation mechanism by *Agrobacterium rhizogenes*

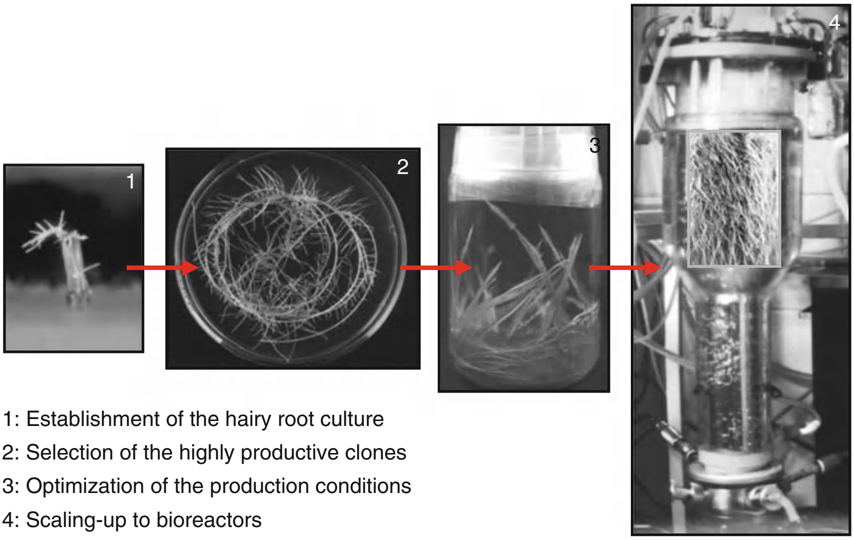
1
2
3
4
1: Establishment of the hairy root culture
2: Selection of the highly productive clones
3: Optimization of the production conditions
4: Scaling-up to bioreactors

Fig. 89.11 Main stages in the development and optimization of a hairy root culture

currently used for the production of phytochemicals, including several terpenoid compounds whose biosynthesis requires a level of differentiation corresponding to the plant root [19].

The main stages for developing and optimizing a hairy root culture are (Fig. 89.11) (a) establishment of the hairy root culture (by infection of the explants with a virulent strain of *A. rhizogenes*), (b) selection and culture of highly productive root clones, (c) optimization of the production conditions (by improving external conditions, culture medium, elicitor and permeabilizing agent treatments, etc.), and (d) scaling up to bioreactors (by adaptation of the bioreactor to the growth of the roots).

Table 89.3 Plant secondary metabolites with biological activity synthesized in plant shoots

Compounds synthesized in shoots	Genus	Pharmacological activity
Vindoline	*Catharanthus*	Antileukemic
Artemisinin	*Artemisia*	Antimalarial
Camptothecin	*Ophiorrhiza, Camptotheca*	Anticancer activity
Atropine	*Atropa*	Anticholinergic
Vinblastine	*Cinchona*	Antileukemic
Quinine	*Cinchona*	Antimalarial
Withanolides	*Withania*	Antitumor, neuroprotective
Indirubin	*Polygonum*	Antileukemic

Compared to biotechnological systems based on undifferentiated cultures, the advantages of hairy root cultures are:

- Fast growth in culture media without PGRs
- Reproducible and predictable levels of the chemically unaltered product
- Genetic stability over long culture periods
- Large-scale cultivation without loss of biosynthetic capacity

2.2.2 Shoot Cultures

Since secondary metabolite production is generally higher in differentiated tissues, as well as root cultures, the cultivation of shoot cultures for the production of medicinally important compounds has been attempted [20]. Undifferentiated cultures often accumulate secondary metabolites to a lesser extent, or sometimes not at all. For example, a potent antimalarial compound, artemisinin, produced by *Artemisia annua* has not been observed to accumulate in undifferentiated cultures, while trace amounts have been detected in shoot cultures [21]. In addition, the development of adventitious shoots in liquid cultures has been shown to provide a highly proliferative and rapid growing system amenable to automated inoculation, mechanical separation, control of the medium components, and efficient delivery to the final stage for plant growth and development [13]. There are a number of medicinal plants whose shoot cultures have been studied for their secondary metabolites (Table 89.3).

2.3 Scaling up to Bioreactor Level

The last step of a bioprocess based on plant cells or organ cultures is the scale-up to bioreactor level. In these processes, it is possible to monitor and control several parameters, including:

Stirring: Normally up to 100 rpm.

Aeration: Measured by partial pressure of oxygen controlled at 50% of the dissolved oxygen.

Temperature: Normally 25 °C.

Foam: It is possible to add antifoam if necessary, but normally cell cultures do not produce high quantities of foam.

pH: Normally controlled to 5.8.

Table 89.4 Comparative study of the main differential traits of microbial and plant cell cultures (Modified from Chattopadhyay et al. [12])

Main traits of the culture	Microbial	Plant cells
Size	Small (2–10 μm)	Big (50–100 μm)
Single cells/clusters	Single cells and clusters	Clusters and single cells
Growth rate	Fast	Slow
Doubling time	Hours	Days
Sensitivity to shaking	Low	High
Product accumulation	Often extracellular	Often intracellular
Culture medium	Simple	Complex[a]
Inoculum density	Low	High (5–10% fresh weight)
Temperature	26–36 °C	25 °C
Aeration rate	High	Low
Foam	Frequently	Rarely
Culture period	Days	Weeks

[a] Including mineral salts, sugars, amino acids, and plant growth regulators

Table 89.4 shows the most important traits of plant cell cultures compared to microbial cultures, which require the reactor, initially designed for the culture of microorganisms, to be adapted to plant cell and organ cultures.

2.3.1 Adaptation of the Bioreactor Design to Plant Cell and Organ Cultures

Due to their different traits, the bioreactors initially designed for microbial cultures have to be adapted to plant cell cultures. As plant cells are very sensitive to shear stress, the first type of modification in the stirred tanks was to change the shape and disposition of the blades of the bioreactor. Big blades present in bioreactors for bacterial and yeast cultures are removed and replaced by a marine propeller, which is less harmful for plant cells. It is also possible to change the big blade at the bottom of the bioreactor for small blades lined up along the entire axis of the stirred system. Similarly, in the paddle system, the large lower blades of the classical bioreactors are replaced by a completely perforated central blade, which moves the culture medium by the peripheral side, or the helix system where the blades are replaced by an axis shaped like a corkscrew (Fig. 89.12).

In bubble columns, sterile air enters through the bottom of the bioreactor and pushes the culture medium and cells by preventing cell sedimentation and causing the diffusion of nutrients and oxygen in the culture medium. The problem is when the bioreactor tank is very big, in which case the air enters too quickly and forcefully in the tank of the bioreactor, and the air bubbles break cells by crashing into them. One modification of the bubble column is the airlift bioreactor (Fig. 89.12). In this case, inside the tank, there is another tube, the draft tube,

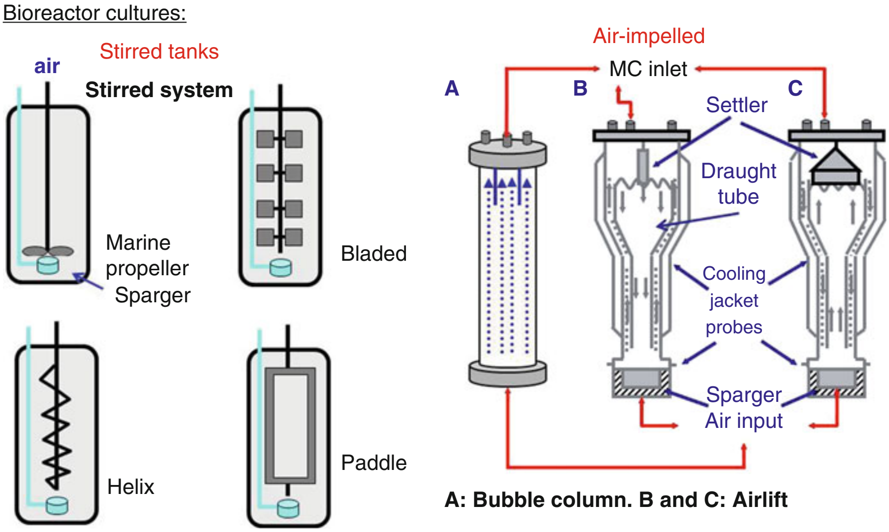
Bioreactor cultures:
Stirred tanks
air
Stirred system
Marine propeller
Sparger
Bladed
Helix
Paddle
Air-impelled
MC inlet
A
B
C
Settler
Draught tube
Cooling jacket probes
Sparger
Air input
A: Bubble column. B and C: Airlift

Fig. 89.12 Adaptations of the bioreactor design to plant cell cultures. Unfortunately, despite the efforts to minimize shaking stress, it is not possible to eliminate it completely in all stirred tanks, which can be substituted by air-impelled bioreactors such as bubble columns and airlift

which creates an outer chamber where the air bubbles rise without crashing directly into the plant cells inside the draft tube.

A new bioreactor model, the wave-bioreactor, has been recently designed, in which the plant cells are introduced in a disposable plastic bag provided with all the probes (oxygen, pH, etc.) [22]. In this system, the disposable bag is placed over a rocker that moves the cells and the culture medium with wave-like motion, thus preventing the settling of the cells and creating a large turbulent surface for correct oxygen transfer (Fig. 89.13d).

Hairy root cultures are even more difficult to scale up than cell cultures because bioreactors are designed for the culture of microorganisms and not plant organs. Roots need two specific requirements: an anchoring system, since it is known that roots moving inside the bioreactor tank cannot grow, although the bioreactor can only begin to work when the roots are immobilized [23], and are protected from the blades of the stirred system. For both reasons, traditional stirred or airlift bioreactors are equipped with a stainless steel mesh that fixes the roots and protects them from the stirring (Fig. 89.13a, b). These problems have led to the design of new devices for optimizing the growth and production of hairy root cultures, such as the spay-bioreactor (Fig. 89.13c). Unlike other bioreactors, the roots in the spray-bioreactor do not grow immersed in the culture medium but are periodically sprayed with culture medium from the top of

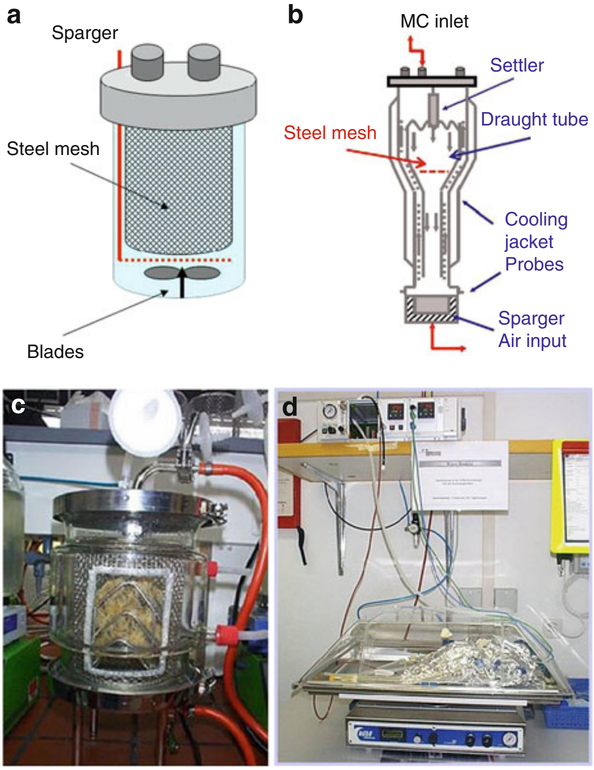

Fig. 89.13 Modifications of the bioreactor design for adaptation to hairy root cultures

the tank. It is as if the roots periodically receive a shower that keeps them moist and fed. The wave-bioreactor is also a good option for root cultures (Fig. 89.13d), and this system has been adopted by ROOTec bioactives Ltd., a company specialized in the production of phytochemicals in hairy root cultures. Also, CBN Biotech from Korea is currently producing and commercializing ginseng extracts from adventitious root cultures from *Panax ginseng* in bioreactors [24].

2.3.2 Industrial Production Systems

For the industrial production of phytochemicals, it is possible to use two kinds of procedures. When the target compound accumulates inside the cells, it is necessary to work in batch cultures (Fig. 89.14). After the growth and production period, the final biomass is harvested from the tank of the bioreactor and normally dried, and then the target compound is extracted from the cells or tissues. On the contrary, when the product is released from the plant cells, it is necessary to work in continuous cultures, in which after the production period, part of the culture

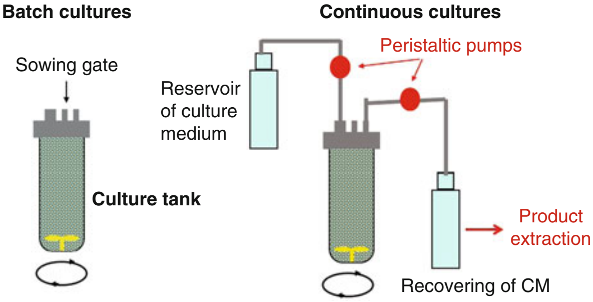

Fig. 89.14 Depictions of industrial process based on plant cell and organ cultures

medium is removed from the tank and replaced by fresh medium in order to rejuvenate the cell biomass (Fig. 89.14). In this system, the product is isolated from the culture medium by a simple procedure.

3 Biotechnological Production of Monoterpenes, Sesquiterpenes, Diterpenes, Triterpenes, and Tetraterpenes

3.1 Monoterpenes

Monoterpenes, the C_{10} class of isoprenoids, are an important class of commercially interesting terpenoids containing colorless lipophilic and volatile compounds. These are mostly found as constituents of essential oils and impart characteristic aromas or fragrances to flowers and flavors to fruits in many plant species. They are also used as herbicides, pesticides, antimicrobial agents, and dietary anticarcinogens. Due to various biological properties, monoterpenes have found extensive industrial applications and have been widely used as flavoring agents in foods and cosmetics, topical medicines, perfumes, and insecticides [25, 26].

Members of the genus *Mentha* are well known for their production of an economically important monoterpene, menthol, which is used as an essential oil throughout the world. Mints have been used and valued as aromatic herbs for thousands of years [27]. There are several examples of menthol production from cell cultures [28–31]. Chang et al. [30] optimized the concentration of chitosan to enhance menthol production in *Mentha piperita* cells and observed that 200 mg L^{-1} gave 166 mg menthol L^{-1} after 12 days of culture. They reported that elicitation with chitosan leads to conversion of pulegone to menthol. Kim et al. [29] studied the effect of a two-phase culture system on cell growth and essential oil formation in *M. piperita*. Their results indicated that the addition of solid second phases (XAD-4, XAD-7, LiChroprep RP-8) affected *M. piperita* cell growth depending on the type and concentrations employed. Cell growth rate was better in the medium containing RP-8 than XAD-4 or XAD-7. Additionally, RP-8 enhanced the peppermint oil formation by 32%. In control cultures (without RP-8), oil content was 89 μg L^{-1}, which was enhanced to 118 μg L^{-1} by a two-phase culture system using RP-8. It is speculated that RP-8 adsorbs the inhibitors of secondary metabolism promoting essential oil biosynthesis. Different strategies like precursor feeding, elicitation, and in situ product removal have been employed, and their synergistic effect has been studied to stimulate menthol production in *M. piperita* [31]. Menthol yield was found to increase by up to 92 and 110 mg L^{-1} in comparison with 77 mg L^{-1} of the control culture upon addition of 60 μM γ-cyclodextrin and 35 μM menthone. Treatment of cells with the fungal elicitor *Aspergillus niger* enhanced the production level up to 140.8 mg L^{-1} in comparison with the control. The synergistic effect of menthone feeding and γ-cyclodextrin treatment followed *by* in situ adsorption with RP-8 has shown a potential way of stimulating menthol production in *M. piperita* cell cultures. The maximum amount of 148 mg L^{-1} menthol has been reported as a result of synergistic effect of these treatments [31].

β-Thujaplicin (also known as hinokitiol), found mostly in trees of the Cupressaceae family, is a novel tropolone responsible for the durability of heartwood [32]. It has also been used clinically and as an ingredient in cosmetics and in toiletry products due to its broad antimicrobial, antifungal, and many other biological activities [33]. Plant cell culture is considered to be an alternative and reasonable approach for the commercial production of monoterpenes as well as tropolone compounds like β-thujaplicin [34–36]. For in vitro production of β-thujaplicin, callus and cell suspension cultures of *Cupressus lusitanica* have been studied and culture conditions were optimized [37, 38]. The concentration of major nutrients, mineral nutrient salts, Fe (II), and vitamins played a role in optimum cell growth and production of β-thujaplicin in cell suspension cultures of *C. lusitanica* [37]. It has been generally found that cell growth is inversely related to secondary product formation. The growth of *C. lusitanica* cells required a sufficient supply of nutrients, but Fe (II) had an inhibitory effect, while insufficient inorganic nutrients and excess Fe were effective for β-thujaplicin production. The type and concentration of the carbohydrate source has also been found to be responsible for monoterpene production. Among the different mono- and disaccharide carbon sources, glucose at a 20 g L^{-1} concentration was found to be the most effective form for β-thujaplicin production in cell suspension cultures of *C. lusitanica* [37]. Sugars are known as a source of carbon as well as acting as an osmotic pressure regulator. Glucose, sorbitol, and mannitol were studied for β-thujaplicin production in the same plant species. A mixture of sugar alcohols (i.e., glucose and sorbitol at 15 g L^{-1}) and small amounts of glucose (5 g L^{-1}) significantly increased the level of β-thujaplicin in the cell culture. Yamada et al. [39] tested the initial cell density for β-thujaplicin production in cell suspension cultures of the same plant species. They found that initial cell density of cultures did not affect β-thujaplicin levels, but the initial addition of β-thujaplicin suppressed its de novo production. They proved that β-thujaplicin synthesis was regulated by a feedback mechanism and that excess accumulation of β-thujaplicin is relieved by its conversion to its methyl ether. Treatment of *C. lusitanica* cells with chemical as well as fungal elicitors can improve the mass production of β-thujaplicin from callus and cell suspension cultures [38, 40]. It has been shown that elicitor treatment with yeast extract induces many novel kinds of terpenes in addition to a significant amount of β-thujaplicin [36].

3.2 Sesquiterpenes

Ambrosia maritima, a perennial herb belonging to the Compositae family, is a source of sesquiterpene lactones. It is used as a molluscicide for the treatment of sites infected with aquatic snails and intermediate hosts of tropical disease. Cell culture systems for these plants have been established for the in vitro production of sesquiterpene lactones [41]. It has been demonstrated that cultured plant cells are able to produce certain secondary metabolites in response to biotic or abiotic stress. Fungal (mycotoxins) and toxic algal extracts (phytoalexins) are the elicitors that act as a biotic stress causing a remarkable increase in active secondary metabolites. It has been reported that toxic algal crude extracts (phytoalexins) promote the production of sesquiterpene lactones (damsin and ambrosin) [41]. To study the effect of a fungal elicitor on sesquiterpene lactone accumulation in this plant species, cell suspension cultures were treated with different concentrations of *Aspergillus niger* [42]. The maximum yield of sesquiterpene lactones was recorded in a culture medium containing 60% fungal extract. HPLC analysis showed that the level of four major sesquiterpene lactones, that is, ambrosin, damsin, neoambrosin, and hymenin, was two times greater than their levels in control cultures. It was concluded from these results that a biotic elicitor with algal and fungal extracts may interact at the enzyme level or with isolated cell components or at the level of tissues, leading to deviations in the metabolic processes of intact plants, tissue culture, or cell suspension culture, versus the biosynthesis and accumulation of secondary metabolites [43].

In the Solanaceae family, bicyclic sesquiterpenoids (rishitin, capsidiol, lubimin, phytuberin, and phytuberol) have been isolated from different plant species including potato, pepper, and tobacco, and they are correlated with the defensive response of plants to invading pathogens [44]. Depending on the plant species, accumulation of the specific sesquiterpenoids occurs when the plant cells are treated with elicitors [45–48]. Chappell and Nable [49] observed an accumulation of large amounts of the sesquiterpenoid capsidiol in tobacco cell suspension cultures upon the addition of an elicitor prepared from fungal hyphae of *Phytophthora megasperma*. They showed the importance of induced HMGR activity, which was responsible for capsidiol production in elicitor-treated tobacco cell suspension cultures.

3.3 Diterpenes

Tanshinones (like tanshinone I, tanshinone IIA, IIB, cryptotanshinone, ferruginol), mainly found in roots and rhizomes of *Salvia miltiorrhiza*, are abietanoid diterpenes with common ortho- or para-naphthoquinone chromophore. They are major bioactive constituents of *S. miltiorrhiza* (Tanshen) with significant pharmacological activities. Tanshen is one of the important herbs used for the treatment of cardiovascular diseases. Other bioactivities include antioxidant, antitumor, and protective effects on the kidney and liver [50, 51]. According to Wang and Wu [52], in vitro cultures are good alternatives for the mass production of tanshinones and other bioactive compounds in *Salvia miltiorrhiza*.

Callus cultures using different explants have been established in *S. miltiorrhiza* by employing Murashige and Skoog medium supplemented with different combinations and concentrations of PGRs [52–54]. Hu et al. [55] studied the tanshinone contents in calli derived from different explants and found only trace amounts of tanshinones in leaf- and petiole-derived calli and a relatively high tanshinone content in calli induced from roots. Calli growth was observed to be faster in light than in the dark. Cell suspension cultures were initiated from calli, and six cell lines were obtained by subculturing the callus cells successively in a liquid MS medium [56]. Ferruginol production in cell suspension cultures was mainly found during the lag phase. Only one cell line was observed to produce cryptotanshinone as well as ferruginol. Medium composition such as the concentration of sucrose, nitrogen source, thiamine, and phosphate had a profound effect on the production of cryptotanshinone and ferruginol in the cell culture [57]. Different strategies like media optimization, elicitor stimulation, immobilization, nutrient feeding, and physical factors (light and dark conditions) have been employed to obtained an enhanced and stable production of tanshinones in *S. miltiorrhiza* in vitro cultures. A two-stage culture method, that is, normal medium for cell growth and then cell transfer to a medium without Fe-EDTA, has been established for a more efficient production of ferruginol from cell culture by Miyasaka et al. [58]. This media was also found to be suitable for cryptotanshinone production in cultured cells. Immobilization of cells from *S. miltiorrhiza* cell suspension cultures was reported to produce 39% and 61% of cryptotanshinone and ferruginol, respectively [59]. Abiotic and biotic elicitors have been applied to stimulate tanshinonene accumulation in *S. miltiorrhiza* cells [60]. Four classes of elicitors, viz., heavy metal ions (Co^{2+}, Ag^{+}·, Cd^{2+}), polysaccharides (yeast extract, chitosan), plant response signaling compounds (salicylic acid, methyl jasmonate), and hyperosmotic stress (sorbitol), were studied for their effects on tanshinone production in cell cultures [60]. Silver nitrate (25 μM), cadmium chloride (25 μM), and yeast extract (100 mg L^{-1}) were found to be the potent elicitors that stimulated the total tanshinone content by more than tenfold (2.3 mg g^{-1} vs. 0.2 mg g^{-1} in control) in *S. miltiorrhiza* cell cultures. Tanshinone biosynthesis in *S. miltiorrhiza* in vitro cultures has been reviewed recently by Wang and Wu [52].

Paclitaxel (Taxol, NSC-125973), obtained from *Taxus* species, is a diterpene with intense antitumoral activity because of its unique mode of action on the microtubular cell system. It is one of the most successful anticancer drugs developed in the past 50 years. Due to several difficulties associated with obtaining Taxol and related taxoids from plants growing in nature, plant cell cultures are considered to be the most favorable and environmentally sustainable approach for its production of at an industrial level. Some pharmaceutical companies, such as the consortium of Bristol-Myers Squibb with Phyton, have developed and commercialized plant cell culture processes for the production of Taxol. Currently, Phyton Biotech is the largest producer of paclitaxel via plant tissue culture, employing a large-scale fermentor with a capacity of up to 75,000 L. Another company, Korean Samyang Genex, uses *Taxus* plant cell cultures to produce paclitaxel with the brand name of Genexol® [6]. The most successful treatments to enhance the Taxol production in different species of *Taxus* involve supplementation of the culture medium with methyl jasmonate. To increase the production of paclitaxel and related taxanes in cell cultures, the effect of methyl jasmonate on free and immobilized cells of *T. baccata* growing in a selected product formation culture medium was investigated by Bonfill et al. [61]. The greatest amounts of paclitaxel (13.20 mg dm^{-3}) and baccatin III (4.62 mg dm^{-3}) were reported upon addition of methyl jasmonate (100 μM) to the culture medium of cells entrapped using a 1.5% and 2.5% alginate solution. Production of paclitaxel and baccatin III has been shown to be affected when *T. baccata* cell lines with different paclitaxel-producing capacities are mixed and cultured in a selected production medium, with and without methyl jasmonate [62]. Through mixing low-, medial-, and high-producing cell lines, paclitaxel productivity in the resulting mixed lines was found to be higher than the mean productivity of the individual lines before mixing, suggesting that the culture components generated by high-producing individual lines within the population might induce paclitaxel production. To study the relationship between the expression of genes from the Taxol biosynthetic pathway and the taxane profile, Onrubia et al. [63] studied the effect of two elicitors, methyl jasmonate and vanadyl sulfate, on the taxane pattern and transcript profile of two key genes (*txs* and *bapt*) involved in Taxol biosynthesis pathway in a selected *T. baccata* cell culture. Methyl jasmonate was found to increase the expression of both *txs* and *bapt* genes as well as significantly activate the production of both Taxol (4-fold) and baccatin III (3.6-fold). However, the presence of vanadyl sulfate in the medium only increased *bapt* gene expression and hence Taxol production (4-fold). These results suggest that analyzing elicitation conditions is a good strategy to improve Taxol production and that the elicitors have different mechanisms of action in Taxol biosynthesis.

3.4 Triterpenes

Triterpenes constitute a very important group of C_{30} terpenes arising from dimerization of two farnesyl pyrophosphate units that produce the intermediate compound, squalene. Among the triterpenes, there are compounds with a primary role in the plant metabolism such as the phytosterols which are involved in the cell membrane formation, whereas others are considered as typical secondary compounds. Some of these secondary compounds, which play a crucial role in the defense and adaptation system of the plant, also show important pharmacological properties and consequently are products of high interest for the chemical-pharmaceutical industry.

Standing out among the bioactive triterpene compounds are the ginsenosides, which are tetracyclic triterpenoid saponins derived from the dammarane skeleton. Ginsenosides are the principal pharmacologically active components of *Panax* spp. roots. The ginseng (*Panax ginseng* Meyer) root has been used for over 2,000 years as a panacea and to promote longevity. Recently, there has been a renewed interest in investigating ginseng pharmacology using biochemical and molecular biological techniques. Pharmacological effects of ginseng have been demonstrated in the CNS and in cardiovascular, endocrine, and immune systems. In addition, ginseng and its constituents have been ascribed antineoplastic, anti-stress, and antioxidant activity. Consequently, ginseng is an herb with many active components, and its beneficial effects are supported by evidence from numerous studies [64].

Ginsenosides are divided into three groups on the basis of their structure: the Rb group (protopanaxadiol including Rb_1, Rb_2, Rc and Rd, and others), Rg group (protopanaxatriols including Rg_1, Rg_2, Re, Rf, and others), and Ro group (oleanolic acid). So far, more than 32 ginsenosides have been identified. It has been shown that the ginsenosides of groups Rb and Rg have different pharmacological properties, some of them being antagonistic, so it is very important to know the exact composition of ginseng material to determine its medicinal activity [65].

Typical ginseng drugs are crude, not well-defined extracts from the roots of 6-year-old plants. The *Panax ginseng* plant is not only widely used as a dietary supplement, but the active ginsenosides are also found in different pharmaceutical forms. In the USA alone, the annual retail sale has reached US $120 million, and the global market is more than US $19.5 billion [66].

Since ginsenosides are accumulated in the root of the plant, mainly after 6 years of growth, their agricultural production is very expensive. Therefore, ginsenoside production by means of biotechnological alternatives has been extensively studied by a number of researchers, using different plant-derived systems such as callus tissues, cell suspensions, and transformed and adventitious root cultures.

The production of ginsenosides in callus cultures started at the beginning of the 1980s. Furuya et al. [67] reported that *Panax ginseng* calli produced considerable amounts of the same kinds of saponins as intact plants and the saponin content was significantly lower in habituated calli than in normal calli. An important European team that studied the ginsenoside production was the Björk group [68], who obtained 33.7 mg g^{-1} DW of total ginsenosides in callus cultures after optimizing the culture conditions. Bonfill et al. [65] achieved total ginsenoside levels of 50 mg g^{-1} DW in root-forming calli, showing that the proportion of Rb group and Rg group ginsenosides depends on the auxin added to the culture.

Panax cell cultures have also been used as a source of ginsenosides since the 1970s. Yasuda et al. [69] started with scaling-up cell cultures to volumes of 600 L. The Japanese company Nitto Denko Corp. established cell cultures at the 20,000–25,000 L bioreactor level, achieving a biomass production of 20 g DW L^{-1} in 4 weeks and also showing that the pharmacological effects of the active ingredients were the same or even higher than the effects caused by the same compounds extracted from the plant roots. Other companies involved in the ginsenoside production by means of cell suspensions are the All-Union Biotechnological Institute from Moscow and PhytoLife in Israel. The effect of the addition of precursors and/or elicitor supplementation on ginsenoside production and pattern in cell cultures has also been assayed. Yue and Zhong [70] have reported that the addition of phenobarbital (1 mM) to the culture medium enhanced the production of ginsenosides belonging to the Rg group but not those of the Rb group. In their studies, they achieved an Rg ginsenoside production of 5.7 mg day^{-1} L^{-1}. Other elicitors such as methyl jasmonate and salicylic acid have also been assayed with good results.

Hairy root cultures have proven to be a good system for ginsenoside production. Mallol et al. [71] obtained *P. ginseng* hairy root cultures producing higher levels of total ginsenosides (5.5 mg g^{-1} DW) than the plant roots (approx. 4.5 mg g^{-1} DW). The same author increased the total ginsenoside production when scaling up the culture from shake flasks to a 2 L wave bioreactor. In fed-batch conditions, the roots produced 2.6 mg ginsenosides L^{-1} day when maintained for 56 days in the bioreactor. Additionally, different reports confirm that ginseng adventitious roots constitute an excellent starting material for ginsenoside production without the problems associated with the presence of foreign genes in the plant genome. Sivakumar et al. [24] showed, after optimizing the culture conditions, that the culture of ginseng adventitious roots in a 10,000 L balloon bioreactor is a good alternative for the production of ginsenosides at an industrial level.

The withanolides are a group of naturally occurring C28-steroidal lactones bearing an ergostane skeleton which are found in species of *Withania*. *W. somnifera* is an important medicinal plant used in Ayurvedic medicine for over 3,000 years. Withanolides are compounds that have shown pharmacological properties such as anti-inflammatory, antitumor, anti-stress, antioxidant, immunomodulatory, hemopoietic, and cardio-protective activities. Recently, Mirjalili et al. [72] have shown that significant levels of withanolide A and withaferin A accumulate in *W. coagulans* transformed roots, suggesting that these roots have considerable capacity to produce withanolides in a scaled-up bioreactor system.

Traditional Mexican medicine has long used *Galphimia glauca* to treat central nervous system disorders. This plant produces nor-seco-galphimines as the main active compounds, which present different pharmacological properties. Osuna et al. [73] reported that free and immobilized cells of this species were able to produce galphimine-B (the most active galphimine compound, with sedative, anticonvulsive, and anxiolytic properties) when cultured on a small scale and in bioreactors. The advantages of immobilized cells include high cell concentration per unit volume of culture, better cell-cell contact, and more favorable conditions for cell differentiation, all promoting cell differentiation and consequently increasing the secondary metabolite production. These authors achieved a 1,381 mg L^{-1} galphimine-B production with immobilized cells growing for 24 days in a stirred bioreactor, with the advantage that all the product of interest, formed inside the cells, was excreted to the medium in its entirety, thus facilitating its recovery.

3.5 Tetraterpenes

Tetraterpenes or carotenoids consist of eight isoprene units derived from isopentenyl diphosphate. Carotenoids are found predominantly in nature as the orange, red, and yellow pigments synthesized by photosynthetic plants, bacteria, and fungi. Chemically divided into xanthophylls and carotenes, carotenoids can serve as precursors to phytohormones and aroma compounds in plants, and they have a multitude of roles in nature [74]. Tomatoes contain a mixture of carotenoids, including carotenoid lycopene, γ-carotene, phytoene, neurosporene, phytofluene, ζ-carotene, β-carotene, and lutein [75]. Campbell et al. [76] used a plant cell culture approach to biosynthesize and radiolabel phytoene and phytofluene for prostate cancer cell culture studies. To induce the biosynthesis and accumulation of the lycopene precursors, phytoene and phytofluene, the herbicide norflurazon was added to established cell suspension cultures of tomato (*Lycopersicon esculentum* cv. VFNT cherry). Norflurazon concentrations, solvent carrier type and concentration, and duration of culture exposure to norflurazon were studied to optimize phytoene and phytofluene synthesis. The maximum yields of both phytoene and phytofluene were observed after 7 days of treatment with 0.03 mg norflurazon/40 mL fresh medium, provided in a 0.07% solvent carrier. Introduction of 14C-sucrose to the tomato cell culture medium enabled the production of 14C-labeled phytoene for subsequent prostate tumor cell uptake studies. In an attempt to increase the carotenoid production in cell cultures of *L. esculentum*, two bleaching herbicides, 2-(4-chlorophenyl-thio)-triethylamine (CPTA) and norflurazon, separately or in combination, were administered during the culture incubation to produce varying ratios of carotenoid lycopene, phytoene, and phytofluene [77]. Norflurazon inhibits phytoene desaturase, leading to the accumulation of phytoene and phytofluene, while CPTA inhibits lycopene cyclase, leading to an increase in carotenoid lycopene accumulation. It has been found that treatment with both herbicides results in an optimal production of all three carotenoids. Screening and selection of high carotenoid-producing in vitro tomato cell culture lines for isotopically labeled [(13)C]-carotenoid production in tomato cell cultures were first achieved by [77]. Different *Solanum lycopersicum* allelic variants for high lycopene production and varying herbicide treatments were compared for carotenoid accumulation in calli as well as cell suspension cultures.

Tomato ripening involves many processes, such as flavor development, color formation, and softening. Regulation of ripening initiation and its progress has been attributed to the influence of many chemical substances. These substances include ethylene, auxins, cytokinins, calcium, copper, ammonia, abscisic acid, polyamines, and oligosaccharides [78]. Lycopene, the red pigment which is involved in ripening of tomato fruits is another example of tetraterpene. Cell suspension cultures of tomato have been used to observe the enhancement of lycopene in tomato cells using auxins (2-(4-chlorophenylthio)-triethylamine (CPTA) and picloram) to confirm the role of CPTA as an enhancer of protein synthesis [79] and as a stimulator of the disappearance of chloroplasts and the accumulation of chromoplasts [80]. Robertson et al. [78] reported the use of cell culture methods for the in vitro observation of in vivo lycopene formation. They reported the use of growth regulators, sucrose, and temperature to regulate lycopene accumulation and found that the native auxin indole-3-acetic acid (IAA) was substantially more effective than 2,4-dichlorophenoxyacetic acid (2,4-D) in promoting lycopene formation, sucrose-inhibited lycopene formation (cell basis), and an optimum temperature of 18–26 °C produced a pattern similar to that observed in the field. Chilling injury of tomato plants is a cellular phenomenon, so cell cultures are a useful model system to study this mechanism. To observe the effect of chilling temperature, tomato cell cultures were kept at temperatures below 10 °C [81]. It was shown that chilling temperatures quickly inhibited the growth of tomato cells but did not kill them.

4 Metabolic Engineering of Terpenes in Plant Cells and Roots

The biosynthetic production levels of many secondary compounds have been increased by the application of metabolic engineering techniques. The stable transfer and integration of genes involved in flux-limiting steps of a biosynthetic route could enhance the endogenous production of the resulting compounds. So it is necessary to understand the relevant biosynthetic pathway(s) and know which enzymes catalyze the sequence of reactions, particularly the slow steps, and which genes encode these enzymes.

Some applications of metabolic engineering to improve terpene production in plant cells and roots are presented here.

4.1 Artemisinin Production in Engineered Hairy Roots

Artemisia annua is an annual herb that produces the antimalarial drug artemisinin, a *sesquiterpene* lactone present mainly in the aerial parts.

As artemisinin has a terpenic structure, its biosynthesis starts in the formation of isopentenyl diphosphate (IPP), as in all the natural terpenoids. In plants, IPP is synthesized either via the mevalonate pathway in the cytosol or via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in the plastid. The IPP derived from the mevalonate pathway is generally used in the biosynthesis of sesquiterpenes (such as artemisinin), phytosterols, and triterpenes, and the IPP derived from the non-mevalonate pathway is employed in the biosynthesis of monoterpenes, diterpenes, and tetraterpenes (Fig. 89.15).

Farnesyl diphosphate (FPP) is the starting molecule for the committed step in sesquiterpene biosynthesis, so artemisinin is synthesized from FPP via the mevalonate pathway in the cytosol (Fig. 89.15). A rational approach has enhanced artemisinin yields by the overexpression of farnesyl diphosphate synthase

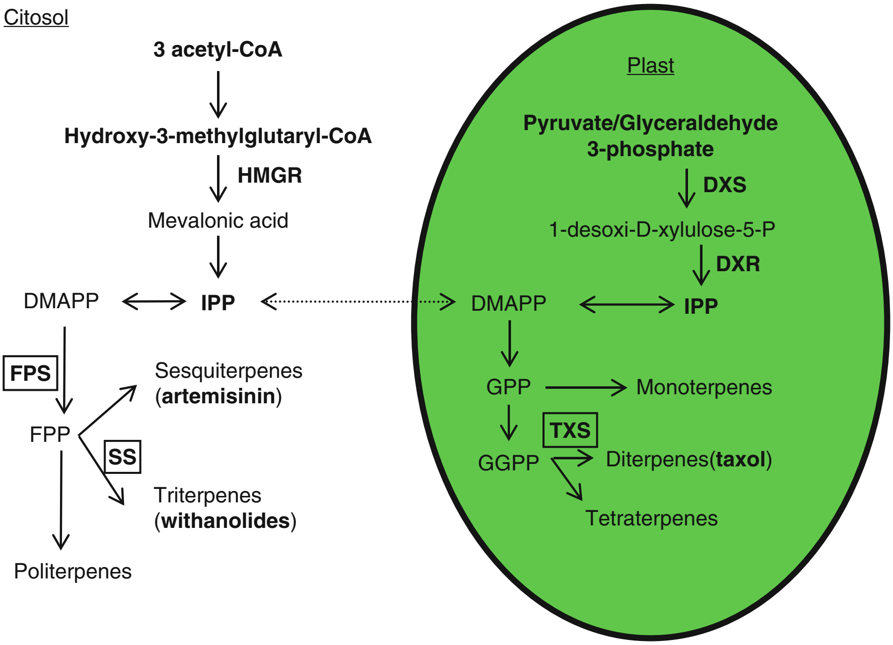
Citosol
3 acetyl-CoA
Hydroxy-3-methylglutaryl-CoA
HMGR
Mevalonic acid
DMAPP
IPP
FPS
FPP
Sesquiterpenes
(artemisinin)
SS
Triterpenes
(withanolides)
Politerpenes
Plast
Pyruvate/Glyceraldehyde
3-phosphate
DXS
1-desoxi-D-xylulose-5-P
DXR
DMAPP
IPP
GPP
Monoterpenes
TXS
GGPP
Diterpenes(taxol)
Tetraterpenes

Fig. 89.15 Biosynthetic pathways of plant terpenes

(FPS, EC2.5.1.1/EC2.5.1.10), the enzyme that catalyzes two consecutive condensations of isopentenyl diphosphate with dimethylallyl diphosphate to form FPP. The cDNA encoding FPS placed under a 35S CaMV promoter was transferred into *Artemisia annua* using *Agrobacterium rhizogenes* to obtain hairy roots with the *fps* transgene. This study has shown that engineered hairy roots can produce 2–3 mg g^{-1} DW artemisinin, a quantity three to four times greater than achieved in the controls [82].

4.2 Taxol and Taxane Production in Engineered Plant Cells

Taxol is a *diterpene* alkaloid with notable antineoplastic properties due to its unique mode of action on the microtubular cell system. The difficulty of obtaining Taxol from its natural source and costly synthetic processes place it among the compounds with highest added value. Taxol can also be produced semisynthetically from more abundant taxoids, but extracting the precursors is also difficult and expensive. Although Taxol is currently being produced in *Taxus* cell cultures on a bioreactor scale, the amount obtained is still insufficient to meet growing world demand. Metabolic engineering of *Taxus* with rate-influencing genes is a potentially successful approach to increasing Taxol production in cell cultures.

As Taxol has a diterpenic structure, its biosynthesis starts in the formation of isopentenyl diphosphate (IPP) via the 2-C-methyl-D-erythritol 4-phosphate (MEP)

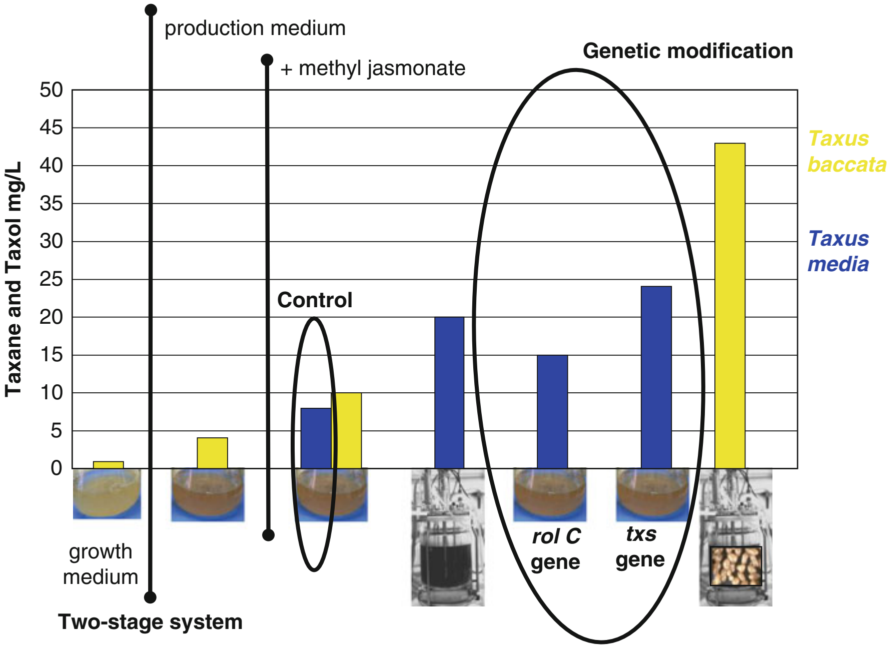

Fig. 89.16 Different approaches to taxane production

pathway in the plastid. Metabolic engineering techniques were applied to establish *Taxus media* transformed cultures harboring the taxadiene synthase (*txs*) gene, which is known to encode taxadiene synthase (TXS, EC 4.2.3.17), the enzyme that controls the first step in the taxane skeleton formation (Fig. 89.15). The stable transfer and integration of this gene could enhance the endogenous production of Taxol.

After direct inoculation of *T. media* seedlings with *Agrobacterium*, two transformed cell lines were studied: RolC, carrying the T-DNA of *A. rhizogenes* 9402 and TXS, carrying both the T-DNA of *A. rhizogenes* and the *txs* transgene of *T. baccata* under the control of the 35S CaMV promoter. The transformed cell lines were cultured in a production medium supplemented with the elicitor methyl jasmonate, and their production was compared with an untransformed *T. media* cell line cultured in the same conditions. The highest taxane production was observed in the TXS cell line when cultured in the optimized production medium with methyl jasmonate, being 265% greater than in the untransformed control and 170% greater than in the RolC cell line [83].

Figure 89.16 compares the different approaches to taxane production using untransformed *T. baccata* cells (yellow) and untransformed and transformed *T. media* cells (blue) under different conditions. An empirical approach to these cultures involved the use of a growth medium, a production medium, and a production medium supplemented with the elicitor methyl jasmonate.

The rational approach involved the genetically modified *T. media* lines (RolC and TXS). These transformed lines showed a higher taxane production than the non transformed line under the same culture conditions. The next step to further increase taxane production could be to culture immobilized cells of these transformed lines in a stirred bioreactor.

4.3 Withanolide Production in Engineered Hairy Roots

The genus *Withania* (*Solanaceae*) includes two medicinally important species, *W. somnifera* and *W. coagulans*, whose antitumor and neuropharmacological properties have been attributed to *triterpenic* steroidal lactones named withanolides. The biosynthetic pathway of withanolides is not fully known, but since they probably derive from cholesterol, they likely follow the mevalonate pathway in the cytosol like all the triterpenes. Among the genes involved in this pathway, the squalene synthase gene (*ss*) is particularly interesting because it encodes squalene synthase (SS, EC2.5.1.21), the enzyme that dimerizes two molecules of farnesyl diphosphate to synthesize squalene, a shared precursor in steroid and triterpenoid biosynthesis in plants (Fig. 89.15). The *ss1* gene from *Arabidopsis thaliana* was introduced in *W. coagulans* under the control of the 35S CaMV promoter together with the T-DNA of *Agrobacterium rhizogenes* A4. The obtained engineered roots carrying the *ss1* gene (SS lines) increased the production of withanolides 2.5-fold in relation to the control roots harboring only the T-DNA from pRiA4 [84].

5 Conclusion

Biotechnology offers an opportunity to sustainably exploit plant cells, tissues, organs, and the entire plant organism, using in vitro cultures and genetic manipulation, for the production of plant secondary metabolites, including important terpene compounds that are beneficial for human health and nutrition. Cultivable land and endangered species would benefit from this alternative practice. As described in this chapter, empirical approaches have been employed for the development and optimization of plant cell-based bioprocesses for terpene production. An empirical approach typically optimizes the plant cell culture system regarding its input factors (cell line, medium, culture parameters, bioreactors, process operations, etc.) and output factors (cell growth, nutrient uptake, productivity, yield, etc.), but what occurs at the cellular and molecular levels remains largely unexplored [85]. In contrast, a rational approach aims to gain knowledge about what is happening in the cells when production increases, but this requires the characterization of genes and proteins involved in secondary pathways, as well as an understanding of their respective regulatory function and role, before finally the biosynthetic pathways can be manipulated for a given application via metabolic engineering. Powerful new tools are currently available, such as those used in studies of functional genomics (transcriptomics, proteomics, and metabolomics).

This has led to an acceleration in the last decade in secondary metabolism research and generated a wealth of new knowledge related with terpene metabolism and other plant secondary pathways [5].

Metabolic engineering in plants, involving the modification of endogenous pathways, has three basic goals: to increase the production of a desired compound, to decrease the production of unwanted compounds, and to produce novel compounds. Strategies for achieving these goals include the engineering of single steps in a pathway to increase or decrease metabolic flux to the target compound, blocking competitive pathways or introducing shortcuts that divert metabolic flux in a particular way. However, this approach has only limited value, and targeting multiple steps in the same pathway could help to control metabolic flux in a more predictable maner. In this context, the use of regulatory genes to establish multiple control over one or more pathways in the cells can constitute an effective strategy [86]. The ability to switch on an entire metabolic pathway in engineered cell cultures by ectopic expression of transcription factors or other regulatory peptides opens new possibilities for secondary metabolic pathways [87], especially those that are not fully elucidated, as is the case of several secondary terpene compounds. This system probably constitutes one of the most promising strategies to develop biotechnological processes for producing high-value terpenoids at an industrial level.

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Taxol Producing Fungi

90

Xiaoran Hao, Jiao Pan, and Xudong Zhu

Contents

1 Introduction 2798
2 Current Methods for Taxol Production 2800
2.1 Extraction of Taxol from Yew Tree Materials 2800
2.2 Taxol from Plant Tissue Culture and Cell Suspensions 2800
2.3 Total Chemical Synthesis of Taxol 2801
2.4 Semisynthesis of Taxol 2801
3 Taxol-Producing Fungi: A Prospective New Source for Taxol Making 2801
3.1 Biodiversity of Taxol-Producing Fungi 2802
3.2 Methods for Screening Taxol-Producing Fungi 2802
4 Molecular Study on Taxol Biosynthesis 2803
4.1 The Biosynthesis Pathway in Yew Tree 2803
4.2 The Biosynthesis of Taxol in Fungi Remains a Puzzle 2804
4.3 Genetic Engineering for Taxol Production 2804
5 Problems and Perspective 2805
References 2806

Abstract

Paclitaxel (Taxol®), a diterpenoid natural product, has emerged as a widely used anticancer drug with its unique mode of action and efficacy against multiple forms of cancers. The fast growing demand in basic research and clinic chemotherapy requires to increase the global supply. Current industrial production of the drug with semisynthesis with the extraction of Taxol precursors from the yew trees cannot meet the market requirement. Thus, alternative techniques have been intensively explored. One of the promising strategies is the use of fungal

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fermentation for Taxol production. This chapter summarizes the recent advances on the study of Taxol-producing fungi, including its taxonomical diversity of the identified strains, the methods for characterization of fungal Taxol, and the molecular basis and heterogenous biosynthesis of Taxol. Current problems delaying fungal production of Taxol in industry were also discussed.

Keywords

Anticancer drug • Taxol • entophytic fungi • diversity • fermentation • biosynthesis pathway

Abbreviations

10-DAB III	10-deacetylbaccatin III
10-DABT	10-Deacetylbaccatin III-10-O-acetyltransferase
AIDS	Acquired immune deficiency syndrome
BAPT	C-13 phenylpropanoid side chain-CoA acyltransferase
CIEIA	Competitive inhibition enzyme immunoassay
CNS	Central nervous system
DBAT	10-deacetylbaccatin III-10-O-acetyl transferase
DBTNBT	3′-N-debenzoyl-2′-deoxytaxol N-benzoyltransferase
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FPP	Farnesyl diphosphate
GGPP	Geranylgeranyl diphosphate
GGPPS	Geranylgeranyl diphosphate synthase
MBC	Metastatic breast cancer
MEP	Mevalonate pathway
NMR	Nuclear magnetic resonance
PAM	Phenylalanine aminomutase
PCF	Plant cell fermentation
PES	Paclitaxel-eluting stent
PTX	Paclitaxel
SM	Secondary metabolism
TAT	Taxa-4(20) 11(12)-dien-5α-ol-O-acetyltransferase
TBT	Taxane 2α-O-benzoyltransferase
THY13α	Taxane 13α-hydroxylase
THY5α	Taxane 5α-hydroxylase
TS	Taxadiene synthase
TSMA	Taxol-specific monoclonal antibodies

1 Introduction

As one of the most successful antitumor drugs, paclitaxel (Taxol®), a diterpenoid natural product, was originally isolated in the bark of the Pacific yew tree *Taxus* *brevifolia* in the mid-1960s [1]. The structure by X-ray crystallography was reported by Wall, Wani, and MacPhail in 1971 [2]. In the later years, an antimicrotubular action of paclitaxel was revealed, i.e., to inhibit disassembly of microtubule and arrest cell cycle at the stage of G2-M phase, thereby causing cell death via apoptosis [3–7]. In addition, Taxol has also been shown to repress angiogenesis by altering cytoskeletal structures and inhibiting endothelial cell migration in vitro and in vivo [7, 8]. Taxanes, a family of natural compounds with the same tricyclic diterpene nucleus that paclitaxel carries, have been increasingly found ever since. Many of them are shown to have a strong antitumor activity and may serve as excellent lead molecules for new drug development [1]. Approximately 10 more taxanes are in either Phase I or Phase II clinical trials, and another 23 taxanes are in preclinical development in PHRMA [9].

Since its approval for treatment of refractory ovarian cancer by FDA in 1992, Taxol has achieved great success in treatment due to its effectiveness and less side effects than other drugs. It has been the best selling drug of its kind in the market. As a broad-spectrum cancer killer, Taxol is currently used in the therapy against ovarian, breast, melanoma, pancreatic, lung, gastric, head and neck carcinomas, and the AIDS-related Kaposi's sarcoma [7, 10]. Besides, new applications of Taxol keep forthcoming, for instance, Taxol protects primary cortical neurons from beta amyloid (Aβ)-induced cell death; thus, it may be used for the purpose of slowing the development of neurofibrillary that leads to the loss of neuronal integrity in Alzheimer's disease [11–13]. Comparing to classic bare-metal stents, paclitaxel-eluting stents (PES) have superb safety and can markedly decrease angiographic and clinical restenosis following percutaneous coronary intervention [14]. The PES system, TAXUS™ Express2™, was granted an approval for clinical application by FDA to Boston Scientific Corporation in March 2004.

Development of novel variant treatments utilizing Taxol has boosted the consumption of Taxol, and as a consequence the demand of Taxol supply in the market keeps growing. Major obstacles for best therapeutic results with Taxol are its poor solubility and consequently difficulties in delivery [15]. Researchers have made attempts to improve the Taxol efficiency by combination with other drugs or modified to develop Taxol analogues [16]. Docetaxel (Taxotere), a modified derivative of Taxol, is easier to formulate and administer due to its greater aqueous solubility. A novel C-seco taxane analogue, IDN 5390, is active against all human tumor xenografts investigated, including the PTX-resistant ones [17, 18]. The anti-vascular endothelial growth factor receptor antibody bevacizumab and sunitinib in combination with Taxol exhibits a better outcome than taxane alone in the treatment of metastatic breast cancer (MBC) [19, 20]. Vorinostat enhances the efficacy of carboplatin and paclitaxel in patients with advanced non-small-cell lung cancer cell lines [21]. The anionic and amide introduction strategy may allow for delivery of Taxol into the central nervous system (CNS) [22].

With more pharmacological applications, the demand for Taxol in the market keeps increasing annually by an estimate of 5–8%. Short global supply exists as a major drawback for the use of the drug as the affordability is concerned.

This chapter focuses on the study of Taxol-producing fungi that promise an alternative way of paclitaxel manufacturing and virtually change the situation of the drug supply in the market.

2 Current Methods for Taxol Production

2.1 Extraction of Taxol from Yew Tree Materials

In the early years after marketed, Taxol was largely extracted from wild yew trees, its bark and other parts. Leading pharmaceutical companies soon started large-scale farming of yew trees. By now, extract of this chemical from raw tree materials is still playing a part in Taxol industry. Unfortunately, Taxol makes up only a small proportion of the total taxoids in *Taxus* trees [23]. Its natural concentration is approximately 0.01% of a dry weight basis in Pacific yew [24]. The taxane contents varied among and within *Taxus* spp. [25–27]. Even in *T. media* Hicksii, a hybrid variety of yew with a highest content of Taxol known so far, Taxol content is between 109 and 112 mg/kg of the dried needles [25]. The problem with this extraction strategy is that Taxol content is significantly influenced by the geographical location, altitude, age, and sex of the trees [23]. The season to harvest and the way to handle the tree materials also have effects on the yield of Taxol [28, 29]. Due to slow growth of yew trees and the low-yield extraction techniques [30], global production of Taxol with this strategy is limited and cannot meet the growing market.

2.2 Taxol from Plant Tissue Culture and Cell Suspensions

Plant cell, tissue cultures, or cell suspension culture of *Taxus* spp. is considered as a comparably fast way to obtain sufficient amount of tree biomass [31–33]. *Taxus* cell cultures have been extensively investigated to increase the content of Taxol via different methods, including screening for the high-taxol-producing genotypes and hybrids [34], nutrients, chemical elicitors (methyl jasmonate, silver thiosulfate), plant growth regulators [35–38], the heat shock [39], mechanical stimulating, two-phase culture [40], and others. The production of Taxol was remarkably improved by these processes. Plant cell fermentation (PCF) technology developed by the German and Canadian biotechnology company Phyton Biotech, Inc., is used for Taxol production (http://www.phytonbiotech.com/news_031215.htm). However, low and unstable Taxol yield, high production cost, and by-product contamination are the main bottleneck for large-scale commercial application of this technology [32]. The overall cost of Taxol production via cell culture or cell suspension remains still high.

2.3 Total Chemical Synthesis of Taxol

Several different synthesis routes of paclitaxel have been published. The total synthesis has been first achieved independently by two teams in 1994, the Holton's and the Nicolaou's [41–43]. The third synthetic route was accomplished by Danishefsky's group in 1996 [44]. Total synthesis of Taxol is a complex task for chemists given the fact that the molecule consists of four complicated rings (A, B, C rings and the oxetane ring) and has 11 chiral centers. The synthesis processed more than 20 steps, and only 0.07% and 2.7% production rates for the Holton's and Nicolaou's routes were obtained, respectively [41, 42]. Mukaiyama et al. have proposed an improved method for the asymmetric total synthesis of Taxol by a different way [45]. An automated synthesizer with a 36-step synthesis sequences for intermediate of Taxol was developed by Doi et al. (2006) [46]. Due to the complexity of Taxol structure, the expensive chemical reagents, and the strict requirement for reaction, the process of the total synthesis of Taxol is multiple-step, costly, and commercially unfeasible for industrial application for Taxol manufacture.

2.4 Semisynthesis of Taxol

Starting with 10-deacetylbaccatin III (10-DAB III), a precursor of Taxol biosynthesis in yew with higher content, several coupling strategies of a phenylisoserine moiety with protected 10-DAB III have been reported by several groups [47–51]. 10-DAB III can be isolated from the needles of *T. media* and *T. baccata* with relatively higher yield [52–55]. The development of Taxol synthesized from baccatin III and C-13 Taxol side chain represents a breakthrough of Taxol industrial production and has become a major source of Taxol and taxotere in industry [47–51]. This semisynthesis has significantly improved the production and global supply of the drug and makes the drug more affordable to patients. However, an unsolved issue of this widely used method is its dependence on the supply of yew tree materials.

3 Taxol-Producing Fungi: A Prospective New Source for Taxol Making

In 1993, Stierle, et al. [56] reported a fungus from the bark of a Pacific yew tree (*T. brevifolia*) that was able to produce Taxol, even when the fungus was grown as a pure culture. This finding stirred up interest of scientists in searching for Taxol-producing fungi all over the world that promises a different approach to produce the compound. Fungal fermentation has been widely used in agriculture, pharmaceutical, and food industries for a long history. It is certainly a good practice for Taxol production at the industrial scale. Advantages of growing fungi over plant cell culture include the medium is simple and inexpensive, the period of fermentation is short, and fungi are convenient for genetic manipulation which is a benefit for breeding to improve the yield of Taxol. Still more, since large-scale fermentation will provide sufficient biomass for Taxol preparation, consumption of yew trees will eventually be eliminated.

3.1 Biodiversity of Taxol-Producing Fungi

Over the past decades, more than 50 Taxol-producing fungal isolates were described by groups from all over the world [57–61]. Most of these strains were isolated from various *Taxus* spp. as endophytes. Yet Taxol-producing fungi were also isolated from bald cypress [62], Wollemi pine [63], *Torreya grandifolia* [64], *Ginkgo biloba* [65], *Podocarpus* [66], *Terminalia arjuna* [67], *Hibiscus rosa-sinensis* [68], and other non-*Taxus* plants. A few epiphytic or saprophytic fungi were also found to make the compound [69]. Noh et al. [70] obtained a Taxol producer, *Pestalotia heterocornis*, from the soil collected in yew forest. Recently, our group isolated a Taxol-producing fungus NK101 from plant debris in the soil as well [71].

Several studies claimed traditional Asian medicinal plants may provide a fertile source for drug discovery. Gangadevi and Muthumary isolated a series of Taxol-producing fungi from medicinal plants [72–75]. Pandi et al. [76] found Taxol in the fermentation broth of an endophytic fungus, *Lasiodiplodia theobromae*, which was isolated from the leaves of *Morinda citrifolia*, a medicinal plant.

By far, identified Taxol-producing fungal strains are distributed to 36 genera: *Achaetomium*, *Alternaria*, *Aspergillus*, *Bartalinia*, *Beauveria*, *Bionectria*, *Botryodiplodia*, *Botrytis*, *Chaetomium*, *Cheatomella*, *Cladosporium*, *Colletotrichum*, *Ectostroma*, *Epicoccum*, *Fusarium*, *Gelasinospora*, *Geotrichum*, *Metarhizium*, *Monochaetia*, *Mucor*, *Ozonium* [57–61], *Papulaspora*, *Penicillium*, *Periconia*, *Pestalotia*, *Pestalotiopsis*, *Phoma*, *Phomopsis*, *Phyllosticta*, *Pithomyces*, *Sporormia*, *Taxomyces*, *Trichothecium*, *Tubercularia*, and *Xylaria*. Some strains remain to be classified [58]. The species in genus *Pestalotiopsis* are the most encountered Taxol producers.

3.2 Methods for Screening Taxol-Producing Fungi

A lack of high throughput screening methods is a drag for identification of fungal Taxol producers. Conventional screening steps include the isolation of fungal strains from collected samples, the culture of fungal isolates, and the extraction of the cultures with organic solvents. The organic phase needs to be concentrated to small volume of 0.5 mL. The residue was subjected to biochemical analysis including chromatography, ultraviolet absorption and mass spectrometry, nuclear magnetic resonance (NMR), and anticancer tests. This is a tedious and time-intensive procedure [71].

A few groups reported the development of Taxol-specific monoclonal antibodies (TSMA) and the enzyme-linked immunosorbent (ELISA) assay for Taxol screening. Jaziri et al. reported the use of TSMA for the detection and the semi-quantification of Taxol in a concentration ranging from 1 to 100 ng [77]. Grothaus developed an indirect competitive inhibition enzyme immunoassay (CIEIA) method in the detection of Taxol and cephalomannine in a concentration as low as 0.3 ng mL^{-1} [78]. Unfortunately, Taxol is a poor antigen, and sophisticated antibody is few in the market. Screening methods based on ELISA are not widely found in literature.

A couple of reports used molecular approaches for the purpose of screening Taxol-producing fungi. For instance, PCR screening procedure was used to amplify fungal genes putatively coding for the Taxol biosynthetic enzymes, such as taxadiene synthase (TS), 10-deacetylbaccatin III-10-O-acetyl transferase (DBAT), and C-13 phenylpropanoid side chain-CoA acyltransferase (BAPT), which are demonstrated to be involved in Taxol biosynthesis in yew trees [79, 80]. Foland constructed a paclitaxel-sensitive budding yeast strain *Saccharomyces cerevisiae* strain AD1-8-tax by introducing a mutated β-tubulin yeast gene that conferred the yeast a sensitivity to Taxol [81]. AD1-8-tax can be used as a convenient tool as a tester in a high throughput screening for Taxol produced in fungal culture. Based on our experience, the low sensitivity of AD1-8-tax strain is a limit of this method as for the time being Taxol concentration in fungal culture is still low.

4 Molecular Study on Taxol Biosynthesis

4.1 The Biosynthesis Pathway in Yew Tree

The biosynthesis of Taxol in *Taxus* spp. has been extensively investigated and basically elucidated due to tremendous efforts by Croteau and coworkers [82, 83]. Based on Taxol structure, it is believed that 19 enzymatic steps are required from the ubiquitous diterpenoid precursor geranylgeranyl diphosphate (GGPP), which is derived from deoxyxylulose-5-phosphate pathway and the mevalonate pathway. GGPP is cyclized by taxadiene synthase to form taxa-4(5),11(12)-diene which is regarded as the first committed step in Taxol biosynthesis. Following stages involve eight steps of cytochrome P450-mediated oxygenations, an array of acylations, oxetane ring formation, and several steps of side chain assembly, resulting ultimately in the synthesis of Taxol. Most of the genes encoding key enzymes involved in this pathway have been cloned and characterized, e.g., geranylgeranyl diphosphate synthase (GGPPS), taxadiene synthase (TS), taxane 5α-hydroxylase (THY5α), taxa-4(20), 11(12)-dien-5α-ol-O-acetyltransferase(TAT), taxane 10β-hydroxylase (DBAT), taxane 13α-hydroxylase (THY13α), taxane 2α-O-benzoyltransferase (TBT), 10-deacetylbaccatin III-10-O-acetyltransferase(10-DABT), phenylalanine aminomutase (PAM), baccatin III:3-amino-3-phenylpropanoyltransferase (BAPT),

and 3′-N-debenzoyl-2′-deoxytaxol N-benzoyltransferase (DBTNBT) [32, 83–87]. Although effective transformation methods have been successfully established in *Taxus* species [88–90], the roles of the genes in Taxol biosynthesis have not been directly demonstrated by genetic manipulation such as gene deletion.

4.2 The Biosynthesis of Taxol in Fungi Remains a Puzzle

The biosynthesis pathway and regulation mechanism of Taxol in fungi are utterly unknown. An intriguing question is whether the pathway is conserved among fungi and plants. Several recent studies suggested using counterpart genes in Taxol biosynthesis in yew trees as molecular markers in the screening for Taxol-producing fungi [79, 80, 91]. A putative taxadiene synthase encoding gene and a 10-deacetylbaccatin III-10-O-acetyltransferase encoding gene have been amplified from Taxol-producing fungi and possessed a high homology to the equivalent genes in *Taxus* spp. [92–94]. However, all reports did not show any direct evidence on the roles of these genes in Taxol biosynthesis in fungi. At least, a targeted knockout mutant of the genes is needed to demonstrate their exact roles in Taxol biosynthesis. Thus, at this stage, genes or pathways related to fungal Taxol biosynthesis are still at large.

4.3 Genetic Engineering for Taxol Production

To construct Taxol biosynthetic pathway in organisms that originally do not produce the chemical has been attempted [95, 96]. Takahashi et al. engineered the baker's yeast *S. cerevisiae* by enhancing the carbon flux to the mevalonate pathway (MEP) and co-expressed terpene synthase genes to accumulate high levels of farnesyl diphosphate (FPP) [96]. Genes in the five sequential steps in early isoprenoid biosynthetic pathway to the intermediate taxadien-5a-acetoxy-10b-ol were expressed in yeast, although the engineered host only yielded a trace level of taxadien-5a-ol [95]. Huang et al. successfully synthesized a precursor of Taxol, taxadiene (1.3 mg L^{-1}), in *E. coli* by expressing genes encoding deoxyxylulose-5-phosphate synthase, isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, and taxadiene synthase [97]. Engels et al. reported an efficient system for the synthesis of taxadiene (8.7 ± 0.85 mg L^{-1}) in yeast [98]. Lately, Ajikumar et al. dramatically increased the titers of taxadiene to approximately 1 g/l (15,000-fold) in an engineered *E. coli* strain by a multivariate-modular approach [99]. These successful cases in taxadiene production suggest that genetic engineering of Taxol biosynthesis pathway in *E. coli* or *S. cerevisiae* may be a shortcut to realize the production of Taxol in microorganisms. The remaining route is still long, however, considering that taxadiene is only the first step toward the final product Taxol in the pathway.

5 Problems and Perspective

A couple of issues delay the application of fungal production of the drug after the first report in 1993. The yield of Taxol in fungal strains is still low for the commercial purpose. The level of Taxol in fungi ranges from a few nanograms to several hundred micrograms per liter of liquid culture. Even worse, a trouble encountered by many groups is the capability of Taxol biosynthesis is unsustainable in the fungal strains after cultured for generations. Several groups provided solid evidence showing that fungal isolates produced genuine Taxol and later the compound disappeared in the same strain. The cause behind this phenomenon may be complicated and is still unclear. Successive transfers of a Taxol-producing fungus *Periconia* sp. resulted in fading Taxol biosynthesis even though fungal growth was little unaffected [64]. A recheck of the first isolate *T. andreanae* by Staniek et al. did not find Taxol in the culture even in scaled up cultures [100]. Wang and his colleagues have observed the same phenomenon in Taxol-producing entophyte *Tubercularia* sp. TF5 [101]. The strain lost the capability of producing Taxol after long-term laboratory culture. The team tried to reactivate the production of Taxol by protoplast mutagenesis and genome shuffling approaches. Nevertheless, Taxol has not been detected again in any mutants.

Many factors may contribute to the instability of Taxol in the culture. Numerous hydrolyzable ester groups and chiral centers on Taxol molecule may undergo hydrolysis or epimerization. Treatment of Taxol with hydrogen peroxide generates 10-deacetylpaclitaxel only. Exposure of Taxol to high-intensity light results in a number of degradants [102]. Tian and Stella studied in detail the degradation kinetics of Taxol as function of temperature, pH 1–12, and buffer components. They found that the compound was stable under acid pH conditions with the optimal stability at pH 4–5 [103–105]. We observed in a culture broth for a Taxol-producing strain that pH value starting with pH 6.0 kept increasing to pH 9.5 at the end of the culture. Under such a condition, Taxol may undergo conversion or degradation.

Loss of the capability to produce Taxol may occur at the genetic level. For example, an aging strain may lose its genetic materials. Transposons may mute genes for Taxol biosynthesis once the chemical is no longer a benefit for competition under laboratory condition. Latest advance in the field of fungal secondary metabolism (SM) revealed that the majority of genes involved in SM stay silenced until an elicitor appears. A similar case was found for an inducible terpenoid defense system in conifers [106]. Stierie and Strobel [107] found that addition of yew needle extract into fungi medium resulted in 100-fold increase in Taxol production. Li and coworkers found that coculture of the cell suspension of *T. chinensis var. mairei* and its endophytic fungus, *Fusarium mairei*, successfully increased the yield of Taxol by 38-fold [108]. Taxol exhibits a strong inhibitory activity against oomycetes including *Pythium*, *Phytophthora*, and *Aphanomyces* [109], implying that Taxol might be originally produced as a protective, antifungal compound against competitors in nature. Environmental elements may play critical roles in activating Taxol biosynthetic genes [100].

It seems evident that fungal fermentation has advantages over the current techniques that are currently used in industrial production of Taxol. Despite the fact that numerous hurdles need to be overcome, application of fungal production of this important antitumor drug is a significant research theme. Studies in this field have generated fruitful results. A great number of Taxol-producing fungi have been found from all over the world. Screening for more fungal isolates from diverse natural niches may facilitate to identify better start strains with higher yield of the compound. On the other hand, to illustrate the genetic basis of the biosynthesis of Taxol in fungi should be put on the top of the working list. Revelation of Taxol genetics will help to find out the mechanism of regulation of Taxol biosynthesis and the exact reasons for its instability in the culture. Hopefully, bioinformatics tools and new genome sequencing techniques will certainly expedite the study toward the solution of existing difficulties.

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Metabolic Engineering of Isoprenoid Biosynthesis

91

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Contents

1 Introduction 2814
2 Biosynthesis and Accumulation of Isoprenoids in Plants 2817
2.1 Isoprenoid Biosynthetic Pathways 2818
2.2 Distribution of Isoprenoids in Plants 2821
3 Isoprenoid Metabolic Engineering 2826
3.1 Aims and Strategy 2826
3.2 Microorganisms as Isoprenoid-Producing Platforms 2828
3.3 Isoprenoid Metabolic Engineering in Plants: Current Achievements and Limitations 2830
4 Conclusion 2835
References 2838

Abstract

Isoprenoids constitute one of the largest families of natural compounds. They play essential functions in plant growth and development and furnish compounds of

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high interest for humans. Here, we present the current knowledge on isoprenoid metabolism before describing the strategies that have been used for isoprenoid metabolic engineering. We discuss the advantages and drawbacks of using micro-organisms and plants as cell platform for the production of isoprenoids of interest.

Keywords

Carotenoid • Essential oil • Isoprenoid • Metabolic engineering • Methylerythritol phosphate • Mevalonate • Terpene synthase • Terpenoid • Transgenic plants • Volatile terpene

Abbreviations

ADS	Amorpha 4,11-diene synthase
CRY2	Cryptochrome 2
DET1	De-etiolated 1
DMAPP	Dimethylallyl diphosphate
DXP	1-deoxy-D-xylulose 5-phosphate
DXR	1-deoxy-D-xylulose 5-phosphate
FPP	Farnesyl diphosphate
FPS	Farnesyl diphosphate synthase
GGPP	Geranyl geranyl diphosphate
GGPS	Geranyl geranyl diphosphate synthase
GPP	Geranyl diphosphate
GPS	Geranyl diphosphate synthase
HMG	3-hydroxyl-3-methylglutaryl
HMGR	3-hydroxyl-3-methylglutaryl reductase
IDS	Isoprenyl diphosphate synthase
IPP	Isopentenyl diphosphate
IS	Isoprene synthase
MEP	Methyl-D-erythritol 4-phosphate
MGT	Multigene transfer
MVA	Acetate/mevalonate
NES1	Nerolidol synthase
NPP	Neryl diphosphate
PSY	Phytoene synthase
PTS	Prenyltransferase
SqS	Squalene synthase
TPS	Terpene synthase
VOCS	Volatile organic compounds.

1 Introduction

Isoprenoids, also called terpenoids, constitute a large class of natural compounds with highly diverse structures and functions. They have been identified in many different organisms including microorganisms and animals but are the most diverse in plants with tens of thousands compounds identified so far [1]. Indeed some terpenoids are essential compounds in plants such as sterols necessary for membrane fluidity, phytols chains and carotenoids involved in photosynthesis and photo-protection, respectively, or hormones, for example, gibberellins and abscisic acid, necessary for plant growth and plant response to non-biotic stresses. However, most isoprenoids are not essential for plant development, but play important functions in the relationship between plants and their environment. Among them, volatile isoprenoids contribute to protective effect under thermic or oxidative stress [2], insect attraction for pollination [3], or the defense against pathogen and herbivore attack [4]. Volatile terpenoids may also serve as signal molecules in plant-to-plant communication [5] and together with fatty acids, amino acids, benzene derivatives, and phenylpropanoids are part of the volatile organic compounds (VOCs) released into the atmosphere by plants in response to biotic and non-biotic stresses [6].

In addition to their role in plants, terpenoids have been the focus of numerous studies as they meet human needs in many different fields, including food industry as colorants (lycopene, β-carotene [7]), "cosmetics and perfumes" industry, production of antimicrobial compounds (reviewed in [8]), and of medicines like the antimalarial compounds artemisinin (dihydroartemisinic acid, [9]) or anticancer drugs [10], for example, taxol [11].

Given the wide range of isoprenoid applications, engineering of this metabolite pathway has attracted a lot of interest. Furthermore in some cases, natural sources of highly valuable terpenoids are limited. As an example, taxol is produced by yew tree in very low amounts (0.02% of dry weight) with an extraction process leading to the death of the tree. Extensive extraction of this molecule in the 1990s has endangered this plant species, making it necessary to find alternative source of taxol [12]. So far, many studies have focused on microorganisms as heterologous system for isoprenoid production (for a recent review [13]). Indeed bacteria have proven to be efficient platforms for carotenoid synthesis [14] yet are limited by several factors including substrate availability, and limited storage for end products which impair their use for industrial production [15]. Aside from carotenoids, other isoprenoids could be produced in bacteria and several ongoing works aim at promoting bacteria as a general platform for isoprenoids production. Despite several improvements, industrial production is not yet affordable using bacteria. Moreover, the huge diversity of isoprenoids of interest is far above the number of plant genes of this pathway actually known, therefore limiting the engineering in heterologous hosts (reviewed in [16]). Yeast [17], algae [18], plant cell culture (reviewed in [19]), and hairy root culture transformed with *Agrobacterium rhizogenes* (reviewed in [20]) offer additional ways for the production of secondary compounds. So far industrial production of carotenoids can be achieved using the microscopic green alga *Xanthophyllomices dendrorhous* for asthaxantin and *Dunaliella salina* for β-carotene production (reviewed in [21]).

Metabolic engineering of higher plants also provides an alternative system to produce high value isoprenoids which may help overcoming the limitations of microorganism-based cell platforms. At the present time, isoprenoid engineering in plants has been successfully performed with the aim to improve plant resistance

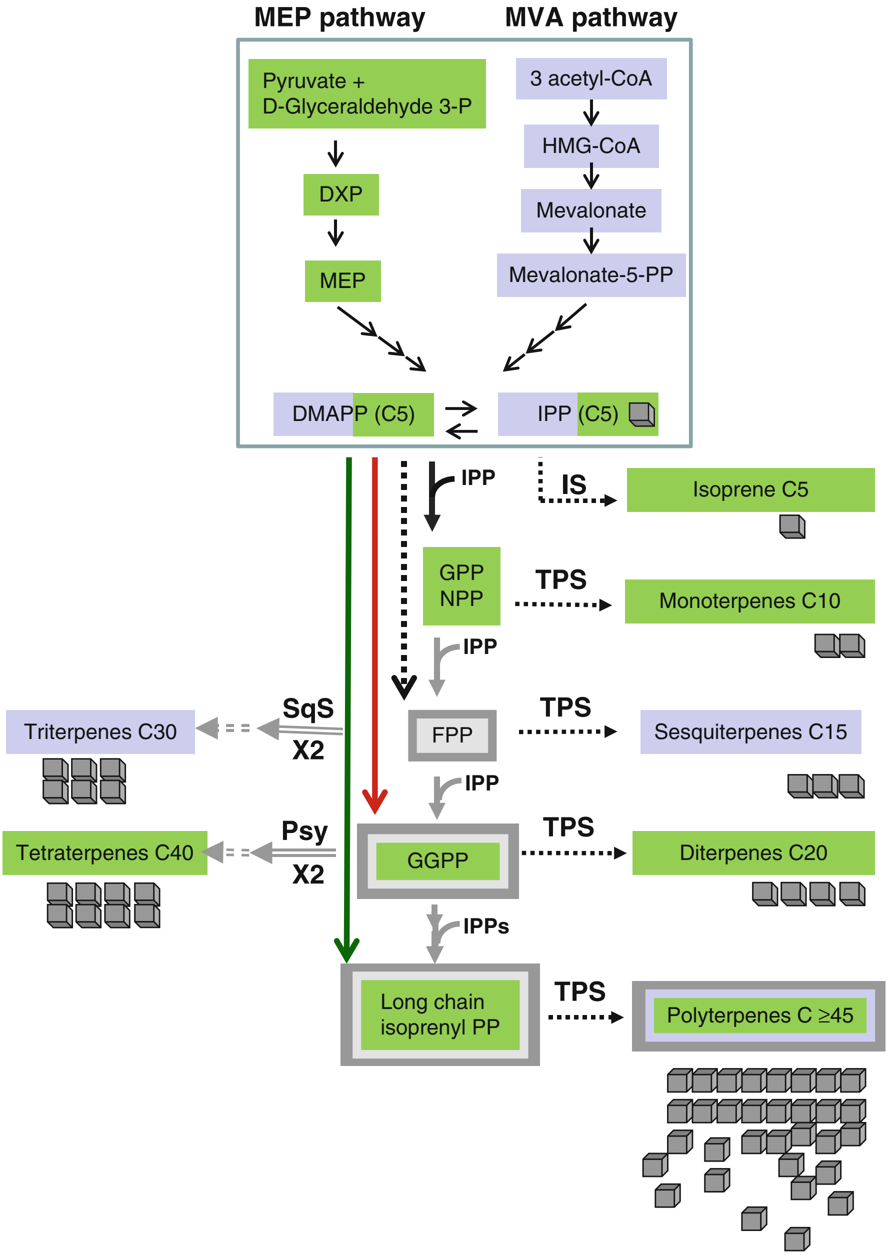
MEP pathway
MVA pathway
Pyruvate +
D-Glyceraldehyde 3-P
DXP
MEP
3 acetyl-CoA
HMG-CoA
Mevalonate
Mevalonate-5-PP
DMAPP (C5)
IPP (C5)
IPP
IS
Isoprene C5
GPP
NPP
TPS
Monoterpenes C10
IPP
SqS
X2
Triterpenes C30
FPP
TPS
Sesquiterpenes C15
IPP
Psy
X2
Tetraterpenes C40
GGPP
TPS
Diterpenes C20
IPPs
Long chain
isoprenyl PP
TPS
Polyterpenes C ≥45

Fig. 91.1 Overview of terpenoids synthesis. Colored frames indicate the cellular localisation of the different pools of metabolites: *green* frames for a plastidial localisation, *blue* frames for a cytosolic localisation, *grey* frames for a mitochondrial localisation. *Black vertical arrow* stands for GPS activity, *doted vertical arrow* for FPS, *red vertical arrow* for GGPS and *green vertical arrow* for long chain prenyl transferase. Cell compartimentalization has been shown according to

to herbivores [22], to modify the fragrance of flowers from ornamental plants or the aroma in plant products (reviewed in [23]), to modulate the content of metabolites having an impact on human health (e.g., carotenoids, reviewed by [24, 25]), or to produce compounds of pharmaceutical interest [26].

In this chapter, we will present various strategies that have been developed for isoprenoid engineering. It is of course beyond the scope of this chapter to present a detailed analysis of isoprenoid biosynthesis. Yet, metabolic engineering of isoprenoid pathways in plants necessitate not only to have access to the genes encoding the proteins that control isoprenoid synthesis but also to acquire a precise understanding of the plant organs and tissues accumulating these molecules as well as of the mechanisms that regulate their accumulation. For these reasons, the first part of this chapter will focus on a general description of isoprenoid pathways. In the second part, different strategies aimed at modifying isoprenoid synthesis in microorganisms and plants will be presented. For extensive analysis of metabolic engineering on specific isoprenoid classes, readers are directed to recent reviews focusing specifically on volatile terpenes [27, 28], triterpenes [29], carotenoids [24], and specific molecules of high health value such as arthemisinin [9] and taxol [30].

2 Biosynthesis and Accumulation of Isoprenoids in Plants

The diversity of terpenoids derives from the complexity of the metabolic pathways governing the synthesis of this group of metabolites. Yet, all terpenes are produced from the C5 basic unit isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). IPP and DMAPP are condensed to form geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15), and geranyl geranyl diphosphate (GGPP, C20) precursors of monoterpenes, sesquiterpenes, and diterpenes, respectively. Higher order terpenoids are formed following the condensation of two FPP molecules (triterpenes, C30) or two GGPP molecules (tetraterpenes, C40), and polyterpenes (C45 and more) synthesis occurs by sequential addition of IPP to allylic diphosphates (Fig. 91.1). In a general way, terpenoid synthesis proceeds through three sequential steps: (1) synthesis of the IPP-DMAPP precursors, (2) production of linear Cn carbon backbones, (3) generation of the tremendous diversity of isoprenoid molecules following specific secondary enzymatic modifications (cyclization, methylation, oxidation,

◀——

Fig. 91.1 (continued) [51]. It does not consider exceptions such as for example, a plastid localized TPS involved in the production of sesquiterpenes [42]. Multi-enzymatic steps are indicated by multiple *arrows*. Each *grey cube* represents a 5-carbon building block constituting the different classes of isoprenoid. *MEP* methyl-D-erythritol 4-phosphate, *MVA* acetate/mevalonate, *DXP* 1-deoxy-D-xylulose 5-phosphate, *HMG* 3-hydroxyl-3-methylglutaryl, *DMAPP* dimethylallyl diphosphate, *IPP* isopentenyl diphosphate, *GPP* geranyl diphosphate, *NPP* neryl diphosphate, *FPP* farnesyl diphosphate, *GPPP* geranylgeranyl diphosphate, *TPS* terpene synthase, *SqS* squalene synthase, *Psy* phytoene synthase, *IS* isoprene synthase, *GPS* geranyl pyrophosphate synthase, *FPS* farnesyl pyrophosphate synthase, *GGPS* geranyl geranyl pyrophosphate synthase epoxidation, etc.). Additional complexity arises from cellular compartmentalization, tissue specialization, and complex regulation of isoprenoid biosynthesis.

2.1 Isoprenoid Biosynthetic Pathways

2.1.1 IPP Synthesis Occurs Following Two Independent Pathways

It has been thought over almost half a century that IPP was only produced from the cytoplasmic mevalonate (MVA) pathway. The discovery that bacteria produced IPP following a totally different pathway, the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, has brought a totally new view on the regulation of isoprenoid biosynthesis [31]. The MVA pathway, which operates in archaebacteria and most eukaryotes, relies on acetyl-CoA supply and is generally considered to provide the precursors for the production of sesquiterpenes (artemisinin, gossypol, etc.), triterpenes (sterols) as well as for protein farnesylation. The MVA pathway has long been described as cytosolic but different data suggest a peroxisomal localization for the full set of enzymes both in mammals [32, 33] and in plants [34]. The MEP pathway is found in most eubacteria [35] and in plants where it is restricted to the plastids [36]. It initiates with the condensation of D-glyceraldehyde-3-phosphate and pyruvate (Fig. 91.1). In plants, where both the MVA and MEP pathways coexist, the MEP pathway typically provides the precursors required for the synthesis of plastidic isoprenoids including isoprene, monoterpenes, diterpenes, and tetraterpenes. However, recent studies suggest that the origin of IPP/DMAPP used for terpene synthesis may depend on the species, tissue, and physiological state of the plant (for a recent review, see [37]). Both cytosolic and plastidic IPP pools have been suggested to contribute to artemisinin synthesis [38], to sesquiterpene synthesis in peppermint [39], in snapdragon [40] and in wild and cultivated tomato species [41, 42]. Thus the subcellular channeling of the isoprenoid pathways might not be as strict as initially thought, although the exact flux of terpenoid precursors between cytosol, plastids, and, in some cases, mitochondria compartments remains to be determined (for a review, see [37]).

2.1.2 Prenyltransferases Catalyze the Production of Isoprenoid Linear Carbon Skeletons

Prenyltransferases (EC 2.5.1.1, PTS) (also called isoprenyl diphosphate synthases, IDSs) catalyze sequential 1′-4 (head-to-tail) associations between IPP and an allylic diphosphate primer substrate (reviewed in [43]). This process requires the initial condensation of IPP with its isomer DMAPP. DMAPP is directly produced through the MEP pathway or obtained from IPP by the enzymatic activity of the IPP isomerase (EC 5.3.3.2). IDSs have been classified as *trans*-prenyltransferases and *cis*-prenyltransferases based on the stereochemistry of the newly formed double bonds (Fig. 91.2) (reviewed in [44, 45]). Hence, IDS form two families of structurally unrelated enzymes that use different mechanisms [46].

In each family, IDS are further separated according to the length of the end product they form. *Cis*-prenyltransferases were initially shown to generate

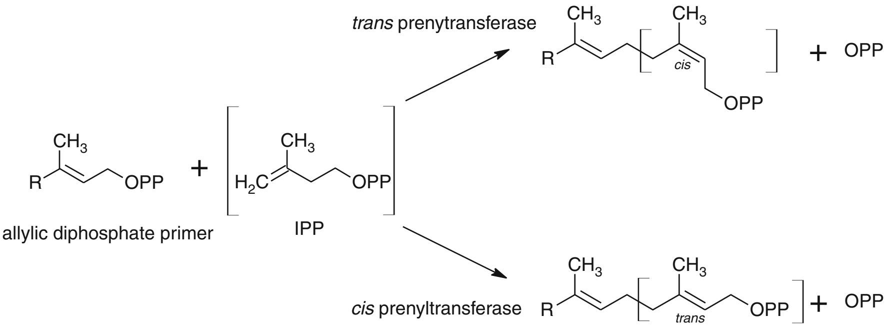

Fig. 91.2 Schematic representation of the reactions catalyzed by *trans*-prenyltransferases and *cis*-prenyltransferases showing the stereochemistry of the double bond formed in the enzyme product. *IPP* isopentenyl diphosphate, *OPP* diphosphate ester

isoprenoid chains longer than C50 (10 isoprene units) by using all-*trans* short chain prenyl diphosphates as allylic primer substrates. *Cis*-PTSs are involved in dolichol [47] and rubber synthesis [48, 49]. Recently, short chain *cis*-prenyltransferases have also been characterized such as the (Z,Z)-FPP synthase [42], and the neryl diphosphate synthase [50] from wild tomato, suggesting that the function of *cis*-PTS in terpene metabolism should be reevaluated [51].

The three central precursors for isoprenoid synthesis, that is, GPP, FPP, GGPP, are essentially produced by IDSs belonging to the *trans*-prenyltransferases group. These enzymes, namely, geranyl pyrophosphate synthase (EC 2.5.1.1, GPS), farnesyl pyrophosphate synthase (EC 2.5.1.10, FPS), and geranyl geranyl pyrophosphate synthase (EC 2.5.1.29, GGPS), play major roles in isoprenoid synthesis. Other *trans*-PTS catalyze the formation of products of more than 20 carbon atoms required for the synthesis of ubiquinone and plastoquinone isoprenoid chains and also specific terpenoids such as chicle (a C500 polymer) [44].

Both *trans*-PTS and *cis*-PTS are poorly conserved at the amino acid level, which makes the prediction of PTS specificity based on sequence analysis difficult [43, 45, 52–55]. Indeed the specificity of PTS appears to be mainly governed by one or two amino acids at the bottom of the enzyme active sites (reviewed in [54]). Yet, some PTS are characterized by enzymatic plasticity that results in the synthesis of more than one compound with different chain lengths [56–58]. For example, the *A. thaliana* solanesyl diphosphate synthase has been shown to accept GPP, FPP, and GGP as substrates, which leads to producing multiple medium/long chain isoprenoids (C25 to C45 in vitro and C35 to C45 in recombinant systems) [59, 60].

Therefore, substrate availability may influence the enzymatic reaction catalyzed by given PTS. In the context of isoprenoid engineering, the choice of the subcellular location for the production of recombinant PTS is an important issue. Indeed, native PTS are present in different cell compartments, that is, plastids, cytosol, mitochondria, and endoplasmic reticulum, depending on their function in cell metabolism.

2.1.3 Several Enzymes Contribute to the Diversity of Isoprenoids

Terpene synthases (TPSs) are responsible for the formation of all isoprenoids using DMAPP, GPP, FPP, or GGPP as substrate. They include isoprene, monoterpene, sesquiterpene, diterpene, triterpene, and tetraterpene synthases which catalyze the first committed step to the biosynthesis of each class of terpenes. Different TPS classifications have been proposed based on their reaction mechanism and products formed (see, e.g., [61, 62]). Among them, squalene synthase and phytoene synthase which catalyze the first committed step, respectively, toward sterol (C30) and carotenoid (C40) synthesis, are structurally distinct from other TPSs [61]. They catalyze the "head-to-head" condensation of FPP and GGPP to form squalene (C30) and phytoene (C40), respectively, with the elimination of both pyrophosphate groups. Other TPSs are evolutionary related to each other [61] and it is not usually possible to predict their product profile on the basis of their primary structure alone.

Most of TPSs catalyze the formation of carbocation intermediates with multiple possible rearrangements of the carbon backbone, eventually leading to multiple products from a single substrate (for a recent review, see [63]). In Norway spruce (*Picea abies*), the diterpene synthase, levopimaradiene/abietadiene synthase, has been shown to produce a mixture of four diterpenes (i.e., abietadiene, levopimaradiene, neobetadiene, and palustradiene). Strikingly, another diterpene synthase from the same species, the isopimaradiene synthase, shares 91% amino acid identity with the former enzyme but products a single-product, isopimaradiene [64, 65]. This example illustrates the high evolutionary plasticity of TPS (for a review, see [62]).

At the time being, genomic and expressed sequence tag (EST) data sets have boosted our knowledge of *TPS* gene families in model plants such as *Arabidopsis* [66], tomato [67], *Medicago* [68], or *Picea* [64, 69]. At one extreme, the genome of the moss *Physcomitrella patens* contains a single functional *TPS* gene encoding a bifunctional copalyl diphosphate synthase/kaurene synthase (CPS/KS; [70]). *TPS* gene families in plant genomes examined so far range in size from 19 to 152 [62]. These arise from cycles of gene duplication, multiple mutations, and functional divergence [61, 62]. To date, the *TPS* gene family has been extensively studied in *A. thaliana*. However, among the 32 potential proteins encoded by the *A. thaliana* TPS genes [66], only 14 have been assigned a biochemical function and classified as monoterpene, sesquiterpene, or diterpene synthase [71–78]. It should be noted that product specificity might also depend on the terpene precursor available. Thus, some sesquiterpene synthases may produce monoterpenes when provided with GPP as starting substrate, and inversely, providing monoterpene synthases with FPP may lead to sesquiterpene synthesis [79]. Therefore, as already discussed in the case of IDS, the subcellular targeting of TPS by determining substrate availability contributes to specifying terpene production [80].

The huge diversity of primary terpene skeletons formed by TPSs can be further modified by the action of various other enzyme classes, such as cytochrome P450 hydroxylases (EC 1.14.14.1), dehydrogenases (EC 1.1; alcohol and aldehyde oxidoreductases), reductases, glycosyl transferases (EC 2.4), and methyl transferases (EC 2.1.1). Among these enzymes, cytochrome P450 hydroxylases play a major role since many terpenes undergo final oxidation steps [81, 82]. Cytochrome P450 monooxygenases, which catalyze substrate-, regio- and stereo-specific oxidation steps, form one of the largest families of enzyme proteins in plants. For example, plant genome sequencing projects revealed 71 full-length P450 genes in the moss *Physcomitrella patens* [83], and 216 in *A. thaliana* [84]. However, according to the most recent survey, the function of more than 70% of the P450s found in the *A. thaliana* genome is still unknown or poorly understood [84]. Deciphering the function of specific cytochromes P450 may be however valuable since the manipulation of cytochrome P450 hydroxylase abundance can be used to modify the proportion of the different terpenoids produced from a common precursor in complex mixture of terpenoids, such as peppermint oil [85, 86]. For example, by reducing the expression of cytochrome P450 menthofuran synthase in peppermint, it has been possible to produce essential oils with a significant reduction in menthofuran content, an undesirable monoterpene oil component [85].

As a conclusion, isoprenoid biosynthesis involves multiple intricate metabolic pathways, operating in parallel in different cell compartments. Indeed most of the isoprenoid biosynthetic enzymes exist as multiple forms with various subcellular localizations and different expression profiles, as revealed by a detailed analysis using *A. thaliana* [51]. Hence, identification of the different genes involved in a specific isoprenoid biosynthesis, which appear necessary for bioengineering experiments, may be quite challenging. Recent reports [60, 80, 87] show that integrated approaches, including combined transcriptomic analysis and biochemical characterization of recombinant enzymes, are highly helpful to associate a function to a gene. Moreover, many enzymes involved in terpenoids synthesis, including some prenyltransferases and terpene synthases, accept more than one substrate and depending on the substrate availability, the product formed may differ [79]. This demonstrates that the substrate availability will ultimately determine the specificity of terpene produced in vivo. As a consequence, for bioengineering experiments, the cellular localization and metabolism context of transgene expression must be carefully taken into account.

2.2 Distribution of Isoprenoids in Plants

The complexity of isoprenoid metabolic pathways relies in part on the diversity of enzymes that participate in this metabolic pathway and on their subcellular localization. Another layer of complexity is brought about by the tight regulation of their synthesis and by their sequestration in various specialized structures. Such specialized structures are essential to consider for metabolic engineering of isoprenoid pathways as they allow high level of terppenoid accumulation. However, it should be noted that some terpenoids do not accumulate in plants. For instance, the simplest terpenoid, the hydrocarbon isoprene (C5H8), is a volatile gas emitted during photosynthesis in large quantities by leaves of many tree species, contributing to VOCs released into the atmosphere [88]. Similarly, a lack of connection between monoterpene synthesis and secretory structures has been reported and plants such as corn and *Phaseolus lunatus*, which are not usually considered as monoterpene essential oil producers, can emit several monoterpenoids when attacked by phytophagous insects ([89], for a review, see [22]).

2.2.1 Isoprenoids are Stored in Plant Tissues

Oleoresin are Produced in Resin Ducts

Volatile terpenes are constituents of the essential oils, floral scents, and resins of aromatic plants, to which they impart their characteristic aromas and flavors [90–92]. Oleoresin, for example, is a viscous and odoriferous liquid found in conifers that contains approximately equal amounts of monoterpenes and diterpenes and smaller amounts of sesquiterpenes [93]. Pine tree resin is one of the best characterized oleoresin and contains large quantities of monoterpenoids, including limonene, pinene, and myrcene, which give the organic solvent turpentine its characteristic sharp odor. However, the ratio of these terpenoid classes and thus the physical and chemical properties of oleoresin can vary with stresses such as air pollution [94], fertilizer application [95], drought stress [96], herbivory [97, 98], and fungal inoculation [99]. Constitutive oleoresin biosynthesis and accumulation in conifers is restricted to various types of resin ducts or blisters (Fig. 91.3a) composed of several layers of epithelial cells with a central storage cavity [100, 101]. Inducing a local terpenoid response by mechanical damage or herbivory leads to the formation of specialized traumatic resin ducts in the stem xylem [98, 102–104].

Various Structures Accumulate Essential Oils

Similarly, essential oils, which can be produced in many plant organs, accumulate in specialized structures. Essential oils are extracted from various aromatic plants belonging to 60 families and mainly localized in temperate to warm areas like Mediterranean and tropical countries where they represent an important part of the traditional pharmacopoeia. Indeed, essential oils are essentially composed of terpenes, mainly mono- and sesquiterpenoids with low quantities of di- and triterpenoids, but also contain aromatic and aliphatic compounds [105]. In most cases, they are characterized by a high concentration of two to three major volatile terpenes whereas secondary molecules are found in trace amounts (reviewed in [8]).

Essential oils can be synthesized by all plant organs (buds, flowers, leaves, stems, seeds, fruits, and sometimes roots, wood, or bark) and are stored and released to the environment by a variety of specialized structures, which include oil cells, cavities, canals, scent glands of flowers, secretory glands (eucalyptus), glandular hairs, or trichomes (labiates) (for a review, see [106]). Besides epidermal hairs, essential oil terpenes may also be accumulated in specialized mesophyll glands, as observed in patchouli (*Pogostemon cablin*) [107].

The simplest secretory system is probably represented by oil cells (Fig. 91.3b) that accumulate essential oils in the vacuole as reported by Lewinsohn et al. [108] on lemongrass leaves. Glandular trichomes represent a more sophisticated system which consists of a stalk composed of one to three cells that supports the head. The number of cells constituting the head has been the basis of glandular trichome

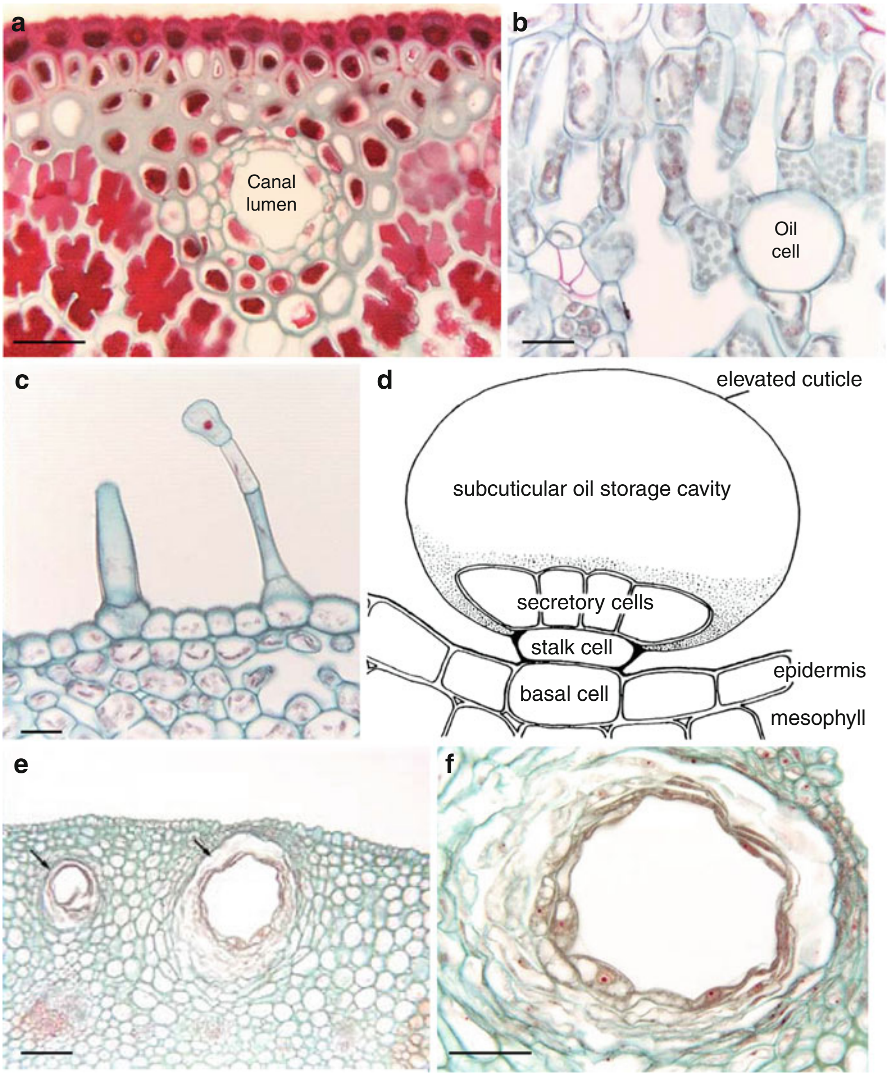
a
Canal
lumen
b
Oil
cell
c
d
elevated cuticle
subcuticular oil storage cavity
secretory cells
stalk cell
basal cell
epidermis
mesophyll
e
f

Fig. 91.3 Some specialized structures for terpene accumulation (**a**) Resin duct on a transverse section of needle leaf of pine (*Pinus*). (**b**) Oil cell on a transverse section of magnolia leaf (*Magnolia*). (**c**) Glandular trichome on a transverse section of foxglove (*Digitalis purpurea*) stem. (**d**) Schematic diagram of a peppermint leaf peltate glandular trichome. (**e**) and (**f**) Oils glands on a transverse section of flower petal of lemon (*Citrus limon*). (**a**, **b**, **c**, **e**, **f**: Reprinted with permission from Mauseth [117]; (**d**) Reprinted with permission from Turner et al. [112] Bars = 20 μm (**a**, **b**, **c**); = 500 μm (**e**); = 100 μm (**f**))

classification that are separated in two main types [109]: the capitate type with a single head cell, and the peltate type that is characterized by a multicellular head as in peppermint [110, 111]. Oils may then simply accumulate within the central vacuole of the secretory cells, or between the cell wall and cuticle (Fig. 91.3c)

[112]. As example, in the peppermint trichomes, monoterpene biosynthesis occurs in the secretory cells and essential oil is stored in the subcuticular storage cavity ([112–114], Fig. 91.3d). Although the early works on glandular trichomes have been conducted on Lamiaceae species, tomato trichomes are now extensively studied and characterized, and Solanaceae could become the reference in terms of trichome biology. Glandular trichomes of *Solanum* range in seven subgroups that differ between species ([115] and references therein). Subgroups distinction is based on the stalk length, cell number of the secretory head as well as on the metabolite content of the trichomes. However, recent extensive transcriptomic and metabolic analyses of *Solanum* trichomes demonstrate quantitative rather than qualitative metabolite differences. Several genes involved in terpenoid synthesis are highly expressed in *Solanum* trichomes and their relative abundance varies between species [80, 116]. Interestingly, genes involved in photosynthesis and carbon fixation are also expressed in *Solanum* trichome, suggesting a possible link between carbon metabolism and requirement of carbon skeleton for terpenoids and probably other secondary compounds synthesis [116].

Finally, oil glands, observed on petals or in the peels of oranges, lemons, and other citrus fruits, represent a clearly distinct class of secretory structure. Oil glands initiate when a small set of cells start producing oil and progressively recruit neighboring cells that in turn start synthesizing terpenoids and so on. In holocrine type of glands, oil cells break down when mature, therefore creating a central lumen. The gland grows larger as more cells become converted to oil-producing cells. Such glands may grow either symmetrically or only on one side, giving either spherical or tube-like structure structures, respectively ([117], Fig. 91.3e).

Laticifers

Another class of terpenoids that accumulate in specialized cells are polyterpenes, such as rubber. Natural rubber is a *cis*-1,4-polyisoprene polymer made from isopentyl pyrophosphate monomers with a C15 *trans* tail derived from the original initiating allylic pyrophosphate. It is one of the most important industrial raw materials in the world, and its sole commercial source is currently the para rubber tree *Hevea brasiliensis* [118]. The biosynthesis of natural rubber takes place in the latex of laticifers, long multinucleate pipes formed from anatomized cells, where it is stored in 0.08–2-μm rubber particles and may reach 40% of the latex dry content [119].

Isoprenoid Intracellular Storage

Many isoprenoids are synthesized by cytosolic pathway; they may then accumulate in the cytosol as cytokinin, or rubber, although the cytosol is deeply modified in this latter case to form latex, or may be incorporated into membranes as sterols [119]. Isoprenoids may also be stored in vacuoles, as observed in the case of essential oils, independantly of the subcellular compartment where constituents are synthesized: in the cytosol (as sesquiterpenes) or in plastids (most of the monoterpenes and diterpenes) [112].

By contrast, carotenoids are synthesized and accumulate in plastids: chromoplasts for fruits, flowers, roots and chloroplasts in photosynthetic tissues.

In chloroplasts, carotenoids (mainly lutein, β-carotene, violaxanthin, neoxanthin, and zeaxanthin) are located in the thylakoid membranes. Their amount and type are relatively conserved across most plant species. In contrast, carotenoid content of nongreen plant plastids may vary considerably [120]: from low (as in white flower petals) to high amounts (as in the dark orange petals of certain marigold varieties). Chromoplast pigments may be those found in the chloroplasts (e.g., lutein of marigold flower petals), be derived from carotenoids (e.g., capsanthin and capsorubin of red pepper fruits, synthesized from violaxanthin), and may be pathway intermediates (e.g., lycopene in red tomato fruits). In those plastids, significant amounts of carotenoids may be stored in membranes, oil bodies, or other crystalline structures within the stroma [120, 121]. Chromoplasts accumulate massive amounts of carotenoids by generating carotenoid-lipoprotein substructures, known as carotenoid-sequestering structures [122, 123] which serve as sinks to sequester excess carotenoids [124, 125].

Hence, the complexity and the compartmentalization of isoprenoid metabolic pathway lead to various terpenoids' localization at the organism and cellular levels. Yet the synthesis and accumulation of isoprenoids is under precise regulatory mechanisms (reviewed in [126]).

2.2.2 Isoprenoid Production is Tightly Regulated in Plants

It has long been described that oil accumulation is tightly correlated with leaf development in many aromatic plants and with the abundance of glandular trichomes [127–130]. Ontogeny also affects oil quality as in *C. martini* characterized by an increase in geraniol content from 65% to 81% until flowering stage at the expense of geranyl acetate [106]. Several reports suggest that the primary control of volatile isoprenoid synthesis during plant development occurs at the transcriptional level. Consistent with this view, a clear correlation has been established between TPS gene expression and the accumulation of the corresponding compounds in leaves and flowers of various plants (see, e.g., [76, 131–134]) that was confirmed by the recent analysis of the tomato TPS gene family [67]. In addition, regulation of isoprenoid synthesis by circadian clock and phytochrome has been reported by many authors and the emission of volatile terpenes, for example, has been shown to follow a diurnal rhythm [126].

Besides being regulated in a development-specific way, essential oil production may be modulated through environmental regulations [135]. In nature, essential oils play an important role in the defense against pathogen and herbivore attack; therefore, they may be produced constitutively and in response to a stimulus (reviewed in [136]). For example, thyme basil (*Acinos suaveolens*) growing in an area regularly grazed by herbivores contains twice the amount of monoterpenoids (15.61 vs. 8.18 mg/g dry matter) as compared to plants growing in inaccessible areas. Moreover *Acinos suaveolens* produces two monoterpenoids (menthone and R-(+)-pulegone) with different toxicities. The more toxic R-(+)-pulegone shows an increase in relative abundance (66.3% vs. 47.5%) in plants exposed to browsing [137]. Yet Opitz et al. [138] showed that terpenoid levels in cotton foliage increased after mechanical damage, herbivory, and jasmonic acid treatments, with a 15-fold increase in E-β-ocimene (monoterpene) amount, for example. These higher levels of terpenoids were achieved by two mechanisms: increased filling of existing glands with terpenoids and production of additional glands. Part of the terpenoids emitted following herbivore attacks attracts predators of these herbivores, therefore contributing to the indirect defense of plants [139]. It should also be noted that volatile isoprenoids are essential components of the plant cellular response to non-biotic stresses being involved in the protection against thermic and oxidative stresses that trigger emission of isoprene and monoterpenes (reviewed in [2]).

Similarly, the levels of carotenoids and their profiles are influenced by many factors, including the developmental stage, environment, and stress: For example exposure of plants to high light intensities leads to an accumulation of zeaxanthin, which has essential photoprotective roles [140]. On the other hand, there are many reports describing changes in carotenoid gene transcript abundance during fruit ripening, flower development, or stress, which coincide with changes in carotenoid content [141]. However, other studies clearly showed that the formation of carotenoid-sequestering structures plays also an important role in regulating carotenoid accumulation (for review, see [142]). Thus, control of the formation of a metabolic sink can offer a novel approach to mediate carotenoid accumulation in food crops. Direct evidence comes from works of Li et al. [143] and Lu et al. [144] on the *Or* gene. *Or* gene expression into wild type cauliflower induces the formation of chromoplasts in the curd cells of transformants whose tissues are colored due to an increased level of β-carotene [144]. Expression of the *Or* transgene in potato tubers leads to increased carotenoid levels as well [144, 145], demonstrating that the *Or* gene controls chromoplast differentiation and could serve as a novel genetic tool to enrich carotenoid content of plants.

As a conclusion, isoprenoids accumulate in structures of various complexities, from sequestration substructures in plastids to specialized cells as laticifers or multicellular structures such as glandular trichomes. Obviously such specialized cells and/or tissues are efficient cell factories that aim at producing and accumulating terpenoids and as such should be considered as essential target for isoprenoid metabolic engineering. On the other hand, knowledge of regulation and genes encoding proteins that control isoprenoid accumulation such as *Or* is also a prerequisite before considering the different strategies that can be developed for isoprenoid metabolic engineering.

3 Isoprenoid Metabolic Engineering

3.1 Aims and Strategy

Metabolic engineering refers to genetic manipulation of cells with the aim to enhance yield and rate of selected metabolite production by remodeling or deregulating metabolic networks [146]. Initial strategies were based on the engineering of single enzyme that was thought to be rate limiting. Today's metabolic engineering allows the transfer of all or part of biosynthetic pathway to selected cell systems [147]. Yet, the main question remains unchanged: to identify key enzymatic steps that impact directly or indirectly metabolite yield. To address this point, stoichiometric modeling of the selected pathway has to be carried out in order to identify key proteins that can enhance product synthesis. Stated another way, metabolic engineering emphasizes metabolic pathway integration and relies on metabolic fluxes as determinants of cell physiology and measures of metabolic control. Metabolic flux control can be pursued in the framework of metabolic control analysis (MCA) developed in the 1970s for the quantitative representation of the degree of flux control exercised by specific enzymes, metabolites, and effectors in the pathway [148] or directly by metabolic flux analysis [149].

An alternative to modeling approaches when pathways are under complex kinetic and/or regulatory interaction lies in the combination of genetic alterations with high-throughput screening [150]. Considering the variety of terpenoids [4], the diversity of enzymes involved in this metabolic network, and the limited knowledge of pathway regulation, engineering isoprenoid metabolism has always been challenging. However, since terpenoids present health-related beneficial properties, many efforts have been undertaken in microorganisms but also in plants to produce specific terpenoids, for example, artemisinin [151] and paclitaxel or to improve the yield and/or composition in terpenoids mixtures such as essential oils or carotenoids [152].

At the present time, an outstanding amount of information concerning biosynthetic genes, enzyme functions, and regulation of isoprenoid biosynthetic pathway is available in bacteria, yeast, and plants [37]. Plant web resources have recently been developed such as the public online databases AraCyc (http://www.arabidopsis.org/biocyc) and KEGG (http://www.genome.jp/kegg) and more recently AtIPD (http://www.atipd.ethz.ch, [153]). These tools provide necessary knowledge for the integrative analysis of isoprenoid metabolism including gene, metabolite, and protein information as well as the subcellular organization of the pathway. All these efforts have contributed to give a boost to research on the engineering of isoprenoid pathways with the aim to industrializing their production via microbial fermentations [154] or plant cell and organ cultures [155]. Transgenic plants or organs [15, 156, 157] with modified terpenoid metabolism have also been developed either with plant model systems such as *Arabidopsis*, rice, and maize or plants that already produce isoprenoids of high added value [10, 24, 30, 158, 159]. So far, different strategies to improve isoprenoid production in microorganism and plant systems have been used with various success: (a) optimization of the available isoprenoid precursor pool, (b) manipulation of pathway by introducing single or combination of genes, or (c) increase storage capabilities for lipophilic isoprenoids, (4) remodeling of the pathway for subcellular organization, and (5) manipulation of regulatory genes.

3.2 Microorganisms as Isoprenoid-Producing Platforms

3.2.1 Advantages of Microorganisms as Cell Factories

Microorganisms are currently considered as cell factories for the synthesis of highly valuable terpenoids [15]. Indeed, they present several advantages over other isoprenoid-producing systems among which the use of low cost carbon sources, the possibility of large-scale fermentation processes, the uncoupling between production phase and growth, and the reduced culture times open the way to industrial production. In addition, some microorganisms are characterized by poor competing metabolic fluxes and co-extraction compound problems as they have limited isoprenoid secondary metabolism [160]. This is the case for *Escherichia coli* and *Saccharomyces cerevisiae*. They have often been used in pathway engineering for isoprenoid biosynthesis as they are well characterized and thoroughly susceptible to genetic manipulation. In these hosts, extended engineering is necessary to build up operational biosynthetic pathways as they barely present isoprenoid synthesis except for central metabolites like quinones or sterols. However, the fair handling of these microorganisms offers a serious advantage over the engineering of poorly characterized native strains since their genome, transcriptome, and metabolome elucidation is nearly completed, supporting deeper engineering investigation [161].

3.2.2 Isoprenoid Engineering in Microorganism and Limitations

Although initially limited by low yield and high costs for large-scale synthesis, engineering of isoprenoid pathway in microorganisms stressed on several breakthroughs during the past 15 years. Of course, the initial cloning and expression of diverse heterologous genes in the downstream isoprenoid biosynthesis pathways led to the first *E. coli* producing plant sesquiterpenes. However, product yield was very poor probably due to low prenyldiphosphate availability which highlighted the limitation of unmodified *E. coli* strains as cell factories for isoprenoid production [162]. This was solved by introducing part or all of the MVA in *E. coli*, leading – as far as lycopene production was concerned – to a tenfold increase in yield compared to the wild type strain counterpart [163]. Introduction of the *S. cerevisiae* MVA pathway in *E. coli* allowed improved isoprenoid precursor production, although this was described to be toxic for the host. The toxicity issue was further resolved by introducing a truncated HMGR, not susceptible to degradation [164]. Alternative strategies to improve precursor supply were based on the engineering of the MEP pathway. Overexpression of DXS and IDI led to an increase in IPP and DMAPP syntheses and as a consequence in the carotenoid yield [165]. More recently, the combination between precursor pathway engineering with the use of improved enzyme variants turned out to be a successful approach to overproduce levopimaradiene in *E. coli* [166]. Such a strategy looks highly promising for the production of other terpenoids of high added value for humans.

Prenyl diphosphate synthesis is also directly depending on the carbon flux diverted from the central metabolic network. Creating new metabolic sinks, that is, uncontrolled isoprenoid biosynthetic pathways, may therefore result in metabolic imbalance and low final product yield. To address this point, *E. coli* strains that control lycopene synthesis in response to carbon availability have been developed making use of the *glnAP2* promoter [14]. This promoter is stimulated by excess glycolytic flux through sensing of acetyl phosphate. This approach led to a significant increase in lycopene synthesis by *E. coli*. Other strategies to enhance the availability of central carbon metabolism precursors for isoprenoid pathway consist in the inactivation of competing pathways at the level of pyruvate and acetyl-CoA. This resulted in an increased carbon flux to IPP and therefore to lycopene with a production which was 45% higher [167]. In yeast, rerouting of the carbon flux into the targeted isoprenoid compound, that is, minimizing competing pathways, also appeared as a workable solution. A fivefold increase in amorphadiene production was reached by downregulating the squalene synthase expression, therefore limiting the sterol branch [168]. Several high-throughput approaches were also used to determine events not predicted by the knowledge base prediction pathway, for example, randomly genetic variations unrelated to terpenoid pathway but modifying it [169].

Additional limitations of metabolic engineering are linked to natural control points in organisms that balance supply and metabolite synthesis. Metabolic engineering struggles to refine these bottlenecks by adjusting the expression level of native/heterologous genes. A striking example of that approach is the case of paclitaxel, the successful anticancer drug, also named taxol. The engineered pathway in *E. coli* consists of its native MEP pathway plus a heterologous isoprenoid downstream pathway. This led to limited yield around 10 mg/L taxadiene [170]. This classical engineering ignores bottleneck effects or intermediate compounds' toxicity. Combinatorial approaches provide solutions to such issues. In this strategy, the complete pathway is split into two modules. Various gene copy numbers and promoters with different strengths drive expression levels of these two modules. *E. coli* strains with various combinations of these two modules were constructed and tested for taxadiene content. The best yield reached 1 g/L, almost 100-fold higher than yields obtained by classical engineering [154]. This example also demonstrates the potential of combinatorial approaches over classical engineering.

3.2.3 Challenges

As discussed in Sect. 2.1, the diversity of isoprenoid relies on the wide range of enzymes that are involved in these metabolic pathways (see Sect. 2.1.1). The sequence information concerning terpene synthase as well as the wide range of additional enzymes, for example, cytochrome P450 hydroxylases, dehydrogenases reductases, glycosyl transferases, etc., which contribute to diversify isoprenoids, is now available [62]. However, one important problem of isoprenoid engineering in microorganism hosts is managing functional expression of heterologous proteins. As an example, *E. coli* cannot functionally express the P450s enzymes that are extensively taking part in the isoprenoid biosynthetic pathway. Plant P450s are mainly membrane bound and they require a reductase partner for their function [13]. However, P450s coming from bacteria can be used. For instance, P450 from *Bacillus megaterium* is a fusion enzyme made of a P450 part and a eukaryote-like reductase domain [171].

Although microorganisms engineering offers many advantages over plant hosts for isoprenoid biosynthesis, many plant isoprenoid biosynthetic pathways are not yet completely known and are therefore not transposable into microbial strains.

3.3 Isoprenoid Metabolic Engineering in Plants: Current Achievements and Limitations

Plants present an interesting alternative to microorganisms since as natural terpenoid producers they possess all requirements for isoprenoid biosynthesis including the complex subcellular organization of this metabolic pathway and storage capacity both at the subcellular and tissue levels (See Sect. 2). In addition, plants have almost unlimited carbon resources insured by photosynthesis which makes them a good alternative to industrial incubator for microorganism production of isoprenoids. Obviously, one important objective of isoprenoid metabolic engineering in plants is to produce compounds of high added value that cannot be extracted in sufficient amount and at low price using natural resources [30, 151] such as the antimalarial compound, artemisinin a sesquiterpene extracted from *Artemisa annua* [9]. In addition, engineering of isoprenoid metabolism in plants is a rapidly expanding research field and there have been many attempts to manipulate monoterpene, sesquiterpene, diterpene, triterpene, or carotenoid syntheses over the last 15 years [24, 27–29].

3.3.1 Constitutive Expression of Selected Genes: A General Strategy to Manipulate Isoprenoid Profiles for Plant Trait Modification

Initial attempts to engineer isoprenoid metabolism in plants relied mainly on the constitutive overexpression of one selected enzyme with the aim to enhance the carbon flux for isoprenoid precursor synthesis or to modify isoprenoid profiles. Indeed, many successful manipulations were reported. As recent examples, the constitutive expression in tomato fruit of a citrus lycopene β-cyclase, which catalyzes the conversion of lycopene to β-carotene, led to a 30% increase in total carotenoid content and a fourfold increase in β-carotene [172], similar to previous attempts using the tomato *Lcyb* gene [173, 174]. Other works focusing on the overexpression of the *PSY1* gene in tomato [175] and in kumquat [176] led to an enhanced accumulation of phytoene, lycopene, and β-carotene. Constitutive expression of PSY1 in tomato was shown to cause competition for precursor availability with other isoprenoid pathways that require GGPP as a starting point. This resulted in abnormal plant phenotypes such as dwarfism due to depletion in gibberellins [175]. Similarly, the ectopic expression during tomato fruit ripening of the lemon basil α-zingiberene led to both mono- and sesquiterpene accumulation while lycopene accumulation was reduced, indicating a competition for plastidic DMAPP and IPP pools [177].

Yet, competition between pathways has been successfully used to redirect carbon flux toward the synthesis of selected isoprenoids by means of the constitutive overexpression of specific monoterpene and/or sesquiterpene synthases. Indeed, modification of monoterpene profiles has been successfully reported, demonstrating that part of the GPP pool could be efficiently redirected to the synthesis of limonene, γ-terpinene, or β-pinene in a series of plants including lavender, eucalyptus, or tobacco [178–180]. The absence of reported effect on plant development as well as the high emission level of the engineered monoterpenes reported is consistent with GPP being not limiting in these studies. In a similar way, constitutive overexpression of the maize *TPS10* gene in *Arabidopsis* plants led to a high level of the sesquiterpenes (E)-β-farnesene and (E)-α-bergamotene emission without detectable effects on sterol or other triterpene accumulation [181], indicative of sufficient FPP availability. This contrasts with other reports clearly indicating a limitation in GPP or FPP availability. Linalool emission in carnation constitutively expressing the *C. breweri* limonene synthase gene did not exceed 6% of total emitted volatiles [182] and minute amount of limonene was produced by tobacco plants expressing the *Perilla frutescens* limonene synthase gene under the control of the strong and constitutive promoter *E2I* [183]. Similarly, tobacco plants constitutively overexpressing a trichodiene synthase, the amorpha-4,11-diene synthase, or even (E)-ß-farnesene synthase only produced trace amounts of the targeted sesquiterpenes, probably due to limited FPP availability [184–186].

Similar strategies were developed to enhance artemisinin production, making use of *Agrobacterium*-mediated transformation of *A. annua* that was reported more than 15 years ago and optimized since that time [187, 188]. Overexpression of single gene, that is, the *IPT* gene from *A. tumefaciens*, the *FPS* gene from *Gossypium arboretum*, or even the gene encoding the enzyme amorpha-4,11-diene synthase (ADS) which catalyzes the first committed step to artemisinin, led to a limited increase in this compound accumulation. This raise ranged between 30% and 100% as compared to control without major differences between the engineered genes [188–191]. Metabolic analysis of the transgenic *A. annua* indicates that there are several bottlenecks that limit the deregulation of this metabolic pathway. These include limitation in the availability of IPP and DMAPP precursors that appear to be furnished by both the MVA and MEP pathways [192, 193]. This issue could be partly solved with the co-transformation of both HMGR and ADS that allowed the coordinate stimulation of precursor synthesis via the MVA pathway and of artemisinin synthesis. Yet the overall efficiency of this approach remained limited due to epigenetic effects, leading to transgene silencing [194].

Indeed, GPP, FPP, and other precursor availabilities may differ between plants, organs, and developmental stages and therefore explain the variability observed between independent experiments performed on tobacco, *Arabidopsis*, maize, and other plants. Consistent with this interpretation, the ectopic expression of enzymes of the MVA or of the MEP pathway led to increased level of MVA-derived isoprenoids (sterols, sesquiterpenoids [195–197]), and MEP-derived isoprenoids (monoterpenoids, chlorophylls, and carotenoids), respectively [86, 198]. Rerouting of GGPP was also possible in the *r* tomato mutant that lacks the fruit-specific phytoene synthase protein and therefore cannot accumulate carotenoid during fruit ripening. In this case, the expression of a taxadiene synthase in the yellow tomato fruits allowed the extraction of 160 mg/kg DW of taxadiene [199]. These plants were often parthenocarpic, produced less fruits and few viable seeds.

Hence, constitutive ectopic expression of selected proteins demonstrates the feasibility of terpene engineering in plants but also highlights several limitations. Among them competition between pathways for precursor availability may lead to unwanted secondary effects on plant development or may result in low product yield. Other limitation not discussed here includes further modifications of the selected product, (e.g., hydroxylation, glycosylation [79, 200]), as well as epigenetic effects (e.g., cosuppression [175]). In addition, constitutive TPS overexpression may lead to inhibitory effects on plant development due to product toxicity. As a conclusion, isoprenoid engineering using strong and constitutive expression of enzymes gives variable results, depending on terpenoid classes, organs, and plants. This demonstrates the requirement of more sophisticated strategies. Such strategies should consider the subcellular localization of the pathway, the necessity to reconstruct part or all pathways to take into account precursor limitation and competition as well as the tissue-specific expression of targeted gene and storage capacity of the products synthesized.

3.3.2 Plant as Cell Platform for the Production of Specific Terpenoids: Reconstructing the Isoprenoid Pathways in Chosen Subcellular Compartment

Terpenoids are mainly produced in the cytosol or in plastids (see Sect. 2). Relocating terpenoid synthesis to a nonnative cell compartment was shown to result in significant yield improvement in yeast [201], suggesting that it could also be successful for other cell producing platform including plants. Following the recent cloning of genes of the artemisinin pathway [202], *Nicotiana* species were used as heterologous hosts both with transient and stable transformation systems [26, 151, 203]. Transient assays using agro-infiltration allowed demonstrating that *Nicotiana benthamiana* could be used as a production platform for the artemisinin precursors, artemisinic and dihydroartemisinic acids, despite a limited accumulation due to internal glycolysation and insufficient oxidation toward the acids. However a real breakthrough was reached following the stable co-expression of up to five plant- and yeast-derived genes involved in the mevalonate and artemisinin pathways in tobacco plants, which led to low levels of artemisinin synthesis in this heterologous system [151]. Indeed, this could be achieved following a combination of favorable factors including the use of a multigene transfer system within a single vector to stably integrate five genes controlled by various promoters, the carbon flux enhancement through the MVA pathway by expressing a truncated form of the HMGR (tHMGR) enzyme that was shown to allow a tenfold increase in sterol biosynthesis [204], and the use of appropriate plant culture conditions. In addition, a major breakthrough was the targeting of amorphadiene synthase (ADS) to mitochondria which led to approximately five- to tenfold increase in artemisinin content as compared to plants with cytosolic ADS. The final yield remained however much lower than in *A. annua*. Further improvement may rely on providing sufficient precursors within the targeted subcellular compartment (i.e., mitochondria in the case presented here).

Consistent with this view, the targeting of FaNES1 to mitochondria only led to the synthesis of traces amount of nerolidol derivatives [139] due to a limited availability of FPP in *Arabidopsis* mitochondria. Inversely, the coordinate targeting to the chloroplast of an avian FPS and a sesquiterpene synthase, the patchoulol synthase (PTS) normally localized in the cytosol, led to a high level of patchoulol synthesis in tobacco leaves which accumulated up to 5–30 μg/g FW [205], above 100 times higher than when addressed to the native cellular compartment. This highlights that precursor availability is indeed a critical issue when redirecting the isoprenoid pathway in a new compartment that may require the co-introduction of several genes for a complete pathway reconstruction.

Multigene transfer (MGT) using *Agrobacterium*-based transformation has been described and a few reports describing this approach for isoprenoid metabolic engineering are available (for a recent review [147]). The best known example, "golden rice," was developed more than 20 years ago by introducing genes encoding the *PSY1* and *LYCB* gene from daffodil together with the bacterial *CRTI* gene which allows the synthesis of β-carotene from GGPP [206]. This strategy led to a limited accumulation of β-carotene (1.6 μg/gDW) that was increased to 37 μg/gDW when the daffodil PSY1 protein was replaced by the corn one which presents a higher activity [207]. More recently, similar approaches were successfully developed in canola [150, 208] or potato [209]. Yet MGT remains a challenging task that requires either to perform co-transformations without possible prediction on the output, retransformation of already transformed lines, or sexual crossing between transgenic lines. For this reason, MGT has often been limited to two to three genes and only few reports described the successful transformation with up to nine genes [150, 208, 210]. A recent technical breakthrough that allows the co-transformation of multigene arrays may provide tools for a routine multienzyme engineering of metabolic pathways in plants [211].

As far as expression in plastids is concerned, the recent development of transformation methods that allow the stable integration of up to six to seven genes in the plastidic genome opens a new alternative for multigene isoprenoid engineering in this cellular compartment. It is not the scope of this chapter to present a detailed analysis of transplastomic, which has been recently reviewed [212]. This approach presents many advantages including limitation of transgene flow and absence of epigenetic effects that may lead to gene silencing. Additionally stacking genes in operon allows the coordinated expression of series of proteins from polycistronic RNA. At the present time, transplastomic has been successfully used to introduce single gene for β-carotene synthesis in tomato [213] and for DXR overexpression in tobacco leaves [214]. More recently, the remodeling of the complete isoprenoid pathway in *N. tabacum* was achieved following the introduction of six genes of the MVA pathway into the chloroplast genome and their coordinated expression [215]. In these plants, both cytosolic and plastidic isoprenoid synthesis was impacted, further supporting the idea of cross talk between both compartments.

3.3.3 Control of Metabolic Pathways: From Promoters to Regulatory Proteins

Although successful in some cases, the use of strong and constitutive promoters for terpenoid engineering may lead to deleterious effects on plants due to competition between the new and endogenous pathways. A pioneering work performed on tomato more than 15 years ago clearly illustrates this point. Constitutive overexpression of the *PSY*1 gene, which encodes a functional phytoene synthase in tomato plants, resulted in an enhanced carotenoid synthesis in most plant organs. However, this also led to dwarfism following the competition created between PSY1 and the ent-kaurene synthase, which catalyzes the first committed step to GA synthesis [175]. This could be easily solved by using a fruit-specific promoter [216]. Indeed many works aiming at modifying the carotenoid profile in plants made use of organ- and/or tissue-specific promoters to limit potential secondary effects. These include the albumen-specific promoters used in rice and corn [150, 206, 207], tuber-specific promoters in potato [209, 217], seed-specific promoters in *Arabidopsis* and *Brassica napus* [208, 218], and promoters specific for tomato fruit ripening [216]. This targeted expression allowed modifying carotenoid profile in specific tissues without impacting other plant traits.

The use of regulatory proteins and mechanisms provides alternative approaches to modulate isoprenoid accumulation. At that time, the general knowledge of the regulatory network that governs isoprenoid accumulation has remained poor. Indeed it is known that both environmental and developmental factors may impact the synthesis of these molecules, depending on their physiological function (see Sect. 2.2), but very few regulatory proteins have been identified so far. The AaWRKY1 transcription factor which appears to be involved in the regulation of artemisinin biosynthetic genes, including the ADS gene, is one rare example of a TF that could be used to manipulate a terpenoid pathway [189]. Identification of transcription factors shall however provide essential tools to modulate all pathways, including steps that were not initially identified as essential for the accumulation of the targeted product. For example, the ORCA3 transcription factor that has been shown to regulate genes involved in terpenoid indole alkaloid synthesis also activates genes initially not anticipated (reviewed in [219]). Interestingly, the phytochrome interacting factors that repress photomorphogenesis are negative regulators of the *PSY* gene and provide an alternative approach to enhance the synthesis of this class of isoprenoid [220]. Light signaling mechanisms have been the recent focus of a tomato TILLING collection screen in order to identify regulatory proteins that impact carotenoid regulation during fruit development and ripening [221].

Other regulatory mechanisms affecting the transcriptional status of genes concern epigenetic mechanisms, that is, histone posttranslational modifications and DNA methylation directed by small RNA [222]. Such mechanisms have been recently shown to impact carotenoid biosynthesis in plants. Recent works demonstrate that carotenoid isomerase (CRTISO) regulation requires the SET DOMAIN GROUP 8 (SDG8) protein which is a chromatin-modifying histone methyltransferase [223]. Moreover, DE-ETIOLATED 1 (DET1), a negative regulator of light signal transduction pathway, affects carotenoid pathway [224, 225] and binds H2B histone in vivo, suggesting that DET1 may affect chromatin condensation states [226]. Furthermore, studies indicate that the abundance of ABA, a carotenoid-derived phytohormone, is in part controlled by HISTONE MONOUBIQUITINATION 1 (HUB1) that mono-ubiquitinates the histone H2B [227], and on the other hand, by DNA methylation mediated by a microRNA (miR402) under abiotic stress conditions [228]. LeSPL-CNR was identified as an epimutant that impacts carotenoid accumulation during tomato fruit ripening together with many other aspects of fruit maturation [229]. Indeed the manipulation of some of these regulatory proteins including DET1 and cryptochrome 2 (CRY2), a blue light receptor, in tomato led to an increased accumulation of fruit carotenoids [225, 230, 231]. Similarly, the repression of DET1 and the overexpression of the microRNA miR 156b resulted in higher accumulation of carotenoids in seeds [232, 233]. These results point out the potential of these approaches to modulate isoprenoid biosynthesis in plants.

Indeed tissue- and stage-specific promoters and regulatory proteins are well suited for modifying plant nutritional traits, for example, carotenoid profile and/or amount. The use of plant cells as platforms for the production of specific isoprenoid remains a more challenging task that requires the precise manipulation of cells capable not only to produce but also to accumulate high level of specific isoprenoids without any effect on cell viability. Plant glandular trichomes present these properties and, as such, are relevant targets for isoprenoid metabolism engineering. Such "green factories" may limit major drawbacks linked to the production of volatile terpenes and diterpenes in plants, that is, cytotoxicity of molecules [8], conversion to conjugated forms that limit the accumulation of the desired product [79, 200], or low accumulation levels due to evaporation of small terpenes. Indeed modifying oils' production in mint glandular trichomes has been achieved with constitutive promoters controlling the expression of the *DXR* gene to enhance IPP synthesis, as well as an antisense metofurane synthase gene to reduce the abundance of the corresponding compound [85, 86]. Similarly, an antisense construct driven by the P35S promoter was sufficient to reduce diterpenoid production in tobacco trichomes [234]. Much better results were however obtained with an RNAi construct using the cembratrienol synthase promoter specifically expressed in tobacco trichomes [235]. So far, the number of available trichome-specific promoters is limited [235, 236] which makes difficult the engineering of series of genes in this cell type.

4 Conclusion

Over the last 15 years, considerable progress has been made in the understanding and engineering of isoprenoid metabolism in both microorganisms and plants. Isoprenoid metabolic engineering pursues two main objectives: (1) the production of highly valuable compounds that can be achieved either using microorganisms or plants and (2) the modification of isoprenoid profiles in plants with the aim to modify agricultural or nutritional properties. In both cases, a general need of pathway knowledge including enzyme functions and regulatory mechanisms is fundamental. However, producing molecules of interest often necessitates a complete knowledge of all enzymatic steps to achieve sufficient yield and easy extraction process, which is not an absolute requirement when agricultural or nutritional traits are targeted.

In this context, genome sequencing programs as well as the public availability of EST resources from a wide range of plant species have boosted the current knowledge of genes encoding enzymes of this pathway. Of particular interest with respect to isoprenoid metabolic engineering, recent works have analyzed the genome wide distribution of enzymes encoding TPS genes using gene model in fully sequenced plant genomes [67], as they contribute to the huge diversity of isoprenoids. Most relevant experiments done are EST sequence analyses and RNA-seq approaches on plant tissues and cells known to accumulate high amounts of terpenoids (see, e.g., [69, 80, 116, 237–239]. Yet, many proteins that are identified as potential TPS have no attributed function. An important challenge will therefore be to develop high-throughput screening methods in order to determine TPS product specificity, which so far has mainly relied on the functional expression of proteins in *E. coli*. In some cases, the correlative analysis of selected gene expression and terpene emission provides information about TPS specificity (e.g., [132, 133, 240]. Indeed, at a larger scale, integration of transcriptomic data together with metabolic profiling of isoprenoids in various plant tissues may provide an efficient way to attribute functions to unknown TPS. Gene network reconstruction provides an alternative approach toward this goal and was recently used to determine the function of a transprenyl diphosphate synthase involved in the synthesis of the solanesyl part of ubiquinone 9 [60]. Function identification of proteins involved in the synthesis of the huge diversity of isoprenoids known so far – still increasing – will be an important research area for this pathway engineering in microorganisms as well as in plants.

A general limitation in isoprenoid production when it is aimed at producing and purifying molecules of interest is due to the low yield of targeted products. Although significant progress has been done in both microorganisms and plant-based systems, in many cases, the final amount of product reached does not allow industrial production. Each isoprenoid is produced via a specific set of reactions that necessitate being identified and transferred to selected hosts before optimizing yield and productivity. Indeed, combinatorial approaches used in *E. coli* have led to encouraging results for taxadiene production [154] and the use of protein engineering, with targeted or random mutations, combined with metabolic engineering was shown to be a relevant approach to achieve high production levels of isoprenoids in bacteria [166]. Successful production of isoprenoids has also been achieved in yeast and although initial experiments were almost exclusively performed in *E. coli*, mostly for the production of carotenoids there have been an increasing number of publications using yeast as a heterologous expression system for the production of functional isoprenoids [200]. Yeast cells present a cellular organization closer to plant cells than do bacteria. Furthermore, the functional expression of P450 enzymes, which is a critical issue for the production of isoprenoids in bacteria turned out to be easier in yeast [13]. This has led to major breakthrough using this cell system by redesigning part of the endogenous isoprenoid pathway with the aim of boosting acetyl-CoA flux toward the mevalonate pathway [241], enhancing IPP synthesis by means of a truncated HMGR [242], and overexpressing FPP and GGPP synthases for increase sesqui-, di-, and polyterpene syntheses [243]. The coordinate use of different intracellular compartments, for example, mitochondria and cytosol [201], allows the scaling up of the targeted isoprenoid production; this in turn opens new possibilities for the metabolic engineering in yeast but also in plants [151]. This highlights the interest of combining complementary approaches with various cell systems.

Indeed, plant-based systems are an appropriate alternative to heterologous system as they naturally produce many different types of isoprenoids and have unlimited carbon resources. Nonetheless, the efficiency of terpenoid metabolic engineering in plant is variable, depending on pursued objectives and isoprenoid classes. In a general sense, the success of a strategy will depend on the ability to redirect carbon flux toward the synthesis of the selected compounds. Hence, precursor availability is a main point that needs to be solved. Of course, in the case of monoterpenes, many successful attempts indicate that GPP might not be limiting although this may vary between organs and developmental stages. Inversely, low sesquiterpene yield suggests that the availability of FPP is often limited, as might be GGPP. Various strategies have been used to circumvent this issue, including overexpression of enzymes of the MVA or of the MEP pathway, the use of a deregulated HMGR enzyme, the spatial and temporal control of isoprenoid biosynthesis gene expression, and recently the retargeting of part of all precursor pathways in a nonnative compartment that may relieve the normal control of carbon flux within this new pathway. At this stage it should be noted that very little work has been focusing on the interface between isoprenoid synthesis and carbon primary metabolism. Yet, both the MVA pathway and the MEP pathway directly depend on the carbon flux provided by the TCA cycle and by the glycolysis, respectively. In this context, it is particularly relevant that genes involved in photosynthesis and carbon fixation are expressed in *Solanum* trichomes that accumulate high terpenoid levels [116]. Furthermore, depletion of sucrose availability in tomato fruit pericarp has been shown to dramatically limit lycopene accumulation, indicating a direct link between carotenoid synthesis and carbon availability [244]. This is consistent with the recent demonstration that impairing PRL1 (PLEIOTROPIC REGULATORY LOCUS 1), which results in sugar accumulation in *Arabidopsis* seedling, enhances isoprenoid accumulation [245]. These results indicate that system biology approaches with the integration of isoprenoid metabolism together with carbon primary metabolism should be developed with the aim to identify important control points involved in the control of terpenoid accumulation. Coordinate regulation of isoprenoid and precursor pathways might also occur at the transcriptional level. Indeed, it is well established that in many cases, isoprenoid accumulation is primarily controlled at the gene expression level although there is no transcription factors identified that control genes of the isoprenoid pathway. Therefore, identifying relevant TFs might provide alternative strategies to deregulated isoprenoid accumulation in plants. Similarly, unraveling the relevance of epigenetic type of regulation in relation with environmental constraints or development cues might also provide new tools to fine-tune isoprenoid production in relation to chromatin remodeling mechanisms.

Efficient terpenoid engineering is also dependent on the storage capacity of end products. This was elegantly illustrated following the characterization of the cauliflower *or* mutant which creates a metabolic sink by enabling chromoplast differentiation, therefore carotenoid accumulation [144]. End product storage is also a critical issue when volatile or diterpenes need to be produced and purified. Indeed reprogramming flower scent or terpene emission as a response to pathogens may not require high storage capability, contrary to the production of molecules of interest such as artemisinin, normally produced in glandular trichome of *A. annua* [202]. Glandular trichomes have been the focus of intense research over the last few years using combined transcriptomic and metabolic profiling approaches [115] and evidence indicates a close relationship between the control of the MEP pathway, volatile terpene synthesis, and trichome density in tomato [246]. No doubt that such approach will allow unraveling the specific metabolism of these cells and provide tools to efficiently use them as "green factories" for the production of highly valuable terpenes.

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Selection of Tomato Rich in Nutritional Terpenes

92

Jaime Cebolla-Cornejo, Salvador Roselló, and Fernando Nuez

Contents

1 Introduction 2854
2 Occurrence 2856
3 Biosynthetic Pathway 2856
4 Breeding Strategies 2862
4.1 Conventional Breeding 2862
4.2 Genetic Engineering 2867
4.3 Limitations and Drawbacks Associated with Analysis and Environment 2871
5 Conclusion 2874
References 2875

Abstract

There is a rising concern about the nutritional and functional properties of food and its use to promote human health and prevent diseases. Considering the high level of consumption of tomato, this vegetable has become an ideal source of health promoting compounds. Among the terpenes, carotenoids have received most part of the attention. The terpenes lycopene and β-carotene have been related to decreased levels of certain heart diseases as well as certain types of cancer. Consequently, a high interest has been placed in the selection of lines with increased levels of these terpenes. The main difficulties in this process have relied on the necessity to separate genotypic effects on the trait from

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environmental or interaction effects. On the other hand, the availability of rapid and efficient methods to determine the content in these compounds has also limited progress in the area. Mutants identified in the cultivated species and wild relatives enable an improved accumulation of total carotenoids or a different distribution among them. Some of these mutants have been used in the development of new cultivars, but usually involve a decrease in yield. The modification of the biosynthetic pathway via genetic engineering has contributed to the dissection of these allelic variants, and to provide new approaches for the development of high carotenoid lines.

Keywords

β-carotene • Breeding • Genetic engineering • Lycopene

Abbreviations

ABA	Abscisic acid
CRTISO	Carotene isomerase
CRTR-B	β-ring hydroxylase
CRTR-E	ε-ring hydroxylase
CYC-B	Chromoplast-specific lycopene-β-cyclase
DXS	1-deoxy-D-xylulose-5-phosphate synthase
EMS	Ethyl methanesulphonate
GGPP	Geranylgeranyl pyrophosphate
IPP	Methylerythritol-4-phosphate
LCY-B	Lycopene-β-cyclase
LCY-E	Lycopene-ε-cyclase
MEP	Methylerythritol-4-phosphate
MVA	Mevalonic acid
NXS	Neoxanthin synthase
PDS	Phytoene desaturase
PSY	Phytoene synthase
ZDS	ζ-carotene desaturase
ZE	Zeaxanthin epoxidase

1 Introduction

The concept of quality for tomato, as well as for other vegetables, has evolved during the last decades. In previous years, the demands of wholesalers and retailers prevailed in the development of new varieties and quality was based on the external appearance of the fruit. Consequently, varieties with high uniformity, deep red color, without green shoulders or cracking scars, and with a long shelf-life were demanded. The focus in breeding programs was set in the external appearance together with high yields and disease resistance, neglecting other aspects such as flavor. As a result, this last trait degenerated progressively and consumers' complaints on tomato flavor became generalized [1]. Ever since, organoleptic quality started to receive more attention by the seed industry.

Following this shift of the quality concept from the external appearance to internal attributes, the health benefits of whole food consumption started to be valued [2]. Accordingly, the nutritional value and functional value of tomato varieties gained importance as breeding objectives. In this area, among the complex family of tomato terpenes, carotenoids and especially lycopene and β-carotene represent the maximum potential for improved internal quality and they have been thoroughly studied during the last two decades. Their antioxidant characteristics have been related to the prevention of degenerative diseases, and thus, they have become a major component of the functional value of tomato.

In this context, a high content in these phytochemicals has gained importance in the tomato marketing. Nevertheless, it is difficult to tell whether consumer interests in healthy products have conditioned the marketing of produces based on health functionality or vice versa [3]. In fact, the correlation between food and health is still open to debate, though it seems that diet plays a major role in the development of different cancers [4]. In a review of more than 200 studies, Block et al. [5] concluded that persons with low fruit and vegetable intake experience twice the risk of cancer compared to those with high intake.

In the case of carotenoids, it has been observed certain inverse relation between their intake in the diet, mainly lycopene and β-carotene, and the development of certain types of cancer [6–8], and a reduced incidence of heart disease [9–11].

In a recent review performed by the U.S. Food and Drug Administration including several studies relating tomato consumption and reduced cancer risk, it was concluded that evidences supporting this correlation were very limited [12]. In the same line, the association of fruits and vegetables intake and the occurrence of coronary heart disease has been questioned [13].

Nonetheless, it should also be considered that it is difficult to prove that a certain component of a complex diet is specifically directly responsible for reduced risk of suffering a disease with a prolonged evolution or a heart disease. The fact that the risk of suffering both types of diseases is determined by a complex mix of different factors combined in different ways in different individuals also adds complexity to the analysis of epidemiological studies and clinical trials.

In fact, recent publications show that carotenoid derivatives would participate in the modulation of transcription systems important in cancer and inflammatory processes [14]. And new large-populations studies again confirm the existence of a relationship between carotenoid intake and the probability of suffering certain cancers. For example, in the case of breast cancer, carotenoid intake would have an inverse relationship with cancer in premenopausal women (especially for smokers), but not in postmenopausal [15].

Despite the absence of clear evidence for a role in prevention of diseases, carotenoids continue to play a role in the diet of consumers and continue to be debated in the light of recent dietary trends [16]. Thus, the research on the effects of carotenoids on health should go on, as well as the efforts to provide new varieties with higher amounts on these terpenes. Here we review the progress made during the last years in the development of high carotenoid lines following conventional and genetic engineering strategies, as well as the limitations found by breeders in the process.

2 Occurrence

Carotenoids include many of yellow, orange, and red pigments present in nature, providing colors to different organs. In the case of tomato, carotenoids are synthesized in leaf tissues, flowers, and fruits. In leaves, β-carotene, lutein, violaxanthin, and neoxanthin are accumulated, with lutein representing the higher concentrations (Table 92.1). In these tissues, carotenoids play an important role in the performance of the plant light-harvesting complex as photoprotectors [19]. In the flowers, the typical yellow color of tomato petals is due to the accumulation of the xanthophylls violaxanthin and neoxanthin [18]. Finally, in the ripe fruits, lycopene is the most abundant carotenoid followed by β-carotene (Table 92.1).

It should be considered though, that the distribution of major carotenoids in the ripe fruit is irregular, with more than twofold amounts of lycopene in the pericarp than in the locules and fourfold higher amounts of β-carotene in the locules than in the pericarp [17].

Carotenoids are synthesized in plastids: in chloroplasts in green tissues, and in chromoplasts in ripening fruits and flowers. During the ripening process, chloroplasts are converted to chromoplasts. The thylakoid structures disassemble, chlorophylls are degraded, and plastoglobules accumulate. Plastoglobules represent the site for accumulation of high amounts of carotenoids [20].

At the mature green stage, all the plastids in the fruit are chloroplasts; at the breaker stage, intermediate chloro-chromoplasts can be identified with both chlorophylls and carotenoids; and at the red ripe stage, only fully developed chromoplasts are found [21]. Recent studies [22] show that following the ripening process, lycopene starts to be found in tomatoes at the breaker stage in tissues of the locular cavities, under the outer pericarp, and it increases in concentration as the process continues (Fig. 92.1). Consequently, the external color changes generally lag behind the internal composition changes.

3 Biosynthetic Pathway

The analysis of tomato color mutants found in *Solanum lycopersicum* or in crosses with related wild species, as well as genetic engineering studies have clarified enormously the biosynthetic pathway of carotenoids. Carotenoids, as other plastid isoprenoids, derive from isopentenyl diphosphate (IPP). IPP used in the synthesis of these compounds may arise at some developmental stages partly from the mevalonic (MVA) pathway [23], but it is mainly synthesized through the methylerythritol-4-phosphate (MEP) pathway [24]. The first enzyme of this pathway,

Table 92.1 Typical carotenoid composition (mg kg^{-1}) in ripe fruits, flowers, and leaves of tomato (Adapted from [17] and [18])

Carotenoid	Ripe fruits	Flowers	Leaves
Phytoene	9.8–29	–	–
Phytofluene	2.1–15.5	–	–
α-carotene	0–0.02	–	–
β-carotene	1.4–11.9	–	19.1
δ-carotene	0–1.8	–	–
γ-carotene	0.5–2.6	–	–
Neurosporene	0–0.3	–	–
Lycopene	78.3–181.2	–	–
Neoxanthin	–	427.4	12.5
Violaxanthin	–	192.6	28.4
Antheraxanthin	–	–	3.2
Lutein	0.9	2.4	50.6
Zeaxanthin	–	–	2.7

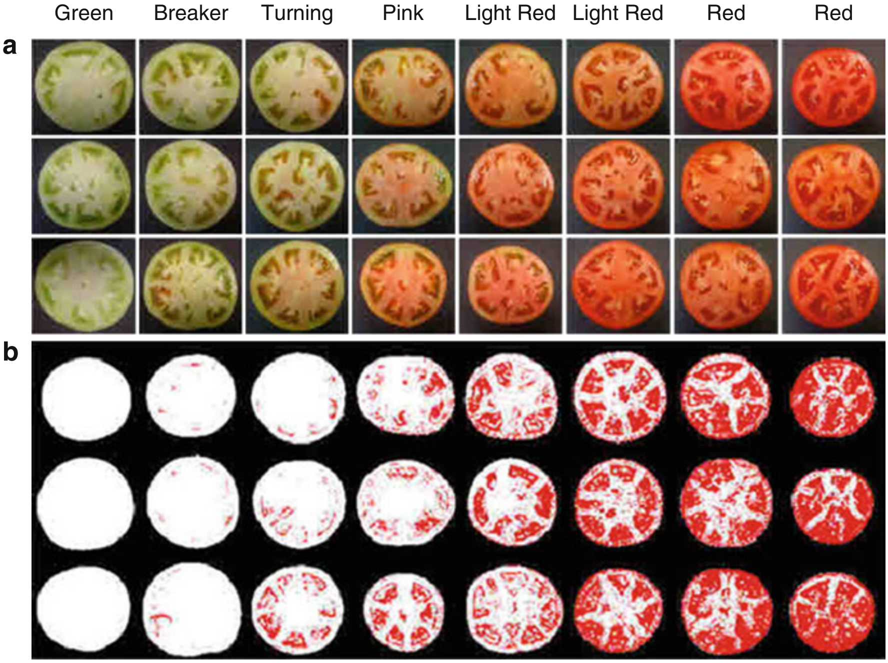


Fig. 92.1 (**a**) Tomato fruit samples at different ripening stages and (**b**) identification of lycopene by Raman Chemical Images in these samples (Adapted from The Journal of Food Engineering, Vol. 107, Qin J, Chao K, Kim MS, Investigation of Raman chemical imaging for detection of lycopene changes in tomatoes during postharvest ripening, pp. 277–288, Copyright (2011), with kind permission of Elsevier)

1-deoxy-D-xylulose-5-phosphate synthase (DXS), has shown a strong correlation with carotenoid synthesis during fruit development and has been proved to catalyze the first regulatory step in carotenoid biosynthesis [25].

The initiation of the carotenoid biosynthesis pathway requires four IPP units that will be joined progressively including isomerization and condensation reactions to form geranylgeranyl pyrophosphate (GGPP), which is the immediate precursor of carotenoids (Scheme 92.1). Two units of GGPP would then be condensed to form 15-*cis*-phytoene. This reaction is catalyzed by the enzyme phytoene synthase (PSY).

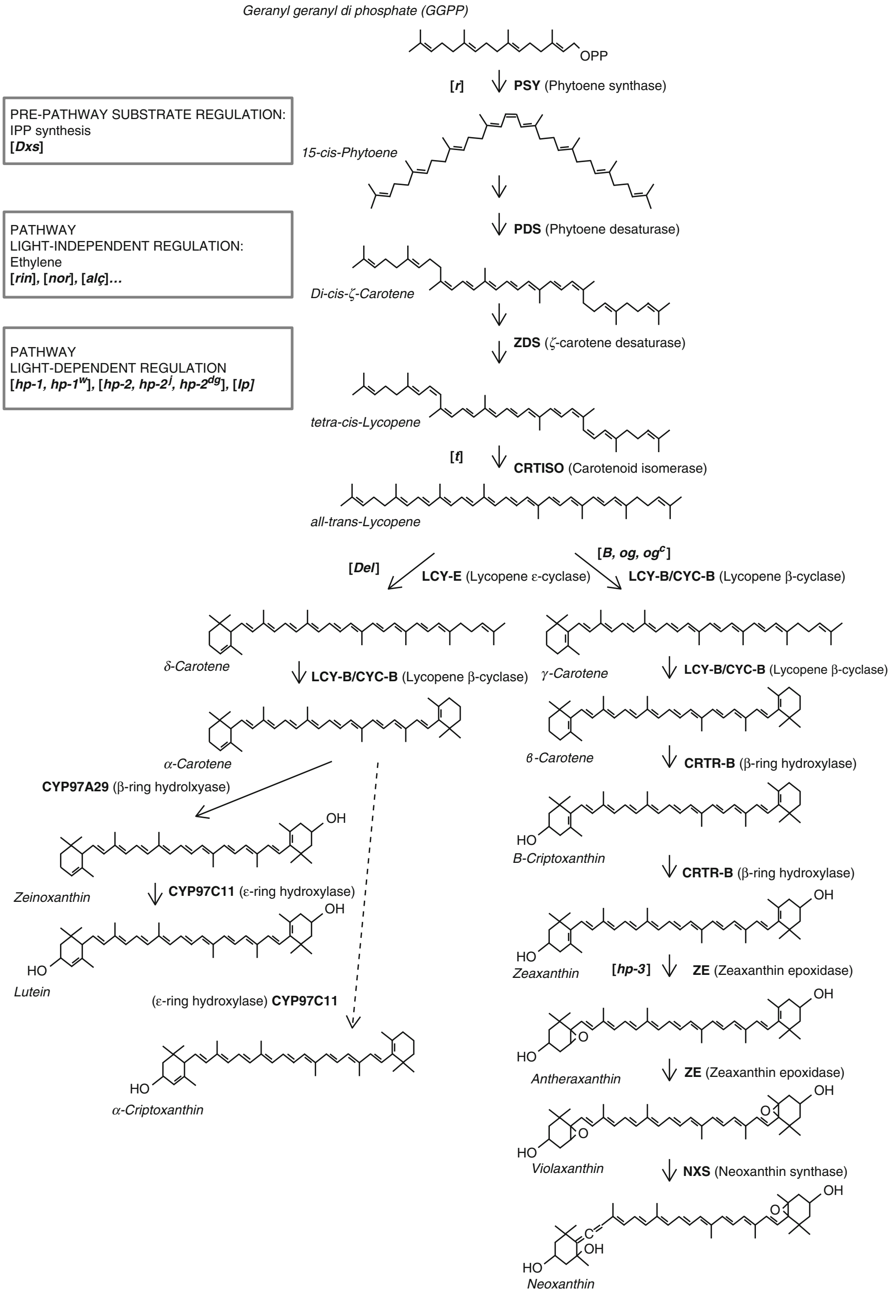
In tomato two PSY encoding genes have been identified: *Psy-1* and *Psy-2*. *Psy-1* encodes the fruit-ripening-specific isoform, which has been proved to be the key enzyme in the control of carotenoid biosynthesis [28]. *Psy-2* encoded enzyme predominates in green tissues (including mature green fruit) and petals and has no role in carotenoid synthesis in ripening fruit [29, 26]. As an example of the role of this enzyme in carotenoid biosynthesis, a null mutation in *Psy-1* in the yellow-flesh (*r*) mutant arrests carotenoid synthesis [30]. In this mutant, the accumulation of the flavonoid rutin in the fruit skin would lead to the development of yellowish fruits.

Continuing the pathway, 15-*cis*-phytoene would suffer then two desaturations carried out by phytoene desaturase, PDS [31], forming phytofluene and ζ-carotene. Two subsequent desaturations are then performed by ζ-carotene desaturase (ZDS) forming neurosporene and finally tetra-*cis*-lycopene (prolycopene).

The analysis of the tangerine (*t*) mutation with orange fruit phenotype due to the accumulation of prolycopene enabled the identification of a carotene isomerase, CRTISO, which is responsible for the conversion of prolycopene to all-*trans*-lycopene [32]. The accumulation of all-*trans*-lycopene as the primary carotenoid in ripe tomato fruits enables the development of red color starting from the colorless precursor phytoene.

Carotenoid pathway continues with the cyclization of lycopene, involving the formation of 6C cyclic end groups. Two major enzymes participate at this point: lycopene-β-cyclase (LCY-B) and lycopene-ε-cyclase (LCY-E). Unusually, tomato contains two lycopene β-cyclases, LCY-B [33] as described above, and also CYC-B, a chromoplast-specific cyclase that would play a major role in carotenoid accumulation in fruits [34]. Lycopene β-cyclase forms initially γ-carotene which only accumulates to traces in ripe fruits as a second β-ring is added by the same enzyme to yield β-carotene.

In red ripe tomatoes, the activity of lycopene β-cyclases is low. In fact, LCY-B transcription level decreases at the breaker stage [33]. On the contrary, in mutants such as Beta (*B*), with orange fruits, CYC-B transcription is dramatically increased, reducing lycopene fruit content and increasing β-carotene accumulation. In fact, the major differences between the wild allele (*b*) and that responsible for the beta phenotype (*B*) exist in the promoter region [34]. Other two alleles of the same gene, the *old-gold* (*og*) and old-gold-crimson (og^c) mutants, represent null alleles arising from two different frameshift mutations. These alleles result in increased lycopene content, as β-carotene synthesis becomes restricted. Another gene linked to *Beta*,


Geranyl geranyl di phosphate (GGPP)
OPP
[r] PSY (Phytoene synthase)
PRE-PATHWAY SUBSTRATE REGULATION:
IPP synthesis
[Dxs]
15-cis-Phytoene
PATHWAY
LIGHT-INDEPENDENT REGULATION:
Ethylene
[rin], [nor], [alç]...
PDS (Phytoene desaturase)
Di-cis-ζ-Carotene
ZDS (ζ-carotene desaturase)
PATHWAY
LIGHT-DEPENDENT REGULATION
[hp-1, hp-1^{w}], [hp-2, hp-2^{j}, hp-2^{dg}], [lp]
tetra-cis-Lycopene
[t] CRTISO (Carotenoid isomerase)
all-trans-Lycopene
[Del] LCY-E (Lycopene ε-cyclase)
[B, og, og^{c}] LCY-B/CYC-B (Lycopene β-cyclase)
δ-Carotene
LCY-B/CYC-B (Lycopene β-cyclase)
γ-Carotene
LCY-B/CYC-B (Lycopene β-cyclase)
α-Carotene
β-Carotene
CRTR-B (β-ring hydroxylase)
CYP97A29 (β-ring hydrolxyase)
OH
Zeinoxanthin
HO
B-Criptoxanthin
CRTR-B (β-ring hydroxylase)
CYP97C11 (ε-ring hydroxylase)
OH
HO
Lutein
HO
Zeaxanthin
[hp-3] ZE (Zeaxanthin epoxidase)
(ε-ring hydroxylase) CYP97C11
OH
O
HO
Antheraxanthin
ZE (Zeaxanthin epoxidase)
HO
α-Criptoxanthin
OH
O
O
HO
Violaxanthin
NXS (Neoxanthin synthase)
OH
O
C
OH
HO
Neoxanthin

Scheme 92.1 Carotenoid biosynthesis pathway in tomato (Adapted from [18], [25], [26] and [27]). Mutants for each enzyme are identified in *square brackets*. Genes involved in external regulation represented in the *squares*

Beta modifier (*mo*$_B$) modifies the expression of *Beta*. In *BB mo*$_B$ *mo*$_B$ genotypes 90 % of the carotenoid content is present as β-carotene and the fruits are orange. On the other hand, in *BB mo*$_B{}^+$ *mo*$_B{}^+$ 50 % of the carotenoids are present as β-carotene and lycopene accumulates up to less than 50 % of total carotenoids, resulting in red-orange fruits [35].

Following an alternative sub-pathway LCY-E using lycopene as substrate introduces an ε-ring forming δ-carotene. Interestingly, LCY-B may also play a role in this sub-pathway introducing an additional β-ring in the other end of the molecule and yielding α-carotene from δ-carotene. In common red fruit cultivars, the transcript level of LCY-E decreases at the breaker stage to undetectable levels in the ripe fruit. In the delta tomato mutant (*Del*), characterized by an orange fruit color due to δ-carotene accumulation, the transcript level of LCY-E is increased 30-fold during fruit ripening [36].

Xanthophylls are formed by the hydroxylation of carotenes. Specific hydroxylases perform its function on β-rings (CRTR-B) or ε-rings (CRTR-E). Regarding β-ring hydroxylases, CRTR-B1 and CRTR-B2 hydroxylases have been described in tomato. The homolog gene encoding CRTR-B1 is present in all tissues, though the highest expression levels are detected in leaves, while CRTR-B2 would be expressed in chromoplasts of flowers and fruits [18].

The action of CRTR-B would yield zeaxanthin from β-carotene following two hydroxylations with beta-cryptoxanthin as intermediate. On the second sub-pathway, it was initially proposed that the joint action of CRTR-E and CRTB2 would produce lutein from α-carotene with α-cryptoxanthin as intermediate [37]. But recent works [26] show that the β-ring hydroxylase CYP97A29 (also CRTR-B1 and CRTR-B2) would produce zeinoxanthin that would be converted to lutein by the ε-ring hydroxylase CYP97C11. Only when β-hydroxylases are impaired or downregulated, α-carotene would be hydroxylated by CYP97C11 yielding α-cryptoxanthin. As β-ring hydroxylases would not be able to hydroxylate the β-ring of α-cryptoxanthin efficiently, no lutein would be produced.

Epoxidation of zeaxanthin by zeaxanthin epoxidase (ZE) would result in the production of violaxanthin via antheraxanthin. From that substrate, the enzyme neoxanthin synthase (NXS) would yield neoxanthin opening the cyclohexenyl 5–6 epoxide ring in violaxanthin [38]. Neoxanthin would be the last product of carotenoid biosynthesis in green parts of the plant, and it would derive in the abscisic acid (ABA) synthesis pathway. The accumulation of neoxanthin and violaxanthin in flowers results in wildtype yellow petals. A defective mutation in the gene encoding CRTR-B2 prevents formation of these xanthophylls, resulting in the white-flower phenotype [18].

Defective ZE mutants may result in an arrest of the ABA biosynthetic pathway that starts from the neoxanthin produced with zeaxanthin [39]. Consequently these mutants may result in wilty phenotypes. In the case of the mutant *high pigment-3* (*hp-3*), coding a defective ZE, it seems that ABA deficiency would increase plastid division, enabling higher biosynthesis and accumulation of carotenoids [40].

The carotenoid biosynthetic pathway described can be regulated at different levels. Some regulations would result in a global increase or decrease of carotenoids, while others would change the natural accumulation profile of carotenoids rather than the global amount.

It must be considered that the development of chromoplasts and plastoglobules is necessary step in the accumulation of carotenoids. The primary growth regulator of the fruit ripening process also plays a major role in the control of the carotenoid synthesis pathway. The key enzyme phytoene synthase (PSY) is under strong positive control of ethylene, and lycopene β-cyclase (LCY-B) is repressed by ethylene resulting in a primary accumulation of lycopene [20]. In fact, during the ripening process, the regulation of the pathway involves an increase in the mRNA levels for the lycopene-producing enzymes phytoene synthase (PSY) and phytoene desaturase (PDS), while the mRNA levels of the genes for the lycopene β- and ε-cyclases, which convert lycopene to either β- or δ-carotene, respectively, decline and completely disappear. Consequently, mutations affecting ethylene synthesis or perception, such as *rin*, *nor*, *alç*, etc., apart from affecting the normal ripening process also result in altered carotenoid content.

Apart from the light-independent component of the carotenogenesis regulation, there is also a light-dependent component. In fact, fruit-localized phytochromes also regulate the extent of carotenoid accumulation [41]. In this context, the mutation *high pigment-1* (*hp-1*), and the allelic *hp-1*^{w}, represents a mutation in a tomato UV-damaged DNA Binding Protein (DDB1) homolog [42]. As a result, the plant would act as perceiving a continuous perception of light. The mutation *high pigment-2* (*hp-2*), and the allelic *hp-2*^{j} and *hp-2*^{dg} [43], affects the photomorphogenesis regulatory gene TDET1 [44], and also affects the light signal-transduction machinery and results in enhanced global carotenoid content. Another mutant, *Intense pigment* (*Ip*) is implicated in a promotion of phytochrome signal amplification [45] and results in 34–38 % more soluble solids and 62.6 % more total carotenoids [46]. Therefore, the modification of light perception may also have a role in the strategies followed in breeding efforts for carotenoid content improvement.

Regarding other modifications that may result in global carotenoid increase, as stated before, an alteration in the ABA pathway, such as in the case of the *high pigment-3* (*hp-3*) mutant, may result in increased carotenoid content, but it may be related with increased plastid division [40].

Instead of a global increase or decrease in total carotenoid content, changes in the enzymes of the biosynthetic pathway may result in a different carotenoid profile. As explained before, the *r* mutant affecting PSY results in an arrest of carotenoid biosynthesis and yellow fruits and the *t* mutant affecting CRTISO accumulates prolycopene and produces orange fruits. After the synthesis of all-*trans* lycopene, the *Del* mutant affecting LCY-E results in δ-carotene accumulation at the expense of lycopene and orange fruits. On the other branch of the pathway, the mutants *B*, *og*, and *og*^{c} affect LCY-B and result in increased β-carotene content at the expense of lycopene (*B*) or increased lycopene at the expense of β-carotene (*og* and *og*^{c}). Finally, the mutant *hp-3*, apart from its side effects related with the ABA pathway, results in decreased content of violaxanthin and neoxanthin.

4 Breeding Strategies

A breeding program for improved carotenoid content in tomato requires, in general, three phases: (a) identification of genotypes with high potential for the accumulation of carotenoids, (b) transfer of the genes controlling the trait into breeding lines, and (c) the evaluation of the expression of the introgressed genes in the new genetic background and selection of elite genotypes with good agronomic performance.

At the moment two main strategies are followed in order to transfer desired genes into breeding lines. The first one involves the exploitation of natural diversity present in the genus. Donor parents are selected among genetic resources of the cultivated species and related wild species, once their carotenoid profile has been evaluated. The genetic control of the trait may be ascertained after the analysis of family progenies. If the genetic control is simple, the efficiency of the breeding program would be higher, though it is not mandatory. Once the donor resources have been identified, the genes responsible for the trait can be introgressed into breeding lines following different hybridization strategies and selection generations. In the process, both carotenoid content and agronomic performance are considered in the selection of appropriate genotypes. Technical advances in genetic engineering have promoted a second strategy during the last decades. Several of the genes controlling high carotenoid content in the genus or in other species and even kingdoms have been cloned and introgressed directly, causing minor alterations of the genetic background of breeding lines. The introgressed genes may alter the biosynthetic pathways and result in increased or decreased contents of compounds related to nutritional or functional value.

Independent of the strategy followed, during breeding programs, two major limitations arise. The first one involves the difficulty of developing a rapid, wide, and precise evaluation of carotenoid contents in breeding lines during the different generations of a breeding program. The second is related to the important environmental influence in the accumulation of carotenoids in tomato [17] that interferes with the identification of the genetic potential of the plants being evaluated. Both factors make extremely difficult an efficient evaluation and selection of elite genotypes and hinder the development of new cultivars with improved carotenoid content.

4.1 Conventional Breeding

The breeding objectives concerning terpenes content and nutritional or functional value have been centered on the development of cultivars with high contents in lycopene and/or β-carotene. These objectives have been pursued for more than 70 years, developing studies targeted to the identification of sources of variability that could be used as donor parents in breeding programs. In this sense, wild species of the genus *Solanum Lycopersicum* related to the cultivated tomato have provided a valuable range of diversity that was lost during the domestication process in the cultivated species.

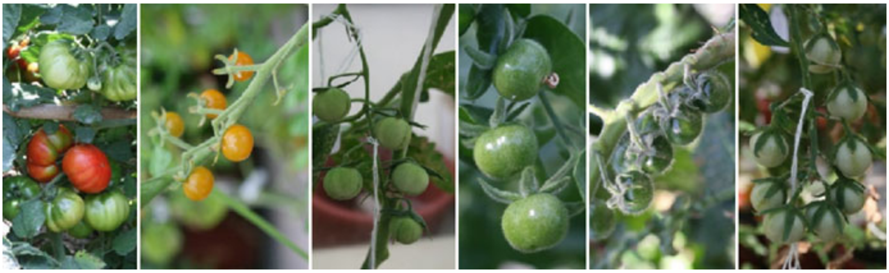


Fig. 92.2 Genetic resources used for the identification of genes modifying carotenoid contents or profile. From *left* to *right*: *Solanum lycopersicum, Solanum chesmaniae, Solanum chmielewskii, Solanum pennellii, Solanum habrochaites, and Solanum chilense* (Photo courtesy of J.V. Valcárcel, COMAV)

Initially, one of the breeding strategies that received great efforts was targeted to the identification of useful variability in the wild species of the section with green fruits (Fig. 92.2), mainly *Solanum habrochaites, Solanum chmielewski, Solanum neorickii, and Solanum pennellii* [48] (Classification following Peralta and Spooner [47] which corresponds to *L. hirsutum, L. chmielewskii, L. parviflorum*, and *L. pennellii*, respectively.). Considering that the lycopene content in these species with green fruits is practically null and the content in β-carotene is low and that in tomato normal contents average 30 mg kg^{-1} [49, 50] and β-carotene 4 mg kg^{-1} [49], it may seem that these sources of diversity would not be the most appropriate. Nevertheless, after several backcrossing generations toward the cultivates species, followed by several selfing generations, an incredible variability was revealed for the accumulation of different carotenoids including the sought lycopene and β-carotene, as well as, phytoene, phytofluene, δ-, α-, and γ-carotene, among others [51, 52]. This new diversity and especially the transgressive segregation of some part of it (especially for lycopene) enabled the selection of genotypes with high carotenoid contents in advanced generations.

This wide range of variability in carotenoid contents relies on considerable differences in the carotenoid biosynthesis pathway between parent species. Therefore, it has been difficult to select in the different progenies genotypes with high carotenoid content. Only after several backcrossing generations, the biosynthesis pathway has been normalized and new phenotypes have been revealed. In fact, this procedure enabled the identification of the mutant phenotypes and genes *B*, mo_B, and *Del* involved in high accumulation of β-carotene and δ-carotene as a result of lycopene cyclization (Table 92.2).

For commercial breeding purposes, the gene *Del* has limited interest, as δ-carotene has no activity as provitamin A. Other mutants such as the mutant *apricot* (*at*) identified in a Mexican population of *S. lycopersicum* conferring an apricot color due to β-carotene accumulation in the absence of lycopene [58] have received little attention. Therefore, most interest has been placed in the selection of genotypes carrying the *B* gene with its modifier mo_B. The most important fresh

Table 92.2 Mutant genes that alter the normal carotene biosynthesis in tomato fruits [30, 34–36, 40, 42, 44, 45, 53–57]

Genes[a]	Species of origin[b]	Gene action	Phenotypic characteristics
t	*S. lycopersicum*	Determinates the poly-*cis* structure of the polyene chain of carotenoid molecules (in opposition to the common all-*trans* carotene structure). Codes a defective CRTISO.	Orange colored fruits due to tetra-*cis*-lycopene accumulation.
at	*S. lycopersicum*	Inhibition of lycopene accumulation without effects on β-carotene synthesis. Probably related to carotenogenesis regulation.	Apricot-like colored fruits.
[B,og, og^c], mo$_B$	*S. chmielewskii (B, mo$_B$), S. habrochaites (B, mo$_B$), S. pennellii (B, mo$_B$), S. neorickii (B, mo$_B$), S. cheesmanii (B), S. galapagoense (B), S. lycopersicum (og, og^c)*	*B* gene increases the β-carotene content at the expense of lycopene by cyclization of lycopene into β-carotene; *og* and *og^c* are null mutants in the locus *B* that block cyclization of lycopene; mo$_B$ is a gene modifier for the *B* gene. *B* encodes CYC-B with altered promoter; *og* and *og^c* encode defective CYC-B	*B* gene induces fruits orange or orange-red in color whereas *og* genes produce dark-red color of fruits even in the early stages of ripening.
Del	*S. chmielewskii, S. chilense, S. pennellii*	Increases the cyclization of lycopene into δ-carotene (increases δ-carotene at the expense of lycopene). Encodes LCY-E.	Orange-red colored fruits due to accumulation of δ -carotene
[hp-1, hp-1^w] *[hp-2, hp-2^j, hp-2^{dg}]*	*S. lycopersicum*	High total carotene content via light regulation.	Higher pigmentation in leafs and fruits. The *hp* genes introduce certain unfavorable traits: slow seed germination and sprout growth, low survival percentage, and breaking plants
Ip	*S. chmielewskii*	High total carotene content via light regulation.	Similar phenotype to *hp* mutants but with lower deleterious effects

(*continued*)

Table 92.2 (continued)

Genes[a]	Species of origin[b]	Gene action	Phenotypic characteristics
r	*S. lycopersicum*	Low total carotene content. Encodes a defective PSY	Yellow pigmentation in fruits by accumulation of the flavonoid rutin.
hp-3	EMS-induced mutant in *S. lycopersicum*	Encodes a defective ZE, blocking the ABA biosynthesis pathway and resulting in increased plastid division	Higher carotenoid accumulation and deep red colored fruits

[a]Genes into brackets have allelic relationships. Genes notated with uppercase are dominant and with lowercase are recessive. *Del* gene is partially dominant.

[b]Classification following Peralta and Spooner [47] (information obtained using with previous classification was actualized).

tomato cultivars developed introgressing these genes have been Caro Red [59] and Caro Rich [60]. These cultivars show orange fruits with β-carotene contents up 50 mg kg^{-1} (roughly tenfold the normal content in standard red cultivars).

In accessions with orangish fruits of the species *Solanum galapagoense, S. cheesmanii*, and *S. pimpinellifolium* (Classification following Peralta and Spooner [47] which corresponds to *L. chesmaniae* (the two first) and *L. pimpinellifolium* respectively.) with high contents in β-carotene, the mutations for the genes *B* and *mo*$_B$ have also been identified [51, 61–65]. But in this case, the carotenoid biosynthesis pathway is similar to the one in cultivated tomato except for the lycopene cyclization mediated by the gene *B*. In these accessions the content in β-carotene is about 60 mg kg^{-1} and lycopene content is very low.

The introgression of these mutations from these species into *S. lycopersicum* is very simple and efficient, as these species have good crossability with cultivated tomato, and it is easier and faster to identify genotypes with interesting pigmentation characteristics.

This approached has been used in the development of the processing tomato cultivar Caro beta (30–40 mg kg^{-1} β-carotene) from a cross between *S. lycopersicum* and *S. galapagoense* [66]. Several indeterminate lines have also been developed with β-carotene contents ranging 60–80 mg kg^{-1} [67]. One accession of *S. lycopersicum* (former var. *cerasiforme*) with orangish fruits has also shown similar β-carotene contents to Caro beta but with standard lycopene contents [68] and shows an additive genetic control [69]. This accession constitutes an alternative and interesting source of variation yet to be exploited.

Unfortunately, the cultivars developed with orange fruits and high β-carotene content have not been commercially successful, as consumers seem to prefer red tomato varieties. Consequently, these nongreen wild relatives, specifically the former var. *cerasiforme* of *S. lycopersicum*, *S. pimpinellifolium*, and *S. cheesmanii*, have been used as sources of variability for high lycopene red-fruited cultivars.

In the former var. *cerasiforme* of *S. lycopersicum* and *in S. pimpinellifolium*, accessions with five- to ninefold lycopene content and three- to fourfold β-carotene content compared to standard cultivars have been identified [52, 70–72]. These materials have great interest in the development of red cultivars with high contents of both compounds [52, 70–72].

The mutants old-gold (*og*) and especially old-gold-crimson (*og*^{c}) identified in *S. lycopersicum* offer another approach increasing specifically lycopene content. These mutants are allelic to the gene *B* [34] and inhibit the cyclization of lycopene to yield β-carotene and result in increases of 30 % in lycopene and a reduction of 40 % in the accumulation of β-carotene [27]. Several cultivars of the crimson type have been developed using these mutants, and despite their lower β-carotene content, they have succeeded commercially due to their intense red pigmentation even when the fruits are not completely ripe, thus satisfying consumer preference regarding external appearance.

Nonetheless, the best results in the development of high carotenoid content cultivars have been obtained using accessions carrying the mutants for the genes *hp-1*, *hp-2*, and *hp-2*^{dg}. These genes improve total carotenoid content over two- and threefold [73–77] without affecting significantly the carotenoid partition of standard cultivars. Cultivars carrying both crimson and high pigment produce fruits with lycopene content up to three- to fourfold that of the standard varieties [78]. Despite the efficiency of these mutants, cultivars with the *hp* genes have associated undesirable pleiotropic effects such as slow seed germination, increased seedling mortality, brittle stems, premature defoliation, and reduced yields. These deleterious effects have limited the success of these varieties, as a yield reduction compensates the benefits of higher carotenoid content.

The mutant *Intense Pigment* (*Ip*) introgressed into *S. lycopersicum* from *S. chmielewski* [79] has effects on carotenoid accumulation similar to those of the genes *hp*, but in this case, the *Ip* gene is dominant, and thus, it is more interesting for the development of hybrid cultivars, avoiding the introgression of the other recessive genes in both parent lines. Additionally, this mutant shows less deleterious effects on seed germination and plant vigor.

The monogenic mutants described have great effects on fruit pigmentation. In fact, several of them have been cloned and sequenced. Therefore, marker-assisted selection is feasible in breeding programs to introgress these mutant genes in parent lines of hybrid cultivars. But despite the advances in the knowledge of these sources of variation, their contributions in the development of new cultivars have been limited. Another alternative to the use of monogenic mutants follows the trends for other quantitative traits with an expression relying in complex metabolic processes. In this context, in addition to phenotypic selection, a great effort is being placed on the identification of quantitative trait loci (QTL) linked to variation in fruit pigmentation. This procedure emulates the approach applied to the selection of cultivars for improved fruit firmness or soluble solid contents. Numerous QTLs introgressed from *S. pimpinellifolium* [80, 81], *S. habrochaites* [82–84], *S. pennellii* [85], *S. peruvianum* [84, 86], and *S. neorickii* [87] or detected in *S. lycopersicum* [88, 89] have been reported.

Nevertheless, it is still necessary to continue in this direction, as the use of QTL-assisted selection may boost the exploitation of the variability present in related species, especially in *S. pimpinellifolium*. The use of markers is a priceless tool to enable selection in early stages of development, avoiding late and costly determinations in ripe fruits.

4.2 Genetic Engineering

A considerable advance has been made during the last decade in the knowledge of the biosynthesis of carotenoids, the metabolic pathway, its precursors, and its regulation. Following this advance, numerous works have been published reporting high carotenoid tomato lines obtained by manipulating the genes controlling several steps via genetic engineering. Most of this works try to emulate the results of the mutants identified using conventional approaches, and some even try to avoid undesirable side effects.

Regarding the manipulation of isoprenoid precursors pathway, it has been attempted to increase the levels of IPP required for the formation of geranylgeranyl diphosphate, the first step in the carotenoid pathway. This has been achieved by intervening in the mevalonic (MVA) pathway expressing constitutively the gene 3-hydroxymethylglutaryl CoA (*hmgr-1*) from *Arabidopsis thaliana* and in the methylerythritol-4-phosphate (MEP) pathway using the gene 1-deoxy-ᴅ-xylulose-5-phosphate synthase (*dxs*) from *Escherichia coli* [90].

The use of *hmgr-1* resulted in elevated levels of phytosterols (up to 2.4-fold), but IPP-derived isoprenoids in the plastid were unaltered. Transgenic lines containing the bacterial *dxs* targeted to the plastid with the tomato *dxs* transit sequence resulted in an increased carotenoid content (1.6-fold), with phytoene and β-carotene exhibiting the highest increases (2.4- and 2.2-fold, respectively). This result emphasizes the idea that IPP used for the carotenoid pathway is mostly obtained from the MEP pathway [24].

In a second approach, several works have been targeted to alter the enzymes that directly participate in the carotenoid biosynthesis pathway. Among these, phytoene synthase, phytoene desaturase, and lycopene cyclases have received most part of the attention.

Fray et al. [91] expressed constitutively a tomato phytoene synthase (PSY1) in tomato, but in this case, high expressers showed reduced height. It was proposed that dwarfism was caused as a consequence of overproduction of phytoene synthase which converts geranylgeranyl diphosphate into phytoene, thus diverting that intermediate away from the gibberellin and phytol biosynthetic pathways. This kind of results stressed the need to utilize promoters, restricting the overexpression or repression of genes to the fruits and during the ripening process.

Fraser et al. [92] also overexpressed the phytoene synthase (CRTR-B) gene, in this case from *Erwinia uredovora*, in tomato under the control of the tomato polygalacturonase promoter, resulting in 2.4-, 1.8-, and 2.2-fold higher contents of lycopene, β-carotene, and lutein, respectively, than the controls. In this case, avoiding constitutive expression, the transgenic plants showed a similar growth and development compared to the wild type.

Following with the next enzyme in the pathway, Romer et al. [93] introduced the gene *crtI* encoding a bacterial (*Erwinia uredovora*) phytoene desaturase, which converts phytoene to lycopene. Although levels of carotenoids were not increased in the transformed lines, β-carotene contents increased threefold without further effects on growth and development. This result was due to an upregulation of carotenoid genes, except phytoene synthase that was downregulated.

The next step in the pathway being modified following different approaches has been the one corresponding to lycopene cyclases. Their importance relies on the role of this step in the regulation of the whole pathway and its role in the partition of the main carotenoids lycopene and β-carotene. The gene silencing of CYC-B using a reverse construct of a fragment of the gene constitutively expressed resulted in flowers with orangish color typical of the *og* mutation with increased lycopene content at the expense of β-β-xanthophyll and deeper red color in fruits due to higher accumulation of lycopene at the expense of β-carotene [34].

Following the opposite approach, plants overexpressing lycopene β-cyclase (LCY-B) cDNA resulted in high contents of beta-carotene at the expense of lycopene, which was almost completely cyclized [94]. The plants did not show collateral effects, instead showed an increased productivity. Total carotenoid content was also increased in some progenies and beta-carotene content was twofold that of the beta mutant.

β-cyclases from other species have also been introgressed via genetic engineering in tomato. Rosati et al. [95] introduced the *A. thaliana* lycopene cyclase gene (*LcyB*) under the control of tomato phytoene desaturase (*psd*) promoter. Apel and Bock [96] introduced the lycopene beta cyclase genes from *Erwinia herbicola* and *Narcissus pseudonarcissus*. The first did not have much effect on β-carotene content, while the second increased the level of beta-carotene in fruits and almost increased total carotenoid content in 50 %, enhancing the flux through the pathway. In green leaves, it increased the levels of the beta branch xanthophylls. Guo et al. [97] constitutively expressed citrus beta cyclase gene (*Lycb-1*) and increased fourfold the content in β-carotene and 30 % total carotenoid content. The alpha branch was repressed until ripening stage, when high contents of α-carotene were obtained. The constitutive expression of this gene altered several different pathways including the synthesis of fatty acids, flavonoids, and phenylpropanoids, among others.

Following a new approach, Ma et al. [98] selectively repressed LCY-B and LCY-E enzymes by reducing mRNA following an RNAi approach. In lines with reduced LCY-E, β-carotene increased while lutein decreased and total carotenoids increased 1.7-fold. In LCY-B reduced RNA lines, β-carotene, lutein, and total carotenoid content decreased. In both lines, an increase of lycopene content up to 4.2-fold was observed.

The metabolic engineering of xanthophyll contents in tomato has also been tried. The overexpression of lycopene β-cyclase (LCY-B) and a *Capsicum* β-carotene hydroxylase (B-CHY) using the fruit-specific *pds* promoter resulted in increased contents of β-carotene (9.2–12.6-fold), β-cryptoxanthin, up to 11 μg/g, and zeaxanthin, up to 13 μg/g [99].

Allelic variants of genes participating in the biosynthesis pathway have also been identified following a TILLING strategy. For example, in the case of phytoene synthase (PSY-1) gene, two mutants with an early stop codon and an amino acid substitution resulted respectively in yellow fruits with similar phenotype to the *r* mutant and yellow fruits until 3 days post-breaker but eventually turning red [100]. These results contribute to the identification of strategies regulating enzyme activity in the fruits.

During recent years, the study of the biosynthetic pathway of carotenoids in tomato has not only been targeted to the study of cDNAs of the genes encoding the responsible enzymes, but also in the study of the corresponding promoters. Though this area of study is still underdeveloped, the tomato PDS promoter has been characterized [101], as well as *S. habrochaites* lycopene β-cyclase (CYC-B) promoter [102]. Other promoters from different species have also been tested in tomato. This would be the case of the zeaxanthin epoxidase (GlZEP) promoter from *Gentiana lutea* [103]. At the same time, an important progress has been made in the identification and characterization of fruit-specific promoters such as ethylene response genes *E8* and *E4m* polygalacturonase and lipoxygenase, mainly acting in the late-ripening stage or the *LA22CD07* and *LesAffx.6852.1.Sl* in green- and red-ripening fruits [104].

Although most part of the effort in the identification and cloning of genes and their genetic engineering has been placed in the carotenoid synthesis pathway, other alternatives have also been considered. As Dellapenna et al. [105] stated, one strategy to obtain more general increases in several metabolites could be to modulate regulatory genes whose products control flux through several biosynthetic pathways.

One of the strategies used in this sense has been to analyze the light-dependent regulation of carotenogenesis. Following this approach, Liu et al. [42] demonstrated that *hp-1* alleles present in the *high pigment 1* mutant represent mutations in a tomato UV-damaged DNA Binding Protein (DDB1) homolog. Trying to emulate this action, the authors proved that repression of LeCOP1LIKE expression resulted in plants with exaggerated photomorphogenesis, dark leaves, and elevated fruit carotenoid content. On the other hand, downregulated LeHY5 plants showed defects in light responses including reduced carotenoid accumulation. Long et al. [106] showed that *hp-1* and *hp-1/og*^{c} lines showed increased levels of carotenoids including phytoene, lycopene, β-carotene, and lutein as well as flavonoids in pericarp and skin tissues. The combination of *hp-1* and *og*^{c} as in the LA3771 mutant would show higher carotenoid contents; thus, it would appear that manipulation of environmental sensing mechanisms are the most effective means of creating high carotenoid/flavonoid fruit [106].

Also dealing with genes encoding components of the light signal-transduction machinery, Davaluri et al. [107] attempted to increase nutritional value in tomato suppressing photomorphogenesis regulatory gene *TDET1* using fruit-specific promoters combined with an RNA interference approach based on inverted repeat constructs. It had been proved previously that *TDET1* is the gene responsible for the phenotype of the mutant *hp2* [44]. This mutant is characterized by an excessive photoreaction involving high flavonoid and carotenoid content but with collateral effects such as causing reduced plant statures, bushiness, and dwarfing that have hindered its exploitation in commercial varieties. Precisely, the targeted suppression in fruits tried to avoid vegetative collateral effects. As DET1 transcripts were specifically degraded in the fruit, carotenoids, phenylpropanoids, and flavonoids content increased significantly (to similar or greater levels than with *hp2*). The increase in the content of two unrelated beneficial groups of plant antioxidants is of great interest in the development of new varieties with enhanced functional or nutritional quality, especially as the analysis of mutants altered in structural genes for carotenoid biosynthesis shows no significant alteration of phenolic or flavonoid contents [106]. Though in this case, minor reductions in fruit weight and Brix were observed, apparently, the collateral effects were much reduced from those observed in the natural mutant *hp-2*.

Giliberto et al. [108] also intervened in the control of photomorphogenesis overexpressing and silencing (virus mediated silencing) the tomato cryptochrome 2 (*CRY2*) gene. Overexpressing plants showed short hypocotyls and internodes, flowering delay, and axillary branches outgrowth but also overproduced chlorophylls and anthocyanins in leaves and flavonoids, carotenoids, and xanthophylls in fruits. Specifically, lycopene, β-carotene, and lutein increased 1.7- 1.3-, and 1.5-fold respectively. The increase in the carotenoid pathway compounds was accompanied by a decrease in the expression of lycopene β-cyclase genes. Virus-induced gene silencing caused minor internode elongation, though Potato virus X infection complicated the interpretation of results.

Other regulatory approaches consisted in the downregulation of abscisic acid (ABA) in tomato during ripening using an RNAi construct of the SINCED1 gene encoding 9-*cis*-epoxycarotenoid dioxygenase driven by the fruit-specific promoter *E8* [109, 110]. This approach caused a reduction in 20–50 % of ABA concentration during ripening. As a result, upstream compounds in the ABA synthesis pathway, such as lycopene and β-carotene, increased their accumulation. This strategy also resulted in an increase of ethylene production. This result may be related to the effects of the *high pigment-3* mutant. In this case, the mutation present in the *hp-3* allele results in a defective zeaxanthin epoxidase that results in ABA deficiency that would cause an enlargement of plastids (twofold larger), probably due to increased plastid division [40].

In addition to the mentioned approaches, some unexpected increase in carotenoid content has been obtained when trying to modify other biosynthetic routes. This was the case of the expression of the yeast *S*-adenosylmethionine decarboxylase gene (ySAMdc;*Spe2*) with the *E8* promoter in tomato plants targeted to increase the levels of the polyamines spermine and spermidine in tomato fruit during ripening [111]. In this case, the increased level of these polyamines was accompanied by higher lycopene concentrations (two- to threefold), prolonged vine life, and enhanced precipitated weight ration and serum viscosity. Recently, the constitutive overexpression of the apple spermidine synthase cDNA in tomato, a key enzyme of the polyamine biosynthesis pathway, also resulted in increased carotenoid concentration, especially in lycopene (1.3–2.2-fold). In this case, the genes encoding the enzymes upstream lycopene formation DXS, PSY, PDS were upregulated while the genes encoding the enzymes downstream LCY-B and LCY-E were downregulated in tomato ripe fruits compared to the wild type. Therefore, authors suggested that a high level of polyamines regulates the steady-state level of transcription of genes responsible for the lycopene metabolic pathway [112].

As it has been stated, genetic engineering can offer an alternative to improve carotenoid content or its profile in tomato. In some cases, this has been achieved with minor or null side effects in the vegetative development. Nonetheless, it should be considered that most part of the evaluations have been made in glasshouse experimental conditions. In fact, in several of the mentioned studies, the results on production of the controls are far from the yields typical of commercial growing conditions. Therefore, it is still necessary to check if these strategies really improve carotenoid content without negative effects of the commercial agronomical performance of the lines.

Additionally, despite the progress being made improving the functional value using genetic engineering approaches, it should be considered that public skepticism on transgenic crops makes rather unlikely that the developed materials could have a commercial use in the near future [3].

4.3 Limitations and Drawbacks Associated with Analysis and Environment

The quantification of lycopene and β-carotene in tomato fruits was initially based on spectrophotometric methods accounting for the relationship between absorbance at certain wavelengths and concentrations of these compounds in organic solvents [85, 113]. Despite the low precision of these non-separation methods compared to chromatography, they have been widely used considering their simplicity and their low operational and capital costs. But at the moment, high-performance liquid chromatography (HPLC) is the most frequently technique used for the analysis of carotenoids in fruit and vegetable extracts [114, 115]. Reversed-phase columns packed with several C_{18} stationary phases still are the preferred chromatographic supports for measuring total carotenoids [116, 117]. However, these chromatographic methods have several shortcomings including the time-consuming conditioning of the columns, the large consumption of solvents, and the high price of the column. In the last years, capillary electrochromatography (CEC) has received much attention as emerging separation technique [118, 119] with high selectivity (as in HPLC) and high efficiency. Nowadays, there are CEC methods available for fast determination of prominent carotenoids in tomato fruits by capillary electrochromatography using methacrylate-ester-based monolithic columns [120] that can be useful in germplasm evaluation.

Nevertheless, during selection processes in breeding programs, it is complex to use these analytical methods, as the sample homogenization, carotenoid extraction

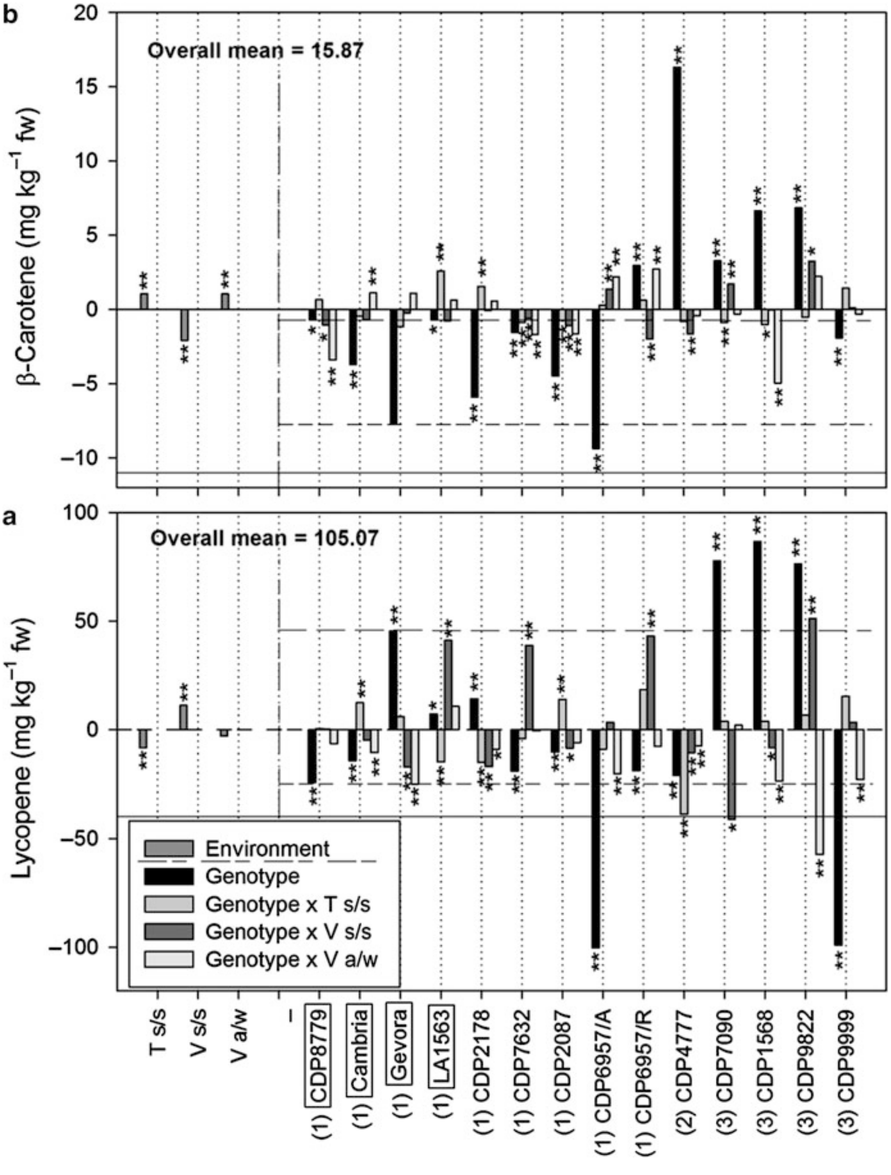


Fig. 92.3 Predicted genotypic, environmental, and interaction effects for lycopene and β-carotene content of the tomato accessions evaluated in three growing environments (Adapted from Journal of the Science of Food and Agricultre, Vol. 91, Roselló S, Adalid AM, Cebolla-Cornejo J, Nuez F, Evaluation of the genotype, environment and their interaction on carotenoid and ascorbic acid accumulation in tomato germplasm, pp. 1014–1021, Copyright (2011) with kind permission of John Wiley and Sons). The growing environments of trial in Spain were: T s/s = Turis, open field, spring-summer cycle; V s/s = Valencia, glasshouse, spring-summer cycle; V a/w = Valencia, glasshouse, autumn-winter cycle. Estimated value significantly different from zero (*t*-test) at P = 0.1, 0.05, and 0.01 level respectively. Controls in X axis are inside a *box*. In *parentheses*:

with organic solvents, and sample preparation, which are common for any of the mentioned analysis, are time consuming. Thus, when hundreds of samples are to be analyzed in a very short time to select the interesting individuals, these approaches offer low efficiency, and the analysis becomes a limiting factor. Consequently, indirect determination using nondestructive methods has been pursued.

D'souza [121] and Hyman et al. [122] proposed colorimetric determination of whole fruits and the use of simple functions such as $(a^*/b^*)^2$ or $(a^*/b^*)^{2.5}$ to determine the contents of lycopene and β-carotene, obtaining determination coefficients of 0.75–0.90 and 0.55, respectively. Nonetheless, the goodness of fit of indirect methods using colorimetric measurements is highly dependent on the genotypes being evaluated. With this methodology, only the external color of the fruit is measured, but as commented in the previous sections, carotenoid content is not limited to the external area of the fruit, and its distribution may vary between genotypes. Therefore, better results are obtained when a specific function is obtained for the specific materials under use [123]. This approach requires the development of complex specific calibration models before analyzing the materials in field. Again this limits the practicability of the methodology.

During the last years, nondestructive methodologies based on VIS-NIR spectroscopy have being developed [124] and they seem quite promising for breeding programs. These methods offer a possibly more reliable prediction, as the measure is not restricted to the surface of the fruit as in the colorimetric approach. In fact, these methods involve prediction errors averaging 3 mg kg^{-1}, but they can be used in nondestructive fast determinations in the field, thus offering a rough but rapid screening of the materials. More precise but time-consuming HPLC or CEC methods may be used in later phases to check the advances achieved and detect minor differences between materials.

Another important matter to consider in carotenoid breeding programs with tomatoes is the influence of the environment on the expression of the character. The content of carotenoids in tomato fruits depends not only on the genotypic potential, but also on the environmental effect and on the interaction between genotype and environment. This environmental influence determines the character heritability and highly influences the success of selections in breeding programs.

It has been reported that the lycopene accumulation depends on temperature and seems to take place at a range of average day temperature between 12 °C and 32 °C [125] or 35 °C [126], with the optimal conditions around 22–26 °C [127]. For β-carotene accumulation, the range of average day temperature is wider than for lycopene. Its biosynthesis is poorly affected by temperatures lower than 12 °C [128] and with temperatures higher than 35 °C; when the lycopene accumulation is

Fig. 92.3 (continued) 1 = *Solanum lycopersicum*; 2 = *S. lycopersicum* (former var. c*erasiforme*); 3 = *S. pimpinellifolium*. Scaling in Y axis is overall mean centered. Reference lines: genotypic *upper* control (*long dashed line*), genotypic *lower* control (*short dashed line*), reported phenotypic average content (*continuous line*). The reported average content considered is: 65, 3.9, and 200 mg kg^{-1} for lycopene [130] and β-carotene [49] respectively inhibited, the conversion of lycopene into β-carotene is stimulated [126]. Nevertheless, the optimal temperature for β-carotene accumulation seems to be around 30 °C [126]. At favorable temperatures, the lycopene and β-carotene biosynthesis increases with the sunlight intensity, probably [127, 129] due to increases in photosynthetic rates. In the case of lycopene, when a harmful direct radiation level occurs (650 Wm^{-2} for 1.5–4 h), its biosynthesis is inhibited.

It is more difficult to assess the effect of genotype-environment interaction (G × E). But this interaction will condition the stability of the expression of the genotypic potential for the trait and, thus, the potential environments recommended for the future cultivar. Therefore, the genetic merit of the accessions tested must be evaluated considering both genotype main effect and G × E interaction, being compared with the genetic merit of the controls for reference (Fig. 92.3). One of the decisions for the breeder would be if it is preferable to develop "high functional value" cultivars with high environmental stability or if it is preferable to develop specific cultivars for specific target environments. This kind of studies requiring multi-environmental evaluation is usually performed in the last phases of the breeding program as they are expensive and time consuming. Nonetheless, it is important to perform them in the early phases of identification of promising sources of variation before deciding to start even more expensive breeding programs to introgress the trait.

In general, the high genetic component responsible for the accumulation of lycopene and β-carotene makes possible the selection of elite genotypes with high content of both carotenoids in tomato breeding programs. The high ratio of genotypic to environmental variance decomposition seems to indicate that high accumulation cultivars with wide adaptation might be successful despite the important environmental effects on carotenoid biosynthesis. Although there is a high genotypic correlation between the carotenoids studied, to perform a joint selection for both carotenoids, it is mandatory to conduct multi-environment trials due to the existence of considerably high negative environmental correlations that may hinder joint selection in a single environment [68].

5 Conclusion

There is an increasing demand in the development of functional food targeted to prevent certain degenerative diseases. Under these premises, antioxidant compounds found in fruits and vegetables have been linked to reduced risks of cancer and cardiovascular diseases. Consequently, seed industry has stressed the need to develop new varieties with high concentrations of these compounds. Among the terpenes present in tomato fruits, carotenoids represent an interesting antioxidant activity. Consequently, numerous efforts have been targeted to improve the content of total carotenoids or of the carotenoid profile of tomato.

Breeders trying to fulfill this objective have encountered two main limitations in their way. The first one relates to the difficulty of determining the carotenoid content in large number of samples typical of breeding programs. Very precise techniques are available, but they require a rather tedious process of carotenoid extraction and are rather expensive. Alternatives for indirect determination via colorimetric measures or VIS-NIR radiation require continuous calibrations which are specific for different materials, and cannot be considered fully reliable for breeding programs. The second limitation arises from the important impact of environment on the accumulation of carotenoids. Consequently, it becomes really difficult to decompose the actual carotenoid content into its genotypic, environmental, and interaction components. In plant breeding, this fact is especially important, as selecting one individual by its measured carotenoid content would not imply that it really has a true genotypic potential.

Despite these important limitations, considerable advances have been made in the selection of high carotenoid lines. In conventional breeding, an important part of this success relies on the use of color mutants identified in the cultivated species, as well as in related wild species. These mutants have altered either enzymes in the carotenoid biosynthetic pathway or in related regulatory systems and result either in increased total carotenoid content or in increases either in lycopene or β-carotene at the expense of the other. Although mutations such as the *high pigments* have been introgressed in commercial varieties offering increased lycopene content, a considerable decrease in yield appears as a side effect.

Advances in the genetic engineering have been obtained following the advances in the knowledge of the biosynthetic pathway and in the genetic analysis of the color mutants. In fact, an important progress has been made using alleles responsible for color mutations combined with promoters specific for fruit tissues and ripening stages. Therefore, side effects arising from the expression of those alleles in vegetative tissues can be prevented. Most part of the results in the area of genetic engineering have been obtained in controlled conditions with very limited productions, and it is still necessary to check if the same results would apply to commercial conditions.

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Volatile Oils from Marine Macroalgae

93

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Contents

1 Introduction 2886
2 Rhodophyta 2886
2.1 Bangiales 2886
2.2 Nemaliales 2887
2.3 Ahnfeltiales 2887
2.4 Gelidiales 2887
2.5 Gracilariales 2888
2.6 Bonnemaisoniales 2888
2.7 Corallinales 2889
2.8 Gigartinales 2890
2.9 Rhodymeniales 2891
2.10 Plocamiales 2891
2.11 Ceramiales 2891
3 Ochrophyta 2893
3.1 Desmarestiales 2893
3.2 Dictyotales 2894

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3.3 Ectocarpales 2896
3.4 Fucales 2896
3.5 Laminariales 2898
3.6 Scytosiphonales 2900
3.7 Sphacelariales 2900
3.8 Sporochnales 2901
4 Chlorophyta 2901
4.1 Ulotrichales 2901
4.2 Ulvales 2901
4.3 Cladophorales 2903
4.4 Bryopsidales 2903
4.5 Codiales 2903
4.6 Caulerpales 2904
5 Discussion 2904
5.1 Pharmacological Activities of Volatile Oils 2906
6 Conclusion 2907
References 2907

Abstract

Volatile oils of algae contain a variety of compounds. The main group, terpenes, contains acyclic: linalool, geraniol, citronellol; monocyclic: limonene, 1-8-cineol, *p*-cymene; bicyclic: *α*- and *β*-pinene, cadinene, aromatic eugenol, and isoeugenol. The others are benzaldehyde, phenol, *p*-cresol, various acids, alcohols, aldehydes, amines, ketones, sulfur containing and halogenated compounds. When volatile oil components of algae and terrestrial plants are compared, the algae were found to be relatively poor. Importantly, halogenated compounds occur only in algae, but not in terrestrial plants. In this chapter, we present briefly but comprehensively the occurrence of various volatile compounds in different classes of algae.

Abbreviations

A	Amines
AC	Acids
ACE	Acetic acid
ACR	Acrylic acid
AD	Amides
AL	Aldehydes
ALC	Alcohols
AM	Amine
ATL	Acetaldehyde
BC	Bromocresol
BE	Benzaldehyde
BH	Bromo-4-hydroxybenzaldehyde
BP	Bromophenol

BU	Butyric acid
BZ	Benzoic acid
BZT	Benzothiazole
CA	Caproic acid
CAP	Caprylic acid
CAR	Carvone
CI	Cineol
CIT	Citronellal
CR	Cresol
CTN	Citronellol
CY	*p*-Cymene
DBA	Dibromoanisole
DBC	Dibromocresol
DBH	Dibromo-4-hydroxybenzaldehyde
DBP	Dibromophenol
DCM	Dichloromethane
DMA	Dimethylamine
DMS	Dimethyl disulfide
EA	Ethylamine
EU	Eugenol
FO	Formic acid
FU	Furfural
FUR	Furfuryl alcohol
GE	Geraniol
HALC	Halogenated alcohol
HC	Halogenated compounds
HDC	Hydrocarbon
IAA	Isoamylamine
IBA	Isobutylamine
IEU	Isoeugenol
IVA	Isovaleric acid
KE	Ketones
LI	Linalool
LIM	Limonene
MA	Methylamine
MFU	Methylfurfural
MMPA	2-Methyl-mercapto-propylamine
PA	Propylamine
PEA	2-Phenylethylamine
PH	Phenols
PI	Pinene
PR	Propionic acid
Pro	Propional
S	Sulfur compounds

TA	Terpenyl acetate
TBA	Tribromoanisole
TBP	Tribromophenol
TE	Terpinolene
TERP	Terpenes
TMA	Trimethylamine
TOL	Terpineol
VA	Valeraldehyde

1 Introduction

Volatile oils comprise all the substances which pass through steam distillation process. The origin of volatile oil is traditional of German work Aetherischen Oele in the first time described by Gildemeister and Hoffmann in 1899 [1]. It is the Quinta essentia (quintessence) which represents the efficient part of every drug. The current name "essential" oil recalls the Paracelsian concept [2]. The quintessential oil originates from the Aristotelian idea that matter is composed of four elements, namely, fire, air, earth, and water. The fifth element, or quintessence, was then considered to be spirit or life force [3]. The term "essential oils" is also used for volatile oils.

History: In the past, volatile oils of terrestrial plants were used in Chinese [4], Assyrian [5], Sumerian [6], Babylonian [7], and Egyptian civilizations [7–9]. Volatile oil was first isolated from marine algae, Fucus, by Heilbron et al. [10] and later in a series of research by Katayama in 1951–1961 [11].

Volatile oil is a mixture of numerous components such as terpenes, amines, sulfur, halogenated compounds, nonterpenic hydrocarbons, and others (acids, alcohols, aldehydes, phenols, etc.) [12].

Some exogenous volatile compounds were found in marine algae, whereas endogenous aliphatic hydrocarbons are originally found in algae [13].

In this chapter, volatile oils have been listed in systematic classification and the compounds in alphabetical order as acids, alcohols, aldehydes, amines, halogenated compounds, hydrocarbons, phenols, sulfurs, terpenoids.

2 Rhodophyta

2.1 Bangiales

2.1.1 Bangiaceae

Porphyra columbina

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Porphyra tenera (Pyropia tenera)

Volatile constituents (dry weight): 0.053 % [11].

AC: FO; ACE; PR; BU; IVA; *n*-CA; CAP [11]; nonanoic; decanoic [15].

AL: PRO; FU; MFU; *n*-VA; BE [11]; ATL; IVA; isobutylaldehyde; tiglic aldehyde; 2-methyl-2-pentanal [16]; (2*E*,4*E*)-octadienal; (2*E*,6*Z*)-nonadienal; (2*E*,4*Z*)-decadienal; (2*E*,4*E*)-decadienal; tridecanal; tetradecanal; pentadecanal; (7Z,10Z)-hexadecadienal; (7Z)-hexadecenal; (8Z,11Z)-heptadecadienal; (8Z)-heptadecenal [15].

ALC: Benzyl alcohol [15].

KE: α-Ionone; *β*-Ionone; 6,10,14-trimethylpentadecan-2-one [15].

Lactone: Dihydroactinidiolide [15].

PH: *p*-Cr [11].

S: Methanethiol [11]; BZT [15].

TERP: LI; GE; 1,8-CI; *p*-CY [11, 17]; α-TOL; α-Cadinol; Cubenol [15]; TE; LIM; α-PI; CAR [17].

2.2 Nemaliales

2.2.1 Galaxauraceae

Galaxaura marginata

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Galaxaura obtusata

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

2.3 Ahnfeltiales

2.3.1 Ahnfeltiaceae

Ahnfeltia plicata

A: MA; TMA [18].

2.4 Gelidiales

2.4.1 Gelidiaceae

***Gelidium* sp.**

Volatile constituents (dry weight): 0.130 % [11].

TERP: LI; GE; 1,8-CI [19].

PH: *p*-CR [19].

Gelidium amansii

Volatile constituents (dry weight): 0.130 % [11].

Gelidium crinale
A: PEA [20–22].

2.4.2 Pterocladiaceae

Pterocladiella capillacea
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Pterocladia capillacea (Gelidium capillaceum)
Volatile constituents (dry weight): 0.28 % [23].
AC: Isobutyric acid [23].
ALC: Nonanol [23, 24]; 3,4-dihydroxybenzyl alcohol; 4-hydroxybenzyl alcohol [25].
TERP: GE; GE formate; GE acetate; TE; CIT; 1,8-CI; α- and β-PI; EU [23].

Pterocladia lucida
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

2.5 Gracilariales

2.5.1 Gracilariaceae

***Gracilaria* sp.**
Volatile constituents (dry weight): 0.094 % [11].

Gracilaria bursa-pastoris
A: PEA [22].

Gracilaria edulis
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Gracilaria secundata
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

2.6 Bonnemaisoniales

2.6.1 Bonnemaisoniaceae

Asparagopsis armata
HC: $CHBr_2Cl$; $CHBr_3$; CBr_4; $CHBr_2I$; CH_2BrCHO; $CHBr_2CHO$; CH_3I; CH_2ClI; $CHCl_3$; CCl_4; $CHBrCl_2$ [26]; eight haloacetones [27–31].

Asparagopsis taxiformis
HC: $CHBr_3$; $CHClBr_2$; CHClBrI; $CHBr_2I$; $CHBrI_2$; CHI_3; CBr_4; four tetrahalopropenes; four polyhalobut-3-en-2-ones, MeCOCH2R (R = Br, I); Br_2C:CHCHO [28]; two haloacetones; eight halobutenoles [27–31] carbonyldiiodide (COI_2);

ICH_2CH_2OH; $BrCH_2CH_2I$; twenty halogenated isopropanols; two halogenatad 2-acetoxy-propanes; $CHBrOCHCHBr_2$; four 1,1,3,3,-tetrahalopropenes; dibromoacrolein; four haloacetamides; eight polyhaloacetone derivatives; seven haloacetic acid derivatives; five haloacrylic acid derivatives [27–30]; 1-octen-3-ones [31].

Bonnemaisonia nootkana

HC: trans-1,3,3,-Tribromo-1-hepteneoxide [32].
ALC: Tetrabromo-2-heptanol; tetrabromo-2-nonanol [32].
KE: 1,1,3,3-Tetrabromo-2-nonanone [32].
AC: Z-3-Bromo-2-heptenoic acid; *Z*-3-bromo-2-nonenoic acid; 3,3-dibromo-*n*-butylacrylic acid; 3,3-dibromo-*n*-hexylacrylic acid [32].

Bonnemaisonia asparagopsis

KE: 5 Chlorinated and brominated 1-octene-3-ones: 1-bromo-2-chloro (1-octene-3-ones); 1-bromo-2,3-dichloro(1-octene-3-ones); 1,2-dibromo-3-chloro(1-octene-3-ones); 1,2,3-tribromo(1-octene-3-ones); 2,3-dibromo-1-chloro (1-octene-3-ones) [33].

Bonnemaisonia hamifera

KE: 1,3-Dibromo-2-heptanone; 1,3,3-tribromo-2-heptanone; 3,3-dibromo-1-iodo-2-heptanone [34].

Delisea pulchra

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Falkenbergia rufolanosa

HC: $CHBr_3$ [27].

2.7 Corallinales

2.7.1 Corallinaceae

Amphiroa anceps

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Cheilosporum sagittatum

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Corallina berteri

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Corallina officinalis

A: MA; TMA; IAA [35].
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

***Corallina rubens* (*Jania rubens*)**
TERP: GE; TE; 1,8-CI [36].

Haliptilon roseum
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

2.8 Gigartinales

2.8.1 Areschougiaceae

Callophycus tridentifer
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

2.8.2 Cystocloniaceae

Cystoclonium purpureum
A: MA; TMA; EA; PA; IBA; IAA; PEA; MMPA [35].

2.8.3 Dumontiaceae

***Dumontia incrassata* (*Dumontia contorta*)**
A: MA; TMA; EA; PA; IBA; IAA; PEA; MMPA [35].

2.8.4 Gigartinaceae

Chondrus crispus
A: MA; TMA; IAA [35].

Gigartina stellata
HC: Polybromomethanes [37].

2.8.5 Phyllophoraceae

***Phyllophora nervosa* (*Phyllophora crispa*)**
Volatile constituents (dry weight): 0.04 % [38].
A: PEA [21].
TERP: GE; 1,8-CI [38].

2.8.6 Polyidaceae

Polyides rotundus
A: MA; DMA; TMA; EA; PA; IBA; IAA; PEA; MMPA [35].

2.8.7 Solieriaceae

Solieria robusta
KE: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

2.8.8 Sphaerococcaceae

Sphaerococcus coronopifolius
Volatile constituents (dry weight): 0.21 % [39].

2.9 Rhodymeniales

2.9.1 Rhodymeniaceae

Lomentaria catenata

KE: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

2.9.2 Halymeniaceae

Halymenia floresii

A: PEA [21].

KE: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

2.10 Plocamiales

2.10.1 Plocamiaceae

Plocamium angustatum

KE: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

***Plocamium vulgare* (*Plocamium cartilagineum*)**

A: MA; TMA; IAA [35].

2.11 Ceramiales

2.11.1 Ceramiaceae

Ceramium rubrum

A: MA; TMA; EA; PA; IBA; IAA; PEA; MMPA [35].

2.11.2 Delesseriaceae

Delesseria sanguinea

A: MA; TMA; EA; IBA; IAA; PEA; MMPA [35].

2.11.3 Rhodomelaceae

Bostrychia calliptera

AL: (*E*)-2-Octenal; nonanal; (*E,Z*)-2,6-nonadienal; (*E*)-2-nonenal; decanal; (*E*)-2-decenal; (*E,Z*)-2,4-decadienal; undecanal; (*E,E*)-2,4-decadienal; dodecanal; tetradecanal; pentadecanal; hexadecanal [40].

ALC: 1-Octen-3-ol; (*E*)-2-octenol; 3,4-dimethylcyclohexanol; 1-nonanol; 1-decanol; undecanol; tridecanol; tetradecanol; pentadecanol; Hexadecanol [40].

HDC: Tetradecene; pentadecene; hexadecane; 1-heptadecene; heptadecane [40].

KE: 2-Decanone; α-ionone; β-ionone; 6,10,14-trimethyl-2-pentadecanone [40].

PH: 2,4-Di-*tert*-butylphenol [40].

TERP: 2,3-Dihydrocitral; β-cyclocitral; β-cyclohomocitral; (*E*)-geranylacetone [40].

Bostrychia radicans

AC: Nonanoic [40].

AL: BE; (*E*)-2-Octenal; nonanal; (*E,Z*)-2,6-nonadienal; (*E*)-2-nonenal; decanal; (*E*)-2-decenal; (*E,Z*)-2,4-decadienal; undecanal; (*E,E*)-2,4-decadienal; dodecanal; tridecanal; tetradecanal; pentadecanal [40].

ALC: 1-Octen-3-ol; (*E*)-2-octenol; 1-octanol; 3,4-dimethylcyclohexanol; 1-nonanol; tridecanol; tetradecanol; pentadecanol; hexadecanol [40].

HDC: 1,3,5-Octatriene; tetradecene; tetradecane; pentadecene; pentadecane; hexadecane; 8-heptadecene; 1-heptadecene; heptadecane [40].

KE: 2-Decanone; α-ionone; β-Ionone; 6,10,14-trimethyl-2-pentadecanone [40].

TERP: 2,3-Dihydrocitral; β-cyclocitral; β-cyclohomocitral; (*E*)-geranylacetone [40].

Bostrychia tenella

AL: BE; (*E*)-2-octenal; nonanal; (*E*)-2-nonenal; decanal; (*E*)-2-decenal; (*E,Z*)-2,4-decadienal; undecanal; (*E,E*)-2,4-decadienal; dodecanal; tridecanal; tetradecanal; pentadecanal; hexadecanal [40].

ALC: 1-Octen-3-ol; (*E*)-2Octenol; 3,4-dimethylcyclohexanol; undecanol; dodecanol; tridecanol; tetradecanol; pentadecanol; hexadecanol [40].

KE: 2-Decanone; α-ionone; β-ionone; 6,10,14-trimethyl-2-pentadecanone [40].

TERP: β-Cyclocitral; β-cyclohomocitral; (*E*)-geranylacetone [40].

HDC: Tridecene; tetradecene; tetradecane; pentadecene; pentadecane; hexadecane; 8-heptadecene; 1-heptadecene; heptadecane; Octadecane [40].

PH: 2,4-Di-*tert*-butylphenol [40].

Chondria crassicaulis

Volatile constituents (dry weight): 0.12 % [39].

Chondria succulenta

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Digenea simplex

Volatile constituents (dry weight): 0.008 % [11].

A: FO; ACE; PR; BU; IVA; *n*-CA; CAP [11].

AL: FUR; MFU; *n*-VA; BE [11].

PH: *p*-CR [11].

S: Methanethiol [11].

TERP: LI; GE; 1,8-CI; *p*-CY [11].

Halopithys incurva

Volatile constituents (dry weight): 0.05 % [41].

TERP: p-CY; EU; IEU; α- and β-PI [41].

Laurencia caespitosa (*Osmundea hybrida*)

TERP: 6-Hydroxycaespitol; caespitane [42].

ALC: Caespitol, Isocaespitol [42].

Laurencia dendroidea

TERP: (1*R**,2*S**,3*R**,5*S**,8*S**,9*R**)-2,3,5,9-tetramethyltricyclo[6.3.0.0^{1,5}] undecan-2-ol; (1*S**,2*S**,3*S**,5*S**,8*S**,9*S**)-2,3,5,9-tetramethyltricyclo-[6.3.0.0^{1,5}] undecan-2-ol [43].

Laurencia nipponica

Volatile constituents (dry weight): 0.82 % [39].

Laurencia glandulifera

Volatile constituents (dry weight): 0.075 % [44].

TERP: Cadelene; azulene [44].

Polysiphonia lanosa

AC: ACR [45].

S: DMS [45].

Polysiphonia morrowii

A: PEA [22].

Polysiphonia tripinnata

A: PEA [22].

***Polysiphonia sphaerocarpa* (*Neosiphonia sphaerocarpa*)**

ALC: CR [46].

HC: $CHBr_3$; CH_3Cl; CH_3Br; CH_3I [47]; 2,4-DBA; 2,4,6-TBA; 3-BH; 3-BC; 3,5-DBC [46].

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [45].

AL: 3,5-DBH; brominated anisole; 3,5-dibromo-4-hydroxybenzaldehyde; brominated 3,4-dihydroxybenzaldehyde [46].

ALC: 3,4-Dihydroxybenzyl; 4-hydroxybenzyl; lanosol [46].

***Polysiphonia urceolata* (*Polysiphonia stricta*)**

A: MA; TMA; EA; PA; IBA; IAA; PEA; MMPA [35].

Rhodomela confervoides

A: MA; TMA; EA; IBA; IAA [35].

3 Ochrophyta

3.1 Desmarestiales

3.1.1 Desmarestiaceae

Desmarestia aculeata

A: MA; DMA; TMA; EA; PA; IBA; IAA; PEA; MMPA [35].

HC: CH_3I; CH_2ClI; $CH_3CH_2CH_2I$; CH_3CHICH_3; CH_2I_2; $CHBrCl_2$; $CHBr_2Cl$; CH_2Br_2; $CHBr_3$ [48].

Desmarestia anceps

HC: CH_3Br; CH_2Br_2; $CHBr_2Cl$; $CHBrCl_2$, CH_2I_2; CH_2ClI; $CH_3CH_2Br_2$ [49].

Desmarestia menziesii

HC: CH_3Br; CH_2Br_2; $CHBr_2Cl$; $CHBrCl_2$; CH_2I_2; CH_2ClI; $CH_3CH_2Br_2$ [49].

Desmarestia viridis

A: MA; DMA; TMA; IBA; IAA; PEA; MMPA [35].

3.2 Dictyotales

3.2.1 Dictyotaceae

Dictyopteris australis

TERP: Dictyopterene A [49–51]; dictyopterene B; dictyopterene C′; dictyopterene D′ [49].

Dictyopteris divaricata

Volatile constituents (dry weight): 3.28 % [39].

HDC: Volatile odoriferous hydrocarbons C_{11} were found as cyclic group: Dictyopterene A′, B, C′, D′; acyclic undecapolyenes as three 1,3,5-undecatrienes, four 2,4,6-undecatrienes, two 1,3,5,8-undecatetraenes.

TERP: Cadiene; 1-cadinol [52]; *β*-elemene; germacrene; cubenol [53].

Dictyopteris latiscula

TERP: Dictyopterene B; dictyopterene C′; dictyopterene D′ [54].

***Dictyopteris membranacea* (*Dictyopteris polypodioides*)**

AC: Dodecanoic; tetradecanoic; hexadecanoic [55].

AL: 2-Heptenal [55].

HDC: 3-Butyl-4-vinylcyclopentene; 3-(1-hexenyl)cyclopentene; 6-(1-butenyl)-cyclohepta-1,4-diene; 6-butylcyclo-1,4-heptadiene; 1,3,5-undecatriene; 2,4,6-undecatriene; heptadecane; 5-octadecene; eicosa-5,8,11,14-tetramethylenoate; eicosa-5,8,11,14,17-pentamethylenoate [55].

KE: 1-Undecen-3-one; 3-undecanone; 1,4-undecadien-3-one; 3-hexyl-4,5-dithiacycloheptanone [55].

S: 3-Oxo-un-4-decenyl [55].

TERP: Dictyoprolene [55].

Dictyopteris plagiogramma

TERP: Dictyopterene A [49–51]; dictyopterene B; dictyopterene C′; dictyopterene D′ [49].

Dictyopteris prolifera

AL: Dictyoprolenol [54].

HDC: *cis*-3-Butyl-4-vinylcyclopentene; *trans*-1-[(1*Z*)-hexenyl]-2-vinylcyclopropane, 4-[(1*E*)-hexenyl]-cyclopentene, 6-[(1*E*)-butenyl]-cyclohepta-1,4-diene [53].

TERP: Dictyopterene A [51]; dictyopterene B; dictyopterene C′; dictyopterene D′ [54]; cubenol [53]; neodictyoprolenol [54].

Dictyopteris undulata

HDC: 4-[(1*E*)-Hexenyl]-cyclopentene; 6-[(1*E*)-butenyl]-cyclohepta-1,4-diene; *cis*-3-butyl-4-vinylcyclopentene; *trans*-1-[(1*Z*)-hexenyl]-2-vinylcyclopropane [53].

TERP: Dictyopterene A [51]; dictyopterene B; C′; D′; neodictyoprolenol; dictyoprolenol; neodictyoprolenol [54].

Dictyopteris zonarioides

TERP: Cadinene [56].

Dictyota dichotoma

Volatile constituents (dry weight): 0.39 % [39].

TERP: Dictyol C; dictyol D [57].

Dictyota dentata (Dictyota mertensii)

Terp: Dictyol C; dictyol H; pachydictyol A [58].

Dictyota dichotoma var. implexa (*Dictyota implexa*)

TERP: Piperitone [59].

Dictyota cervicornis (Canistrocarpus cervicornis)

TERP: Dolastane [60, 61].

Dictyota divaricata (*Dictyota implexa*)

Volatile constituents (dry weight): 3.28 % [39].

TERP: Dictyopterene A [51]; cadinene [53].

Homoeostrichus sinclairii

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Lobophora variegata

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Taonia atomaria

TERP: 4-Cadainene; Cadinane-4; (-)-germacrene D; (-)-cubenol; 4-epi-cubebol [60].

HDC: 5-Diene [62].

3.3 Ectocarpales

3.3.1 Chordariaceae

***Sphaerotrichia japonica* (*Sphaerotrichia divaricata*)**

Volatile constituents (dry weight): 0.21 % [39].

3.4 Fucales

3.4.1 Cystoseiraceae

Cystoseira barbata

AD: Dimethylformamide [63].
HALC: $CH_2Cl.CHBrOH$ [63].
HC: $CH_2Cl.CH_2Br$; $CHCl_2.CH_2Cl$; $CHCl_2$. $CHCl_2$; hexachlorobutadiene [63].
TERP: 1,8-CI; TOL; GE; α-PI [64].

Cystoseira crinita

ALC: 2,3-Butanediol [65]
AC: Chloroacetic; Chloroacetic acid ethyl ester [65].
S: DMS [65].
TERP: Crinitol [57, 66–68]; geranylgeraniol [67].

Cystoseira stricta var. amentacea

ALC: Hexanol; Octanol [69].
TERP: Cubenol [69].

***Cystoseira stricta var. spicata* (*Cystoseira amentacea var. spicata*)**

HC: 2-[(2′*E*,6′*E*,14′*E*)-10′,11′-Dihydoxy-3′,7′,11′,15′-tetramethylhexadeca-2′,6′,14′-trien]-1,4-dihydroxy-6-methylbenzene [70].

KE: Quinone [70].

***Cystoseira trinodis* (*Sirophysalis trinodis*)**

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

3.4.2 Fucaceae

Ascophyllum nodosum

HC: Polybromomethanes [37].

Fucus distichus

HC: CH_3I; CH_2ClI; $CH_3CH_2CH_2I$; CH_3CHICH_3; CH_2I_2; $CHBrCl_2$; $CHBr_2Cl$; CH_2Br_2; $CHBr_3$ [46].

Fucus serratus

A: MA; TMA [35].

Fucus spiralis
A: MA; TMA [35].

Fucus vesiculosus
A: MA; TMA [34].
HC: CH_3CH_2I; $CHBr_3$; CH_2Br_2; CH_2Br_2Cl; CH_2I_2; C_2H_5I; C_3H_7I (1-, 2-); C_4H_9I [71]; polybromomethanes [37].

3.4.3 Hormosiraceae

Hormosira banksii
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

3.4.4 Sargassaceae

Cystophora intermedia
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

***Cystophora moniliformis* (Esper) Womersley & Nizamuddin**
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Halidrys siliquosa
A: MA; TMA [35].

***Hizikia fusiformis* (*Sargassum fusiforme*)**
Volatile constituents (dry weight): 0.053 % [11].

Hormophysa cuneiformis
HDC: 2-octene [54].

Sargassum sp.
Volatile constituents (dry weight): 0.062 % [11].
AC: FO; ACE; PR; BU; IVA; *n*-CA, CAP [11].
ALC: FUR [11].
AL: FU; MFU; *n*-VA; BE [11].
PH: p-CR [11].
S: Methanethiol [11].
TERP: 1,8-CI; *p*-CY; LI; GE [11].

Sargassum confusum
Volatile constituents (dry weight): 0.19 % [39].

***Sargassum erosum* (*Haraldiophyllum erosum*)**
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Sargassum horneri
Volatile constituents (dry weight): 0.04 % [39].

Sargassum globulariaefolium
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Sargassum lophocarpum
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Sargassum neurophorum
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Sargassum polyacanthum **(*Sargassum siliquastrum*)**
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Sargassum vulgare
Volatile constituents (dry weight): 0.037 % [72].
TERP: GE; 1,8-CI; α-PI [72].

3.4.5 Seirococcaceae

Phyllospora comosa
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

3.5 Laminariales

3.5.1 Alariaceae

Alaria crassifolia
AL: (*E*)-2-Nonenal; (*E,Z*)-2,6-nonadienal; (*Z,Z*)-3,6-nonadienal [73].
ALC: (*E*)-2-Nonenol; (*E,Z*)-2,6-nonadienol [73].
TERP: Cubenol [73].

Undaria pinnatifida
Volatile constituents (dry weight): 0.030 % [39], 0.054 [11].
AL: (*E*)-2-Nonenal; (*E,Z*)-2,6-nonadienal; (*Z,Z*)-3,6-nonadienal [73].
ALC: (*E*)-2-Nonenol; (*E,Z*)-2,6-nonadienol [73].
TERP: Cubenol [73].

3.5.2 Chordaceae

Chorda tomentosa **(*Halosiphon tomentosus*)**
A: MA [34].

3.5.3 Costariaceae

Costaria costata
AL: (*E*)-2-Nonenal; (*E, Z*)-2,6-nonadienal; (*Z, Z*)-3,6-nonadienal [73].
ALC: (*E*)-2-Nonenol; (*E, Z*)-2,6-nonadienol [73].
TERP: Cubenol [73].

3.5.4 Laminariaceae

***Kjellmaniella crassifolia* (*Saccharina sculpera*)**

AL: (*E*)-2-Nonenal; (*E*,*Z*)-2,6-nonadienal; (*Z*,*Z*)-3,6-nonadienal [73].
ALC: (*E*)-2-Nonenol; (*E*,*Z*)-2,6-nonadienol [73].
TERP: Cubenol [73].

Laminaria sp.

Volatile constituents (dry weight): 0.051 % [11].
AC: FO; ACE; PR; BU; IVA, *n*-CA, CAP [11, 72]; ACR [74].
AL: FU; α-MFU; *n*-VA [11]; BE [11, 75]; PRO; VA [75].
ALC: FUR [11].
AM: TMA [75].
HDC: Hencikosane [11].
KE: Acetone [75].
PH: *p*-CR [11].
S: Methylmercaptan [70]; methanethiol [11]; DMS [74].
TERP: α-LIM; 1,8-CI; *p*-CY; LI; GE; α-PI; EU [11, 75].

***Laminaria angustata* (*Saccharina angustata*)**

AC: FO; ACE; PR; BU; Valeric; CA; CAP [76, 77].
AL: (*E*)-2-Nonenal; (*E*,*Z*)-2,6-nonadienal; (*Z*,*Z*)-3,6-nonadienal [73]; VA; FU; α-MFU; BE [76, 77].
ALC: (*E*)-2-Nonenol; (*E*,*Z*)-2,6-nonadienol [73].
PH: *p*-CR [74].
TERP: Cubenol [73]; 1,8-CI; LI [77]; *d*-LIM [76]; GE; EU [77].

Laminaria digitata

A: MA; DMA; IBA; IAA [35].

Laminaria hyperborea

A: MA; DMA; IBA; IAA [35].

***Laminaria japonica* (*Saccharina japonica*)**

AC: FO; ACE; PR; BU; valeric; CA; CAP [74].
AL: (*E*)-2-nonenal; (*E*,*Z*)-2,6-nonadienal; (*Z*,*Z*)-3,6-nonadienal [73]; VA; FUR, α-MFU; BE [74].
ALC: (*E*)-2-Nonenol; (*E*,*Z*)-2,6-nonadienol [73].
PH: *p*-CR [73].
TERP: Cubenol [73]; 1,8-CI [76, 77]; *d*-LIM [76]; LI; GE [76, 77]; EU [77]; α-PI [76].

***Laminaria saccharina* (*Saccharina latissima*)**

A: MA; DMA; IBA; IAA [35].
HC: CH_3I; CH_2ClI; $CH_3CH_2CH_2I$; CH_3CHICH_3; CH_2I_2; $CHBrCl_2$; $CHBr_2Cl$; CH_2Br_2; $CHBr_3$ [46].

3.5.5 Lessoniaceae

Ecklonia cava

AL: (*E*)-2-nonenal; (*E,Z*)-2,6-nonadienal; (*Z,Z*)-3,6-nonadienal [73].
ALC: (*E*)-2-nonenol; (*E,Z*)-2,6-nonadienol [73].
TERP: Cubenol [73].

Ecklonia radiata

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

3.6 Scytosiphonales

3.6.1 Scytosiphonaceae

Analipus japonicus

TERP: Dictyopterene B [78]; dictyopterene D′ [78].

Colpomenia bullosa

TERP: Cubenol; *β*-elemene; *β*-ionene; dictyopterene A [78]; dictyopterene B (-)-hormosirene [78, 79]; dictyopterene C′; dictyopterene D′ [78].

Colpomenia sinuosa

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

***Endarachne binghamiae* (*Petalonia binghamiae*)**

HC: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

***Scytosiphon* sp.**

Volatile constituents (dry weight): 0.053 % [11].

***Scytosiphon lomentarius* (*Scytosiphon lomentaria*)**

Volatile constituents (dry weight): 0.02 % [39].
A: MA; TMA; IAA [34].
AL: Hexanal; dodecanol; tridecanal [77].
TERP: Cubenol; *β*-elemene; *β*-ionene; dictyopterene A [78]; dictyopterene B (-)-hormosirene [78, 79]; dictyopterene C′; dictyopterene D′ [78].

3.7 Sphacelariales

3.7.1 Sphacelariaceae

***Cladostephus spongiosus* (Hudson) C. Agardh**

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

***Halopteris paniculata* (*Stypocaulon paniculatum*)**

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Halopteris platycena

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

***Heterochordaria abietina* (*Analipus japonicus*)**

Volatile constituents (dry weight): 0.01 % [39].
TERP: Cubenol; β-elemene; dictyopterene A [78]; dictyopterene B [78]; dictyopterene D′ [77].
KE: β-Ionene [78].

3.8 Sporochnales

3.8.1 Sporochnaceae

Sporochnus comosus

PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

4 Chlorophyta

4.1 Ulotrichales

4.1.1 Ulothrichaceae

***Acrosiphonia centralis* (*Acrosiphonia arcta*)**

A: MA; TMA; IAA [35].

4.2 Ulvales

4.2.1 Ulvaceae

Enteromorpha sp.

Volatile constituents (dry weight): 0.021 % [11].
AC: FO; ACR; ACE; PR; BU; IVA; *n*-CA; CAP [11].
AL: FU; MFU; *n*-VA; BE [11, 80].
ALC: FUR [11].
PH: *p*-CR [11].
S: DMS [11].
TERP: LI; LIM; GE; TE; 1,8-CI; *p*-CY; α-PI, CAR [11, 80, 81].

***Enteromorpha clathrata* (*Ulva clathrata*)**

TERP: d-LI; LI; TE; 1,8-CI; α-PI; CAR [80, 81].

***Enteromorpha compressa* (*Ulva compressa*)**
A: MA, TMA, EA, IAA [35].

***Enteromorpha intestinalis* (*Ulva intestinalis*)**
S: DMS [82].

***Enteromorpha linza* (*Ulva linza*)**
Volatile constituents (dry weight): 0.02 % [35].
HC: Polybromomethanes [37].

Ulva lactuca
A: MA; TMA [34].
AC: 4-Hydroxybenzaldehyde, 4-hydroxybenzoic acid, 4-hydroxyphenyllactic acid, 4-hydroxyphenylacetic acid [45].
PH: 2,4,6-TBP; P [43]; 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].
2,4-DBA; 2,4,6-TBA [45]; polybromomethanes [37].

Ulva pertusa
Volatile constituents (dry weight): 0.019 % [11].
AC: FO; ACE; PR; BU; IVA; ACR; *n*-CA; CAP [11].
AL: FU, MFU, *n*-VA, BE [11], hexanal, (*E*)-2-octenal; (*E*)-2-nonenal; (*Z*,*E*)-2,6-nonadienal; (*E*,*E*)-2,4-decadienal; (*Z*,*Z*)-8,11-heptadecadienal; (*Z*,*Z*,*Z*)-8,11,14-heptadecatrienal; (*Z*)-8-heptadecenal; heptanal [83].
ALC: FUR [11]; 1-penten-3-ol; 2-penten-1-ol; (*Z*)-3-hexen-1-ol; 1-octen-3-ol [84].
HDC: (8*Z*)-Heptadecenal [84, 85]; (8*Z*,11*Z*)-heptadecadienal [84, 86]; (8*Z*,11*Z*,14*Z*)-heptadecatrienal [84–86]; pentadecanal [84]; hencikosane [11].
KE: *β*-Ionone [87].
PH: *p*-CR [11].
S: DMS [11, 87, 88].
TERP: 1,8-CI; *p*-CY; LI; GE [11, 87]; CI; CIT; EU; LIM; safrole; terpenylacetate; α-PI; *d*-LIM; CAR; α-TOL [89].

Ulva rigida
AL: Acetaldehyde; nonenal [90].
ALC: 2-Pentanol; 2,3-butandiol; 2-ethylhexanol [90].
HC: DCM; $C_3H_6Cl_2$; $CHBr_3$; dichloromethane [90].
S: DMS [90].

4.2.2 Monostromataceae

Monostroma nitidum
AL: Pentadecanal; 8,11,14-heptadecatrienal; 8-heptadecenal; 2,4,7-decatrienal [84]; 2,4,7-decatrienal [91].

4.3 Cladophorales

4.3.1 Cladophoraceae

***Cladophoropsis herpestica* (*Cladophora herpestica*)**
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Cladophora rupestris
A: MA; TMA; IAA [35].

4.4 Bryopsidales

4.4.1 Halimedaceae

Halimeda cuneata
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Halimeda discoidea
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Halimeda opuntia
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

4.4.2 Udoteaceae

Chlorodesmis major
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

4.5 Codiales

4.5.1 Codiaceae

Codium fragile
Volatile constituents (dry weight): 0.031 % [11].
A: MA; TMA; EA; IBA; IAA [35].
AC: FO; ACE; ACR; PR; BU; IVA; *n*-CA; CAP; BZ [11].
AL: FU; MFU; *n*-VA; BE [11].
ALC: FUR [11].
HC: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].
PH: *p*-CR [11].
S: DMS [11].
TERP: 1,8-CI; *p*-CY; LI; GE; TE; EU [11].

Codium galeatum
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

Codium lucasii
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

4.6 Caulerpales

4.6.1 Caulerpaceae

Caulerpa cactoides
PH: 2-BP; 4-BP; 2,4-DBP; 2,6-DBP; 2,4,6-TBP [14].

5 Discussion

The origin of volatile oils in algae was discussed by various authors [83, 87, 92–96]. The pleasant-odorous compounds of marine algae are terpenes and unpleasant components are especially aldehydes, amines, halogenated, sulfurous, and other compounds.

Many aliphatic acids, such as FO, AC, PRO, BUT, VAL, and rare-aromatic acids, such as BZ, were found in marine algae. The origin of these acids has not been reported. The origin of acrylic acid was shown to result from hydrolysis of dimethyl-β-propiothetin [82]. Nonvolatile fatty acid amounts in algae were previously published [97].

Short- and middle-chain aliphatic aldehydes and aromatic aldehydes were found in algae. Volatile long chain aldehydes (C_{15}-C_{17}) in *Ulva pertusa* are formed from the fatty acid through the formation of the corresponding 2-hydroperoxy acids [88, 98]. Long chain aldehydes in green algae are formed by decomposition of acids [96]. Some of them are metabolites and some are biosynthesis products. Long chain aldehydes, such as tetradecanal, pentadecanal, (8*Z*,11*Z*)-heptadecadienal, and (8*Z*)-heptadecenal, were shown to be formed enzymatically in the filament of the algae [15].

Marine algal amines were first investigated by Steiner and Hartmann [18] and reviewed [20, 21]. Amine compounds such as MA; TMA; DMA; EA; PA; IBA; IAA; PEA were found in algae [35]. Volatile amines in algae occur from decarboxylation of amino acids [99]. TMA and TMA oxides were also found in marine algae [100]. PEA was found in red algae *Ceramium rubrum, Cystoclonium purpureum, Desmarestia aculeate, Dumontias incrassate* [20]; it was also detected in only 6 of 17 algae species of red algae G*elidium crinale, Polysiphonia morrowii, P. tripinnata, Gracilaria bursa-pastoris, Halymenia floresii* (Clemente) C. Agardh*, Phyllophora crispa* but PEA was not found in brown and green algae [22].

There are numerous halogenated compounds in algae. Only volatile halogenated compounds have been included in this chapter. Many volatile biogenic halogenated compounds were synthesized by algae and emitted into atmosphere and can produce halogen by photo degradation, then destroy ozone. Therefore, these compounds are important for atmospheric chemistry [101, 102]. The highest release of brominated compounds was caused by *Laminaria saccharina* [46].

Bromophenols as 2-and 4-BP, 2,4-and 2,6-DBP, and 2,4,6-TBP were found in 87 samples of red, brown, and green marine algae. 2,4,6-TBP was found in all samples [14]. Bromo peroxidases are capable of brominating organic substances in the presence of bromide and hydrogen peroxide in the biosynthesis of bromophenols in marine algae [103]. It was assumed that CBr_2Cl_2 and $CHBrCl_2$ are possibly formed by macroalgae [104]. The biosynthesis of all the bromo compounds was not clearly explained.

Iodinated compounds (CH_2I_2) were reported in *Laminaria saccharina* and *Fucus distichus* and for $CHBr_3$ in *Laminaria saccharina* and *Desmerestia aculeata*. The richness of halogenated compounds CH_2I_2 in *Laminaria saccharina* and *Fucus distichus; and* $CHBr_3$ in *Laminaria saccharina* and *Desmerestia aculeata* is noticeable.

The main difference between volatile oils of terrestrial plants and algae is halogenated compound content.

Marine algae can synthesize hydrocarbons and some aliphatic hydrocarbons [13]. On the other hand, marine algae can take up hydrocarbons from seawater contaminants such as petroleum derivatives [13, 105–108], phthalates [109], and pesticide [110]. Recently, volatile compounds like 86 aliphatic, 56 cyclic oil components have been identified in algae [104]. These exogenous compounds found in algae led to erroneous results. The problem in this field is the differentiation of autochthonous and exogenous hydrocarbons in algae. For example, some of the substances reported in the literature are 2,4,5-trimethyl-1,3-dioxolane; 1,3-dioxan-5-ol; 2-ethoxybutane; 2-ethylhexanol; 1-methyl-3-(1-methylethyl)-benzene; 2-phenoxy-ethanol; benzothiazole, etc. The origins of these and other suspicious compounds should be proven as autochthonous or exogenous [105–107, 111].

Some of the volatile odorous components of algae, such as dimethyl sulfide, are unpleasant and are mainly distributed in Chlorophyta and also in some Rhodophyta [112]. Dimethyl sulfide and acrylic acid have resulted from the enzymatic cleavage products of dimethyl-β-propiothetin (dimethyl-2-carboxyethylsulfonium hydroxide), from *Enteromorpha intestinalis* and *Acrosiphonia centralis* [113], which is a metabolite of methionine [45] released into the sea water [112].

The important pleasant odorous constituents of volatile oil in algae are terpenes which are isoprene units, hooked together. The precursor, isoprene (3-metyhl-1.3-butadien), is not found in a free form in plants. Terpenes are classified according to the number of isoprene units. In volatile group terpenes, mainly acyclic, mono-, and dicyclic groups are present. Many nonvolatile group terpenes were found in algae, but only volatile oil terpenes are included in this chapter. In acyclic group of the terpene constituents contains generally most pleasant odorous compounds. The important compounds for perfumery found in algae are in acyclic group: linalool, citral, geraniol, terpinolene; in cyclic group: 1,8-cineol; in bicyclic group: α-pinene and β-pinene, and aromatic group eugenol and isoeugenol. As can be seen, only a small number of terpene compounds were reported in algae. Since there is no sufficient number of reports, we cannot estimate which algae contain the highest levels and distributional differentiation of these terpene compounds. Terpenes acyclic series are labile compounds and rapidly transformed in free form by various factors such as light, temperature, air, and microorganisms but oxidation and reduction products of terpenes were not found in algae. A number of literatures have been published on the transformation of terpene compounds. By these factors, macrobiological and fungal conversion of citral and geraniol to geranic acid was reported [114]. To prevent the biotransformation of terpenes, it is necessary to work with fresh algae.

5.1 Pharmacological Activities of Volatile Oils

The general activities of volatile oils are antiseptic, antiflogistic, expectorant, sedative, and spasmolytic [115]. Anisole showed spasmolytic activity [115]. 1,8-Cineol showed acetylcholine esterase inhibitor activity [115].

Many reports have been published on antibacterial effects of volatile oils from algae. However, antibacterial activity of the active constituent of the volatile oil has not been reported. We used only the first publication on antibacterial activity of volatile oils in algae and excluded the subsequent ones. Various fractions obtained from *Enteromorpha* showed antibacterial activity, and inhibited *Escherichia coli* and *Staphylococcus aureus*. Antibacterial action of this alga was at least partially attributed to its volatile components [17, 80]. Essential oil of *Pterocladia capillacea* was found to be active against bacteria and fungus [24].

Acrylic acid showed a broad spectrum of antibacterial activity and inhibited both Gram-positive and Gram-negative bacteria at similar concentrations. Acrylic acid feed levels between 0.01 % and 1.0 % caused an apparent increase in the growth rate of chicks, whereas 10 % levels caused anorexia and death [116].

Two sesquiterpenes (1*R**,2*S**,3*R**,5*S**,8*S**,9*R**)-2,3,5,9-tetramethyltricyclo [6.3.0.0^{1,5}] -undecan-2-ol; (1*S**,2*S**,3*S**,5*S**,8*S**,9*S**)-2,3,5,9-tetramethyltricyclo-[6.3.0.0^{1,5}]undecan-2-ol isolated from *Laurencia dendroidea* were not active against eight bacteria strains and the yeast *Candida albicans*, but showed some antioxidant activity [43].

Volatile compounds of *Laminaria japonica, Kjellmania crassifolia, Gracilaria verrucosa* and *Ulva pertusa* showed antimicrobial activities [117].

Aroma profiles of fresh *Ulva pertusa* were examined and it was found that 7-heptadecene, hexanal, (*E*)-2-octenal, (*E*)-2-nonenal, (*Z,E*)-2,6-nonadienal, (*E,E*)-2,4-decadienal, (*Z,Z*)-8,11-heptadecadienal, (*Z,Z,Z*)-8,11,14-heptadecatrienal, and (*Z*)-8-heptadecenal were responsible for the sensory characteristic aroma of the Ulvales [83].

Volatile fractions obtained from *Ulva pertusa* were tested for anthelmintic activity using santonine solution as the control. Dimethylsulfide fraction was the most powerful against the earthworms which were killed at 1,000, 2,000, and 4,000 times dilutions as compared to santonine, terpene, and carbonyl fractions [118]. The effects of volatile constituents of marine algae (*Ulva pertusa*, *Enteromorpha*, *Codium*, and *Sargassum*) on muscle contraction of earthworms were also investigated. Middle segment of a test worm was dissected, fixed on a hook, and dipped in Ringer solution. Carbonyl and terpene fractions at 0.001 % dilutions had strong contractive effect, while the muscle did not seem to react to dimethylsulfide and fatty acids. Furfural, present in carbonyl fraction, caused muscle contraction at 0.1 % concentration [119]. The active principle in the terpene fraction was unknown.

Volatile oil of *Ulva pertusa* showed feeding attractants activity [120].

6 Conclusion

Volatile oils of algae contain a variety of compounds. The main group, terpenes, contains acyclic: linalool, geraniol, citronellol; monocyclic: limonene, 1-8-cineol, *p*-cymene; bicyclic: α- and β- pinene, cadinene, aromatic eugenol, and isoeugenol. The others are benzaldehyde, phenol, *p*-cresol, various acids, alcohols, aldehydes, amines, ketones, sulfur-containing compounds, and halogenated compounds. These compounds except halogenated ones were also found in terrestrial plants. The volatile oil components of algae did not show noteworthy pharmacological activity.

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Ecological Functions of Terpenoids in Changing Climates

94

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Contents

1 Introduction 2915
2 Ecological Functions of Major Groups of Terpenoids 2917
2.1 Hemiterpenoids 2917
2.2 Monoterpenoids 2918
2.3 Sesquiterpenoids 2920
2.4 Diterpenoids 2921
2.5 Triterpenoids 2921
3 Distribution of Terpenes Within Plants and in Environment 2923
3.1 Localization in Cells and Organs 2923
3.2 Volatile Compounds in the Atmosphere 2924
3.3 Accumulation and Functions in Soil 2924
3.4 Aquatic and Marine Environments 2925
4 Terpenoids and Plant Adaptation to Climate Change-Related Abiotic Stresses 2926
4.1 Temperature Changes 2926
4.2 Drought and Flooding 2927
4.3 Changing Atmospheric CO_2 2928
4.4 Eutrophication and Nutrient Availability 2929
4.5 Ozone and Oxidative Stress 2929

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4.6 UV-B Radiation 2930
4.7 Multiple Abiotic Stresses 2930
5 Ecological Functions of Terpenoids under Abiotic Stresses 2931
5.1 Plant–Animal Communication 2931
5.2 Plant–Plant Interactions 2936
5.3 Plant–Fungi Interactions 2936
5.4 Terpenes and Ecosystem Function 2937
6 Conclusions and Future Prospects 2938
References 2938

Abstract

Terpenoids comprise the largest and most structurally diverse class of organic compounds. Terpenoids have a multitude of ecological functions, which might be perturbed under changing environmental conditions. The best-known functions of terpenoids in-, and volatilized from, plant tissues are related to adaptation to abiotic stresses and biotic stressors, such as herbivores and pathogens. However, the high volatility and reactivity of some terpenoids may also affect the atmosphere composition. Volatility of terpenoids provides, for sessile plants, a tool for communication with other organisms such as neighboring plants, pollinators and foes of herbivores, via air-borne infochemicals. We review the main properties of biogenic terpenoids, from various scales, including the plant cell, leaf surface and canopy. We also explore links to ecological functions and atmospheric behavior, including the chemical transformations of terpenoids. The functionality of the terpenoids could be strongly influenced by the predicted changes of climatic conditions upon proceeding global climate change. On the other hand, volatile terpenoids released by large forested areas continues to have a significant effect on local climatic conditions e.g., by formation of light-scattering blue haze which is composed of terpenoid-derived secondary aerosol particles. Such processes might also be altered by climatic changes.

Keywords

Climate • defense • herbivory • pollination • pollutants • resins • secondary aerosols • storage • terpenes • volatiles

Abbreviations

BVOC	Biogenic volatile organic compound
CBSC	Carbon-based secondary compound
DMADP	Dimethylallyl diphosphate
DMNT	(E)-4,8-dimethyl-1,3,7-nonatriene
IDP	Isopentenyl diphosphate
MBO	Methyl butenol or 2-methyl-3-butene-2-ol
MEP	2-C-methyl-D-erythritol-4-phosphate pathway
MT	Monoterpene
MVA	Mevalonic acid pathway

NO_x	Oxides of nitrogen
O_3	Ozone
SQT	Sesquiterpene
TMTT	(E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
VOC	Volatile organic compound

1 Introduction

Terpenoid compounds comprise a major group of secondary compounds and are the most diverse among those synthesized by organisms, with 30,000 members [1]. All terpenoids are derived from the five carbon unit (C_5) skeleton of isoprene, and therefore terpenoids can referred to as isoprenoids. Terpenoid classification is dependent upon the number of five-carbon units present in each compound. For example, isoprene is a hemiterpenoid; ten-carbon terpenoids with two C_5 units are called monoterpenoids. Other compound groups are sesquiterpenoids (C_{15}), diterpenoids (C_{20}), triterpenoids (C_{30}), tetraterpenoids (C_{40}) and polyterpenoids ($C_{[n>40]}$). If terpenoids are pure hydrocarbons formed from isoprene units, without removal or addition of methyl groups or addition of oxygen atoms, they are called terpenes. Irregular homoterpenes, C_{11}-homoterpene DMNT and C_{16}-analog TMTT are common floral odor constituents and herbivore-induced compounds in vegetative parts of plants.

The importance of terpenoids to life is highlighted by the fact that two separate pathways have been found to produce the terpenoid precursor C_5 units isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP). The mevalonic acid (MVA) pathway is functional in archae, animals and fungi, 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway is found in green algae, and terpenoids are produced by both pathways in bacteria and plants [2]. In plants the MVA pathway is active in the cytosol and it provides C_5 units for sesquiterpene, triterpene and polyterpene biosynthesis whereas the MEP pathway occurs in plastids and produces C_5 units for isoprene, monoterpenes, diterpenes and carotenoids [1]. Recent reports have indicated metabolic crosstalk between biosynthesis pathways and e.g., the homoterpene DMNT may originate from both pathways.

Terpenoids can be found in various plant organs. Concentrations of terpenoids in plant tissues are controlled by the availability of substrate and the activity and type of biosynthesis enzymes. Emission rates of volatile terpenoids from plant leaves are controlled by their synthesis rates and compound-specific physicochemical characteristics, mainly their solubility, volatility and diffusivity. These are affected by physicochemical constraints caused by temperature, stomatal conductance and leaf structure. Storage of terpenoids could be isolated from other plant tissues with specialized structures such as secretory cavities, resin canals (Fig. 94.1), latex canals (lactifers), and glandular trichomes.

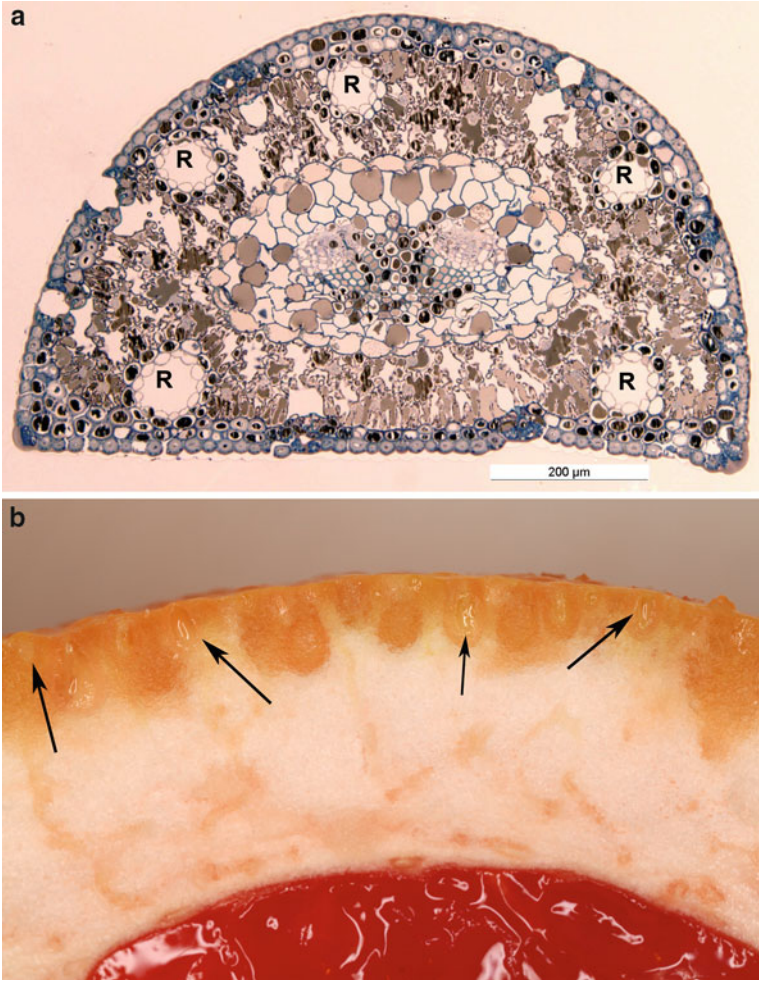

Fig. 94.1 Examples of terpenoid storage structures embedded in plant tissues. (**a**) Cross-section of (*Pinus halepensis*) needle showing four resin canals (*R*) in the needle mesophyll layer (micrograph provided by Dr. Minna Kivimäenpää). (**b**) Secretory cavities of red grapefruit (*Citrus paradisii*) peel for storage of monoterpenes, mostly limonene

Highly volatile terpenoids have a small storage pool and their emission rates are directly responsive to light- and temperature-dependent synthesis rates. Less volatile terpenoids have a large storage pool and total plant emission rate is a combination of emissions from direct synthesis and temperature-dependent evaporation from storage pools.

2 Ecological Functions of Major Groups of Terpenoids

Enormous diversity of terpenoids found in nature suggests that they are important compounds in plant evolution and ecology. The chemical properties of terpenoids and their derivates are manifold. The same chemical compound may have different ecological functions when appearing in solid, liquid or gaseous phase.

2.1 Hemiterpenoids

The most important C_5 hemiterpenoid is the highly volatile isoprene, which is not stored, but emitted from plant chloroplasts in a temperature- and light-dependent manner. Total global isoprene emissions from vegetation are estimated to be 412–601 $\times$ 10^{12} g C annually [3]. Occasionally isoprene-bound carbon emission can represent up to 5% of the photosynthetic net carbon uptake in an individual plant. In the plant kingdom, species of the same genus can represent a wholly different strategy in their capacity to synthesize and emit isoprene; a certain species might not emit isoprene whereas in a closely-related species isoprene can be the dominant VOC emitted. Other biogenic sources of isoprene emissions are breath of humans and animals.

A multitude of functions for isoprene are proposed to explain why plants invest in costly isoprene production to then release such a large amount of recently fixed carbon back into the atmosphere. Improved thermotolerance and enhanced tolerance against ozone and other reactive oxygen species (ROS) are the most often associated responses with the enhanced isoprene production in plants [4]. In contrast, as isoprene emission also consumes metabolites, it has been proposed that plants use isoprene emission as a "safety valve" to get rid of unwanted metabolites. Isoprene is not generally known to affect herbivory, but some evidence indicates that transgenic isoprene emitting plant species, which normally do not emit isoprene, are able to deter caterpillars from feeding and also repel parasitic wasps [5]. Isoprene oxidation products, methacrolein and methyl vinyl ketone are common constituents of the atmosphere above isoprene-emitting trees.

Another common hemiterpenoid in conifer forest atmospheres is methylbutenol (MBO), a five-carbon alcohol produced and emitted only by species of pines (*Pinus* spp.) native to western North America and northern Europe. MBO is biochemically synthesized in chloroplasts by MBO synthase from same precursors as isoprene. Ecophysiological and ecological functions of MBO are poorly understood as this compound has been detected only from pines.

Other hemiterpenoids are e.g., hemiterpenoid glucosides which are found to accumulate in plant roots under nutrient deprivation and carbon flux overflow.

2.2 Monoterpenoids

Monoterpenoids with C_{10} skeleton is not the most diverse group of terpenoids, but they can be considered to be the most important because of their abundance and frequent appearance in nature. Most common monoterpenes e.g., α-pinene can be detected from nearly every green plant since chloroplasts produce monoterpenes, even by those species which do not synthesize isoprene. Plastids of other cells such as epithelial cells of resin duct in conifers produce α-pinene and other monoterpenoids in storage structures. Resin of conifers is a mixture of monoterpenes and diterpenes. The word "terpene" is derived from the word "turpentine," which is mostly an essential oil containing α-pinene distilled from tree resins, originally from "turpentine tree" *Pistacia terebinthus*.

Molecular structures of monoterpenoids are present as three major types: acyclic, monocyclic and bicyclic. Oxygenated derivatives of *acyclic monoterpenes* are more widespread in nature than acyclic monoterpenes themselves. Such derivatives are the monoterpene alcohols citronellol and geraniol, or the monoterpene aldehydes citronellal and geranial. *Mononocyclic monoterpene hydrocarbons* are exemplified by limonene, which is a major component of orange and lemon peel oils, and β-phellandrene, which is emitted by conifer trees under biotic stress. These compounds are relatively common in nature. *Bicyclic monoterpenes* are represented by the aforementioned α-pinene, one of the most abundant and important monoterpenes.

High volatility of monoterpenes has been recognized for a long time and humans have used steam distillation to separate monoterpene-rich essential oils from plants for perfume and flavoring purposes. Monoterpene-rich spicy plants have monoterpenes stored in glandular trichomes on leaf surface (Fig. 94.2). Humans have used spice plants to flavor food, because warm food releases monoterpenoids from plant storage. Songbirds use a similar strategy by adding fresh spicy plants such as *Lavandula stoechas* in their nest [6]. Warmth of nestlings increases monoterpene emissions from plants, reduce bacteria growth in nest and repel bird ectoparasites from nestlings. Perhaps this avian use of monoterpenoids also mark nests and play a role in behavioral ecology.

Monoterpenes produced in chloroplasts have similar functions as isoprene to control thermotolerance and ozone tolerance of plants. Owing to their lower volatility, monoterpenes can be adsorbed on plant membranes or even taken up by neighboring non-emitting plants for temporary storage when temperature is below +20 °C. Plants that do not store monoterpenes emit these compounds mostly in a light–dependent manner while species storing monoterpenes can emit them in darkness if temperature is sufficiently high.

There are a multitude of ecological functions of monoterpenoids. The best known functions are as signal molecules in flower scent to attract pollinators, and as defense-related roles against herbivores and plant pathogens. Very important here is indirect defense signaling to attract natural enemies of herbivores and allelopathic effects between higher plants to improve plant performance in plant communities. Although monoterpenes are repellent or feeding deterrents for many

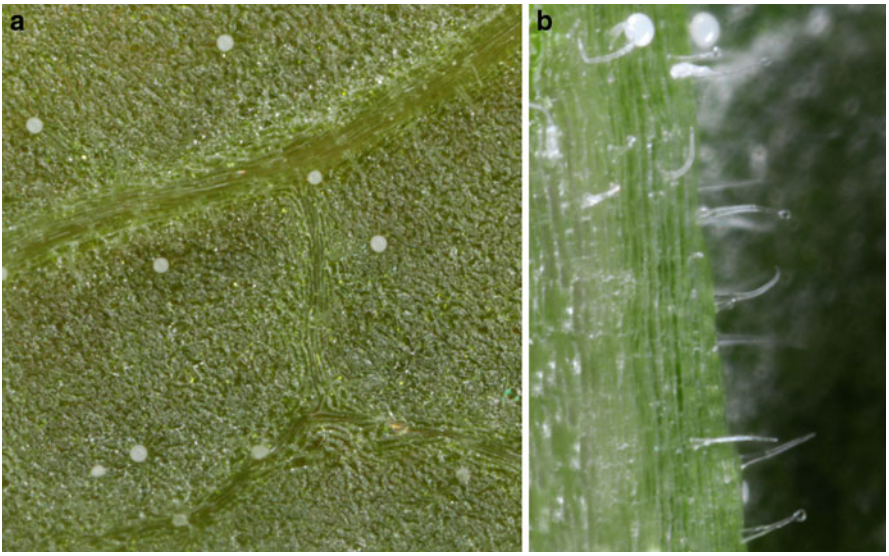

Fig. 94.2 Glandular trichomes of sage (*Salvia officinalis*) store essential oil composed of monoterpenes. (**a**) Peltate glandular trichomes forming oil glands on lower leaf surface of sage. (**b**) Peltate glandular trichomes, capitate glandular trichomes and non-glandular trichomes on leaf veins of sage

herbivore species, insects as well as mammals, there are many animal species that are particularly adapted to feed on monoterpene-rich plant species. The ratio of specific monoterpenes or their enantiomers can often act as the cue for herbivores to identify the most suitable plant species and individuals for food. Herb species in the family Apiacea differ in their relative concentration of the same common monoterpenes and the host selection of species specific psyllids (Homoptera: Psyllidae) are associated to monoterpene composition of Apiacea host species. Several Australian marsupial species feed on *Eucalyptus* leaves almost exclusively. In the boreal conifer forests vole species and moose consume the monoterpene-rich Scots pine. Also humans consume fruits, vegetables and spicy leaves rich in monoterpenes, which indicate the low toxicity of many common monoterpenoids to mammals, and perhaps a metabolic benefit to consumers.

Conifer resin, which is a mixture of monoterpenes and diterpenes is an important protective compound against bark beetles and other conifer herbivores. The volatile monoterpenes emanating from a specific tree is often the cue for bark beetles to find a tree where the tree defenses could be compromised from abiotic or biotic stresses. The synchronized mass attack is the strategy bark beetles use to reduce the effects of resin-based defenses in conifers. Aggregation hormones released by bark beetles are oxidized monoterpenoids such as ipsdienol, ipsenol and verbenol. It is believed that these compounds can be oxidation products of host plant monoterpenes such as myrcene. Recently it has been observed that most of the monoterpenoid aggregation pheromone components are biosynthesized *de novo* in bark beetles [7].

Plant feeding insects are able to store host plant monoterpenes and use the compounds in their own defense. Pine sawfly larvae store mono- and diterpenes of host plant resin for defensive purposes (see diterpenes). Larvae of Chrysomelid leaf beetles feeding on *Salix* and *Populus* are able to store and sequester precursors of iridoids (cyclopentane monoterpenoids) derived from host plant. In addition to this sequestration iridoids may even have a dual origin as larvae of *Plagiodera versicolora* and *Phratora laticollis* have the potential to de novo produce iridoids from the precursors in their food [8].

Flower scent is extremely variable between plant species, but often monoterpenes are the dominating group of compounds in floral odor bouquet. Flowers of one plant species can emit more than 100 scent compounds. Linalool, β-ocimene and β-myrcene are monoterpenes that have been most-oft reported from flowers. Proportion of monoterpenes of the total scent is variable depending on the age of flowers and the part of the flower analyzed, but their proportion in many cases exceeds 50% of the total flower emission. The ratio of different compounds in flower scent affects the composition of pollinating insect communities. Wasps, flies, beetles and butterflies have a different odor preference in addition to preferences of visible flower colors. Specialized pollinator species use the combination of visual and olfactometric cues of flowers to detect their specific host species.

2.3 Sesquiterpenoids

Sesquiterpenoids are the largest and most diverse class of terpenoids having more than 6,500 described compounds [1]. The structure is based on the union of three C_5 groups leading to C_{15} substances and their derivates. Most of the sesquiterpenes are semivolatile liquids and often comprise plant essential oils together with monoterpenes. Molecular structures may be acyclic or contain rings, like the monoterpenes. Farnesene is an example of common *acyclic* sesquiterpenes, which with oxidation can become a sesquiterpenoid such as sesquiterpene alcohol farnesol. Example of a *monocyclic* sesquiterpenes is humulene which is common in the essential oil of hops (*Humulus lupulus*). *Bicyclic* sesquiterpene β-caryophyllene, with an additional cyclobutane ring, is a sesquiterpene found in flowers of many species and it has a significant function as an alarm pheromone in aphids. Alarm pheromones are released by attacked aphids to encourage other members of the aphid colony to escape from their enemies. *Tricyclic* sesquiterpenes, such as longifolene from longleaf pine, have a third ring in their structure.

Oxygenated tricyclic sesquiterpene alcohols ledol and palustrol are sticky semivolatiles produced high amounts e.g., by circumpolar *Rhododendron tomentosum* (formerly *Ledum palustre*) known also as wild rosemary or northern Labrador tea. These sesquiterpene alcohols are toxic to humans in high concentrations but act as effective repellents against blood-feeding insects and ticks. Recently these semivolatile compounds have been found in condensate on the leaf surfaces of neighboring plants in their natural growth habitats during nighttime and reemitted to the atmosphere during warm daytime conditions. These properties are associated to repellency of herbivorous insect from neighboring plants suggesting an “extended chemical defense” by these sesquiterpenoids on plant community level [9].

Derivatives of sesquiterpenes have an important role in plant-herbivore interactions. Drimane-type dialdehyde sesquiterpenes such as polygodial and warburganal are known feeding deterrents for several herbivorous insect species and human perceive these as hot tasting [10]. Sesquiterpene lactones are characterized by a lactone ring and many representatives are toxic to herbivores from several insect orders. These compounds are frequently found from glandular trichomes or latex ducts of members of the Compositae family. Gossypol in cotton is a phenolic sesquiterpene dimer produced by the pigment glands of cotton. Gossypol is toxic to several herbivorous insects and acts as a feeding deterrent to a variety of important defoliators of cotton.

2.4 Diterpenoids

Diterpenoids are C_{20} compound formed of four C_5 units. Basic diterpenes are mostly non-volatile compounds found from plant resins (e.g., in the Coniferae) and latex (e.g., in the Euphorbiaceae). In conifer resin abietic acid and other diterpene carboxylic acids are solids mixed with essential oils when formed in resin ducts. The viscosity and flow of resin is controlled by the proportion of essential oils, which are composed of monoterpenes and sesquiterpenes. When these oily substances evaporate, resin acids crystallize and form a solid protective layer on the damage site. Bark beetle attack induces resin flow on conifer bark (Fig. 94.3).

Resin acids in conifer are important feeding deterrents for many polyphagous herbivores. However, specialist herbivores of conifer trees are adapted to detoxify diterpenes and other terpenoids of resin or store these substances for defense against their own natural enemies. Pine sawfly larvae are serious defoliators which store diterpene and monoterpene mixtures in special storages connected to foregut. When attacked by predators sawfly larvae discharge a droplet of resin towards the enemy with a rapid backward movement (Fig. 94.4). After smeared with a sticky resin the predator often abandons pursuit of sawfly larvae.

2.5 Triterpenoids

Triterpenoids, C_{30} compounds with basic skeleton composed of six isoprene units, is a large and diverse group of compounds which have several ecological functions. Triterpenoids produced by plants can appear in plants mixed in resins, latex, corks or in leaf waxes. They often act as feeding deterrent for herbivores (including humans), and may interfere with insect growth and development. Major groups of ecologically significant triterpenoids produced by plants are cucurbitacins, limonoids, saponins and cardenolides (cardiac glycosides) [10].

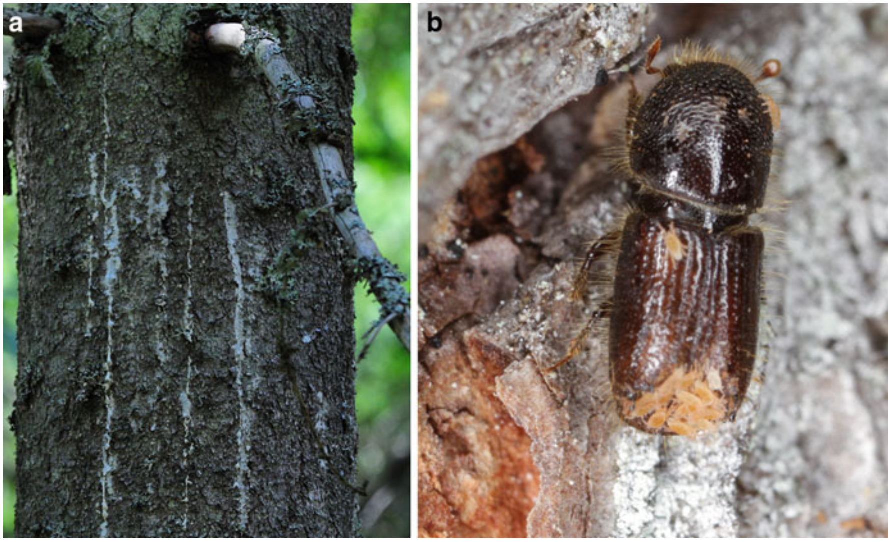

Fig. 94.3 Conifer tree defense against bark beetles with induced resin production. (**a**) Resin flow on Norway Spruce (*Picea abies*) bark after attack by bark beetles. (**b**) The European spruce bark beetle (*Ips typographus*), the most serious bark beetle pest of Norway spruce, attacking spruce bark with phoretic mites, which are probably involved in the transmission of fungal tree pathogens

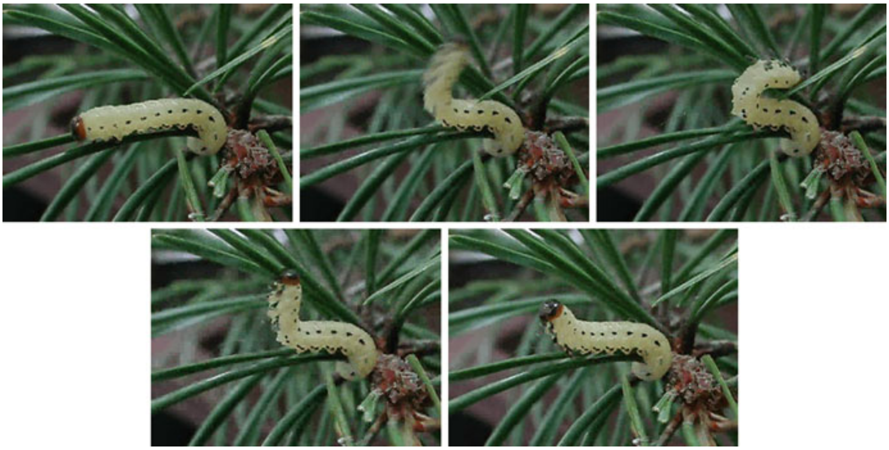

Fig. 94.4 When disturbed, e.g., by birds or other predators, the pine sawfly *Diprion pini* larva shows a rapid defense movement and discharges a droplet of resin which is chemically identical mixture of mono- and diterpenes as resin produced by the pine host *Pinus sylvestris*

Cucurtbitacins are tetracyclic compounds produced by plants of Cucurbitaceae family and humans sense their taste as bitter. Some specialized cucumber leaf beetles of can efficiently detoxify these compounds. Limonoids are a large group of oxygenated triterpenoids produced by plants in Rutaceae and Meliaceae families.

Limonoid azadirachting is produced by the neem tree and China berry and is very strong feeding deterrent for several insect species and acts as a natural insecticide that is effective against the desert locust. Saponins are a group of triterpenoid glycosides with soap-like properties. This is because of the presence of hydrophobic triterpene and hydrophilic sugar group in one molecule will give saponins detergent-like properties. Cardenolides are steroidal glycosides, triterpene derivatives that are referred to as cardiac glycosides because of their heart-arresting properties. Many of these compounds are bitter-tasting and lethal to mammals in high concentrations. Often cardenolides induce vomiting in mammals, which reduce the risk of fatal poisoning. Specialist herbivorous insect species adapted to feed on milkweeds (*Asclepias*) such as the Monarch butterfly, are often bright colored and they are able to sequester host plant cardenolides for defensive purposes [11].

Other ecologically important triterpenes are the phytoecdysteroids, which are plant derived ecdysteroids, triterpene analogues to insect molting hormone ecdysone. Many ferns and gymnosperms contain these compounds in high concentration and they are expected to give protection against insect herbivores. Papyriferic acid is a triterpene in the resin of birch (*Betula*) species that protects bark against winter browsing by mammals.

3 Distribution of Terpenes Within Plants and in Environment

3.1 Localization in Cells and Organs

Concentrations of terpenes in plants are enormously variable among individuals and even organs within populations. Genetic control of terpene synthesis affects the composition of terpenes in each individual. Environmental and temporal variation in each organ could be very high e.g., some specific compounds in young leaves can be replaced with other compounds in older foliage. This variation is also affected by abiotic or biotic factor inducibility.

One terpene localization strategy by plants is production in specialized structures such as resin and latex ducts, secretory cavities and glandular trichomes. In these structures terpenoids are synthesized by secretory cells and stored in very high concentrations, which could be autotoxic to other plant tissues. Ecological interactions are influenced by locale and type of structures in plant organs. For example organs such as leaves face threats against different herbivores than in wood. Conifer needles produce monoterpenes and diterpenes protect against totally different insect herbivore or fungal pathogen species than in woody stem phloem.

Terpenes volatilization can be sensed by herbivores as they approach a potential host plant with implications for the defense strategies of plants. Volatile compounds can repel most potential generalist herbivores at some distance from the plant, but the same emitted VOCs have an opposite function in attracting host plant-specific herbivores. Terpenes secreted on plant surfaces as waxes or resins or deposited in glandular hairs may give direct protection against many small insect and mite herbivores and inhibit the germination of fungal spores and growth of pathogenic fungal hyphae. Latex and resins ducts located in leaf or needle mesophyll layer are efficient upon herbivore wounding of tissues feeding. Defense compounds are rapidly released from pressurized and damaged ducts. Sticky compounds can physically or chemically halt feeding.

3.2 Volatile Compounds in the Atmosphere

Isoprene, monoterpene and sesquiterpenes are volatile terpenes which have several functions after release from plants. Monoterpenes and sesquiterpenes are important part of flower scents to attract pollinator species. Different flower parts such as sepals, pets, anthers and pollen have different scent profiles of volatile compounds. Pollinators can use this volatile profile to detect the optimal availability of pollen. With pollen several less-volatile terpenoid compound can spread over long distances from the original host plant.

Vegetative isoprene and monoterpene emissions have strong light- and temperature-dependence because of large amounts of biosynthesis in chloroplasts. Changes in temperature and photosynthetically active radiation (PAR) affect emission rates of these volatiles. Emission from species having monoterpenes emitted from storage, and emissions of sesquiterpenes are more strongly related to changes in temperature than light. Also other abiotic factors influence emission rates, but the most distinctive changes are caused by biotic factors which induce the production of volatile terpenoids. Herbivore-induced terpenoid emissions affect defense responses of neighboring plants and behavior of other herbivores, but more importantly they affect the efficiency of natural enemy search for herbivore hosts. Homoterpene emissions are particularly typical for herbivore-damaged plants.

Different mono- and sesquiterpenes have variable lifetimes in the atmosphere depending the other reactive compounds in the atmosphere. In atmospheres rich of oxides of nitrogen (NO_x) volatile terpenes participate in the formation of ozone (O_3). If the concentrations of NO_x are low, monoterpenes and sesquiterpenes react with O_3 leading to lower concentration of phytotoxic O_3. The reaction products can be liquid or amorphic solid nano-scale particles which later aggregate into larger aerosol particles [12]. Reaction products of VOCs are called secondary organic aerosol particles (SOA), because they are formed in the atmosphere from gases and fumes.

3.3 Accumulation and Functions in Soil

In the rhyzosphere terpenoids can affect seeds and roots of competing plants, soil microbes and animals. Terpenoids spread through volatilization from plant rhizosphere, leaching from above-group parts of plants, or by decomposition of plant debris. When terpenoids accumulate in soil in high concentrations from terpene-rich plant species, plant species sensitive to phytotoxic terpenes will gradually disappear in ecological time, leading to changes in plant community composition. Understory vegetation of conifer forest is adapted to monoterpene and dipterpene-rich needle litter which may release degradation products several years after needle cast. Monoterpenes of plant essential oil, sesquiterpenes, particularly sesquiterpene lactones have been reported to have biological effects on soil fauna and microbes.

Plant communication via volatile sesquiterpenes at the third trophic level, natural enemies of herbivores, has been shown in soil ecosystems. Root beetle damages on maize root system will induce production and emissions of (*E*)-β-caryophyllene, also known as an aphid alarm pheromone. This compound spreads through soil pores where it attracts entomopathogenic nematodes that, in turn, control root beetle larvae [13].

Monoterpenes in the coniferous soil atmosphere have been shown to affect microbial activities and net nitrogen mineralization and nitrification in nitrogen poor soils. These observations suggest that terpenes together with condensed tannins in soil, may have a crucial role in microbial processes related to soil C and N cycling. One of the proposed mechanisms is binding of proteins and certain other organic N-containing compounds [14].

3.4 Aquatic and Marine Environments

Photosynthesizing vegetation in terrestrial ecosystems is known to be important source of isoprene and monoterpenes released into the atmosphere. Globally the average net primary production expressed as a dry mass produced per area unit per year (g m^{-2} $year^{-1}$) is even higher in marine algal beds and reefs than in tropical rainforests. Therefore, it is astonishing that only very recently it has been observed that algae in an active phytoplankton bloom of the ocean can be a significant source of monoterpene emissions into the atmosphere [15]. In low chlorophyll regions of oceans, monoterpenes over the sea surface are mostly below detection limits. In laboratory conditions cyanobacteria produce isoprene in a significantly higher emission rate than diatoms and the isoprene emission rates are related to the actual cell volume.

Less volatile terpenes have been described from macroalgae. Recently it has been shown that macroalgae are able to damage corals using hydrophobic allelochemicals present on their surfaces. The monoterpene lactone, loliolide, in red algae and diterpene derivates from the green alga *Chlorodesmis fastigiata* have been described as potent allelochemicals that induce damage in corals in site-specific spots of algal contact. Corals in global decline, such as *Acropora*, are most severely affected [16]. Several marine invertebrates cover their bodies with species of seaweeds, polyps, sponges, or bacteria that contain diterpene alcohols. These "clothing" species give protection against the predatory fishes. Fish- repellant properties of the diterpene alcohols produced by the companion organisms are the chemical mediators of this mutualistic behavior [17]. The advantage for the "covering" mutualist species is that the mobile invertebrate host will improve their food availability when searching for its own food sources.

4 Terpenoids and Plant Adaptation to Climate Change-Related Abiotic Stresses

4.1 Temperature Changes

Temperature is a major driving factor for the formation of ecosystems and biomes. Geographical distribution of species largely depends on their adaptation to a local temperature range. Warming or cooling of local climate will lead to changes in the distribution of biotic organisms. Distribution changes occur more rapidly for mobile animals than in sessile plants. Therefore, plant adaptation to changing climatal temperatures is predicted to be slower than that of animals. Herbaceous plant species with a massive production of plumed seeds for long-range wind-dispersion exemplifies one strategy for coping with rapid changes in local climate; guerilla adaptation. At the other extreme of adaptation capability to climate warming are trees with very long life cycles with local seed dispersal; phalanx adaptation. Guerilla adaptation is more suited for annual invaders whereas phalanx adaptation is seen more in climax species.

Global climate warming is a result of extensive use of fossil energy which has resulted in continuously increasing concentrations CO_2 and other greenhouse gases in the atmosphere. In the greenhouse effect, solar infrared radiation warms the lower atmosphere and surface of the earth efficiently owing to absorption of warming infrared radiation by higher concentration of greenhouse gases. Climate warming also affects annual precipitation in large areas and together these factors have a strong impact on the survival of plant and animals in ecosystems.

The biosynthesis of secondary metabolites in various organisms is directly dependent on temperature. Rapid changes in the physiological status e.g., stomatal function and allocation of resources (i.e., substrate availability) at high temperatures could have dramatic changes in production of terpenes by plants. Volatile terpenes also have higher volatility at higher temperatures, which affects emission rates, but also affects concentrations in tissues. At higher temperatures isoprene and monoterpene emission have consistently increased reported emission rates in monitoring studies [18]. This is a result from more active synthesis in leaves and needles, but partly higher release rate from storage, which may lead to lower concentrations in the tissues, even though emission rates are higher.

In deciduous trees even elevated night temperatures have resulted in higher daytime emissions rates of isoprene, monoterpenes, sesquiterpenes and a homoterpene on a leaf-area basis. This increased emission per leaf area unit was associated with substantial increase of total leaf area of tree saplings. The strong correlation of plant growth rate and terpenoid emission rates from plants with temperature indicates that there are likely increased terpenoid emissions under warmer climate conditions. For plant species which defend against herbivores with semivolatile compounds adsorbed on their leaf surfaces, higher night temperatures coupled with a loss of defensive cover could mean increased susceptibility to herbivores.

There is less knowledge of the responses of diterpenes to changing temperature. However, the evidence of resin acid concentrations of conifer needles and bark at elevated temperature suggests slight increase in concentrations, but the situation is not as clear as for monoterpene response. The production of triterpenes of tropical plants did not follow the significant temperature dependence of tannin in some plant species. It is possible that the terpenes with higher number of isoprene units are not as responsive to temperature changes as other plant secondary metabolites, because plant allocate substrates to less costly isoprene and monoterpene synthesis.

4.2 Drought and Flooding

Drought stress is a typical seasonal situation in many geographical; particularly affecting agriculture. Some plants go dormant and some are structurally adapted to low water availability. During climate warming the timing of rainy and dry seasons have changed in large areas such as the horn of Africa in the eastern part of the continent, where long drought periods in recent years have resulted in serious yield losses and famine. Often changed climatic conditions will lead to more frequent heavy rains and flooding in the geographical areas where the vegetation has not adapted to such high precipitation. Of course, precipitation timing also affects plant growth and development.

Long dry periods lead often to accumulation of salts in soil and create an additional stress for plant roots. Drought and salt-induced stress in root systems also results in leaf stomata closure, and thus creates biochemical limitations of photosynthesis. Both the reduction of photosynthesis and the stomatal closure are expected to negatively affect carbon supply into the MEP pathway and reduce terpenoid biosynthesis. Against this hypothesis, experimental evidence has shown that isoprene is not reduced by increasing drought stress until the stress becomes heavy and almost completely inhibits photosynthesis. Carbon labeling experiments have shown that under stress isoprene synthesis in plants continue by using alternative sources of carbon earlier fixed and carbon stores are possibly related to starch breakdown. This change of isoprene precursor is temporal and rewatering of plants leads to isoprene emission bursts when photosynthesis-dependent isoprene production is reactivated [4].

In Scots pine and Norway spruce saplings episodes of severe drought has resulted in the increased concentrations of several individual monoterpenes and diterpene resin acids in the xylem storage vesicles. Elevated monoterpene concentrations have been detected from the needles of Norway spruce. Emission analysis of inducible volatile terpenes of maize after simulated moth damage showed variable responses of individual mono-, sesqui- and homoterpenes to decreasing soil water availability. During drought conditions, the emissions of monoterpene linalool and sesquiterpenes β-caryophyllene and (*E*)-nerolidol and homoterpene TMTT were decreased whereas emissions of sesquiterpenes α-farnesene and β-bisabolene showed the opposite trend.

In California chaparral and Mediterranean bush vegetation, monoterpenes produced by above and below-ground plant parts accumulate in soil. Sequestration to soil is related to plant allelopathy, which improves root competitive ability to the host.

Dry and hot periods promote monoterpene volatilization from soil and may also increase flammability. As a result, recurrent dry periods may lead to more frequent and more rapidly spreading bushfires when monoterpene-rich soil supports the spread of fire, again removing competitors.

Flood-simulating root water-logging does not affect needle monoterpene concentrations of Norway spruce. Root anoxia as a result of flooding results in increased emission of many stress marker volatile compounds such as ethanol, acetaldehyde and nitric oxide (NO), but less is known about the influence of flooding on monoterpene and isoprene emission of plants.

4.3 Changing Atmospheric CO_2

Climate warming is a result of increased greenhouse gas concentrations in the atmosphere. Carbon dioxide is crucial for plant photosynthesis and therefore changes in atmospheric CO_2 concentration have direct effects for plant physiology and concentration of carbon-based compounds. The average atmospheric CO_2 concentration was approximately 320 ppm in the mid-1960s and reached the level of 393 ppm in early 2012 at the Mauna Loa Observatory, Hawaii. Three-quarters of the increase was likely the result of greater fossil fuel use and the remainder of increase from changes in land-use such as increased deforestation.

Increased atmospheric CO_2 concentrations with more carbon available for photosynthesis increases plant productivity and indirectly increases capacity of vegetation to produce terpene per area unit. One typical characteristic for plants grown at elevated CO_2 concentration atmospheres is thicker leaves. Emission of volatile terpenoids per leaf area unit could therefore be higher at elevated- than ambient CO_2 concentration. However, current observations from elevated CO_2 exposure experiments have reported the opposite [18]. In particular isoprene emission has decreased at elevated CO_2 and monoterpene emissions have responded in a variable manner depending on the individual monoterpene compound being monitored. Reduced isoprene and monoterpene emissions at high CO_2 environments might suggest the uncoupling of isoprene synthesis from photosynthesis, similarly to that under drought stress, leading to consequent inhibition of isoprene emission at the leaf level.

Evidence from terpenoid analyses of plant tissues support the observations of the effects of elevated atmospheric CO_2 concentrations on volatile terpenoid emissions. Responses of stored monoterpenes and diterpenes in the needles of Scots pine and Norway spruce sapling to elevated CO_2 were opposite when compared to temperature effect; significantly reduced concentrations of many compounds have been detected [19]. Elevated CO_2 had less influence on the concentrations of the stored monoterpenes and diterpenes in the stem bark, phloem and xylem. In Scots pines grown at elevated CO_2 and elevated temperature environments in large closed-top-chambers, elevated CO_2 resulted in consistently reduced total monoterpene concentration while temperature effects were more variable [20].

4.4 Eutrophication and Nutrient Availability

Eutrophication of coastal areas results in an increase of phytoplankton aquatic ecosystems and the potential of rapidly increased emissions of isoprene and monoterpenes from the algae and cyanobacteria in the bloom. Eutrophication, therefore, could be a result of higher density and biomass of phytoplankton per area unit, but unfortunately studies of terpenoid production of single species under the excess nutrients have not been performed.

In terrestrial plants the allocation of carbon to carbon-based secondary metabolites, particularly phenolic compounds, is often attenuated when higher amount of nitrogen and other nutrients are available for plant growth. Terpenoids, such as diterpenes and monoterpenes found in resin, sequestered in special storage structures, normally responds variably. When compared to nutrient deficient plants, fertilized plants grown faster and develop larger organs. It has been observed that under improved nitrogen availability pine needles grow larger and they form increased number of resin canals. This means higher resin concentration in well-developed needles and may result in higher emissions rates of monoterpenes by diffusion from the storage.

4.5 Ozone and Oxidative Stress

In the troposphere, the lowest layer of atmosphere, volatile terpenes perform a dual action, depending on the presence of anthropogenic pollutants such as oxides of nitrogen (NOx). When NOx concentrations are low, volatile terpenes react with ozone to reduce the atmospheric concentration of this phytotoxic gas. When air is contaminated with NO_x formed e.g., by combustion, volatile terpenes participate in reactions which results in formation of tropospheric ozone.

There is very wide variation in the reactivity and the atmospheric lifetime of volatile terpenes with ozone in standardized conditions at an average ambient O_3 concentration of 30 ppb. Among the shortest lifetimes of phytogenic terpenes are 1 min for the monoterpene α-terpinene and 2 min for the sesquiterpene β-caryophyllene. The longest lifetimes are more than 235 days for the monoterpenoid camphor, more than 110 days for the monoterpene monoterpene 1,8-cineole and more than 33 days for the sesquiterpene longifolene. In similar conditions isoprene has a lifetime of 1.3 days and the monoterpene α-pinene, for 4.6 h [21].

It has been suggested that volatile terpenes play a similar dual action inside the leaves and participate in ozone quenching in the intercellular spaces before they are released into the atmosphere. In plants exposed to ozone either a reduction or an increase of isoprene and monoterpene emission is observed. Particularly high ozone concentrations induce the terpene emission often as a result of cellular damage. There is evidence that volatile terpenes are able to reduce ozone damage and to quench ozone and reactive oxygen species (ROS). Furthermore, it has been demonstrated in tropical forests a large fraction of total plant sesquiterpene emissions undergo within-canopy ozonolysis, which may benefit plants by reducing ozone uptake and oxidative damage to the foliage.

Exposure of terpene-storing conifers to moderately elevated ozone concentration does not lead to substantial changes in monoterpene or diterpene concentrations in needles.

4.6 UV-B Radiation

Solar UV-B radiation has been increased in the areas of stratospheric ozone hole in the polar area. Some observations of isoprene emission from vegetation experimentally exposed to supplemental UV-B simulated 20–30% depletion of stratospheric ozone, which suggests increased emission of isoprene under elevated UV-B radiation conditions.

4.7 Multiple Abiotic Stresses

Most scenarios of future climate change includes several abiotic factors affecting plant growth changing at the same time relative to current conditions. Very few studies have attempted to investigate volatile terpene emissions under multiple abiotic stresses, either in the laboratory or field. In one of these, [19] terpenoid concentration and emission studies were performed on trees in chamber and open field experiments. In these experiments exposures to elevated temperature and CO_2 concentration, to elevated ozone and elevated CO_2 concentration and to elevated ozone concentration and elevated temperature were combined. It is expected e.g., that combination of warmer growth temperature and doubled ambient CO_2 concentration could improve plant capacity to produce costly terpenoids for defense and other essential functions. However, monoterpene content of needles of Scots pine trees grown at elevated CO_2 and elevated temperature environments and their combinations in large closed-top-chambers was lowest in the combination treatment. Interestingly, analysis of monoterpene emission from the same combined CO_2 and temperature treatment indicated significantly increased monoterpene emissions compared to ambient conditions [19]. This observation suggests that increased monoterpene emission rates from conifer needles may indicate reduced storage in needles.

European aspen grown in field site in Finland with combined doubled atmospheric ozone levels and increased average temperature of +1 °C had a differential response in isoprene and monoterpene α-pinene emissions depending on genotype [22]. Isoprene emission was decreased at elevated temperature in one clone, but ozone exposure compensated for this decrease. Both clones had slightly reduced α-pinene emission rates at elevated ozone, but elevated temperature treatment compensated for the ozone effect.

A long-term large field exposure of North American deciduous trees to doubled atmospheric ozone and CO_2 in Wisconsin was studied relative to terpenoid responses. Elevated ozone reduced the gene expression coding the isoprene synthase activity and the amount of this enzyme in American aspen. Combining elevated ozone and elevated CO_2 treatments demonstrated that elevated CO_2 cannot compensate for this effect [23]. Both treatments combined or singly reduced isoprene emission from the ozone sensitive clone.

5 Ecological Functions of Terpenoids under Abiotic Stresses

5.1 Plant–Animal Communication

Plant-animal communication that occurs between the primary producers (plants), primary consumers (herbivorous animals) and secondary consumers (carnivorous animals) is one of the most intriguing and sensitive processes in ecosystems. In particular volatile terpenoids play an important role as chemical cues in these interactions below- and above ground and changes in the abiotic environment may disturb this interplay in different phases (Table 94.1, [24]).

5.1.1 Types of Cues and Signals and Signal Perception

Herbivores can typically sense suitable host plants using olfactory cues from long distance. Many volatile terpenoids bear the essential information in their molecular structure. Different stereo isomers of the same compound may result in different response when sensed by insect antennae or the olfactory sensors in the nose of vertebrate animals. Another important factor affecting signal perception and behavioral response in herbivore is the relative proportion of different volatile compounds, terpenoids or other volatiles, in the odor plume released by a plant. Combination of certain monoterpenes and sesquiterpenes are very distinctive in certain plant families. Specialist herbivore species can separate these combinations from similar monoterpenes released by other plants, because of their strict ratio in the host species.

Table 94.1 Types of chemical plant defenses against herbivorous animals, and the type of functions and advantages for the plant. Terpenes are functional in all type of defenses

Type of defense	Functionality	Advantages for the plant
Constitutive defense	Continuous production of defense compounds	Cost effective, if plant is continuously under herbivore attack
Inducible defense	Production of defense compounds is activated after herbivore damage	Cost effective, if there is not a great herbivore load
Direct defense	Defense compounds directly affect the damaging herbivore by reducing feeding (also termed bottom-up effects)	Direct defense can be constitutive or inducible
Indirect defense	Chemical compounds attract natural enemies of an herbivore to locate a suitable prey after attack (also termed top-down effects or multitrophic interactions)	Indirect defense is normally inducible and thus cost effective under a low herbivore environment. Effective only if natural enemies are within the range of spreading signals

If communication is based on the combination of extremely reactive and less reactive volatile compounds, the original ratio of compounds could soon be corrupted under environmental disturbances that affect reactivity of volatile terpenes. A signal could still be reliable for short distances, but in longer distance communication, the original proportion of individual compounds and the information they transmit may be lost.

5.1.2 Direct Herbivore Defense

Volatile terpenoids released by a plant can act as a repellent for herbivorous species which do not prefer the odor. To keep herbivore away before probing is a strategy to avoid occasional biting and induced damage, but repellents also give protection against plant virus diseases transmitted by many sucking insects. In many cases short stylet probing is sufficient for virus transmission from an insect to plant. The weakness of this strategy is that the same volatiles that repel most of the polyphagous species could signal the presence of the plant to certain potential specialist herbivores.

Orientation of herbivorous insects could be disturbed if environmental factors reduce synthesis and emission of repellent compounds or destroy the molecules soon after their release into the atmosphere. This will affect the efficiency of repellent compound to prevent herbivorous insect recognition of the plant for attack. Weakness of the volatile signals may reduce their repelling efficiency against generalist herbivore species. On the other hand, the pressure from specialist herbivore species could be reduced when their detection rate of suitable host plants is reduced.

Terpenoids sequestered on plant surfaces, such as glandular trichomes, resins on stem and bark and compounds bound in the epidermal waxes have the potential to prevent herbivory or other exploits. In particular, females of many fly and butterfly species walk on the surface of a herbaceous plant to taste the quality of plant to decide if it is a suitable host. These insects have contact chemoreceptor hairs in the tarsal segments of their legs for tasting. Insects making test bites for tasting can be deterred from feeding with high concentration of bad-tasting terpenoids in plant tissues.

Changes in environmental conditions affect nutrient availability and uptake by plants. Variability of nutrient availability and particularly nitrogen influences plant physiology and growth affecting available plant biomass for herbivores. An extensive meta-analysis [25] of resource availability effects on carbon-based secondary metabolites (CBSCs) in plant foliage demonstrated that concentrations of most CBSCs were reduced when nitrogen availability improved. Terpenoids did not follow this rule. Improved nitrogen availability increased concentration of monoterpenes and diterpenes in conifers. In general, CBSCs were not influenced by phosphorous availability from soil. Performance of herbivores is affected more by the nitrogen-carbon (N/C) ratio in the foliar tissue than by the actual concentration of terpenes and other CBSCs in the foliage, making the assessment of terpenoid effects on herbivores under nutrition deficiency condition difficult.

Elevating atmospheric CO_2 concentrations affect plant N/C ratio conversely to that of nitrogen fertilization, but in the same direction as nitrogen deficiency. Plants grown in enriched CO_2 atmosphere have "nitrogen dilution effect" leading to lower foliar nitrogen content than in plants grown in similar condition in the current ambient CO_2 concentration (ca. 390 ppm). This have frequently resulted in higher consumption rate and food intake by insect herbivores, their lower growth rate and often delayed developmental time and reduced pupal weight. Increased food uptake by herbivores also result in their increased exposure to CBSCs, including terpenoids, although terpenoid concentrations in green tissues could be decreased. In conifers increased resin flow in mature pine trees growing under elevated CO_2 and moderate soil fertility have been detected [26] This result suggests that under elevated CO_2 atmospheres conifers trees could become better protected against bark beetles, because excessive resin flow is a major defense system in conifers against bark beetles.

Elevated temperature is related to increased atmospheric CO_2. A meta-analysis of the combined effect of elevated CO_2 and temperature [27] on plant-herbivore interaction revealed that plant terpenoids are not significantly affected by CO_2, but increase of temperature or temperature in combination with CO_2 resulted in increased terpenoid concentration. In woody plants the woody tissues are more responsive than green tissues. Insect herbivore performance has been reduced at elevated CO_2 and improved at elevated temperature, but the combination of these treatments has mostly canceled these effects.

Phytotoxic ozone (O_3) has a similar increasing trend as CO_2 in atmosphere, but it is more variable spatially and appears in much lower concentrations (e.g., in range of 30 ppb–80 ppb). Impact of elevated O_3 on plant terpenoids and herbivore performance has not been studied as extensively as CO_2. A meta-analysis [28] of recent literature of combined O_3 and CO_2 effects on plants indicated that O_3 alone did not affect primary metabolites, but concentrations of terpenoids were significantly increased by 8% and combination with elevated CO_2 intensified O_3 impact. Although terpenoids were increased, elevated O_3 improved some indices of insect performance such as higher pupal mass and shorter larval development time, but these effects were counteracted by elevated CO_2.

As O_3 is a strong oxidant and it will affect terpenoid compounds found on the cuticular wax layer of plant leaves, even at the concentrations that do not penetrate in plant mesophyll through stomata. Waxes and their terpenoid content affect plant resistance against larval stages of some moth species, including the most globally-distributed pest of cultivated plants, diamondback moth (*Plutella xylostella*). Changes in triterpene composition of cuticular waxes have been found as a result of elevated O_3 exposures. In general, the combined effects of climate change factors on plant defense chemistry and ecology of herbivores could be very complex and generalizations are therefore difficult to make with any confidence.

5.1.3 Indirect Herbivore Defense

Volatile compound spectrum induced in plants by herbivore feeding is most often dominated by terpenoids. Among other common induced compounds are fatty acid-derived C_6 green leaf volatiles (GLVs) and their derivatives, phenylpropanoid aromatic compounds (such as methyl salicylate (MeSA) and indole), and certain alkanes, alkenes, alcohols, esters, aldehydes and ketones. These induced compounds emitted by plants are responsible for the attraction of natural enemies of herbivores. This "cry for help" with herbivore-induced VOCs is called indirect plant defense against herbivores [1]. The composition of volatile bouquet released by plants differs between inducing herbivore species and particularly between insects with different feeding habits. The emission profile after feeding by chewing insects could have more terpenoids than other compounds. Aphid feeding on the same plant species may result in methyl salicylate-dominated emissions.

Herbivore-induced volatile terpenoids have more functions in plant physiology, defense and modifiers of atmospheric properties beyond being carnivore attractants. The same compounds shown to be functional with only few plant–herbivore–carnivore combinations may have several other functions in the species interactions in natural communities; e.g., controlling growth of fungal pathogens or the behavior of hyperparasitoids attack on first order carnivores. Therefore, these compounds should be taken in the context of an infochemical web that overlays food webs of a community [29]. Volatile compounds known as herbivore-induced compounds can be induced also by fungal attackers, although the ratio of terpenoids and other compounds such as methyl salicylate could be different than for those induced by herbivores.

The mechanism of how climate change-related abiotic factors affect the efficiency of indirect defenses in plants is still poorly understood. The role of volatile emissions for indirect defense is crucial in terrestrial ecosystems. Currently, the research activity on plant volatile compounds and response to climate change factors is extensive. The research is driven to better understand the possible role of VOCs in global climate change. Abiotic factors related global climate change on volatile terpenoids and type of growth and physiological responses of plants on these stresses is summarized in Table 94.2.

There are very few reports of cases when indirect defense function of volatile terpenes has changed under climate change factors. Elevating temperature may increase the volatility of herbivore-induced terpenes, but not necessary their synthesis. If carbon is allocated more to terpenoids and isoprenes to mitigate temperature stress, their overproduction could negatively affect induction of sesquiterpenes and homoterpenes, and even result in reduced indirect defense.

Moth-damaged cabbage plants grown at elevated CO_2 (720 μmol mol^{-1}) had only minor reduction in the emission of homoterpene DMNT and sesquiterpene (*E,E*)-α-farnesene when compared to plants grown at ambient CO_2. However, this decrease was sufficient to disturb volatile signaling to the third trophic level. A generalist, the predatory pentatomid bug, and a specialist Braconid parasitoid of the moth were not able to discriminate between the odors of intact and moth-larvae damaged plants when both types of plants were grown at elevated CO_2. The same species preferred volatiles from damaged plants when plants were grown at ambient CO_2. [31]. On the contrary, when grown at elevated CO_2 oilseed rape

Table 94.2 Reported effects of abiotic factors on volatile, non-stored volatile terpenoids.

Abiotic factor	Plant physiological response	Terpenoid emission response
Elevated temperature (moderate)	Enhanced growth and metabolism, higher photosynthesis	Mostly increased, but no effect or decrease also reported
Short-term high temperature	Stomatal closure, inhibition of photosynthesis, heat shock protein formation	Increased
Elevated CO_2	Enhanced growth, stomatal response, decreased leaf area	Mostly decreased, but no effect or increased also reported
Elevated O_3	Oxidative stress, lesion formation and cell death, retarded growth, flower abortion	Increased emission upon induction and decreased atmospheric concentrations due to high reactivity
Long-term drought	Retardation of growth, decreased leaf area, enhanced stomatal closure, enhanced root growth, earlier abscission	Variable, depending on plant species
General nutrient deficiency	Reduced growth	Decreased to no effect
Nitrogen deficiency	Reduced growth	Increased to no effect
Salt stress	Stomatal response, osmotic effects	Decreased or increased
UV-B radiation	Impaired photosynthesis, reduced growth	Increased to no effect

References: [18, 24, 30]

plants increased their induced terpenoid emissions. Parasitoid wasps were able to orientate to host-damaged plants independent of plant type or CO_2 concentration.

Elevated O_3 reduces the concentration and shortens lifetime of herbivore-induced volatile terpenes in the atmosphere. This effect has influenced the behavior parasitoid wasps on genetically engineered oilseed rape plants for Bt toxin overexpression. Under elevated O_3, moth-damaged non-Bt plants attracted 75% of the parasitoids, but only 36.8% of parasitoids orientated to moth-damaged Bt plants compared with controls [32].

5.1.4 Pollination Biology

The attraction of floral colors for pollinating insects has long been known. Also floral scent from volatile compounds, including terpenoids, is an important cues for attracting pollinators. Pollinators sense flowers from long distance by odorants, whereas they use both visual and scent cues at closer distances from the target flower (Fig. 94.5). Both generalist and specialist pollinators use volatile cues [33]. Bees (*Apis* sp.) are especially adept at recognizing specific volatile bouquets [34], while moths rely more on longer range location strategies [35].

In addition to aromatic phenolic compounds and sesquiterpenes, the common monoterpenes linalool, β-myrcene and β-ocimene in flower scent have high signaling value for pollinators. Recently it has been calculated that as the result of their reactivity with ozone, these monoterpenes have had their signaling distance to bees

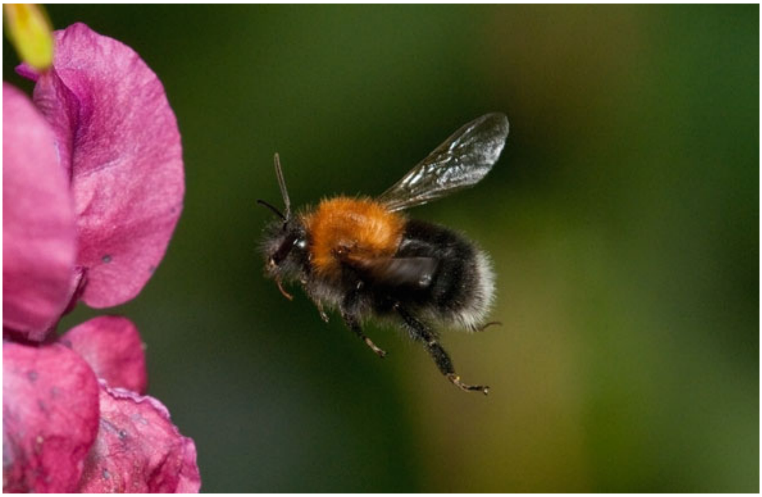

Fig. 94.5 Bees, bumblebees, butterflies and other pollinators use volatile terpenoids as cues when they orientate their flight towards flowering plants. Tree bumblebees (*Bombus hypnorum*) keep their antennae stretched out when approaching a flower to collect odorants

and other pollinators reduced from several kilometers in pre-industrial times to less than 200 m in the more ozone-polluted environments today [36]. Pollution causes them to lose their way en route to flowers, their food.

5.2 Plant–Plant Interactions

Plants are able to send warning signals of herbivore attack to their neighbors with herbivore-induce volatiles. Intact receiver plants can activate their defenses when exposed to these signals and thus be better prepared if herbivores will damage them. Recently [37] it has been shown that at elevated O_3 concentration of 80 ppb at an ozone-rich atmosphere can significantly degrade monoterpene β-ocimene and homoterpenes DMNT and TMTT released by spider mite-damaged lima bean plants. The decreased concentration of these terpene compounds in O_3-rich atmosphere reduced the distance over which signaling occurred between herbivore-damaged and intact plants. The measure of signaling response in receiver plants was induced production of extra floral nectar which is an arrestant and alternative food source for predatory mites.

5.3 Plant–Fungi Interactions

Various fungal species are able to synthesize and emit mono- and sesquiterpenes [38]. These can be detected from hyphae and fruiting bodies such as mushrooms.

Terpene production in fungi may be related to inhibition of growth of other microbes, but also for attraction of animals to spread the fungal spores. On the other hand, growth of hyphae and germination of spores of many plant pathogenic fungi is inhibited by high concentrations of volatile monoterpenes produced by host plant [39]. For the growth of most plant pathogenic fungi sufficient humidity is needed. Under hot and dry conditions fungal growth is inhibited by low humidity. Elevated temperature will increase volatility of plant monoterpenes and sesquiterpenes and may intensify growth inhibitory effects of the volatile plant terpenoids against fungal pathogens. In many plant species fungal infection induce similar bouquet of inducible volatiles compounds as herbivore feeding. Due to their microbial induction and antimicrobial effects the compounds induced by fungi and bacteria can be called phytoalexins. There is evidence the some inducible VOC could stimulate the sporulation of fungi that could be pathogenic to e.g., herbivorous spider mites. This tritrophic communication could be disturbed by VOC degrading effects of elevated O_3 concentrations.

5.4 Terpenes and Ecosystem Function

It has been shown that plant volatiles distributed in the environment have several functions in ecosystems. Many of the plant-emitted volatile terpenes have been reported to shape ecological interactions occurring among species within single or between multiple trophic levels. Recently reported adsorption of semivolatile sesquiterpenoids by birch foliage from a neighboring shrub species [9] indicated that up to 25% of the compounds emitted by an individual plant in the morning could be volatiles originally synthesized by other plant species in the same community. Because these compounds have a potential to give protection against herbivores by an associative way the results suggest that semivolatile terpenoids will have important role to affect plant species distribution and community level dynamics.

Plants are able to control oxidative capacity of atmosphere by the emissions of reactive volatile terpenes. The capability to control tropospheric ozone formation and degradation by reactive volatile emissions makes the major monoterpene emitters such as conifer trees, ecosystem engineers that can affect the atmospheric quality. If boreal conifer forests adapt to global warming by substantial increases in reactive monoterpene emissions they may have potential to add secondary organic aerosol formation in the forested areas. Higher aerosol density has been shown to increase light diffusion, promote cloud formation and increase cloud albedo and an overall cooling impact on climate. Increased diffusion of solar radiation has been shown to improve light penetration into conifer tree canopies. Finally, enhanced photosynthesis will improve CO_2 uptake within a conifer canopy and result in ameliorated carbon sequestration and higher net primary production of the ecosystem.

6 Conclusions and Future Prospects

Based upon the current knowledge of plant terpenoids reviewed here, we can be reasonably sure that factors related to global climate change will have an impact on terpenoid synthesis in plants and the dispersal of terpenoids in the atmosphere and soil. The ecological functions of terpenoids could be severely disturbed e.g., when atmospheric ozone degrades the important volatile information compounds such as monoterpenes and sesquiterpenes.

The efforts for more efficient food production to feed the increasing human population require new and efficient, environmentally sustainable and pollinator-friendly pest control strategies in crop plant protection. Terpenoids produced by crop plants or their companion plants could be one important tool to advance to that direction. Applications that minimize the harm for the environment and risk of development of resistant pest populations are needed. The principles of terpenoid-based natural indirect herbivore defense should be assessed more thoroughly to steer plant breeding to better include these properties in future cultivars.

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Hairy Root Culture: An Alternative Terpenoid Expression Platform

95

Jocelyne Trémouillaux-Guiller

Contents

1 Introduction 2942
2 Terpenoid Biosynthesis in Plants 2944
3 *Agrobacterium rhizogenes:* A Natural Engineer of Genetic Transformation 2948
4 Emergence and Development of Hairy Roots 2950
5 Ri-Plants Taken as Bioreactors 2950
6 Transgenic Plant Devoid of Hairy-Root Phenotype 2952
7 Novel Genes Revealed by Ri-T-DNA Activation Tagging and Transcriptome Analysis 2953
8 Engineering of Terpene Metabolism Pathways 2954
9 Hairy Roots: Fascinating Routes to Produce Terpenoids 2955
10 Elicitation Treatment: A Booster System of Secondary Products 2956
11 Hairy Root Cultures on a Large Scale: A True Challenge 2958
12 Therapeutic Merits of Plant Terpene/Terpenoids 2959
13 Conclusion 2961
References 2962

Abstract

A fantastic source of natural products encompassing the vast group of the terpenes, alkaloids, and phenolic compounds is provided by the plants. About 25,000 of these secondary metabolites hold a strong potential in a large domain of industrial and pharmaceutical applications. Plant families, such as *Lamiaceae, Asteraceae, and Taxaceae*, synthesize several terpenoid classes with a great economic value. Hairy roots, resulting from *Agrobacterium rhizogenes*–mediated transformation of plant cells, can be harnessed for terpenoid production on a large scale. *A. rhizogenes* over other transformation systems makes possible an

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easy regeneration of transgenic plants. Based on the deletion of *rol* genes located on the wild-type Ri-T-DNA, disarmed versions are created in order to overcome abnormalities of Ri-plants. Terpenoid accumulation from hairy roots can be optimized by elicitation treatment with jasmonates. Ri-T-DNA activation tagging system and transcriptome analyses are powerful tools to isolate new function genes involved in the terpenoid biosynthesis. Such genes encoding enzymes involved in the secondary metabolism can be inserted between the T-DNA borders. Lastly, some pharmacological properties of these plant terpenes/terpenoids involved in the therapy of more or less severe pathologies are reported.

Keywords

Activation tagging • *Agrobacterium rhizogenes* • Elicitation • Hairy roots • Metabolic engineering • Pharmacological properties • Ri-plants • Ri-T-DNA • Secondary metabolite production • Terpenes • Terpenoids

Abbreviations

2, 4-D	2, 4-Dichlorophenoxyacetic acid
Ac-MVA	Acetate mevalonate pathway
BABA	β-aminobutyric acid
BAP	6-Benzylaminopurine
CPPU	4-*N*-(2-chloro-4 pyridyl) –N′-phenylurea
DMAPP	Dimethylallyl pyrophosphate
DXP	Deoxyxylulose 5-phosphate
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
HMGR	3-Hydroxy-3-methylglutaryl CoA reductase
IAA	Indole 3 acetic acid
IPP	Isopentenyl pyrophosphate
Kin	Kinetin
MeJA	Methyl jasmonate
MEP	2-C-methyl-D-erythritol 4-phosphate pathway

1 Introduction

Plants represent most structurally vast and varied source of natural products, although less than half of the estimated 200,000+ secondary metabolites have been identified at the present time [1]. In nature, a plethora of phytochemicals have specialized functions in ecological interactions and are frequently produced in response to pathogen or abiotic stresses [2]. These natural secondary compounds can play a beneficial or deleterious role in the plant-insect, plant-pathogen, and plant-plant relations [3]. Owing to their large number and great diversity, the secondary metabolites hold an immense potential in industrial and/or medicinal application fields [4], notably as cosmetics, food additives, perfumes, and biopharmaceuticals for the human and animal healthcare [5]. Based on their biosynthetic origins, these secondary products can be divided into three major groups including the alkaloids (±12,000 types), phenolic compounds (±8,000 types), and terpenes (±25,000 types) which do not seem to be all directly involved in the development and growth of plants [4, 6]. The terpenes that are hydrocarbons exclusively composed of carbon and hydrogen and the terpene-derived terpenoids (or isoprenoids) by oxidation or some alteration of the carbon skeleton encompass more than 40,000 structures and constitute the largest and mostly diverse class of all known natural molecules [7]. Among them, a great chemical diversity is composed of primary metabolites (chlorophylls, carotenoids, cytokinins, gibberellins) and more than 25,000 secondary or specialized metabolites have harmful or beneficial effects on other organisms [3, 6]. Specialized terpenes/terpenoids have a myriad of important industrial applications notably, as flavoring agents, perfumes, insecticides, and biopharmaceuticals [8]. Higher plants biosynthesize commonly terpenes via two IPP (isopentenyl pyrophosphate) sources generated from two independent pathways: acetate-mevalonate (Ac-MEV) (in the cytosol) and 2-C-methyl-D-erythritol 4-phosphate (MEP) or deoxyxylulose 5-phosphate (DXP) (in the plastids) pathways (Fig. 95.1), present in vegetative tissues, flowers, and more occasionally in roots [9]. The early steps of terpene/terpenoid biosynthesis in the plants start by the formation and assembly of C5 isoprene units (1 isoprene unit = a monomer form) that result from two separate pathways. Polymerization of C5 isoprene units leads, for example, to monoterpenes C_{10} (2 isoprene units), sesquiterpenes C_{15} (3 isoprene units), and diterpenes C_{20} (4 isoprene units) [9] (Fig. 95.1). Indeed, terpene biosynthesis occurs, in the cytosol or the plastids, through the condensation of the five-carbon precursor isopentenyl pyrophosphate (IPP) (active isoprene unit) and its allylic isomer dimethylallyl pyrophosphate (DMAPP). The sequential head-to-tail addition of IPP units to DMAPP leads to the prenyl diphosphate geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). These three previous components serve respectively as precursors for sesquiterpenes, triterpenes (in the cytosol) and monoterpenes, diterpenes and tetraterpenes (in the plastids) (Fig. 95.1). Several terpenoids of a great industrial/medicinal interest are accumulated in the plant roots [10].

In the past three decades, hairy root cultures have been extensively investigated in vitro as a fascinating and alternative research tool for producing secondary metabolites at levels comparable to those found in the wild roots [11–20] and/or for revealing novel biomolecules [21, 22]. Hairy roots offer many advantages over cellular suspension cultures, notably their high growth rate, genetic stability, and hormonal independence [23]. Compared with transformed roots, undifferentiated cell cultures show most often a permanent instability responsible for low levels of secondary products and/or a rapid loss of the accumulation potential [24–27].

This chapter focuses on the terpenoid production from plant species, hairy root cultures, and Ri-plants; the terpenoid increase by hairy root elicitation; and recent strategies as T-DNA activation tagging/transcriptome analyses for isolating novel genes and hairy root engineering in order to improve terpene metabolism pathways. Lastly, therapeutic properties of some plant-derived terpenoids are reported.

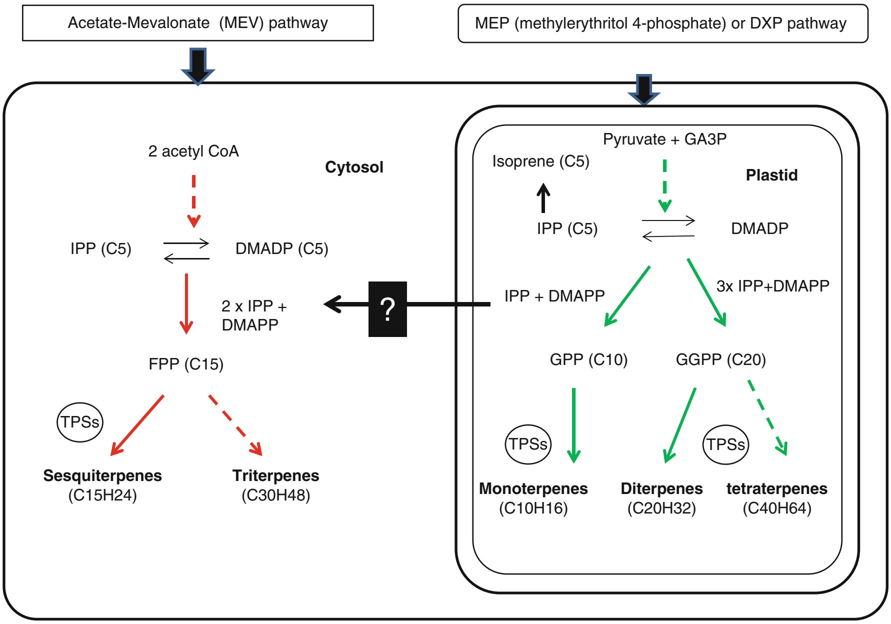
Acetate-Mevalonate (MEV) pathway
MEP (methylerythritol 4-phosphate) or DXP pathway
2 acetyl CoA
Cytosol
IPP (C5)
DMADP (C5)
2 x IPP +
DMAPP
FPP (C15)
TPSs
Sesquiterpenes
(C15H24)
Triterpenes
(C30H48)
?
Pyruvate + GA3P
Isoprene (C5)
Plastid
IPP (C5)
DMADP
IPP + DMAPP
3x IPP+DMAPP
GPP (C10)
GGPP (C20)
TPSs
TPSs
Monoterpenes
(C10H16)
Diterpenes
(C20H32)
tetraterpenes
(C40H64)

Fig. 95.1 *Terpene biosynthesis pathways and their subcellular localization in the plants*. Different classes of terpenes are respectively formed in the cytosol or the plastid by two independent pathways in the plants, that is, acetate-mevalonate pathway (MEV) (cytosol) and methylerythritol 4-phosphate (MEP) or deoxyxylulose 5-phosphate pathway (DXP) (plastid). Monoterpenes, diterpenes, and tetraterpenes are derived from IPP and DMAPP from the plastidial MEP or DXP pathway. Sesquiterpenes and triterpenes are biosynthesized from IPP and DMAPP from the cytosol pathway. *Black square* with a white question mark suggests a possible transport of IPP (isopentenylpyrophosphate) from the plastid to the cytosol. Other metabolites involved in the different steps are *DMAPP* dimethylallylpyrophosphate, *FPP* farnesylpyrophosphate, *GA3P* D- glyceraldehyde- 3-phosphate, *GPP* geranylpyrophosphate, *GGPP* geranylgeranylpyrophosphate. TPSs in the *circle* correspond to terpene synthases. *Broken arrows* show several enzymatic steps (Adapted from Aharoni et al. [8] and Sallaud et al. [154])

2 Terpenoid Biosynthesis in Plants

Plant species are capable of manufacturing different types of secondary products which can be harnessed by humans for their beneficial properties in a large domain of industrial or medicinal applications [4]. World Health Organization (WHO) estimates that up to 80% of people rely mainly on traditional herbs as remedies for their medicines [28, 29]. Extracted from entire plants, secondary products are used by food and pharmaceutical industries, although most often numerous natural plant-derived molecules remain undiscovered or unexplored for their pharmacological properties [1]. Nowadays, the distribution of terpenes/terpenoids in the nature is extensively studding [30] and numerous terpenoids, presenting tremendous industrial and therapeutic properties, have already been identified in perennial herbs or woody plant species (Table 95.1). A large number of highly efficient terpenes, including

Table 95.1 Different terpene/terpenoid classes synthesized via the Ac Mevalonate/MEV pathway + (in the cytosol) and via the MEP or DXP pathway ° (in the plastid) in some plant species that are known for their therapeutic properties

Terpene class	Plant species	References
Monoterpenes (C10H16)/monoterpenoids		
Menthol	*Mentha piperita* L.	[9]
Menthol-geraniol	*Anethum graveolens* L.	[124]
Monoterpene alcohols (Linalool, geraniol, nerol. . .)	*Vitis vinifera* L.	[32]
Essential oils (E.O)	*Centaurium erythraea* Rafn	[99]
Oxygened monoterpenoids (E.O.)	*Pelargonium graveolens* L'Her Ex Ait	[153]
Volatile terpenoids (Cavacrol, p-cymene)	*Ocimum basiculum* L.	[36]
Thymol; para-cymene; α, γ terpinene	*Ocimum gratissimum* L.	[35]
Volatile oils	*Panax ginseng* CA Meyer, *Prunus japonica*	[47]
Monoterpenoids	*Salvia sclarea* L.	[40]
Sesquiterpenes (C15H24)/Sesquiterpenoids +		
Sesquiterpenoids	*Solanum tuberosum* L.	[97]
	Solanum truncatula L.	[93]
	Solanum habrochaites	[154]
Sesquiterpene lactone/bilobalide	*Ginkgo biloba* L.	[155]
	Salvia sclarea L.	[140]
Sesquiterpene volatiles	*Vitis vinifera* L.	[31, 32]
Sesquiterpene lactone, with an endoperoxide bridge)	*Artemisia annua* L.	[129]
(Artemisinin)	*Artemisia indica* Willd	[42, 54]
Terpene aldehyde – sesquiterpenoid (gossypol)	*Gossypium barbadense*	[135]
Diterpenes (C20 H32)/diterpenoids °		
Ginkgolides/diterpene trilactones	*Ginkgo biloba* L.	[73, 155]
Abietane diterpenes – Diterpenoid: tanshinones	*Salvia scalrea* L.	[40]
	Salvia miltiorrhiza Bunge	[41, 119]
Complex diterpenoid: diterpene-alkaloid	*Taxus cuspidata* Sieb. &Zucc.	[57]
	Taxus media Hicksii	[58]
	Taxus baccata L.	[59]
	Taxus brevifolia Nutt Sieb	[56]
Diterpenoids:	*Panax ginseng* CA Meyer	[44]
Triterpenes (C30 H48)/Triterpenoids +		
Ginseng triterpenoid saponins – Ginsenosides	*Panax ginseng* CA Meyer	[49, 120, 158]
Saponins	*Salvia sclarea* L.	[40]
Saponins-glycyrrhizin	*Glycyrrhiza glabra* L.	[157, 161]
	Glycyrrhiza radix L.	
Dammarane-type saponin glycosides	*Gynostemma pentaphyllum* (Thunb.)	[50, 51]
Aralia saponins V	*Aralia elata* Miq	[103]

(*continued*)

Table 95.1 (continued)

Terpene class	Plant species	References
C-13-norisoprenoid – Lancemaside A	*Codonopsis lanceolata* Trautv.	[104, 160]
Triterpenoid glycosides (saponins) Escin	*Aesculus hippocastanum* L.	[43]
	Aesculus indica L.	[151]
Tetraterpenes (C40 H64)/Tetraterpenoids °		
Carotenoids	*Aesculus hippocastanum* L.	[42, 43]

monoterpenes and sesquiterpenes, play a significant role in the interactions of plants with other environmental organisms [8]; notably terpene volatiles, carrying out important information, permit the pollinators to locate flowers and protect reproductive tissues against diverse attacks [31]. Monoterpenoids, sesquiterpenes, and norisoprenoids of low molecular weights, which have commonly been identified as volatile compounds or volatile oils emitted from flowers, fruits, and leaves of numerous plants, possess important values, such as flavor, fragrance, and aroma compounds. Likewise, other terpenoid volatiles, mainly monoterpene alcohols, for example, linalool, geraniol, nerol, and terpineol, have been considered as flavor and aroma products of grapevine berries and vine [32]. The composition of floral volatiles, for example, pollen volatiles from several grapevine varieties serve as attractants for pollinators, but also may function to deter herbivore attacks and defend the flowers against the pathogen, desiccation, or UV-light damages. Likewise, sesquiterpene volatiles at the highest levels were detected in this floral organ of *Vitis vinifera* L. cv. Cabernet Sauvignon immediately before bloom and at bloom [31]. Aromatic herbs, such as *Ocimum basilicum* L. (sweet basil), *Thymus vulgaris* L., and *Ocimum gratissimum* L., of the family Lamiaceae are largely utilized because of multiple properties as distinctive aroma additives to food or as medicinal herbs [9, 33–35]. These aromatic oils, stored in the leaves, are composed of aroma monoterpenes (eugenol, thymol, carvacrol, and linalool) and exhibit potent antioxidant properties [36]. Essential oils, that is, green pesticides are an excellent alternative to synthetic pesticides in order to reduce negative impacts to human health and environment [37].

Plant species, belonging to different genera and synthesizing several under classes or types of terpenes/terpenoids, are succinctly described below (Table 95.1). The first genus *Salvia*, the largest genus of the family Lamiaceae (or Labiatae), distributed throughout the world, offers a great and cosmopolitan group of about 236 genera and 900–1,000 species [38] and is phytochemically characterized by their diterpene contents [39, 40]. *Salvia* species that are of widespread interest owing to their large spectrum of medicinal activities can be cultivated in several countries of Europe [41]. Turkey is one of the major centers of diversity for genus *Salvia*, with 93 species of which 54 % are endemic [38]. Two subgenera *Salvia* and *Sclarea* possess abietane diterpenes, whereas *Calosphace* has clerodane diterpenes and *Leonia*, abietane and clerodane diterpenes. Considered as compounds specific to this species the diterpenoid tanshinones of *Salvia miltiorrhiza* (Chinese sage), contained in the rhizomes, have received more attention [41]. The aerial parts of *Salvia sclarea* contain mostly monoterpenoids and sesquiterpenoids, whereas twelve diterpenoids and two triterpenoids have been identified in the roots of this species. Most of the diterpenoids, abietane or arranged abietane-type, show various biological activities [40]. Genus *Aesculus* belonging to Hippocastanaceae or Horse-chesnut family regroups 100 species or varieties. *Aesculus* sp. are deciduous trees and shrubs among which several species, such as *Aesculus hippocastanum, Aesculus chinensis, Abronia turbinata, and Acalypha indica*, have been cultured as pharmaceutical crops for providing Standardized Therapeutic Extracts (STEs) [42]. More than 210 biomolecules have been isolated from the genus *Aesculus* and identified as belonging to different terpenoid classes, triterpenoids, triterpenoid glycosides (saponins), and tetraterpenoids (carotenoids) [42, 43] (Table 95.1). Two Eurasian species, of which aescin and individual compounds have been identified and other *Aesculus* sp. exhibit potent antitumor, antiviral, antioxidative, anti-inflammatory effects. The root of *Panax ginseng* CA Meyer is largely used as traditional medicine since ancient time due to its stimulating and tonic properties [44]. Genus *Panax* (Araliaceae family), regrouping about 11 slow-growing species, that is, perennial plants with strong roots, has been used for therapeutic aspects for centuries. Among them *Panax japonicus* (Japanese ginseng), *Panax quinquefolius* (American ginseng), and *Panax ginseng* (Korea or Asia ginseng) [45, 46] are cultured in Japan, China, Korea, but also in the United States and Canada [47]. *P. ginseng* is the most required species for its restorative properties [48], and its roots contain active principles detected as ginseng triterpenoid saponins and ginsenosides (Rx), that is, saponin glycosides, the most prominent constituents of ginseng [49]. The chemical compounds of different *Panax* species are relatively similar and approximately 38 types of ginsenosides have been identified with variable levels according to the *Panax* species [46]. *Panax ginseng* is close to *Gynostemma pentaphyllum* because of the secondary metabolite production and pharmacological properties that its chemical entities confer on this plant species. *Gynostemma pentaphyllum* Thunb. or jiaogulan (Chinese name), a member of the family Cucurbitaceae and genus *Gynostemma*, is an oriental medicinal herb biosynthesizing numerous Dammarane-type saponin glycosides, and about 90 compounds known as gypenosides that have been isolated and considered as responsible for the pharmacological properties recently attributed to this species [50, 51] (Table 95.1).

Lastly, two other important genera regroup precious species biosynthesizing phytochemicals involved in the treatment of mortal pathologies. In first, the genus *Artemisia* is one of the largest of the family Asteraceae with more than 500 species and includes a large number of aromatic plants. Genus *Artemisia* is mostly composed of perennial plants and distributed worldwide, mainly across the temperate zones of the Northern Hemisphere, but a few species can also be observed on the Southern Hemisphere [52]. Overall *Artemisia* species are considered as herbs (*Artemisia annua* L., *Artemisia vulgaris* L.), subshrubs (*A. changaica* Krasch, *A. crithmifolia* L.), or shrubs (*A. tridentate* Nutt.) with strong aroma [52, 53]. Artemisinin, a sesquiterpene lactone with an endoperoxide bridge [42, 54], has been isolated from species *Artemisia annua* L., a model plant owing to its small genome, which is widely used to treat the malaria disorders [42] (Table 95.1).

Another important genus *Taxus*, composed of small coniferous trees and shrubs, belongs to the family Taxaceae including many species, such as *Taxus baccata*, *Taxus brevifolia*, *Taxus chinensis*, *Taxus Canadensis* or *Taxus cuspidata* or still *Taxus media*. These coniferous trees, relatively slow-growing, can reach between 1 and 40 m in length and produce red berry-like fruits. Known commonly as yews, these species are poisonous because most of the trees produce taxol. Taxol or Paclitaxel (its generic name) that was isolated in 1971 [55] from the bark of the yew tree (*Taxus brevifolia* Nutt) is a complex diterpenoid (diterpene alkaloid) possessing very strong antineoplastic efficiency due to its unique mode of action on the cell microtubules [56–58]. Considered as an alternative product to taxol, taxotere or its generic name "docetaxel" is a chemical entity, semi-synthetically via the conversion of non-cytotoxic molecules: baccatin III and 10-deacetylbaccatin III precursors of taxol extracted from the needles of *Taxus baccata* L., the European yew [59] which has provided an interesting and renewable source of taxanes with the similar action mode for treating the same variety of cancer pathologies.

In the last decades, numerous biotechnological investigations have made possible the establishment of hairy root cultures from the plant species, previously described, in order to produce terpene/terpenoids of pharmaceutically great interest.

3 *Agrobacterium rhizogenes:* A Natural Engineer of Genetic Transformation

Phytopathogen gram-negative soil bacterium, *Agrobacterium rhizogenes*, is able to infect wounded plant cells [60] for inducing a tumorous pathology, referred to as "hairy root syndrome" [61, 62] and revealed by the emergence of adventitious roots at or near to the inoculation site of the agrobacteria [63] (Fig. 95.2). Pathogenicity of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* depends respectively on a large and extrachromosomal Ti- (for tumor inducing) or Ri- (for root-inducing) plasmid carrying a DNA region termed Ti-T-DNA or Ri-T-DNA respectively, [64] that can be horizontally transferred and stably integrated into the nuclear genome of higher plants [62], as dicotyledon, gymnosperm, and some monocotyledon host species [65]. Ri-T-DNA harbors between its specific border sequences ($T_{R\ (R=\text{right border})}$ and $T_{L\ (L=\text{left border})}$) of 25-bp repeats, notably the T_L-DNA region carrying the root loci (*rol*) genes responsible for the hairy root phenotype. Ri plasmids, such as *pRi*A4 or *pRi*1855, belonging to the hypervirulent agropine-type *Agrobacterium rhizogenes* strains, possess a T-DNA formed of two independent regions [66] termed T_R-DNA and TL-DNA. The T_R-DNA region carries the aux1-2 genes that encode enzymes involved in auxin synthesis and play an ancillary role in the transgenic root formation [67]. Eighteen open reading frames (*Orfs*) have been identified from the TL-DNA region, among them the 10, 11, 12, and 15 *Orfs* correspond respectively to the *A, B, C*, and *D rol* genes [68]. Other genes, termed *ops* genes, are equally located in the T_R-DNA and code for opine synthesis, that is, particular amino acid derivatives serving for specific food to the bacteria [69–72]. Genetic transformation of recalcitrant species, as

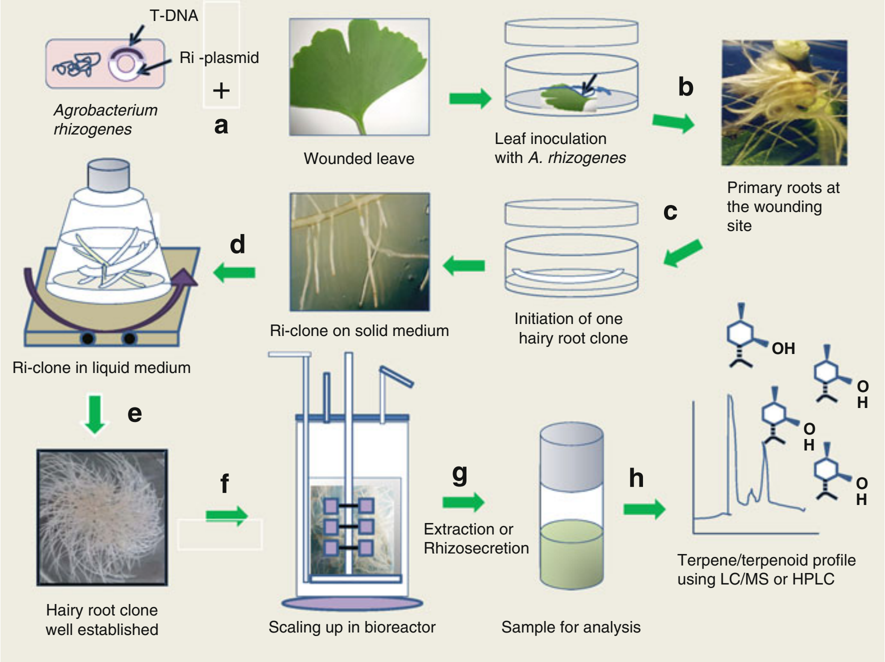
T-DNA
Ri -plasmid
+
a
Agrobacterium rhizogenes
Wounded leave
Leaf inoculation with A. rhizogenes
b
Primary roots at the wounding site
c
Initiation of one hairy root clone
Ri-clone on solid medium
d
Ri-clone in liquid medium
e
Hairy root clone well established
f
Scaling up in bioreactor
g
Extraction or Rhizosecretion
Sample for analysis
h
OH
OH
OH
OH
Terpene/terpenoid profile using LC/MS or HPLC

Fig. 95.2 *Establishment, culture on a large scale and terpene/terpenoid production from hairy roots.* (**a**) Both partners involved in the genetic transformation: *Agrobacterium rhizogenes* (a pathogen gram-negative soil bacterium) harbors a large Ri-plasmid carrying the wild-type T-DNA (*black arrows*) or an engineered T-DNA that can be transferred into the plant genome (*black arrow*) and one wounded leave. (**b**) After 48–72 h of co-culture with bacterium/plant cells, emergence of adventitious primary roots at or near the infected site. (**c**) Each primary root excised on the leaf explant and placed on solid medium grows vigorously for generating a true clone of hairy root (HR). (**d**) A crucial step corresponds to the transfer of the root tip segments of HR into the agitated liquid medium. (**e**) One well-established HR clone grows rapidly and shows the characteristics of the HR phenotype, that is, plagiotropic growth, high branching in hormone-free medium. (**f**) Scale-up of hairy root culture in specific bioreactor for commercial exploitation of valuable terpenoids. (**g**) Separation and identification of terpene/terpenoids by liquid chromatography/mass spectrometry (LC/MS) or by high performance liquid chromatography (HPLC)

Ginkgo biloba, required the use of hypervirulent agropine-type strains [73]. On the contrary some Ri plasmids, as *Ri*8196 or *pRi*2659, have a simple T_L-DNA capable of initiating hairy roots and producing different opine types (mannopine, mikimopine, or cucumopine) according to the *A. rhizogenes* strain [74]. Fundamental studies have improved our understanding of the different molecular events resulting in the T-DNA transfer through the host cytoplasm up to nuclear plant genome. Nevertheless, the final steps of T-DNA insertion into the plant genome, involving host factors, are still unknown. The Ti/Ri plasmids possess *vir* genes (*vir* for virulence) that encode Vir proteins responsible for the plant-bacterium attachment, formation, and protection of the simple and mobile copy of the T-DNA for its translocation from bacterium to the host nucleus [75–77]. Most *vir* genes, carried by the Ti/Ri plasmids and involved in the T-DNA secretion system, are similar. However, the Ri plasmid possesses GALL genes coding for two full-length GALLS (-FL and -CT) proteins, instead of the *vir*E2/*vir*E1 genes present in *A. tumefaciens* [78, 79].

4 Emergence and Development of Hairy Roots

In the previous decades, the strong power of *A. rhizogenes* to transform various plants and induce hairy roots has been largely demonstrated [71]. About 200 species of higher plants (at least 30 families), mostly dicotyledonous species, could be genetically transformed with *A. rhizogenes* and led to the development of adventitious roots [80]. This efficient mode of bacterial transformation is depending on several parameters, including the strain type, bacterial concentration, co-culture time of the bacterium/explant, temperature, light/darkness, as well as the plant species and explant type [81]. Different surface-sterilized explant types (i.e., leaf discs, leaves, stem segments, petioles, roots, embryos, or plantlets) are wounded and then inoculated with *Agrobacteria*. After 2–4 weeks primary adventitious roots, coming from the differentiation of one single plant cell transformed by one bacterium [62], appear at, or near, wound site of plant cells infected by *A. rhizogenes* [82] (Fig. 95.2). Roots, reaching about 1–1.5 cm in length, are excised and placed separately on solid or semi-solid hormone-free medium for initiating a true clone. Hairy root phenotype is characterized by a fast hormone-free growth, an unusual ageotropism, a profusion of root hairs with abundant lateral branching [83–85] (Fig. 95.2). Hairy root cultures can present more and less than root thickness, root length, number of root hairs and root branching, according to plant species and *A. rhizogenes* strain used for hairy root induction [86]. Sometimes, Ri-roots can become brown and stop their growth in liquid medium, because the most crucial step results from the transit of the hairy root clones into agitated liquid medium [87] (Fig. 95.2). But the morphology of *A. rhizogenes*–induced hairy roots is similar in structure to wild-type roots with a few notable exceptions [66]. A recent paper underlined that the A. *rhizogenes*–mediated transformation has made possible the establishment of hairy root cultures from 116 plant species [88].

5 Ri-Plants Taken as Bioreactors

A. rhizogenes–mediated transformation can be exploited for producing terpenoids using plants as bioreactors. Advantages of transformation with *A. rhizogenes* over other methods encompass adventitious organs/roots and rapid shoot regeneration reducing the risk of somaclonal variation [89]. Moreover, this transformation is that it enables the remarkable development of Ri-plants via a marker-free selection through the observation of the hairy root morphology, as the primary indicator of genetic transfer [90]. Lastly, another fundamental advantage from the wild-type *A. rhizogenes strains*, compared with the *A. tumefaciens* ones, is that the hairy root creation can naturally lead to the plantlets. Thereby such plants are free from the legal controls of GMOs in Japan [91]. One pioneered plant regeneration, performed successfully from *Brassica napus* hairy roots, displayed that fertile Ri-plants could sexually transmit the hairy root phenotype to their progenies [83]. Such a heredity of Mendelian type has recently been confirmed by Crane et al. [92] from Ri-plants of *Medicago truncatula*, an interesting legume, that emits a large spectrum of volatile terpenoids, including monoterpenes, sesquiterpenes, and tetranor-terpenoids, induced by herbivore attacks [93]. Studies reported that transgenic plants were regenerated from hairy roots of 62 different taxa, representing 53 plant species from 24 families [89] or still up to 79 plant species [94].

Besides, Ri-plants potentially can equally offer promising horticultural/floricultural applications. Classical breeding in floriculture serves to create new plant varieties, although the available gene pool for introducing novel agronomic or aesthetic features is genetically limited to the parental background [95]. Appeared after the *rol* gene insertion into the plant genome, cytological abnormalities make possible the creation of the novel floricultural varieties with exciting characteristics. For instance, Ri-regenerants of *Kalanchoe blossfeldiana*, an economically important plant in Europe, exhibited a dwarf and compact growth habit compared to an elongated growth habit, commercially disadvantageous, of the control plants [90].

An overview of the literature reveals that plant regeneration from hairy root segments can take, spontaneously, directly or indirectly (after a callusing phase before shoot regeneration) several developmental ways. Thereby, buds were initiated from adventitious Ri-root parts with or without exogenous growth regulators [66], for example in *Aesculus hippocastanum*, a plant species producing triterpenic glycosides [43], buds emerged from hairy roots placed on the solid medium devoid of exogenous hormones [96]. Nevertheless, the number of *A. hippocastanum* buds was found superior when the Ri-roots were placed on the medium supplemented with BAP. Rooting of these shoots was initiated more rapidly with IBA (in 80% of explants) than IAA (in 40% of explants) [96]. Another regeneration model was reported from genus *Solanum* which is one of the largest *genus* of family Solanaceae comprising about 84 genera and 3,000 species and producing numerous flavonoids and terpenoids, such as sesquiterpenoids in *Solanum tuberosum* [97] (Table 95.1). *Solanum nigrum* hairy roots, maintained on a hormone-free medium, developed spontaneously plantlets capable of producing in vitro flowers and fruits, whereas control plantlets remained in the vegetative state [89]. *Pelargonium graveolens* hairy roots, coming from Ri-T-DNA-mediated transformation of leaves and internodal segments, expressed their shoot regenerative capacity from both explant types [98]. *Centaurium erythraea* Rafn (*Gentianaceae*) is a traditional medicinal plant of Croatia that accumulates monoterpenoids in the aerial parts [99] (Table 95.1). *C. erythraea* hairy roots, placed in the medium added with IAA and BAP, formed shoots which were rooted at 68–97% without any hormonal supplement [100]. The Ri-plants regeneration could be associated to the formation of embryogenic calli [100]. *Panax ginseng* CA Meyer possesses numerous pharmacological activities, mostly attributed to one type of its constituents, namely, ginsenosides belonging to triterpenoid saponins [46] (Table 95.1). Placed on the medium without any growth regulator and in the dark, shoots and yellow calli in *Panax ginseng* appeared simultaneously from hairy root clones. Embryogenic calli, transferred to light, gave rise to somatic embryos leading to shoots/plantlets [101]. Transformed callus-derived embryogenic cell lines of *Panax ginseng* have been able to develop embryos and plantlets arising from only *A. rhizogenes rol* C-gene expression [102]. Root segment-derived hairy root lines of *Aralia elata*, a wood shrub producing pharmaceutically important metabolites as triterpenoids (*Aralia* saponins V) [103], and cultured on nutritive medium supplemented with 2, 4-D could generate embryogenic calli able to develop somatic embryos up to the cotyledonary stage without any phytohormone. Embryo germination led to plantlets and 100% of them were successfully acclimatized under greenhouse conditions [103]. Embryogenic calli, cotyledonary embryos and plantlets were developed from hairy root segments of *Codonopsis lanceolata* (Campanulaceae family) cultured in medium supplemented with 2, 4-D and accumulated, in the stems, higher contents of three major triterpenoids (lancemaside A, foetidissimoside A, and aster saponin Hb) than wild-type plants [104].

The third organogenesis type requires an intermediate callus phase before the formation of shoots and plantlets. Thereby, hairy root–derived calluses of *Mentha piperita* proliferated abundantly in the presence of 1 μM NAA, 10 μM CPPU and 10% coconut powder in the culture medium. Placed onto solid hormone-free medium *M. piperita* calli gave rise to shoots and roots [105].

6 Transgenic Plant Devoid of Hairy-Root Phenotype

Ri-Regenerants, arising from genetic transformation by the T-DNA insertion, exhibit frequently typical abnormalities due to the *rol* gene expression as wrinkled leaves, short internodes, dwarfness, numerous lateral shoots, flowers, and plagiotropic roots with an extensive branching [94, 106, 107]. Nevertheless, normal morphology of the leaves, a good stem elongation, and an extensive root system could be observed from Ri-regenerants of *Alhagi pseudoalhagi*, and similarly of *Hypericum perforatum* [108]. In contrast, transgenic somatic embryo-derived plantlets of *Aralia elata* exhibited an aberrant hairy-root phenotype compared to the mother plants [103]. This particular aspect of Ri-plants can be discouraging for initiating certain programs of applied research, notably, in the agronomy fields. Easy protocols have been established to overcome undesirable abnormalities of the Ri-plants by creating disarmed versions, as it has been achieved with *A. tumefaciens* [66]. The abnormal hairy-root phenotype can be suppressed after deletion of the oncogenes, that is, *rol* genes, located on the wild-type Ri-T-DNA and responsible for these morphological singularities. This deletion is performed without any consequence for the T-DNA insertion, since with exception of the right and left borders none of the other T-DNA regions are indispensable to the horizontal transfer of foreign genes into the host genome [109]. Therefore, the *Rol* genes carried by the wild-type Ri-T-DNA may be replaced by additional genes overexpressing secondary metabolites/recombinant proteins or encoding the resistance to herbicides, insects, and various pathogens [110]. Two vector types, co-integrative and binary vectors, can be used as genetic transformation systems [111]. A binary vector system contains, for example, the Ti plasmid carrying only virulence genes, whereas its T-DNA has been removed [112] and the second plasmid carries the foreign gene inserted between the border sequences of the Ri-T-DNA with or without deleting the *rol* genes [113]. Plants coming from leaf explants of *Centaurium erythraea* Raf co-cultured with the *A. rhizogenes* strain LBA 9402, carrying a binary vector, were reported by Piatczak et al. [100]. Using binary vectors, deleted oncogenes, the problem of the surprising hairy root phenotype may be bypassed.

7 Novel Genes Revealed by Ri-T-DNA Activation Tagging and Transcriptome Analysis

Hairy roots, coming from the genetic horizontal transfer of the wild or recombined Ri-T-DNA vectors in the host plant genome, represent an ideal engineering platform for exploiting new biotechnology tools (Fig. 95.2). DNA activation tagging [114] and RNA silencing processes followed by transcriptome analyses are recent powerful tools capable of revealing novel genes, notably those involved in the secondary metabolism, but also, of identifying gene function [114]. Activation tagging process permits to generate gain-of-function mutations, whereas gene silencing leads to loss-of-function approaches [115]. RNA silencing corresponds to a natural mechanism of genetic control taking place in virus resistance, genome maintenance, and developmental control in plants [116]. The classical loss-of-function process can be limiting because of the absence of visible phenotypes which restrict the identification of mutants [16]. Thus, one of the most frequently applied approaches is DNA-activation tagging insertional mutagenesis [117] which can circumvent many disadvantages of loss of function [44]. The *Agrobacterium* spp. T-DNA vector, carrier of multimerized transcriptional enhancers of the cauliflower mosaic virus (CaMV) 35 S promoter, is considered as a powerful mutagen [44] capable of discovering novel dominant or semi-dominant mutations observed in primary transformants [117]. Activation tagging system leads to a random genomic insertion, in the plant genome, of Ri-T-DNA constructs harboring constitutive enhancer sequences positioned next to the right- or left-hand T-DNA border, which are able to cause transcriptional activation of flanking genes [22, 117, 118]. For example, Choi et al. [44] generated activation tagged *P. ginseng* hairy root lines in order to dissect, isolate genes responsible for ginsenoside and triterpene lactone biosynthesis and evaluate the ginsenoside profile of these hairy root lines [119].

Another recent approach based upon the methyl jasmonate inducible system that was applied to hairy root cultures of *Panax ginseng* made possible the identification of new genes through the analysis of 3,134 expressed sequence tags (ESTs) [120]. From the isolated transcripts several would correspond to the genes encoding enzymes as Cytochrome P450, glycosyltransferases, and oxidosqualene cyclase (OSC) involved in the ginsenoside (i.e., triterpene glycosides) biosynthesis [120]. G*inseng* (or other plant species) ESTs data set can provide important information on genes involved in the regulation and biosynthesis of secondary metabolites, only, when their accumulation can be induced by MeJA treatment [120]. Besides, Jian et al. [121] emphasized the fundamental role of the *A. rhizogenes*–mediated transformation system coupling, at once, genetic transformation and regeneration for gene function testing in *Lotus corniculatus*.

8 Engineering of Terpene Metabolism Pathways

The fortification of numerous steps by overexpression of multiple biosynthesis genes or transcription factors for controlling the expression of genes is a fascinating strategy to alter the accumulation of secondary metabolites [122]. Metabolic engineering of terpenoids biosynthesized in plants can be directed toward two major objectives: to increase the plant resistance to predators and diseases or still to increase the production of medicinal or industrial compounds and to make possible a better knowledge of genes encoding enzymes involved in the terpenoid metabolism pathways or its regulation [8]. Understanding the function of genes involved in terpene production can lead to discovery of novel compounds or metabolic pathways, which might reveal favorable properties for human applications [4]. Thereby *Nicotiana tabacum* plants displayed an increase of terpene emission from leaves after insertion of monoterpene synthase genes [4]. The red pigment present in *Salvia militiorrhiza* (*Lamiaceae*) roots is composed of many diterpenoid tanshinones. In this plant, diterpenes are synthesized through the MEP pathway in the plastids (Fig. 95.1) and DXR (DXP (1-deoxy-D-xylulose 5-phosphate) reductoisomerase) is an enzyme catalyzing the first step of this pathway. Wu et al. have cloned and sequenced the gene coding for the DXR protein [41]. Functional expression of the *dxr* gene was controlled by genetic complementation with the lethal *dxr*-deficient *E. coli* mutant strain. Besides, the RNA-blot analysis, from hairy root cultures of *S. militiorrhiza*, elicited with a hyperosmotic stress and/or yeast extract, showed a strong induction of the *dxr* gene transcription that was correlated with the diterpenoid tanshinone increase in these cultures. The same authors concluded that the *dxr* gene involved in the MEP pathway may be a target for the metabolic engineering of diterpenoid tanshinone biosynthesis [41]. The regulation of terpenoid metabolism and, particularly, the regulation of factors controlling genes encoding enzymes of the secondary metabolism require subsequent investigations [123]. *S. militiorrhiza*, taken as a model plant owing to its small genome, made possible the identification of 40 terpenoid biosynthesis-related genes, of which 27 are novel. It was reported that 20 of the 40 genes could be involved in the tanshinone biosynthesis [124].

9 Hairy Roots: Fascinating Routes to Produce Terpenoids

Secondary metabolites of low molecular weights are widely biosynthesized throughout the plant Kingdom [122] and a large biological diversity of them is accumulated in the roots [85]. In the past few years, a great interest for the natural products led private companies to consider the progresses of the biotechnologies as an alternative strategy for producing biomolecules of high added value. Numerous plant species, genetically transformed with the natural vector *Agrobacterium rhizogenes*, are good candidates to accumulate terpene/terpenoids in organ culture systems [23, 40]. Important research investigations that use differentiated cultures, instead of cell suspension cultures, have been focused on transformed (hairy) roots [23] which offer a flexible and versatile system for a large-scale production of phytochemicals [125]. Hairy roots, because of their high differentiation, allow a stable and extensive production of secondary compounds [126]. Moreover, owing to their culture mode in a sterile, confined, and controlled environment avoiding the transgene dissemination, hairy roots present serious advantages compared with entire plants cultured in the open field [18, 22]. The transformed root cultures can be propagated indefinitely in liquid medium and conserve, at once, a morphological integrity and biochemical stability [86, 115]. Besides, the *rol* genes are now known to possess a stimulating effect on the secondary metabolism with a durable stability, over long-term culture [127]. After its insertion in the plant genome, the T-DNA appears to be a remarkable element in the gene regulation [128]. As described below, some hairy root cultures have shown their capacity to accumulate terpenes/terpenoids in vitro. Thereby, chemical analysis displayed that the contents of three major triterpenoids in hairy roots of *Codonopsis lanceolata* Trautv were similar to those found in wild roots harvested from soil [104]. Sterile 4-week-old shoots of *Salvia sclarea* transformed with the *A. rhizogenes* strain, LBA 9402, led to hairy root cultures from which four diterpenoids (ferruginol, salvipisone, aethiopinone, and 1-oxoaethiopinone) and two ursine-type triterpenoids were isolated [40]. The light enhanced significantly the abietane diterpenoid levels and affected slightly the triterpenoid spectrum in transgenic cultures. Kuzma et al. [40] concluded that *S. sclarea* hairy roots, accumulating terpenoids at higher levels than the roots of field-grown plants, could significantly provide a commercial source of diterpenoids. Hairy roots of *Artemisia dubia* and *Artemisia indica* arising from sterile plantlet-derived stems were transformed with both *A. rhizogenes* strains, LBA 9402 and 8196. Artemisinin levels and fresh root biomass were significantly enhanced in the Ri-roots compared with the control roots and according to the *A. rhizogenes* strain and *Artemisia* species used. Higher levels (0.603 % and 0.753 %) of this natural sesquiterpene lactone were detected in *A. dubia* hairy roots when the stems have been respectively inoculated either with LBA 9402 or 8196 *A. rhizogenes* strains [129].

Despite numerous positive results, some transformed root systems accumulated less secondary metabolites than plant tissues in vivo. The *A. hippocastanum* zygotic embryos are known to be the major site of aescin, a group of chemically related triterpenic glycosides (saponins) involved in the treatment of peripheral vascular disorders as venous insufficiency [43, 96]. Androgenic embryos of *Aesculus hippocastanum* (horse-chestnut), induced from anthers and inoculated with the *A. rhizogenes* strain A4GUS, led to hairy root clones [43]. The substantial percentages (3.50 % and 4.1 %) of aescin, detected in two hairy root clones, remained inferior to those found in the cotyledonary embryos placed on the hormone-free medium [43]. These authors concluded that the androgenic embryos of *A. hippocastanum* cultured with 2, 4-D is a better strategy for the aescin production on a large scale. Besides, Zdravkovic et al. [96] reported that the hairy root clones of horse chestnut were subsequently able to regenerate transformed plants which may be useful in horticulture for the bonsai establishment. Another tremendous advantage of hairy roots results from the feasibility of regenerating transgenic plants, whereas the regeneration of tissues transformed with *A. tumefaciens* is proved to be often more problematic. Overall secondary metabolites are biosynthesized and accumulated in the roots/hairy roots; nevertheless, some of them can be produced and/or stored in the aerial parts of the plant. Thereby, the total spectrum of secoiridoids that were detected in the non-transformed plants could be provided in vitro at eightfold superior levels in the roots and aerial parts of *Centaurium erythraea* Ri-plants [100]. Likewise, essential oils were mostly detected in the leaves and stems of *Mentha*, but the highest oil levels of *M. piperita* are found in the leaves of Ri-plants compared with those of the wild-type plants [130]. Moreover, the dwarf regenerants of *Mentha* species displayed the hairy root phenotype abnormalities, which does not present any negative impact on the essential oil production [105]. Most hairy root–derived rose-scented geranium plants (*Pelargonium* species) provided a large panel of monoterpenoids (essential oils) similar to those of the wild-mother plant, at the exception of two Ri-plants that biosynthesized two volatile monoterpenoids possessing better olfactory value offering an improvement from a commercial point of view [98]. Green hairy roots can accumulate some secondary products that are normally synthesized in green aerial parts of the plant [130].

10 Elicitation Treatment: A Booster System of Secondary Products

Plants are capable of producing and releasing active allelochemicals targeted in defense against pathogen attacks and stress conditions [2, 131]. Allelochemicals are accumulated under the impact of stress molecules, called elicitors, and then released by the plants into the environment by root exudation, volatile emissions from leaves and other aerial parts [132]. Several natural elicitors have been observed as efficient inducers for boosting the production and secretion of secondary metabolites in cultures in vitro, notably, when they are added to the hairy root cultures [115]. Thereby, the secondary plant messenger methyl jasmonate (MeJA), and its relatives, from the oxylipin family, is a stress hormone able to induce the improvement or optimization of secondary product contents [10, 18, 97]. In 1962, MeJA was discovered as a sweet-smelling biomolecule present in *Jasminum* *grandiflorum* L. flower extracts [133, 134] and described as “hormone” because of the cellular responses of elicitation, at low levels, distant from its site of biosynthesis. The jasmonate elicitors are synthesized via the octadecanoid pathways, beginning at linolenic acid and ending at (+) – 7- epi-JA and its conjugates and isomers [134]. There are some examples of elicitation processes for stimulating the secondary metabolite accumulation. Gossypol (sesquiterpenoid) is a toxic phytoalexin biosynthesized in *Gossypium barbadense* (a cotton plant) that possesses anticancer, antiviral, antimicrobial, and antiparasitic properties and is routinely isolated from cottonseed or from cotton roots. This terpene aldehyde interacts with various anticancer agents for increasing the efficiency of their pro-apoptic proteins, detoxification enzymes, and signaling kinases. Only MeJA was found to able to stimulate the contents of gossypol and methylated forms in cotton hairy root cultures. Moreover, the distribution of enantiomers was not found to be similar in elicited or control transformed roots of *G. barbadense* [135].

Taxol or Paclitaxel (generic name), a complex diterpenoid, is one of today’s better known antineoplastic drugs [56] and acts as a strong antitumor agent owing to its unique mode of action on the cellular microtubules. Its extraction from its natural source and costly synthetic process make it one of the products with the highest added value [58]. Taxotere or Docetaxel (generic name) is a chemical entity semi-synthetically performed via the conversion of non-cytotoxic molecules: baccatin III and 10-deacetylbaccatin III precursors of taxol extracted from the needles of *Taxus baccata* L., the European yew. Paclitaxel can equally be semi-synthesized from its natural precursor, 10-deacetyl baccatin III. As a promising alternative, cell suspension cultures of various *Taxus* species have been harnessed for the efficient production of taxol and its related taxane compounds [57]. The impact of explant source and growth regulators was tested in order to optimize the induction and selection of fast growing callus lines of *Taxus baccata* (European yew tree) [136]. In *Taxus baccata* L. (European yew tree), Ashrafi et al. [136] tested different combinations of growth regulators and various explants (stems, needles, and base of needle) for callus production. Taxol and baccatin III were respectively increased by 3.1- and 5-fold in the callus-derived cell suspensions cultured in medium added with 2, 4-D + Kin, at different concentrations according to both expected secondary metabolites [136]. *Taxus media* cell lines, transformed with *A. rhizogenes* and termed “TXS” and “Rol C” (*txs* corresponding to the transgene encoding enzyme taxadiene synthase of *T. baccata*), were treated with MeJA for optimizing taxane production. The highest taxane level was observed in the “TXS” cell line and resulted from the activity of enzyme taxadiene synthase and MeJA [58]. To date, the most favorable proceeding for a sustainable production of taxol and related taxoids at industrial level is provided by plant cell cultures [58]. Although production has been scaled-up, a serious drawback of the cell suspension cultures is their instability and unpredictability of the expected secondary products [137]. Due to their great genetic stability and potentially unlimited propagation in vitro, hairy roots as well-differentiated organs, are able to accumulate of important taxanes. For example, three strains of *A. rhizogenes* (R1000, A4 and 15834) were compared for the initiation of *Taxus cuspidata* hairy root lines. As regards its growth capacities, a selected line was treated with MeJA and produced a total paclitaxel content of 52, 5 mg/l over 2 weeks of incubation and at 20-L culture scale. These taxol levels produced after MeJA addition were similar to those detected in the cell suspension cultures of *Taxus* [57]. As another example, taxol and 10-deacetylbaccatin III (10-DAB III) were increased in hairy root cultures of *Taxus* x *media* var. Hicksii Rehd in presence of the BABA precursor, used alone or in combination with MeJA [137]. To date, only two reports have been published for the production of paclitaxel and its related compounds by hairy root cultures [137].

Other molecules, biotic or abiotic, can act as elicitors for successfully boosting the accumulation and/or secretion of secondary products. A non-protein amino acid, that is, β-aminobutyric acid (BABA), used against pathogen attacks and added separately or in combination with the yeast elicitor for enhancing the tanshinone production in *Salvia miltiorrhiza* hairy roots, showed that BABA could strongly potentiate elicitor-induced secondary metabolism in plant organ cultures [138]. From another experimental work, Ge and Wu [139] studied the separate and combined action of biotic (yeast=YE) and abiotic (Ag+) elicitors on the secondary metabolism activities of *Salvia miltiorrhiza* hairy roots. These authors showed that the YE-induced tanshinone production seemed to be mainly derived from a non-MVA pathway encompassing DXS (1-deoxy-D-xylulose 5-phosphate synthase) since YE did not stimulate HMGR (3-hydroxy-3-methylglutaryl CoA reductase). In contrast, Ag+-induced tanshinone production seemed to be dependent on both pathways (MVA and non-MVA pathways), because it was suppressed by the inhibitors of both pathways [139]. Elicitation, but, also cell permeabilization and trapping processes have been investigated to increase the secretion of secondary products in the culture media [18].

11 Hairy Root Cultures on a Large Scale: A True Challenge

Hairy root offers a flexible and versatile culture system that is a promising biotechnology for the large-scale production of valuable phytochemicals [125]. The transfer in bioreactors is the key step toward a commercial production of terpenes/terpenoids by hairy root cultures. Bioreactors offer several advantages for the mass culture of plant cells or organs, for example, a constant regulation of conditions at various stages of the culture [28]. To scale up hairy root cultures, several operational factors, including inoculum size, medium, growth rate, secondary product recovery (intracellular/extracellular), and reproducibility of results, must be overpowered before the technology is ready for commercialization [23]. Thereby a perfect sterility, maintenance of the homogenization of roots as well as biomass measurement, agitation, oxygenation, and nutrition availability are the major goals to reach for scaling-up hairy root cultures [23, 130, 140]. In fact, growth of transgenic roots in liquid medium results in dense and packed root biomass, leading to the formation of stagnation which slows down the fluid nutrient circulation and limits oxygen availability [86]. Mechanical agitation generates wounding of hairy roots and callus formation [130]. To overcome these limiting hurdles, hairy roots can predominantly be grown in spray or droplet bioreactors and mist bioreactors in which the roots are immobilized on mesh support and exposed to humidified air or a gas mixture and nutrients [86]. Recent bioreactors with their different advantages/drawbacks for producing hairy roots, on a large scale, have been detailed and classified by Srivastava and Srivastava [23] as liquid-gas, gas-phase, or hybrid reactors, for example, airlift bioreactors, bubble column reactors, and nutrient mist bioreactors. Conductivity measurements between small- and large-scale reactor cultures of hairy roots were compared [141]. Scaling studies showed that at 1 L *Artemisia annua* and *Arachis hypogaea* hairy roots had better growth in the mist reactor than shake flasks. The mass production of *Panax ginseng* hairy root cultures has been established in bioreactors [142] and the ginsenoside accumulation from transformed root cultures through large-scale reactor system was assessed to 1–10 t [28, 143]. Roots cultured in bioreactors have been shown to release therapeutically active compounds into the liquid medium of the bioreactor, for example, the antineoplastic drug isolated from various *Taxus* species [29].

12 Therapeutic Merits of Plant Terpene/Terpenoids

Numerous terpenoids, such as taxol, ginkgolides, digoxin, gossypol, and artemisinin, have been found efficient in the prevention and therapy of severe pathologies encompassing cancer and other severe diseases. Nevertheless, a vast proportion of higher plants have not still been screened for medicinal activities. Drug discovery from plant species remains an essential strategy in the search for future medicines [144]. From pharmaceutical point of view, terpenoids achieve a rich pool for harnessing new drugs and leading products. Cancer, after cardiovascular pathologies, is the second cause of death [145]. In fact, numerous terpenoids exhibit cytotoxicity against a wide variety of tumor cells as well as anticancer efficacy in preclinical animal models [146]. Terpenoids, a large class of mevalonate-derived phytochemicals, suppress the multiplication of neoplastic cells and the growth of implanted tumors; for example, geraniol, a volatile isoprenoid, is an acyclic dietary monoterpene which exerts an antineoplastic activity against different cancer cells both in vitro and in vivo. This antineoplastic property has successfully been assessed on TC-118 human tumors transplanted into Swiss nu/nu mice [145]. Likewise, it has been exemplified by diterpenoid drug taxol relating to the cancer [123]. Indeed, taxane biomolecules coming from genus Taxus represent one of the most powerful types of compounds among all the chemotherapeutic drugs efficient to treat a variety of cancers (encompassing ovarian, breast, lung, head and neck carcinomas, and the AIDS-related Karposi's carcinoma) [56, 57]. The antitumor property results of the binding of drug to the human β-tubulin, such a chemical interaction leads to inhibit the depolymerization of the microtubules [147]. Docetaxel (taxotere) and Paclitaxel (taxol) offer some different characteristics in their molecular pharmacology, which are potentially responsible for their clinical activity and toxicity profiles [136, 148]. Taxotere displays the greater affinity to β-tubulin, targeting centrosome organization and acting on cells in three phases of the cellular cycle (S, G2, M), whereas Taxol causes cell damage by altering the mitotic spindle at the beginning of the mitosis [57, 136]. Artemisinin (a sesquiterpene lactone) isolated from *Artemisia* sp. is an antimalarial drug efficient against multidrug-resistant strains of the malaria parasites: *Plasmodium* sp. [42], notably, *Plasmodium falciparum* that is the responsible agent for one of the world's most severe pathologies causing at least 500 million cases globally each year and resulting in more than one million deaths [149].

Pharmaceutical properties of several plant species can be due to the association of terpene/terpenoids with other active molecules. That will explain the large spectrum of therapeutic activity of these plant species. *Gynostemma pentaphyllum* (Thunb.) or jiaogulan (Chinese name), a member of the family Cucurbitaceae and genus *Gynostemma*, is an oriental medicinal herb characterized by a large spectrum of pharmacological activities, of which antitumor, antioxidant, cholesterol-lowering, immunopotentiating, and hypoglycemic effects could be observed [50]. Genus *Aesculus* (Horse chesnut) encompasses more than 210 compounds from which different classes have been isolated, in particular triterpenoids, triterpenoid glycosides (saponins), flavonoids, and long fatty chain molecules [42]. Standardized therapeutic extracts (HCSE: Horse chesnut seed extract) and aescin (a saponin mixture) of *Aesculus* sp. are considered as the main source of triterpenic glycosides, a group known as aescin and traditionally used against rheumatism, skin, and peripheral vascular disorders [43]. In Europe, the bark, leaves, HCSE, and aescin have been used in the treatment of chronic venous insufficiency, hemorrhoids, and postoperative edema [42]. Several other medicinal properties have been ascribed to saponins, such as immunostimulant, hypocholesterolaemic, and anticarcinogenic effects [150]. But, one of the most interesting properties is the antitumor action of some novel saponins found recently from the *Aesculus pavia* species [42]. The *Aesculus indica* extract, used against the MCF-7 breast cancer cell line, displayed a significant antiproliferative activity, and dose-dependent effect ranging from 34.2 % (at 10 μ-g/ml) to 94 % (at 500 μ-g/ml) could be observed [151]. Inflammation is a natural response of the mammalian body to a variety of pathologies or hostile agents. Rheumatoid arthritis is one of the challenging diseases associated with inflammatory manifestations. In order to treat similar disorders, various molecules have been isolated as, for example, a potent anti-inflammatory analgesic molecule termed "Aspirin" which was harnessed from bark of *Salix alba* L. Recent findings of several species such as *Achillea millefolium* (Asteraceae) or *Aspilia Africana* C.D. Adams (Asteraceae) and *Bacopa monnieri* (Scrophulariaceae) showed an anti-inflammatory activity due to their chemo-profiles revealing the presence of essential oils/sesquiterpenes, terpenoids (α-pinene, limonene), and triterpene, respectively [152]. Aromatic oils, stored in the leaves, are composed of aroma monoterpenes, such as eugenol, thymol, carvacrol, and linalool, and exhibit potent antioxidant properties which may prevent in vivo oxidative damages such as lipid peroxidation associated with cancer, premature aging, atherosclerosis, and diabetes [36]. Besides, the essential oils, that is, green pesticides, are excellent alternatives to synthetic pesticides in order to reduce negative impacts to human health and the environment [37], notably the essential oils of *Pelargonium graveolens* showed insecticidal effects with a maximal mortality rate of 100 % [153].

Since ancient times, genus *Salvia* (*Lamiaceae*) has been used throughout the world, owing to its large spectrum of beneficial effects, such as its antineoplastic, antiviral, cardioactive, antidiabetic, and antiplasmodial activities [39, 40]. Among these species, *Salvia miltiorrhiza* (Chinese sage) with a rhizome possessing medicinal powers. Different molecules of *S. miltiorrhiza* (or Danshen in Chinese) are widely recommended in modern and traditional medicine for the treatment of various disorders, for example, blood-circulation diseases. The red pigment of its roots is mainly composed of numerous diterpenoid tanshinones, such as tanshinone IIA, major bioactive molecules of the *Salvia* species offering numerous pharmacological activities notably antineoplastic effects [41]. Tanshinone IIA induces apoptosis and inhibits proliferation, migration, and invasion of a number of neoplastic cell types belonging to hepatoma, osteosarcoma, leukemia, prostate cancer, gastric cancer, breast cancer, colon cancer, and glioma. Besides, the prevention and treatment of cerebral infarction by *S. miltiorrhiza* Bunge involves several action modes, including anti-atherosclerosis, anti-hypertensive, anti-platelet aggregation, anti-inflammatory, and anti-oxidative effects. Plant-derived natural products have provided many bioactive terpenoids, some of which have led to important pharmaceuticals available on the market or promoted in clinical trials by the emerging "biotech" companies [144].

13 Conclusion

Plants hold a strong potential for pharmaceutical and industrial applications, resulting from the great richness in secondary metabolites, notably in terpenes/terpenoids which represent the largest group of natural products. Potentially terpenoids/terpenes remain to be identified in numerous plant species, known or still unexplored. Plant transformation by *Agrobacterium rhizogenes* leads to the formation of hairy roots, that is, of well-differentiated roots presenting at once a high genetic stability, an unlimited propagation, and spontaneous regeneration in media devoid of growth regulators. Hairy roots and *A. rhizogenes*–derived plants are potentially promising bioreactors which can be used for producing biomolecules of high added value. Ri-plants make possible the production of secondary metabolites exclusively accumulated in the aerial parts of the plant. In the *Catharanthus roseus* leaves, the final step of terpenoid indole alkaloid (TIA) biosynthesis leads to vinblastine and vincristine (bisindole alkaloids), whereas their two precursors are produced in the roots. Moreover, Ri-regenerants can allow the creation of new varieties in horticulture/floriculture. Bacterial Ri-T-DNA, responsible for the genetic transformation, is a true secretion system based on the genetic horizontal transfer of foreign genes into the host plant genome. Thereby, hairy roots open large avenues for engineering terpenoid metabolism pathways or revealing novel genes by recent approaches as T-DNA activation tagging or MeJA-inducible system following transcriptome analyses. Nevertheless, due to the great complexity of these pathways, numerous terpenoid biosynthesis-related genes are always to be isolated and identified in plants. Overall, hairy roots produce terpenoids/terpenes at levels comparable to or higher than those found in the wild roots. The cultures of hairy roots assure serious safety advantages compared to those of plants cultured in the open field. Biotic or abiotic elicitors have been used to enhance terpenoid contents. Most often methyl jasmonate was found to be efficient to improve the secondary metabolite synthesis. Nevertheless, the major challenge for commercial exploitation of the hairy roots is scaling up at industrial level. Based on the cell suspension culture biomass concept, specific reactors have been altered to overcome the hurdles limiting the hairy root culture on a large scale. Nowadays medicinal plants provide therapeutically active terpenoids for treating more and less severe pathologies. A vast pool of higher plants biosynthesizing secondary products remain again to be identified. Certain biomolecules of known plants have not been screened for therapeutic properties. In the near future, drug discovery from plant species is a crucial investigation to treat, notably, severe pathologies.

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Part XI

Terpenes: Classes - Occurrence, Biosynthesis, Structure and Chemistry, Distribution

Regular Monoterpenes and Sesquiterpenes (Essential Oils)

96

Remigius Chizzola

Contents

1 Introduction 2974
2 Occurrence 2975
3 Phytochemistry 2977
3.1 Isomers and Stereochemistry 2977
3.2 Classification 2979
3.3 Rearrangements and Transformations 2989
4 Biosynthesis 2991
4.1 Biosynthesis of the Isoprene Units 2991
4.2 Biosynthesis of Monoterpenes 2992
4.3 Biosynthesis of Sesquiterpenes 2992
5 Biological Activities 2992
5.1 Aroma Impact 2994
5.2 Antimicrobial Activity 2994
5.3 Anthelmintic Activity 2997
5.4 Anticancer Activity 2997
5.5 Antioxidant Activity 2997
5.6 Other Activities and Applications 2998
5.7 Toxicity 2998
5.8 Allergenic Properties 2999
5.9 Ecological Functions of Volatile Mono- and Sesquiterpenes 3000
6 Biotechnological Approaches 3001
7 Conclusion: Prospects 3002
References 3003

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Abstract

Monoterpenes resulting from the combination of two and sesquiterpenes from three branched, unsaturated C_5 units (isoprene) represent a large class of natural products with a wide range of biological activities. They include unsaturated hydrocarbons and their oxidation products as alcohol, aldehydes, ketones, and rarely ethers. Volatile compounds are constituents of essential oils that are accumulated by numerous plants in special structures in the tissue. Besides the conspicuous aromatic properties, a wide range of biological activities has been documented allowing a wide field of applications. In an ecological context, mono- and sesquiterpenes play an important role in the relations between organisms, for example, as attractants of pollinators or deterrents of herbivores. On a large-scale, monoterpene emissions from vegetation in nature can have ecosystem-wide influences. The further investigation and documentation of this high biodiversity and its sustainable use remains a promising task. This requires the further development of analytical and production techniques and the exact definition and characterization of the plant sources.

Keywords

Aroma compounds • Biological activity • Essential oil • Monoterpene • Sesquiterpene

1 Introduction

Terpenoids represent a large class of natural products with numerous biological functions. Although terpenoids are usually regarded as secondary (plant) products, some of them are widespread and are involved in basic biological processes as electron transport in mitochondria and plastides. The name terpenoids is derived from turpentine, the aromatic resinlike exudations obtained from various coniferous trees. The common feature of all terpenoids is that they are built up by branched C5 units, isoprene (= 2-methylbutadiene). Terpenoids derived from just one isoprene unit are called hemiterpenes.

Monoterpenes are built up by two isoprene units, sesquiterpenes by three. Figure 96.1 shows how two isoprene units may be joined together.

The most common linkage is the head-to-tail binding that results in the regular monoterpenes. Irregular monoterpenes display a tail-to-middle binding displaying a cyclopropane structure as, for instance, in the pyrethrins, natural insecticides from *Chrysanthemum* species. Tail-to-tail bindings occur in diterpenes as squalene where two monoterpenes are linked together [1].

Iridoids are monoterpenoids with a cyclopentane fused to a tetrahydropyran ring. In secoiridoids, the cyclopentane ring is opened. Most of these compounds are nonvolatile glycosides and will be reviewed elsewhere.

This chapter has its main focus on mono- and sesquiterpenes as volatile compounds in essential oils. Since the mid of the 1990 as reliable and easy to operate

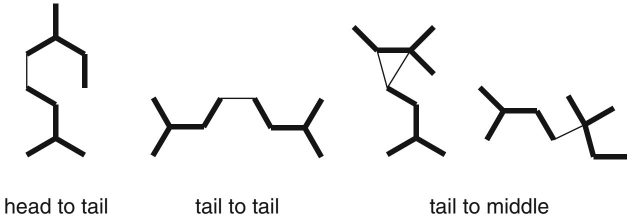


Fig. 96.1 Linking isoprene units together

gas chromatography–mass spectrometry systems became widespread, a great wealth of data on essential oils and volatile substances from all biological matrices has been generated and has become available in respective journals (e.g., Flavour and Fragrance Journal, Journal of Essential Oil Research, Journal of Essential Oil Bearing Plants, and Natural Product Communications) and handbooks [2–5].

Essential oils are complex mixtures of volatile constituents isolated from the plant material by hydrodistillation or steam distillation or expression in the case of citrus fruits. Extracts with apolar solvents yielding also the apolar terpenoids should not be designated as essential oils. Besides mono- and sesquiterpenes, essential oils may contain nonterpenoid hydrocarbons, phenylpropanoids, esters, lactones, phthalides, nitrogen- or sulfur-containing constituents, and isothiocyanates [5].

Plants rich in essential oils are mainly found in species of the families Apiaceae, Asteraceae, Cupressaceae, Lamiaceae, Lauraceae, Myrtaceae, Pinaceae, Piperaceae, Rutaceae, Santalaceae, and Zingiberaceae. As lipophilic compounds, essential oils are accumulated in particular structures as oil cells, secretory ducts, or glandular hairs. Frequently, the essential oils are associated with resins or gums.

Additionally, monoterpene glycosides have been reported in some plants. They are regarded as precursors of the volatile oil components [6]. As an example, among the glycosidically bound volatiles isolated from *Juniperus communis* were the monoterpenoids thymoquinone and p-cymen-8-ol [7].

The most conspicuous characteristics being their lipophilic and aromatic properties, essential oils are used as flavors and fragrances with a wide range of biological activities and applications.

2 Occurrence

Mono- and sesquiterpenes are produced by a wide range of organisms. The highest diversity has been recorded in higher plants, but terpenoids are also synthesized in bacteria, fungi, algae, and animals including marine organisms. Some prominent examples are given below:

Fungi: Among the odor compounds found in fungi, various oxidized sesquiterpenes have been reported from the mushroom *Lactarius hatsudake* in Japan [8].

As nonvolatile compounds, trichothecenes are prominent sesquiterpene mycotoxins produced mainly by *Fusarium*, *Trichoderma*, *Stachybotrys*, and

Myrothecium species. These compounds bear a tetracyclic structure with a 9,10 double bond and a 12,13-epoxy group. There are 8-hydroxytrichothecenes (e.g., T2 toxin), 8-ketotrichothecenes (e.g., nivalenol), and macrocyclic diesters [9].

Various marine organisms have been reported to produce terpenoids often with unique structures including monoterpenoids, sesquiterpenoids, sesquiterpene isonitriles, and mero-sesquiterpenoids. The latter are formed by condensation of a terpenoid with a nonterpenoid aromatic moiety [10]. Marine algae (sea weed) are living in an environment presenting a high availability of halogene ions as chloride and bromine. From these organisms, various halogenated terpenoids have been described [10].

Monoterpene hydrocarbons were produced and emitted by marine phytoplankton including α-pinene, β-pinene, myrcene, camphene, sabinene, and *p*-ocimene [11].

Liver mosses: the chemistry of liver mosses has been studied intensively during the past years. Among the volatile components, a range of terpenoids, often with a unique structure, have been reported [12]. (−)-Bicyclogermacrene has been identified as a constituent of various liverworts such as *Plagiochila asplenioides*, *Diplophyllum albicans*, and *Mylia* species [13].

Mosses: Among 38 volatiles identified by HR-SPME and GC/MS in the bryophyte *Rhodobryum giganteum* were α-pinene, limonene, camphor, and α-farnesene [14]. Besides various nonterpenoid volatiles, a wide range of mono- and sesquiterpenes have been found in the essential oils from the mosses *Tortula muralis*, *Homalothecium lutescens*, *Hypnum cupressiforme*, and *Pohlia nutans* [15].

Higher plants: Most of the reported mono- and sesquiterpenes origin from higher plants where a high diversity of these compounds has evolved. Examples of the occurrence are given below along with the presentation of the various compounds. The production of terpenoids and the accumulation of essential oils are governed by a range of factors as physiological variation concerning the plant development, type of secretory structure, seasonal variation, mechanical or chemical injury, or environmental factors as climate, diseases and pests, and edaphic factors [16]. Terpenoid pattern may be different in the various plant parts. As an example in *Laser trilobum*, limonene and perillaldehyde are the main compounds in the fruits whereas in the leaves and stems bornyl angelate dominated [17]. Often higher proportions of sesquiterpenoids as compared to monoterpenes were found in leaves than flowers and fruits. The pattern of oil components may change dramatically during fruit development as in the case of bitter fennel (*Foeniculum vulgare*) where with the formation of the fruit from the flowers, the percentages of fenchone and estragole in the essential oil increased while limonene, α-and β-phellandrene, and fenchyl acetate decreased [18]. Also within a plant, leaves of different age inserted at different heights of the stem may display distinct essential oil compositions. For instance, in oregano (*Origanum vulgare* ssp. *hirtum*), the *p*-cymene content was higher in the lower leaves and the carvacrol and γ-terpinene content were higher in the upper leaves [19].

Plants from a given species but of different origin may display quite different patterns of secondary compounds. These chemically different types that usually cannot be differentiated by morphological characters are called chemotypes.

The occurrence of chemotypes is quite frequent in essential oil-producing plants. For instance, in thyme (*Thymus vulgaris*), at least six different chemotypes according to the main monoterpenes in the essential oil are known [20, 21]. The respective monoterpenes are limonene, 1,8-cineol, linalool/linalyl acetate, sabinene hydrate, geraniol, thymol, and carvacrol.

The delimitation of chemotypes is often difficult. For practical reasons, often the distinction is done according to the main oil compounds, but the biogenetic pathways should also be considered.

Among the nonvolatile terpenoids, there is also a great variability of iridoids mainly in the families Apocynaceae, Gentianaceae, Lamiaceae, Loganiaceae, Plantaginaceae, Rubiaceae, Schrophulariaceae, Valerianaceae and Verbenaceae and of sesquiterpene lactones, especially in Asteraceae [9].

Some mono- and sesquiterpenoids are also found in animals. The most prominent examples are defensive secretion of insects. Iridoids are present in various beetles and cantharidin, a monoterpene derivative with skin irritating properties, in meloid beetles. Bitter tasting iridoid glycosides are found in some caterpillars. Also, the toxins of termites contain mono- and sesquiterpenes [9].

Anthozoa: Recently, 15 new guaiazulene type sesquiterpenes have been described in a Chinese gorgonian *Anthogorgia* species [22].

Mollusks: From *Aplysia kurodai* (Cyclopteridae), the monoterpene halogenated cyloethers aplysiapyranoid A–D have been isolated [9].

3 Phytochemistry

As mono- and sesquiterpenes contain 10 or 15 carbon atoms arranged in various branched and often cyclic structures, the chemical systematic nomenclature according to IUPAC [23] results in complex names that are used in handbooks and databases. For a practical use for frequently occurring or used compounds, common names have been applied, mostly derived from prominent plant sources, as pinene from *Pinus*, the genus of the fir trees or eucalyptol from the eucalyptus oils.

3.1 Isomers and Stereochemistry

The presence of double bonds and the possibility of an oxygen atom at different positions in the molecule account for the occurrence of isomers. There are structural isomers and frequently stereoisomers. Structural isomers as well as *cis–trans* isomers are usually separated in gas chromatography with standard columns, the method of choice for the analysis of essential oils. However, the separation of enantiomers needs specific chiral columns based on cyclodextrins. Therefore, in most publications where these columns were not used, there is no differentiation between the enantiomers.

Enzymes producing the final monoterpene are often stereospecific, so that in many cases only one of the two possible enantiomers is produced or predominates.

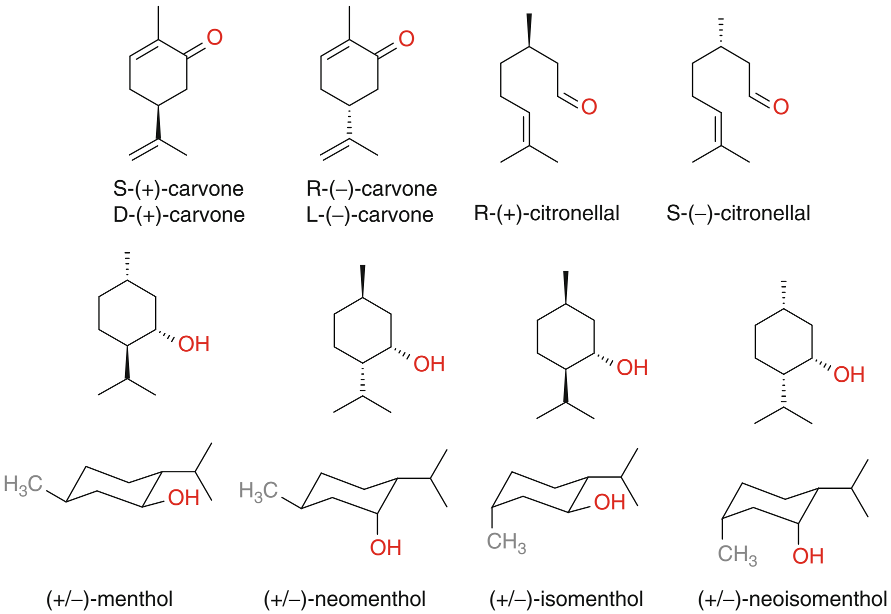

O
O
O
O
S-(+)-carvone
D-(+)-carvone
R-(–)-carvone
L-(–)-carvone
R-(+)-citronellal
S-(–)-citronellal
OH
OH
OH
OH
H3C
OH
H3C
OH
OH
CH3
CH3
OH
(+/–)-menthol
(+/–)-neomenthol
(+/–)-isomenthol
(+/–)-neoisomenthol

Fig. 96.2 Examples of isomers occurring in monoterpenes

Table 96.1 Ratio of R-(+)-citronellal to S-(−)-citronellal in various essential oils with citrus aroma [24]

Plant species	%[a]	(+)-citronellal (%)	(−)-citronellal (%)
Melissa officinalis	3–31	97–99	1–3
Cymbopogon nardus	1.1	85	15
Eucalyptus citriodora	71.2	56	44
Litsea cubeba	0.3–0.9	33–53	47–67
Nepeta cataria var. *citriodora*	0.4	0.1	99.9
Backhousia citriodora	0.1	17	83

[a] Percentage of citronellal in the essential oil

The following examples illustrate the significance of stereoisomers in this substance group (Fig. 96.2).

The two carvone enantiomers are a prominent example. They differ in optical rotation, circular dichroism, and aroma impact. S-(+)-carvone with typical caraway odor occurs in the fruits caraway (*Carum carvi*), whereas R-(−)-carvone is a minty aroma component in spearmint (*Mentha spicata*).

Table 96.1 presents the ratio of R-(+)-citronellal to S-(−)-citronellal in essential oils of various origin. The nearly exclusive occurrence of the (+)-form is a characteristic of lemon balm (*Melissa officinalis*) oils and can be used as criterion to detect adulterations [24].

Menthol composed of a cyclohexane ring substituted with a methyl- and an isopropyl group in para-position and an OH group displays three carbons centers with different ligands so that eight stereoisomers in four pairs of enantiomers exist (Fig. 96.2).

Humans were able to differentiate the odors of the (+) and (−) enantiomers of α-pinene, carvone and limonene but failed to differentiate between (+)- and (−)-menthol. Both menthol enantiomers have a peppermint odor quality but (−)-menthol has a much stronger cooling effect than (+)-menthol [25]. S-(−)-limonene has a turpentine odor impact and R-(+)-limonene an orange odor impact [25]. Neomenthol, isomenthol, and neoisomenthol do not present a cooling effect.

The enantiomeric distribution of chiral monoterpenes may greatly vary with the individual compounds. For instance, in higher plants, germacrene D is present as (−)-enantiomer, while the (+)-enantiomer is more likely to occur in lower organisms [22]. In a *Myrtus communis* essential oil from Algeria limonene was exclusively present in the (+)-enantiomeric form, α-pinene with more than 95% in the (+)-form, while β-pinene, β-phellandrene, and neomenthol occurred rather as a racemate [26].

3.2 Classification

Mono- and sesquiterpenes are often classified according to the structure of the carbon skeleton, acyclic or the various cyclization types, and the oxidation status as hydrocarbons, alcohols, aldehydes, ketones, or esters.

3.2.1 Monoterpenes

Acyclic

Hydrocarbons: Myrcene, *Z*-β-ocimene, and *E*-β-ocimene are branched hydrocarbons with three double bonds occurring in many essential oils (Fig. 96.3).

Oxygenated compounds: Linalool is a tertiary alcohol and can be found in many essential oils; it is accompanied by linalyl acetate. Linalool is the main compound in the oils from lavender (*Lavandula angustifolia*), coriander fruits (*Coriandrum sativum*), bergamot (*Citrus aurantium* var. *bergamia*), orange flowers (*Citrus sinensis*), and the linalool chemotype of thyme (*Thymus vulgaris*). Geraniol and nerol are two *cis–trans* isomers. Geraniol is widespread in essential oils, occurring mainly in *Cymbopogon* species. Geraniol is widely used in perfumery. Geranyl acetate is found in *Callitris* and *Eucalyptus* species.

Citronellol occurs free or as esters in many essential oils as in the leaves of *Pelargonium odoratissimum* and fresh flowers of *Rosa gallica* and *R. damascena* [27].

The aldehydes geranial (= *E*-citral, citral A) and neral (= *Z*-citral, citral B) are two *cis-trans* isomers with lemonlike scent present in lemongrass (*Cymbopogon citratus*) and the fresh peel of lemon (*Citrus limon*). They are used as food flavoring and in perfumery.

Citronellal, a further aldehyde, is a main constituent in citronella oils (*Cymbopogon nardus*) and can also be found in *Melissa officinalis* and in various *Eucalyptus* species as *E. citriodora*. It is mainly used in perfumery [27].

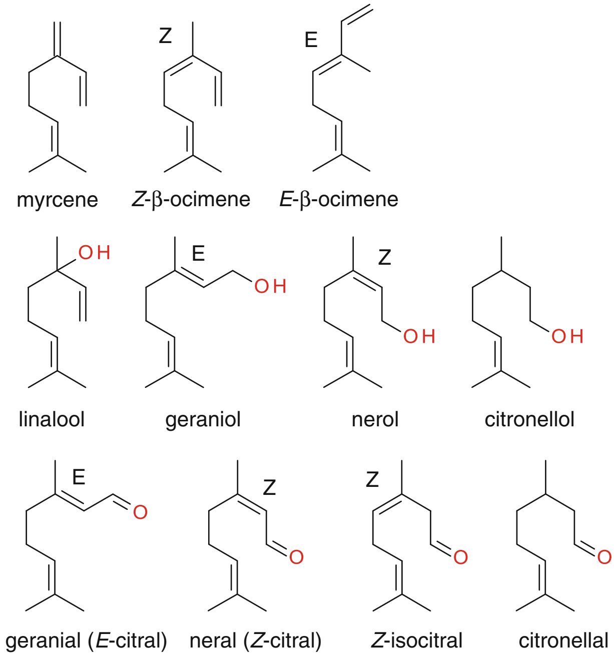


Fig. 96.3 Acyclic monoterpenes

Monocyclic Compounds with *p*-Menthane Skeleton

p-Menthane is the 1-methyl-4-isopropyl-cyclohexane skeleton (Fig. 96.4). Menthadienes result from different rearrangements of the α-terpinyl cation differing in the position of the two double bonds. As hydrocarbons, they comprise α-terpinene, γ-terpinene, α-phellandrene, β-phellandrene, α-terpinolene, and limonene and are frequent compounds in many essential oils. (+)-Limonene is a main compound in the expressed oil from *Citrus* fruits peels. (−)-Limonene occurs in needle oil of young fir twigs (*Abies alba*) and in some mint oils (*Mentha* sp.). α-Terpinene is a component of the cardamom fruit (*Elettaria cardamomum*). *p*-Cymene is an aromatic monoterpene. It occurs together with γ-terpinene and its hydroxylated derivatives thymol and carvacrol in *Thymus* and *Origanum* species.

The most important monoterpene alcohols are menthol, *cis*- and *trans*-piperitol, *cis*- and *trans*-pulegol, isopulegol, *cis*- and *trans*-carveol, thymol, carvacrol, terpinen-4-ol, and α-terpineol. The latter two bear a tertiary alcohol function. Terpinen-4-ol is a major antibacterial component of tea tree oil (*Melaleuca alternifolia*). α-Terpineol is a major compound in cardamom oil. (−)-Menthol is a main constituent in mint oils (*Mentha x piperita* and other *Mentha* species). Terpinen-4-ol is typically found in marjoram oils (*Origanum majorana*); α-terpineol occurs in various essential oils including those from *Melaleuca viridiflora* and nutmeg (*Myristica fragrans*). Thymol and carvacrol are

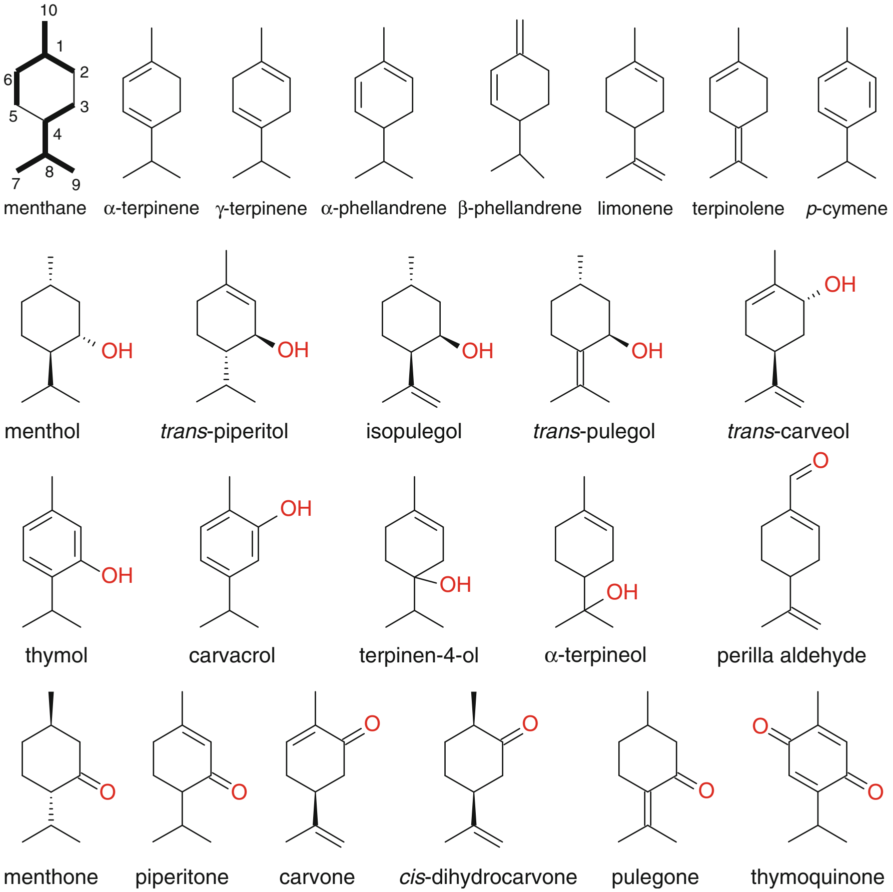

10
1
2
6
3
5
4
8
7
9
menthane
α-terpinene
γ-terpinene
α-phellandrene
β-phellandrene
limonene
terpinolene
p-cymene
menthol
trans-piperitol
isopulegol
trans-pulegol
trans-carveol
thymol
carvacrol
terpinen-4-ol
α-terpineol
perilla aldehyde
menthone
piperitone
carvone
cis-dihydrocarvone
pulegone
thymoquinone

Fig. 96.4 Monoterpenes with menthane structure

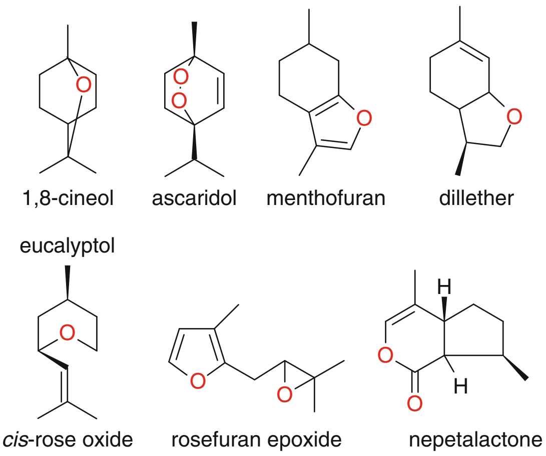
characteristic compounds of various *Thymus* species. Also the *Origanum* species are ordinarily rich in carvacrol. From India, a *Coleus aromaticus* oil rich in carvacrol has been reported [28].

Perillaldehyde is derived from limonene. In fruits of *Laserpitium siler* and *Laser trilobum*, it may account together with limonene for more than 80% of the essential oil [16]. It occurs also in *Perilla argata* and *Citrus reticulata* [27].

In analogy to the mentioned alcohols, the corresponding ketones are menthone, also present in *Mentha* oils, piperitone, pulegone, and carvone. (+)-Pulegone is the main constituent of pennyroyal oil (*Mentha pulegium*); piperitone occurs in *Mentha* oils, *Cymbopogon sennarensis*, *Lippia alba*, and *Eucalyptus dives*.

Thymoquinone is a major compound in the seed oil of *Nigella sativa* with antioxidative and protective properties.

Fig. 96.5 Oxidized monoterpenes with O-heterocycles



Mono- and Bicyclic Compounds with an O-Heterocycle

In the biosynthesis of some compounds with menthane structure, an intramolecular reaction of oxygen-containing groups leads to the formation of a second heterocylic ring (Fig. 96.5).

1,8-Cineol, eucalyptol with an ether bridge between C$_1$ and C$_8$, is the main compound of eucalyptus oil (*Eucalyptus globulus* and other species). 1,8-Cineol is as well component in numerous other essential oils, mainly in species of the genera *Thymus*, *Achillea*, and *Artemisia*.

Ascaridol presents a peroxide structure and displays anthelmintic activity and some toxicity to mammals. It is found in *Chenopodium ambrosioides* and some *Achillea* and *Artemisia* species.

A 5-membered oxygen containing heterocycle fused to the cyclohexane ring is the characteristic structure of menthofuran and dill ether. Menthofuran is an atypical compound of pennyroyal oil (*Mentha pulegium*) and appears in small amounts in mint oils. Dill ether is a major compound in the distilled oil from dill herb (*Anethum graveolens*) providing the typical dill odor.

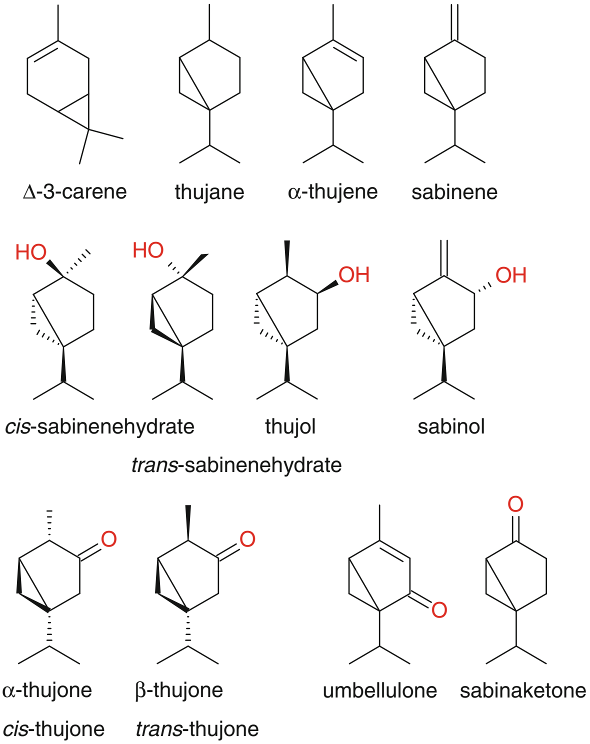
Cis- and *trans*-rose oxide bear a tetrahydropyran ring and can be found in small amounts in *Pelargonium graveolens*, *Citrus paradisi*, and *Citrus sinensis* oils. Rosefuran epoxide occurs in small quantities as aroma component in *Alyosia triphylla*, and *Agastache rugosa* oils.

Nepetalactone as main oil component from catmint species (*Nepeta* sp.) occurs in three isomers (4aα,7α,7aα; 4aα,7α,7aβ; 4aα,7β,7aα) and is a powerful attractant and stimulant for cats. It is produced from iridodial.

Bicyclic Compounds with Carane and Thujane Skeletons

These compounds present a cyclopropane ring beside the cyclohexane structure (Fig. 96.6). In the carane skeleton, the cyclopropane structure arises from an additional bond between C3 and C8 of the menthane structure. The major

Fig. 96.6 Monoterpenes with carane and thujane structure


Δ-3-carene
thujane
α-thujene
sabinene
HO
HO
OH
OH
cis-sabinenehydrate
thujol
sabinol
trans-sabinenehydrate
O
O
O
O
α-thujone
β-thujone
umbellulone
sabinaketone
cis-thujone
trans-thujone

compound of this group is δ-3-carene that can be found in turpentine oils obtained from *Pinus*, *Picea*, and *Abies* species.

In the thujane derivatives, the cyclopropane occurs within the cyclohexane structure. The main hydrocarbons with these skeletons are α-thujene and sabinene, occurring in many essential oils.

Cis- and *trans*-sabinene hydrate, formerly called thujanol, are the two main tertiary alcohols. They are typical compounds in the marjoram oil (*Origanum majorana*) and a specific chemotype of thyme oils (*Thymus vulgaris*). Sabinol is found in the oil of *Juniperus sabina* and other *Juniperus* species. These alcohols occur as free compounds or as acetates.

α-Thujone (*cis*-thujone) and β-thujone (*trans*-thujone) are ketones and occur in various essential oils. The most prominent example is absinth from absinth wormwood (*Artemisia absinthium*), but they are also present in other *Artemisia* species, *Thuja occidentalis*, and *Tanacetum vulgare*. These compounds are of toxicological concern.

Bicyclic Compounds with Pinane Skeleton

Pinanes bear the [3.1.1] bicyclic structure and therefore a cyclobutane ring. α-Pinene and β-pinene are the hydrocarbons that occur widely in essential oils

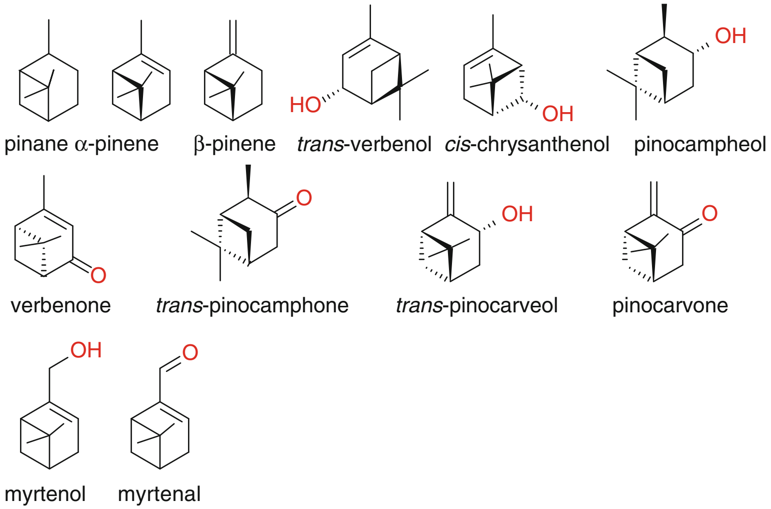


Fig. 96.7 Monoterpenes with pinane structure

and are abundant in turpentine oils from *Pinus* species (Fig. 96.7). Depending on the position of the double bond and the OH group, there are the secondary alcohols *cis*- and *trans*-verbenol, *cis*- and *trans*-chrysanthenol, *cis*- and *trans*-pinocarveol, and pinocampheol occurring occasionally in essential oils. For instance, *trans*-pinocarveol is a major compound in *Chenopodium ambrosioides* essential oil. The corresponding ketones are pinocarvone, verbenone, and chrysanthenone. Myrtenol is a primary alcohol and myrtenal the corresponding aldehyde.

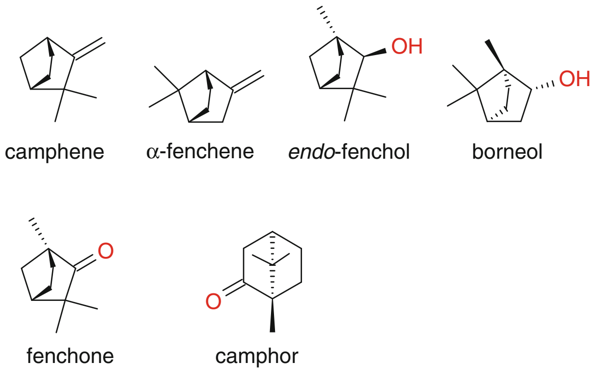
Bicyclic Compounds with Bornane, Camphane, and Fenchane Skeletons

Compounds of these groups possess a [2.2.1] bicyclic structure (Fig. 96.8). Camphene and fenchene are hydrocarbons occurring in various essential oils. (+)-Borneol is the main component in borneol camphor oil isolated from *Dryobalanops aromatica*; (−)-borneol occurs in Ngai camphor oil from *Blumea balsamifera*. Additionally, borneol and bornyl acetate are found in various oils including rosemary (*Rosmarinus officinalis*) and nutmeg (*Myristica fragrans*). Fenchyl alcohol (fenchol) occurs as endo-fenchol (α-fenchol) and exo-fenchol (β-fenchol) as minor components in various essential oils. (+)-Fenchone occurs in fennel fruits (*Foeniculum vulgare*) and *Lavandula stoechas* essential oil, (−)-fenchone in *Thuja occidentalis*. Camphor builds intensively smelling crystals. It is widespread in essential oils including those from the camphor tree (*Cinnamomum camphora*), feverfew (*Chrysanthemum parthenium*), and *Lavandula* and *Artemisia* species.

Tricyclic Skeletons

Tricyclene has a tricyclic [2.2.1.0] saturated hydrocarbon structure and is the main compound of the essential oil from the leaves of *Gossypium barbadense* [29].

Fig. 96.8 Monoterpenes with bornane, camphane, and fenchane structures



3.2.2 Sesquiterpenes

Acyclic Sesquiterpenes

The farnesenes are branched hydrocarbons with four double bonds (Fig. 96.9). *E,E*-α-farnesene and *E*-β-farnesene are widespread in essential oils, whereas *E,Z*-α-farnesene and *Z,Z*-α-farnesene are not reported to occur in nature. *Z,E*-α-farnesene is a constituent of the trail pheromone of the red fire ant. The oxidation products, farnesols as primary alcohols, as well as *E*- and *Z*-nerolidol as tertiary alcohol are occasional essential oil constituents.

The aldehydes α-sinensal and β-sinensal with a very low odor threshold occur as minor compounds in essential oils from various *Citrus* species.

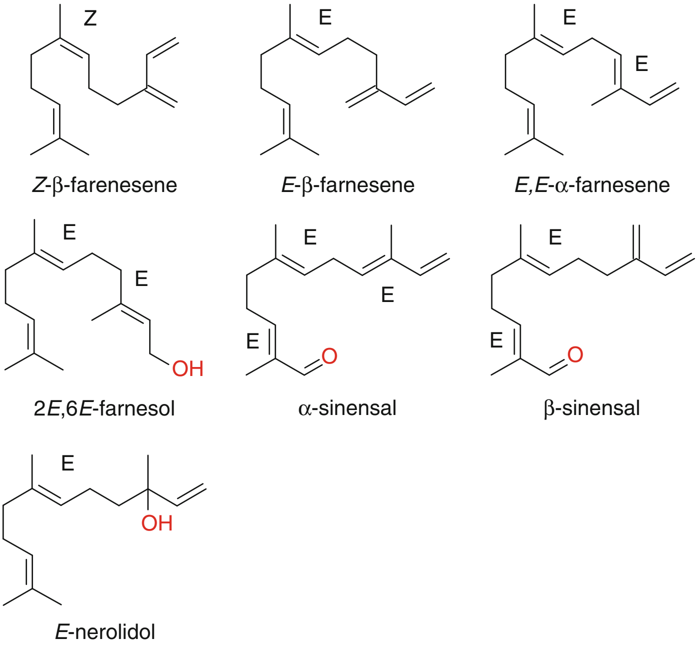
Cyclic Sesquiterpenes

The folding of the farnesyl chain during biosynthesis allows to build up a great variability of cyclic skeletons. The most important are displayed in Fig. 96.10. In some structures as in the elemane and eremophilane skeletons, rearrangement occurs so that the original head-to-tail binding of the isoprene units is no longer obvious.

Monocyclic Compounds

The bisabolane derivatives bear a C6 ring formed in analogy to the menthane skeleton (Fig. 96.11). The bisabolene isomers (*Z*-α-bisabolene, β-bisabolene, *E*-γ-bisabolene, and *Z*-γ-bisabolene) occur in various essential oils. α-Zingiberene is a main constituent of ginger oil (*Zingiber officinale*). The pungent taste of this drug is due to the gingerols (phenols). β-Curcumene is a typical compound of curcuma oils (*Curcuma aromatica* and *C. longa*). The aromatic *ar*-curcumene is found in *Curcuma* species and ginger.

The oxidized bisabolene derivative α-bisabolol with antiphlogistic properties is a main active compound in German chamomile (*Matricaria recutita*). Depending on the origin of the plants and the chemotype or variety, varying proportions of the oxides (bisabolol oxide A, bisabolol oxide B, or bisabolone oxide A) may be present in the respective oils [30]. α-Bisabolol can also be found in larger



Z-β-farenesene

E-β-farnesene

E,E-α-farnesene

2*E*,6*E*-farnesol

α-sinensal

β-sinensal

E-nerolidol

Fig. 96.9 Acyclic sesquiterpenes

proportions in some other species as the candeia tree (*Eremanthus erythropappus*), *Populus deltoides*, *Plinia cerrocampanensis* [31], and the roots of *Laserpitium zernyi* [32].

The germacrenes bear a 10-membered ring system. Germacrene D is widespread in plants and often found in leaf oils while germacrene A and germacrene B occur occasionally in essential oils.

α-Humulene presents an 11-membered ring. It is found in the essential oils of hop (*Humulus lupulus*), in cannabis (*Cannabis sativa*), and in small amounts in many other essential oils.

Bi- and Tricyclic Compounds

Some bi-and tricylic sesquiterpenes are frequently found in essential oils:

The bicyclic bergamotenes display like the pinenes, a cyclobutane ring. *Trans*-α-bergamotene occurs in lemon oil, in lodgepole pine (*Pinus contorta*), and as main compound in the oil from *Pimpinella affinis* [33].

Cadinane, muurolane, and amorphane derivatives possess decalin ring system substituted with an isopropyl and two methyl groups. Of these, β-cadinene is one of the most common in essential oils. It is abundant in conifer oils.

β-Caryophyllene (*E*-caryophyllene) derived from α-humulene presents a C9 ring fused to a cyclobutane ring. It is widespread in plants including food plants. In the essential oils from cannabis, oregano, and rosemary, it represents a major compound. It is found in the vegetative parts of many plants. β-Caryophyllene

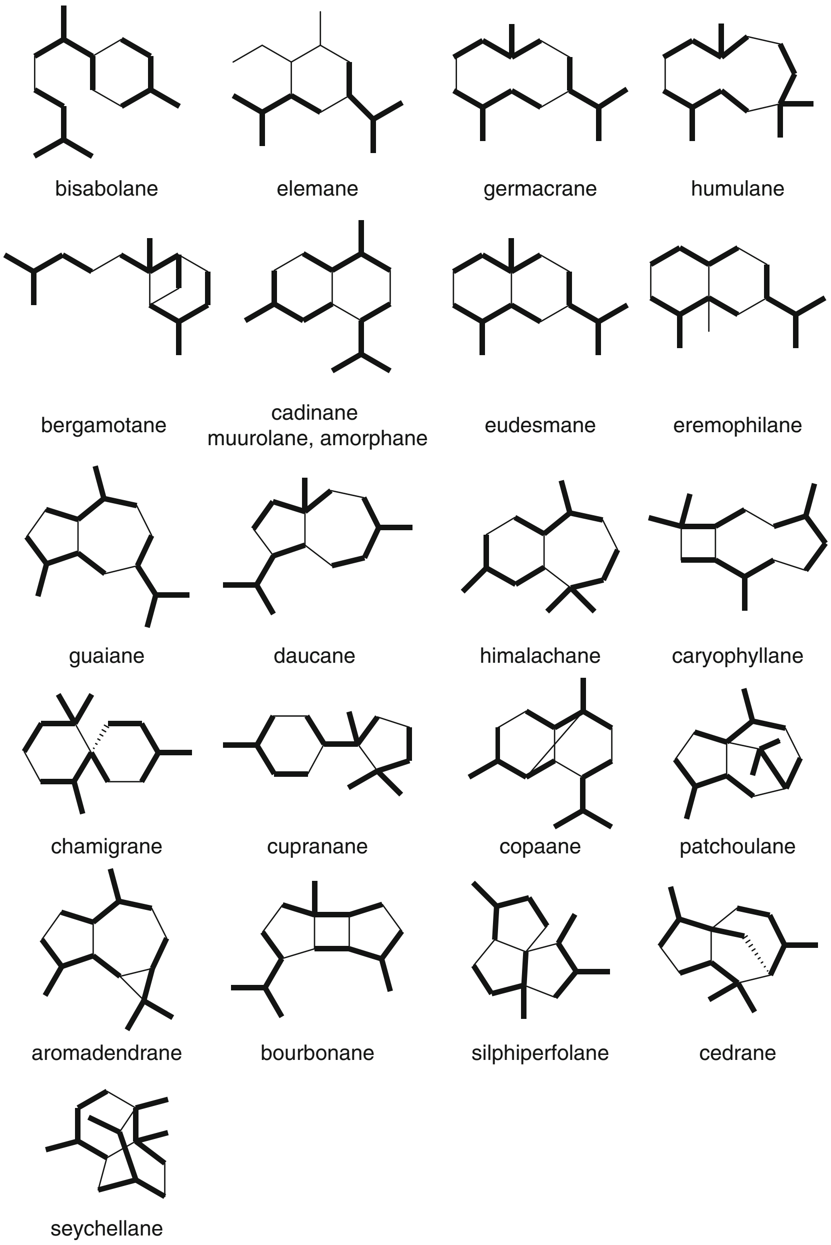

bisabolane
elemane
germacrane
humulane
bergamotane
cadinane
muurolane, amorphane
eudesmane
eremophilane
guaiane
daucane
himalachane
caryophyllane
chamigrane
cupranane
copaane
patchoulane
aromadendrane
bourbonane
silphiperfolane
cedrane
seychellane

Fig. 96.10 Important cyclic sesquiterpene skeletons

selectively binds to the CB(2) receptor so that it act as a functional CB(2) agonist. Also in vivo, this natural product exerts cannabimimetic effects with anti-inflammatory action [34]. Isocaryophyllene (*Z*-caryophyllene) occurs in clove oil and *Jasminum sambac* oil.

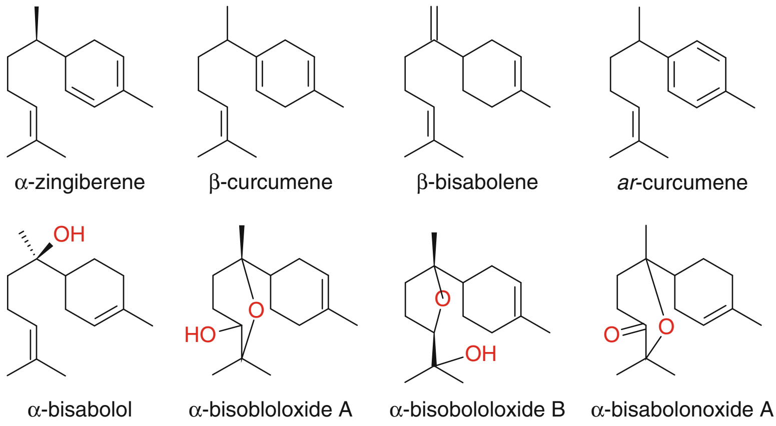


Fig. 96.11 Important bisabolane sesquiterpenes

The spiro compound α-chamigrene has been isolated from *Schisandra chinensis* fruits and β-chamigrene from *Chamaecyparis taiwanensis* [27]. β-Chamigrene was the main compound in *Trichilia connaroides* root oil [35].

Further bi- and tricyclic sesquiterpene hydrocarbons occurring in various essential oils are β-bourbonene, copaene, α-patchoulene (besides patchouli alcohol in patchouli oil from *Pogostemon cablin*), daucene, cyclosativene, β-cedrene (conifers, *Juniperus* sp.), bicyclogermacrene, aromadendrene, aristolene, α-ylangene, and longifolene.

The bicyclic C14 aromatic hydrocarbon chamazulene with antiphlogistic properties is a characteristic compound present in German chamomile and some *Achillea millefolium* agg chemotypes. Due to its system of conjugated double bonds, it confers a deep blue color to the essential oil. A blue azulene derivative is also present in the mushroom *Lactarius indigo*.

Oxidized Compounds

Among the oxygenated sesquiterpenes (Fig. 96.12), the oxidation product from β-caryophyllene, caryophyllene oxide, is widespread in plants. Also spathulenol is a widespread sesquiterpene alcohol. For example, it occurs in German chamomile and together with γ-eudesmol as major oil component in *Eucalyptus oleosa* [36]. Daucol and carotol are aroma components found in the fruit oils from carrots (*Daucus carota*) [27].

Davanone and related compounds bear a tetrahydrofuran ring. Davanone is a main compound in davana oil obtained from *Artemisia pallens* (Asteraceae). The oil has a unique flavor profile and is used in combination with various other natural flavors [37]. Davanone occurs also in specific chemotypes of other *Artemisia* species (Fig. 96.12).

Sesquiterpene lactones are nonvolatile compounds that do not occur in essential oils. Occurring mainly in Asteraceae, the sesquiterpene lactones that have interesting biological properties will be presented elsewhere.

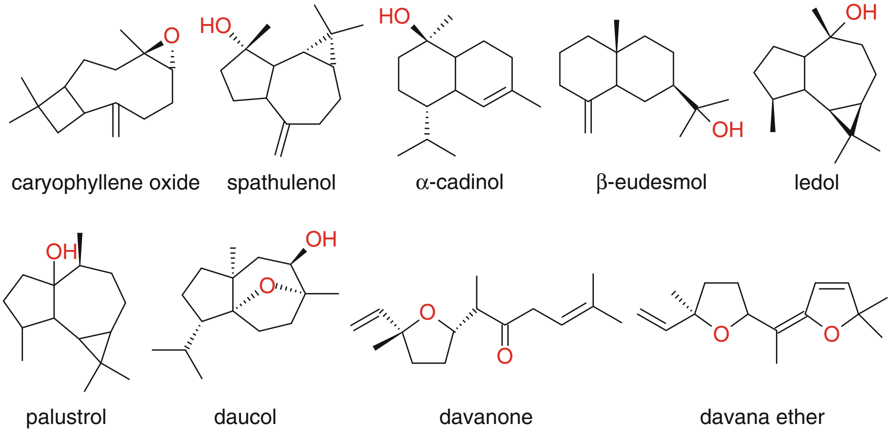


Fig. 96.12 Further selected oxidized sesquiterpenes

3.2.3 *nor*-Sesquiterpenoids

nor-Sesquiterpenoids are derived from sesquiterpenes by the loss of one or more carbon atoms. Some examples are given below (Fig. 96.13):

Ptaquiloside is an instable glucoside present in the widely distributed bracken fern (*Pteridium aquilinum*). Under alkaline conditions, it is converted in a conjugated diene-one which is readily added to various nucleophilic compounds. Therefore, it has a carcinogenic potency [9].

Geosmin and dehydrogeosmin are emanations from microorganisms (*Streptomyces* sp.) with an earthlike, musty smell. Geosmin is also present in some wines and the floral scent of cacti of the genus *Turbinicarpus* and some other genera [38].

(*E*)-4,8-dimethylnona-1,3,7-triene, a C11-nor sesquiterpene, is among the most widespread natural scent components [38].

Damascone, damascenone, and ionone are ketone aroma compounds derived from the breakdown of carotene. Damascone has a roselike aroma and is used in perfumery. β-Damascenone has been identified as an aroma compound in red wines with concentrations around 1–1.5 μg/L. The odor threshold is strongly dependent from the matrix, being very low in aqueous alcoholic solutions and much higher in wines. It acts as possibly enhancer of fruity aromas [39].

3.3 Rearrangements and Transformations

Terpenoids with double bonds can undergo various rearrangement and transformations as well as oxidation reactions. Some reactions proceed easily; others may be induced by acid, heat, or irradiation treatment. Some examples are given below:

β-Caryophyllene (*E*-caryophyllene) is oxidized when exposed to air, and after 5 weeks, nearly 50% of the original compound was consumed. The main oxidation product was caryophyllene oxide [40]. Limonene is easily oxidized to limonene

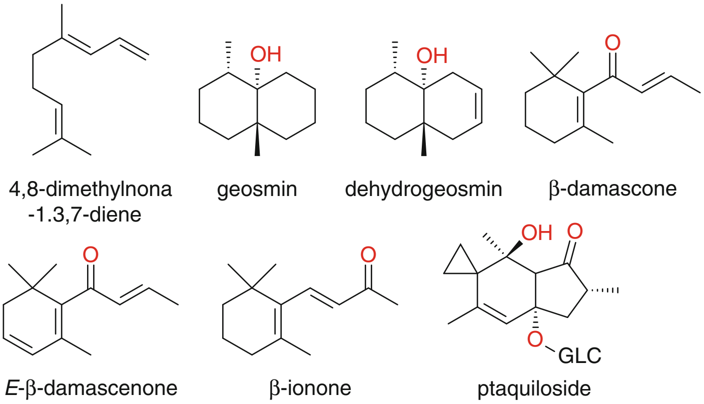

4,8-dimethylnona
-1.3,7-diene
geosmin
dehydrogeosmin
β-damascone
E-β-damascenone
β-ionone
ptaquiloside
OH
O
GLC

Fig. 96.13 Selected *nor*-sesquiterpenoids

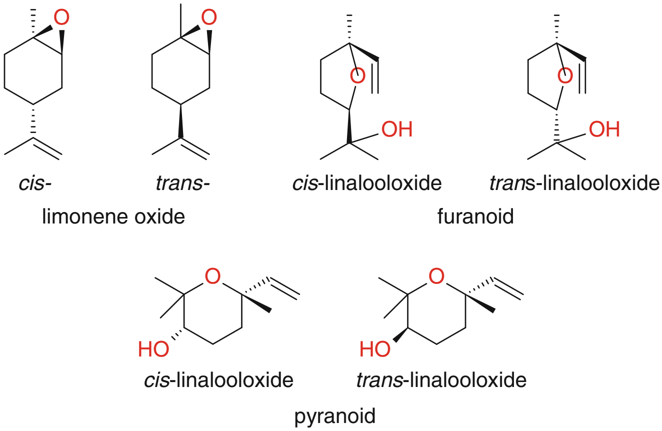

cis-
trans-
limonene oxide
cis-linalooloxide
trans-linalooloxide
furanoid
cis-linalooloxide
trans-linalooloxide
pyranoid
O
OH
HO

Fig. 96.14 Oxidation products from limonene and linalool

oxide, carveol, and carvone. Also linalool undergoes oxidation leading to various oxides as presented in Fig. 96.14.

Acid catalyzed rearrangements of *cis*- and *trans*-sabinene hydrate yielded terpinen-4-ol (56%), γ-terpinene (18%), α-terpinene (9%), terpinolene (3%), *p*-cymene (1%), α-terpineol (1%), and E/Z-*p*-menth-2-en-1-ol (1%) [41].

Major acid catalyzed rearrangements products of germacrene D include α-muurolene, (+)-γ-muurolene, (−)-α-amorphene, α-ylangene, α-copaene, selina-4(15),6-diene, opposite-4(15),7-diene, 4(15)-cycloaxene, isodauca-4,6-diene, and various cadinene isomers with the cadinanes predominating [22]. Some of such rearrangement products are eventually found as natural metabolites.

The transformation of terpenoids in food products can be the origin of "off-flavors" as in the case of orange juice where the metal catalyzed or photooxidation

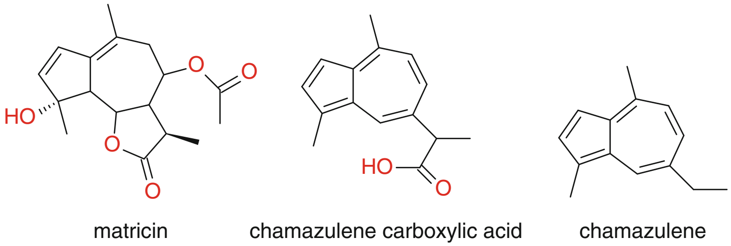


Fig. 96.15 Formation of chamazulene during hydrodistillation

of the sesquiterpene valencene affords nootkatone or when the juice gets a terpene note through the oxidation of limonene to carvone [42].

During production of essential oils through hydrodistillation, some transformations have been characterized. The most prominent example is the formation of the blue chamazulene in essential oils from German chamomile or yarrow (Fig. 96.15). The starting compound in the plant is the nonvolatile sesquiterpene lactone matricin that is converted to chamazulene carboxylic acid which in turn is decarboxylated to chamazulene [43].

Similarly, terpinen-4-ol present in the essential oil of marjoram (*Origanum majorana*) arises from rearrangement of sabinene hydrate during distillation [41].

4 Biosynthesis

The biosynthesis of terpenoids has been reviewed in a range of handbooks and compendia [1, 43–45]. A special chapter within this series will be devoted to this topic.

4.1 Biosynthesis of the Isoprene Units

As mentioned above, terpenoids can be seen as built up by isoprene units. In vivo, these units are the interconvertible isomers isopentenyl diphosphate IPP (isopentenyl pyrophosphate) and dimethylallyl diphosphate (DMAPP) (dimethylallyl pyrophosphate).

There are two ways to produce these biological isoprene units:

1. The mevalonic acid pathway producing IPP from acetyl CoA takes place in the cytosol of the cells. The steps include the formation of 3-hydroxy-3-methylglutaryl-CoA from three acetyl CoA, the reduction to mevalonic acid, the subsequent activation with ATP resulting in the formation of mevalonate diphosphate, and finally the decarboxylation of mevalonate diphosphate to release IPP.
2. The deoxyxylulose phosphate (DXP) pathway or more precisely the methylerythritol phosphate (MEP) pathway or non-mevalonate pathway in the plastids. The pathway begins with the condensation of glycerinaldehyde-3-phosphate

with pyruvate to yield 1-deoxy-D-xylulose-5-phosphate which is then reduced to 2-C-methyl-erythrose-4-phosphate (MEP). Then follows in three reactions the conversion to 2-C-methyl-D-erythritol-2,4-cyclodiphosphate and subsequently a reduction to form the linear 1-hydroxy-2-methyl-2(E)-butenyl-4-phosphate. The latter is finally converted to IPP and to a lesser extent to DMAPP.

4.2 Biosynthesis of Monoterpenes

The first step in monoterpene synthesis is the formation of geranyl diphosphate (geranyl pyrophosphate, GPP) from one IPP and one DMAPP by isoprenyl diphosphate synthases. The reaction mechanism of the monoterpene synthases starts with the ionization of GPP to give a carbocation that is involved in various cyclizations, hydride shifts, or rearrangement reactions before the reaction is terminated by deprotonation or water incorporation. For the production of cyclic monoterpenes, the geranyl cation is first converted to a linalyl cation intermediate that can yield the initial cyclic intermediate, the α-terpinyl cation. This α-terpenyl cation can subsequently be converted to the wide range of cyclic monoterpenes [46]. Terpene synthases have been intensively studied during the last two decades. The high number of monoterpenes produced arises from the high diversity of the terpene synthases and from the ability of these enzymes to produce more than one end product.

4.3 Biosynthesis of Sesquiterpenes

Sesquiterpenes are formed from three C5 units where a further molecule IPP reacts with GPP to form farnesyl diphosphate (farnesyl pyrophosphate, FPP). FPP is further converted to linear or cyclic products. By cleaving the diphosphate group, an allylic cation is produced that underlies an electrophilic attack on the central or distal double bond leading to cyclization. Due to the increased chain length and an additional double bond, a huge number of structures can be built up. Sesquiterpene cyclase enzymes typically produce a major product accompanied by a range of related structures [44].

5 Biological Activities

Due to their physiological and aromatic properties, a wide field of applications has been established for plants and plant products containing mono- and sesquiterpenes. To underline the importance of these compounds, an estimated world consumption of the main essential oil types is presented in Table 96.2. The traditional uses as spices and seasoning of food, the uses as flavoring agents in household products and in perfumery are well known. Uses in the so-called wellness area as additives in bath, oil lamps, or in massages encounter an increasing popularity.

Table 96.2 Estimated world consumption of major essential oils [5]

Oil type	Botanical species	Tons/year	Main terpenoids
Orange	*Citrus sinensis*	50,000	Limonene (92–97%)
Corn mint	*Mentha arvensis*	25,000	Menthol (70%)
Peppermint	*Mentha piperita*	4,500	(−)-Menthol (30–55%), menthone (14–32%)
Eucalyptus	*Eucalyptus globulus*	4,000	1,8-Cineol (>70%)
Lemon	*Citrus limon*	3,500	Limonene (60–80%), geranial (0.5–2%), neral (0.2–1.2%)
Citronella	*Cymbopogon winterianus*	3,000	Citronellal (30–45%), geraniol (20–25%), citronellol (9–15%)
Citronella	*Cymbopogon nardus*		Geraniol (15–23%), citronellol (3–9%), geranial (3–6%)
Eucalyptus	*Eucalyptus citriodora*	2,100	Citronellal (70–87%), citronellol (5–10%)
Spearmint	*Mentha spicata*	2,000	(−)-Carvone (50–80%)
Cedarwood (Virginia)	*Juniperus virginiana*	1,500	α-Cedrene (22–53%), thujopsene (10–25%), cedrol (5–30%)
Lime	*Citrus aurantifolia*	1,500	Limonene
Lavandin	*Lavandula angustifolia x L. latifolia*	1,000	Linalool (24–35%), lavandulyl acetate (28–38%)
Litsea	*Litsea cubeba*	1,000	Geranial (38–45%), neral (25–33%)
Cedarwood (China)	*Cupressus funebris*	800	α-Cedrene (13–29%), thujopsene (18–31%), cedrol (10–16%)
Coriander	*Coriandrum sativum*	700	(+)-Linalool (65–78)
Grapefruit	*Citrus paradisi*	700	Limonene
Patchouli	*Pogostemon cablin*	600	Patchoulol (27–35%)
Basil (Reunion type)	*Ocimum basilicum*	500	Estragol (75–87%), linalool (0.5–3%)
Basil (European type)	*Ocimum basilicum*		Linalool (45–62%), estragol (0–30%), eugenol (2-15%)
Mandarine	*Citrus reticulata*	500	Limonene (65–75%), γ-terpinene (16–22%)

Rational phytotherapy relies on active substances from plants. Classical essential oil-bearing medicinal plants are German chamomile (*Matricaria recutita*), thyme (*Thymus vulgaris*), peppermint (*Mentha x piperita*), caraway (*Carum carvi*), and fennel (*Foeniculum vulgare*) [43, 47]. They are not only used to cure human disease but are of increasing interest in veterinary phytotherapy.

Aromatherapy is mostly applied to procure alleviation of chronic pain, depression, cognitive disorders, anxiety, insomnia, and stress-related disorders [48]. In this context, the influence of essential oils on the nervous system is studied [49]. Linalool-containing essences are traditionally used as sedatives, analgesics, and anxiolytics [50].

Useful pharmacological effects of essential oils have been studied intensively and reviewed [51–53] and presented also in handbooks and textbooks of pharmacognosy [43, 47, 54]. However, many effects described in primary literature were observed in vitro or in specific test systems and are therefore not yet directly applicable. In the following, only a limited selection of activities can be presented.

5.1 Aroma Impact

The aromatic properties of essential oils are perhaps the most conspicuous and account for a wide range of traditional applications. Many terpenoids have characteristic odors, some of them are presented in Tables 96.3 and 96.4 [55–57]. The odor thresholds however may vary greatly and depend on the matrix. (−)-Limonene with citrus-like aroma is a key odorant of orange juice and neral/geranial the key odorants of lemons. 1-*p*-Menthene-8-thiol present in grapefruit juice and responsible for the grapefruit aroma has with 0.02 ng/L one of the lowest odor threshold values [42].

5.2 Antimicrobial Activity

The need of exclusion or elimination of pathogenic microorganisms, the appearance of resistances against antibiotics, and the request of ecological safe compounds has driven the research of antimicrobial activity of natural compounds. Within the last years, an increasing number of scientific papers describing the composition and biological activity of essential oils of various sources have been published. Various modes of action in the bacterial cell have been discussed including degradation of the cell wall, damage to cytoplasmic membrane and membrane proteins, leakage of cell contents, coagulation of cytoplasm, and depletion of proton motive force [51, 58–60]. The lipophilic character of the terpene skeleton combined with the hydrophilic character of the functional groups is essential for activity. Thus, a rank of activity has been proposed as follows: aldehydes > ketones > alcohols > esters > hydrocarbons [61]. In many instances, gram-negative organisms were slightly less susceptible than gram-positive bacteria. Effective components displayed minimum inhibitory concentrations (MICs) of 0.05–5 μl/ml in vitro [51].

Testing 25 different bacterial strains including animal and plant pathogens, food poisoning, and spoilage bacteria, the component with the widest spectrum of activity was found to be thymol followed by carvacrol, α-terpineol, terpinen-4-ol, eugenol, linalool, (−)-thujone, δ-3-carene, geranyl acetate, (*cis* + *trans*) citral, nerol, geraniol, menthone, β-pinene, (−)-limonene, α-pinene, α-terpinene, borneol, (+)-sabinene, γ-terpinene, citronellal, terpinolene, 1,8- cineole, bornyl acetate, carvacrol methyl ether, myrcene, β-caryophyllene, α-bisabolol, α-phellandrene, α-humulene, β-ocimene, aromadendrene, and *p*-cymene, in that order [58]. Antibacterial and antifungal activity was also described in oils with high levels of sesquiterpenes as cadinene, *Z*-β-farnesene, γ-muurolene, spathulenol, and α-selinene [61]. Linalool and citral were very efficient in inhibiting a wide range

Table 96.3 Odor impact (aroma descriptor) of selected monoterpenes [55–57]

Compound	Odor
Borneol	Woody-camphoraceous, dry-minty
Isoborneol	Camphoraceous, weak peppery and woody
Camphene	Camphoraceous, mild-oily
Camphor	Camphoraceous, fresh, warm-minty, ethereal
δ-3-Carene	Resinous, sweet, refined-limonene-like, spicy
Carveol	Fresh, caraway- and spearmint-like
S-(+)-Carvone	Spicy, caraway-like
R-(−)-Carvone	Spearmint-like
Citronellal	Minty, citrus-like
Citronellol	Floral, roselike
Cumin aldehyde	Sharp, acid, pungent, woody, oily
Cuminyl alcohol	Floral, oily-spicy, dillseed- and caraway-like
Cymen-8-ol	Weak citrus-like
p-Cymene	Fresh, weak citrus-like, lemon and bergamot notes
Geranial	Lemon, sweet
Geraniol	Floral, lemonlike, minty
Limonene	Fresh, citrus-like, mild lemon and orange notes
Linalool	Fresh, floral, clean, sweet, lemon notes
Myrcene	Mild, sweet, balsamic, plastic note
Neral	Sweet, lemon
Nerol	Rose, sweet
cis-β-Ocimene	Herbal, warm-herbaceous, sweet-floral, neroli-oil-like
trans-β-Ocimene	Herbal, weak floral
α-Phellandrene	Spicy, herbaceous, minty, peppery-woody, fresh, citrus
β-Phellandrene	Peppery, minty, refreshing, citrus-like
α-Pinene	Pine-like, sharp, woody, turpentine-like
β-Pinene	Dry-woody, pine-like, resinous-terpene-like, spicy
Piperitone	Fresh, minty, camphoraceous
Sabinene	Warm, oily-peppery, woody-herbaceous, spicy
cis-Sabinene hydrate	Mild, pleasant, warm, woody-balsamic
trans-Sabinene hydrate	Warm, balsamic-woody, mild
Terpinen-4-ol	Nutmeg-like, spicy, woody-earthy, liliac-like
α-Terpinene	Refreshing, lemon-citrus-like
γ-Terpinene	Fresh-herbaceous, citrus-like
α-Terpineol	Floral, liliac-like
Terpinolene	Sweet-piney, oily, petroleum-like
α-Thujene	Green, herbal, woody

of *Candida albicans* isolates. The terpenoids arrested the cells at different phases of the cell cycle, that is, linalool and linalyl acetate at G1 and citral and citronellal at S phase [62]. Also various synergistic and antagonistic effects have been observed. 1,8-Cineol and camphor had a synergistic antimicrobial effect on *Candida albicans* [61].

Table 96.4 Odor impact (aroma descriptor) of selected sesquiterpenes [55–57]

Compound	Odor
trans-α-Bergamotene	Weak woody, warm, tea-leaf-like
Bicyclogermacrene	Green, spicy, mushroom-notes, dry-woody
β-Bisabolene	Sweet, warm, balsamic, woody, spicy
α-Bisabolol	Sweet, mild floral
β-Bisabolol	Mild, sweet, weak herbal
Bulnesol	Weak woody, weak spicy
Cadina-1,4-dien-3-ol	Weak fruity, weak woody, spicy
Cadina-1,4-diene	Fruity, mango-like, spicy, woody
δ-Cadinene	Dry-woody, weak medicinal
α-Cadinol	Weak woody, medicinal, dry
Calamanene	Weak floral, weak spicy
β-Caryophyllene	Woody, spicy, terpene notes
Caryophyllene oxide	Weak woody, warm, mild, weak spicy
α-Copaene	Vegetable-like, hops-like, woody
α-Cubebene	Spicy, herbal
β-Cubebene	Herbal, woody
Cubenol	Spicy, herbal, green-tea notes
β-Elemene	Fresh, warm, weak woody
γ-Elemene	Mild, woody
δ-Elemene	Lime-oil-like, woody
β-Eudesmol	Woody
trans-β-Farnesene	Warm, mild, sweet
trans,trans-α-Farnesene	Mild, warm, sweet
cis,trans-α-Farnesol	Mild, sweet, oily, floral, fresh green
trans,trans-α-Farnesol	Weak fresh, fresh green, weak floral
Germacrene B	Earthy, mushroomlike
Germacrene D	Weak spicy, weak fruity, apple-like, weak dry-woody
α-Guaiene	Sweet-woody, balsamic, peppery
β-Guaiene	Balsamic, woody, spicy
Guaiol	Woody-balsamic, mild, sweet, weak floral
α-Gurjunene	Balsamic, woody
α-Humulene	Weak woody, weak spicy, herbal
γ-Muurolene	Weak spicy, weak herbal, woody
T-Muurolol	Weak woody, weak spicy, mild
trans-Nerolidol	Woody-floral, weak green
Spathulenol	Weak fruity, weak herbal
α-Zingiberene	Spicy, fresh, sharp

Among the antifungal activities, the antiaflatoxigenic effects of some essential oils merit attention. Out of various tested oils, *Thymus vulgaris* and *Citrus aurantifolia* inhibited the aflatoxin production and growth of *Aspergillus parasiticus* while *Carum carvi* inhibited the aflatoxin production but not the fungal growth [63].

Antiviral properties have been reported from several essential oils. Oils from *Melaleuca alternifolia* and *Santolina insularis* showed activity against *Herpes simplex* virus types 1 and 2. Some of this activity was also present in oils from peppermint and lemongrass [61].

5.3 Anthelmintic Activity

Ascaridol contains a 1,4-peroxide bridge. It is the main constituent of the volatile oil of American wormseed (*Chenopodium ambrosioides* var. *anthelminticum*, Chenopodiaceae). It has anthelmintic properties but is also toxic to mammals. Ascaridol can also be found in chemotypes of some *Achillea* species.

5.4 Anticancer Activity

Tumorigenesis is a complex multistep process influenced by many factors. There are some population-based studies indicating that a diet rich in isoprenoids play a role in the ability to avoid cancers [61]. In various experiments and test systems, some mono- and sesquiterpenes showed activity, where peril alcohol and D-limonene were of greatest interest. α-Bisabolol, the anti-inflammatory compound of chamomile oil, showed a strong time- and dose-dependent cytotoxic effect on glial cells [61]. β-Caryophyllene showed some potentiating effect on the anticancer activity of α-humulene, isocaryophyllene, and paclitaxel on various cell lines [53]. Further studies are necessary to evaluate the anticancer potential of thymoquinone [53].

5.5 Antioxidant Activity

Natural antioxidants have been studied intensively during the past years which are mainly phenolic compounds. Among the many essential oils with antioxidative properties, those containing phenolic compounds as thymol and carvacrol showed the highest activities [61, 64–66]. Various test systems have been used, and it has to be differentiated between the complete essential oil and individual components. α-Pinene and limonene show low activity in the DPPH (2,2-diphenyl-1-picrylhydrazyl) test system while terpinene and terpinolene display a high hydrogen-donating capacity against DPPH [53]. Geraniol has a high hydrogen-donating capacity toward DPPH, whereas terpinen-4-ol is a weak antioxidant. Germacrene D with its three double bonds as electron rich centers showed good ability to scavenge superoxoid radical anions [61]. However, linalool or nerolidol may also display pro-oxidant activity [64].

In many cases, the antioxidative activity of essential oils cannot be attributed to the main compounds; minor compounds and synergistic effect may significantly contribute to the activity.

5.6 Other Activities and Applications

Antinociceptive and Analgesic Effects. An example of analgesic effects is (−)-menthol which was able to increase the pain threshold, whereas (+)-menthol did not show such activity [53].

Enhancing the skin penetration of medicaments is an interesting topic in pharmacy. Among the essential oils tested, those from *Camellia oleifera*, fructus *Evodia*, rhizoma *Acori tatarinowii, s*emen *Myristicae*, and pericarpium *Citri reticulatae* showed to be effective [53].

Considerable research has been carried out to investigate the insect repellent properties of essential oils, a field that is of special interest in tropical developing countries. Out of 38 undiluted essential oils tested, those of citronella (*Cymbopogon nardus*), patchouli (*Pogostemon cablin*), clove (*Syzygium aromaticum*), and *Zanthoxylum limonella* were the most effective in repelling the mosquito *Aedes aegypti* [67]. However, the main problems for an efficient application arise from the fact that the oils are too volatile for a long-lasting effect and the short distance of repellency [53].

Within the last two decades, numerous trials have been carried out with aromatic plants as feed additives in animal husbandry. Firstly, the intention was a use of sensory additive and flavoring and appetizing substances to substitute for antibiotic growth promoters that have been forbidden and to promote digestion. Usually, the effects on growth performance are nonexistent or marginal. Nevertheless, various positive effects on the animals have been alleged and partly also achieved. In this context, improvements at three levels are aimed including the feed characteristics, optimization of digestion and performance, as well as the characteristics of animal products as, for example, oxidative stability of meat and eggs [68]. The influence of essential oils on ruminal fermentation is an example with promising outcomes. In in vitro experiments, thymol was able to inhibit the production of lactate and of the greenhouse gas methane [69]. Also the essential oil from *Salvia officinalis* showed some inhibition on methanogenesis [70].

5.7 Toxicity

Essential oils may display irritating properties. When used externally, some products as turpentine increase capillary blood flow, cause rubefaction, and give a sensation of heat. However, the acute toxicity by the oral way is generally low. Most oils have LD50 values above 5 g/kg [47]. The most toxic essential oils are those from boldo (*Peumus boldus*) containing ascaridol and causing convulsions, *Chenopodium*, thuja, and pennyroyal (*Mentha pulegium*). Some constituents as the thujones, pulegone, carvacrol, and carvone show LD50 values below 1 g/kg in animals [47]. However, serious intoxications are rare. Formerly, most accidents occurred with camphor.

Pulegone and menthofuran are hepatotoxic. Pulegone is first metabolized to menthofuran and then to electrophilic metabolites that may give adducts with cellular proteins. Therefore, the menthofuran content of peppermint oil should be as low as possible. Maximum levels for pulegone in foodstuff and beverages to which flavorings have been added were set to 25 mg/kg in foodstuff, 100 mg/kg in beverages, with the exception of 250 mg/kg in peppermint or mint flavored beverages, and 350 mg/kg in mint confectionery. Tolerated daily intake (TDI) of menthofuran and pulegone was set to 0.1 mg/kg body weight [71].

The thujones have neurotoxic properties leading to dose-dependent tonic-clonic seizures in animals [72]. Thujones from *Artemisia absinthium* are in the drink absinthe, which has been banned in most countries. However, a new reexamination of the concentrations of thujones in various absinthes puts into question whether the dosages for toxicity were achieved [73]. When using traditional herbal medicines containing thujone, the thujone intake should not exceed 3.0 mg/day and the duration of use should be limited to 2 weeks [74].

Ledol and palustrol are two aromadendrane derivatives in the essential oil from Labrador tea (*Ledum palustre*). This oil causes vomiting, gastroenteritis, and excitation in man but is also toxic for animals [9]. Recently, fraganol and fraganyl acetate, rare monoterpenes, were described as toxic essential oil components in *Achillea umbellata* [75].

5.8 Allergenic Properties

The search for allergens of plant origin has made considerable progress within the last years. Among the terpenoids, there are some sesquiterpene lactones being potent sensitizing agents including artemisifoline, anthecotulide, and alantolactone and others, showing an exocyclic methylene group on the lactone ring [9].

Alleged allergenic monoterpenes and sesquiterpenes are citral (neral + geranial), limonene, geraniol, linalool, citronellol, and farnesol. Among the sesquiterpene lactones, there are potent allergens [9].

Fragrances may also act as contact allergens. Among 26 substances tested, the terpenoids listed in decreasing frequency of sensitization were hydroxycitronellal, farnesol, citronellol, geraniol, linalool, and limonene [76].

Some monoterpenes such as limonene, linalool, and geraniol autoxidize on exposure to air and may form allergenic compounds. Geraniol itself is considered as weak contact allergen and is used for fragrance allergy screening among dermatitis patients. Compared to oxidized linalool and limonene, oxidized β-caryophyllene appeared to be a rather rare sensitizer [40].

The toxicity of terpene alcohols when used as fragrance ingredients has been intensively reviewed [77]. At concentrations likely to be encountered by consumers, these chemicals are considered nonirritating to human skin. At the present maximum use concentrations, their potential for eye irritation is considered minimal. 6,7-Dihydroxygeraniol is a potent skin sensitizer and has been prohibited in fragrance materials. Linalool may contain impurities or oxidation products that are strong sensitizers. Farnesol is a weak sensitizer so that its use in fragrance materials has been restricted [77].

Concentrations of α-pinene and β-pinene in indoor air range from 1 to 45 μg/m^3 (50 percentile values), and in Germany, based on toxicological data, indoor air guide values for bicyclic monoterpenes have been proposed including the health hazard guide value of 2 mg/m^3 (RW II) and the health precaution guide value of 0.2 mg/m^3 (RW I) [78]. Recently, the respective indoor air guide values for monocyclic monoterpenes (limonene) have been set at of 10 mg/m^3 (RW II) and 1 mg/m^3 (RW I) [79].

5.9 Ecological Functions of Volatile Mono- and Sesquiterpenes

In an ecological context where a community of individuals and species is considered, terpenoids play an important role. The most conspicuous relationship might be the emission of floral scents as complex mixtures to attract pollinators to flowers, guiding them to food resources and allowing them to discriminate between flowers. Within a species, the level of scent emission changes in response to endogenous diurnal rhythms, flower age, pollination status, and environmental conditions such as light, temperature, and moisture status [80, 81]. The observation that in many essential oil plants the highest oil contents occur during the preblooming or blooming stages may be related to this function.

Furthermore, volatiles are produced in response to damage and herbivore attack; they may directly affect herbivores' physiology and behavior due to their toxic, repelling, or deterring properties. They can also attract enemies of attacking herbivores, such as parasitic wasps, flies, or predatory mites, which can protect the signaling plant from further damage [80, 82]. Such semiochemical mono- and sesquiterpenes elicit the antennal response of the interacting insects, which can be studied by gas chromatography coupled to electroantennography [61]. For instance, α-farnesene and germacrene B are volatile signaling compounds in spruce (*Picea abies*). In gymnosperms, monoterpenes as α-pinene, β-pinene, myrcene, and limonene accumulate in resin ducts and are toxic to numerous insects including bark beetles [83].

The attack of herbivores induces changes in the volatile emission of plants. For example, in water mint (*Mentha aquatica*), undamaged plants emit pulegone, which attracts the beetle *Chrysolina herbacea*. Upon feeding, the plants start to build up and emit menthofuran, which is a repellent for the beetle [84]. Another aspect is that volatiles emitted from insect damaged plants attract the natural enemies of these herbivores [82]. In cotton (*Gossypium hirsutum*), terpenoid accumulation and synthesis is induced by foliar damage through mechanical injury or herbivore attack. The higher level of terpenoids was achieved by filling existing glands and production of additional glands [85]. Complex relationships exist between the Chinese Masson pine (*Pinus massoniana*), the sawyer beetle (*Monochamus alternatus*) which is the vector of the pinewood nematode (*Bursaphelenchus xylophilus*) causing pine wilt disease, and the fungi (*Sporothrix* sp.) which in turn are the food source of the nematode. In this interplay, the amount and ratio of α-pinene to β-pinene plays an important role [86].

Volatile emissions from plants may also have an ecosystem-wide impact when quantitative estimations of the emissions are considered. Within the last decades, it has been shown that natural vegetations emit considerable amounts of organic volatiles. Conifers release huge amounts of isoprene and monoterpene hydrocarbons [44]. Emissions may be dramatically enhanced when the plants are damaged. So it has been shown that wounded *Copaifera officinalis* emits a complex mixture of sesquiterpenes which is comparable to the sesquiterpene pattern found in the leaves determined by extraction with organic solvents [87]. Large amounts of volatiles are emitted by plants. The annual global emissions of isoprenes are estimated to 500 Tg [88] including isoprene and α-pinene. Biogenic emissions of volatile organic carbons from the Finnish forests are dominated by monoterpenes, mainly α-pinene and δ-3-carene [89]. Interactions with solar radiation are investigated especially in connection with the formation of ozone. The emissions are chemically oxidized or otherwise transformed into different aerosol compounds. The global significance of volatile emissions from boreal forests in atmosphere chemistry is discussed since these emissions may lead to aerosol formation and therefore have an overall cooling impact on climate [90].

6 Biotechnological Approaches

As mono- and sesquiterpenes are important natural products with a wide application range, great efforts have been undertaken for the biotechnological production of valuable compounds.

Although a range of transformations of terpenoids is accessible by the way of classical chemical synthesis [10], the use of (micro)organisms to perform such reactions yielding valuable products is nowadays again of great interest as these procedures meet the requirements of a environmentally friendly so-called green chemistry. The enormous progress achieved in this field has been recently reviewed [91, 92]. The transformation of the sesquiterpene valencene to nootkatone which is the grapefruit aroma that is used in foods, cosmetics, and pharmaceuticals is a classical example and can be achieved by various microorganisms [92]. The regiospecificity of microbial biocatalysts offers the possibility to obtain specific products. As an example, depending on the microorganism used, the biotransformation of D-limonene can yield carveol, carvone, perillalcohol, perillaldehyde, perillic acid, isopiperitenol, α-terpineol, limonene-1,2-epoxide, limonene-1,2-diol, or limonene-8,9-epoxide [93].

However, in the biotechnological production of specific mono- or sesquiterpenes, manifold difficulties have to be resolved including the lipophilic character of substrates and/or products; their toxicity, especially on the membranes of the microorganisms; and the formation of unwanted or toxic by-products. Additionally, in many cases, the targeted products are built at low rates, and the reactions depend on weakly active cofactors that are difficult to manage [94, 95].

Metabolic or pathway engineering aims to establish biosynthetic pathways for compounds of interests in heterologous organisms such as microbes and higher plants. Metabolic engineering of terpenoids in plants is attempted with various purposes including not only the production of a specific metabolite but also enhanced disease resistance, weed control by producing allelopathic compounds, better pest management, production of medicinal compounds, increased value of ornamentals and fruit, and improved pollination [96, 97]. By using bioinformatics and molecular databases, metabolic engineering largely contributes to a better understanding of biosynthetic pathways [88].

As microbial hosts, *Escherichia coli* and *Saccharomyces cerevisiae* have often been employed in pathway engineering of functional isoprenoids [95, 98].

Another example is the expression of *Fragaria vesca* nerolidol synthase 1 in *Arabidopsis thaliana*. The transgenic plants produced high levels of linalool and expressed also *E*-8-hydroxy linalool, *Z*-8-hydroxy linalool, and *E*-8-hydroxy-6,7-dihydrolinalool [96].

A promising approach is the prospect to upregulate terpene synthesis in the tree *Copaifera langsdorfii* which could result in an increase in the diesel-like resin that might prove beneficial to the global market of biofuels [88, 99]. A further example is transgenic *Eucalyptus camaldulensis* that has been established by introducing two expression constructs containing *Perilla frutescens* limonene synthase cDNA, whose gene products were designed to be localized in either the plastid or cytosol. The transgenic plant yielded 2.6–4.5 times more limonene and increased also the 1,8-cineol and α-pinene production as compared to the wild type [100].

7 Conclusion: Prospects

The biodiversity of essential oils including the low molecular terpenoids remains a fascinating field of research, and the sustainable use of this bounty in a wide field of applications presents some challenges in future [101].

Screening, characterizing, and managing this enormous biodiversity will need a continuing development of highly sensitive, high-throughput analytical methods including new advanced techniques.

The identification and authentication of the starting plant materials containing the active terpenoids for plant production and/or product manufacturing is an essential aspect that is often not sufficiently observed in quality assurance. This can be assessed by traditional morphological and phytochemical methods as used in pharmacognosy, as well as by new emerging DNA-based molecular methods. To give an example of possible confusions, under the name “oregano,” there are in the world dozens of species belonging not only to the genus *Origanum* but also to other genera and plant families but having similar sensorial properties as oregano [68].

The optimization and promotion of the cultivation and breeding of special crops producing terpenoids is a field that will be well established in the future. This plant production should be in accordance with good agricultural practice (GAP) [102].

The further development of biotechnology with the genomic and metabolomic analyses and genetic engineering will advance a variety of fields involving bioactive terpenoids ranging from food and animal nutrition to plant protection.

Exploiting new sources containing bioactive terpenes includes developing new methods to produce hemi-synthetic terpenoid components from waste materials arising in agriculture or in the food processing industry.

The high volatility and limited stability represent drawbacks of the storage and applications of essential oils and complicate the in vitro tests as well [59]. To overcome this, new developments are targeted on delivery systems as (micro) encapsulation as a way to control the release of volatile compounds with various purposes including pharmaceuticals as well as consumer products [103].

Although many biological activities as antimicrobial or antioxidative and other effects have been intensively studied and well documented, further tests are needed to evaluate interactions between individual compounds of the multicomponent mixtures [59]. The methods of systems biology offer a new approach to study these complex relationships.

When used as food and feed additives or as food supplements, new quality criteria must be defined regarding the identity of the material used and characteristic marker substances or/and chemical or DNA-based fingerprints.

Much effort must be done in promoting the transferability of in vitro to in vivo results.

In conclusion, the trend of the last years in the use of herbal products has shown that quality aspects and confidence in safe and healthy products including pharmaceuticals, cosmetics, household products, as well as foodstuffs of plant and animal origin have become a major issue. So, actually a well-balanced risk–benefit assessment of bioactive essential oils is one of the great challenges, and policymakers must be convinced that research on natural products as the volatile terpenoids in essential oils is an important task to guarantee future human and animal welfare [101].

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Monoterpenes: Iridoids

97

Biswanath Dinda and Sudhan Debnath

Contents

1 Introduction 3010
2 Occurrence 3011
3 Structural Classification 3011
4 Isolation, Chemistry, and Structural Elucidation 3013
5 Examples of Different Types with Physical Constants and Plant Source 3015
6 Synthesis 3033
7 Biosynthesis 3035
8 Pharmacology 3042
8.1 Antiallergic Activity 3042
8.2 Antiarthritic Activity 3044
8.3 Antibacterial Activity 3045
8.4 Anticancer Activity 3047
8.5 Anticoagulant Activity 3051
8.6 Antidiabetic Activity 3052
8.7 Anti-inflammatory Activity 3053
8.8 Anti-osteoporosis Activity 3053
8.9 Antiprotozoal Activity 3054
8.10 Antiviral Activity 3055
8.11 Immunomodulatory Activity 3057
8.12 Insecticidal Activity 3057
8.13 Melanogenesis Inhibitory Activity 3057
8.14 Nerve Growth Factor-Potentiating Activity 3057
8.15 Neuroprotective Activity 3058
8.16 Wound-Healing Activity 3059
9 Conclusion 3059
References 3059

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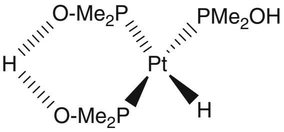
Abstract

The chemistry and pharmacology of iridoids, a special class of cyclopentanoid monoterpenes, are highlighted. Occurrence of some typical members of iridoids, secoiridoids, and their derivatives from different plant sources with their physical constants is listed in Table 97.1. Structural classification, synthesis, biosynthesis, and important pharmacological activities of iridoids and secoiridoids such as antiallergic, antiarthritis, antibacterial, anticancer, anticoagulant, antidiabetic, anti-inflammatory, anti-osteoporosis, immunomodulatory, insecticidal, melanogenesis inhibitory, nerve growth factor-potentiating, neuroprotective, and wound-healing activities are briefly discussed.

Keywords

Biosynthesis • characterization • chemistry • classification • iridoids • pharmacology • secoiridoids • synthesis

Abbreviations

CDP-ME	4-Diphosphocytidyl-2-C-methylerythritol
CDP-MEP	4-Diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate
DMAP	Dimethylaminopyridine
DMP	Dess-Martin periodinane
DXR	1-Deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	1-Deoxy-D-xylulose-5-phosphate synthase
Ghaffar-Parkins catalyst	
IBX	2-Iodoxybenzoic acid
ME-cPP	2-C-methylerythritol-2,4-cyclopyrophosphate
NMDA	N-Methyl-D-aspartate
Otera's catalyst	$(XR_2SnOSnR_2Y)_2$ $(ClBu_2SnOSnBu_2Cl)_2$
TBDPSCl	tert-Butyldiphenyl chlorosilane
$TMSCHN_2$	Trimethylsilyldiazomethane
$TMSOT_f$	Trimethylsilyl trifluoromethanesulfonate
Trost ligand	(1*R*, 2*R*) (+) 1,2-Diaminocyclohexane-N,N′-bis-(2-diphenylphosphinobenzoyl)

1 Introduction

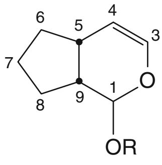
Iridoids represent a special and interesting class of cyclopentan [*c*] pyran monoterpenoids. The name "iridoid" is a generic term derived from the names of compounds iridomyrmecin, iridolactone, and iridodial, which were isolated from the defense secretion of the species of *Iridomyrmex*, a genus of ants. They are usually of plant metabolites and provide biologically and chemotaxonomically a structural link between terpenes and alkaloids [1]. More than 3,000 iridoids in various subclasses have been reported in the literature. Their potential pharmacological properties have created special attention to natural products chemists, biologists, and pharmacologists for understanding of their physiological functions in plants and animals and importance in human health and better use as herbal drugs.

2 Occurrence

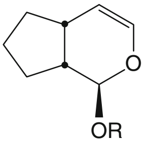
Iridoids and their [2, 3] seco-derivatives (secoiridoids) are found usually as glucosides in a number of plants in families Acanthaceae, Apocynaceae, Bignoniaceae, Caprifoliaceae, Gentianaceae, Labiatae, Lamiaceae, Loasaceae, Loganiaceae, Oleaceae, Plantaginaceae, Rubiaceae, Saxifragaceae, Scrophulariaceae, Valerianaceae, and Verbenaceae. Plants of genera *Viburnum* and *Sambucus* (Caprifoliaceae), *Penstemon* (Scrophulariaceae), and *Valeriana* and *Patrinia* (Valerianaceae) are the rich sources of valeriana-type iridoids, whereas plants of genera *Allamanda* and *Plumeria* (Apocynaceae) and *Morinda* and *Pentas* (Rubiaceae) are the important sources of plumeria-type iridoids. Secoiridoids are found mainly in the plants of genera *Gentiana* and *Swertia* (Gentianaceae); *Lonicera* (Caprifoliaceae); *Fraxinus*, *Jasminum*, *Ligustrum*, and *Syringa* (Oleaceae); and *Galium* and *Isertia* (Rubiaceae). The plants of genera *Premna* (Verbenaceae) and *Paederia* (Rubiaceae) are the important sources of bis-iridoids. The plants of several genera of Rubiaceae and Scrophulariaceae are the potential source of simple iridoids. They are found in the plants which have folk medicinal values for the treatment of various kinds of diseases in different countries. Chemotaxonomically, they are useful as biochemical markers for the study of phylogeny of the plants.

3 Structural Classification

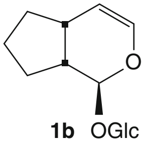
Structurally, iridoids are known as cyclopentan [c] pyran with a hydroxyl or glucosyloxy group at C-1 position of the pyran ring (**1**). Iridoids with a hydroxyl or acyl group at C-1 position are known as iridoid aglucones (**1a**) and with a glucosyloxy group as iridoid glucosides (**1b**). Iridoids may be monomeric, dimeric, trimeric, etc. according to the number of basic aglycone units present in a molecule. Monomeric iridoids are subgrouped into four types according to the number of carbons in the basic skeleton of the aglycone part: eight-carbon basic skeleton (**1c**), nine-carbon basic skeleton (**1d**), ten-carbon basic skeleton (**1e**), and 14-carbon basic skeleton (**1f**). Iridoids in each subgroup differ from each other by the presence or absence of hydroxy, epoxy or alkoxy, halo substituents in both cyclopentane and pyran rings and alkyl or alkyl-derived substituents (e.g., $-CH_3$, $-CH_2OH$, $-CHO$, $-COOH$) in C-4 and/or C-8 position. A β-*cis*-fused (H-5/H-9β) ring juncture is the most common structural feature of these compounds.



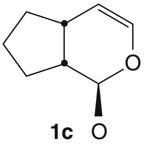
1 R = H or Glc



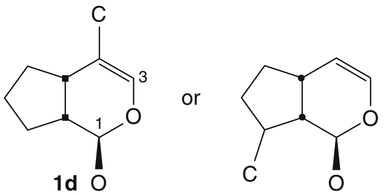
1a: R= H or acyl



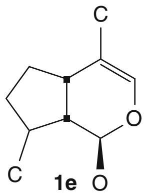
1b



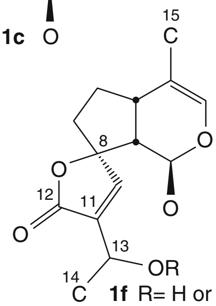
1c



1d

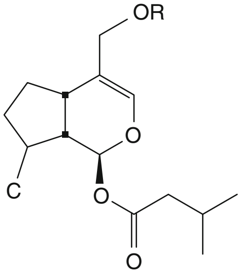


1e

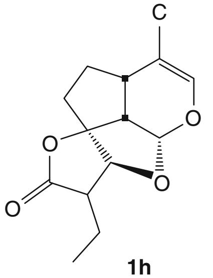


1f R= H or acyl

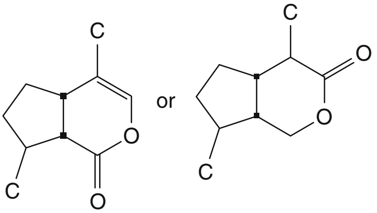
Monomeric iridoids may be linked to each other through glucosyl hydroxyl group or phenolic or terpene hydroxyl group forming bis-, tris-, and tetrakis-iridoids. Sometimes monomeric iridoids are attached to alkaloid or terpene unit, producing iridoid alkaloid or iridoid terpenoid. Iridoids having isovaleroyl group at C-1 oxygen and glucosyl group at C-11 oxygen are known as valeriana-type iridoids (**1g**). Iridoids having five-membered spironolactone ring at C-8 position are called plumeria-type iridoids (**1f**), and if the spirolactone ring forms an ether linkage with C-1 oxygen, the iridoids are known as plumericin type (**1h**). If pyran ring of iridoids is converted into pyrone ring, iridoids are called iridolactone (**1i**).



1g

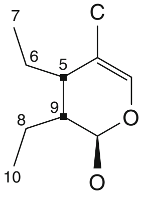


1h

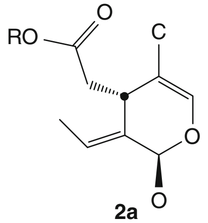


1i

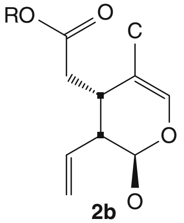
(7,8)-Secoiridoids are represented by the structure **2**. Secoiridoids are subgrouped accordingly to the arrangement of C-7 and C-8 carbons. If C-7 carbon is esterified and C-8 carbon is connected to C-9 carbon by a double bond, secoiridoids are called oleosides (**2a**), and if C-8 carbon is connected to C-10 carbon by a double bond, these are called secologanin type (**2b**). If both C-7 and C-8 carbons are attached together by hemiacetal linkage, these are called morronisides (**2c**), and by a six-membered lactone ring, these are called kingisides (**2d**). When C-7 carbon is attached to C-11 carbon through a lactone ring, these are called sweroside type (**2e**), while if C-7 carbon is attached to C-1 by a lactone ring, these are called isoswerosides (**2f**). When both C-7 and C-8 carbons are attached together through a seven-membered lactone ring, these are called jasmolactone type (**2g**). Some iridoids are cleaved at C-3 position of pyran ring; these are called cerberidol type (**2h**). When cleaved at C-1 position, these are called crescentoside type (**2i**) or jiofuran type (**2j**). Some secoiridoids are cleaved in both cyclopentane and pyran rings (**2k**).



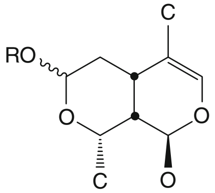
2



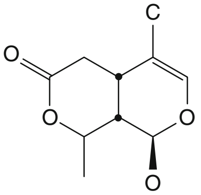
2a



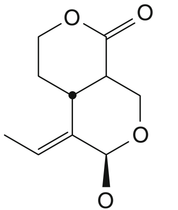
2b



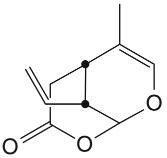
2c



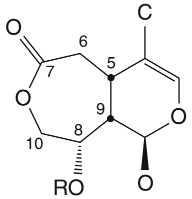
2d



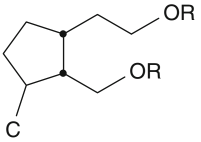
2e



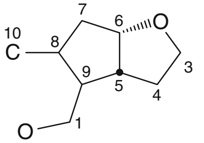
2f



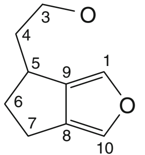
2g



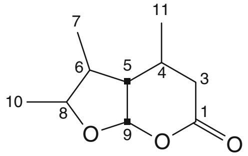
2h R = H or Glc



2i



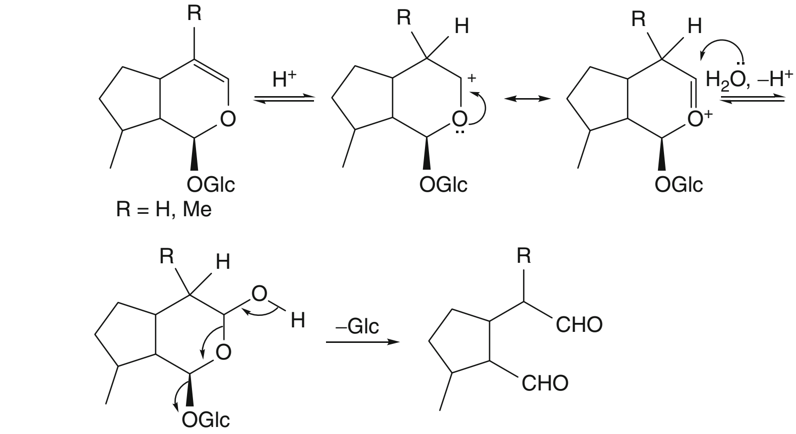
2j



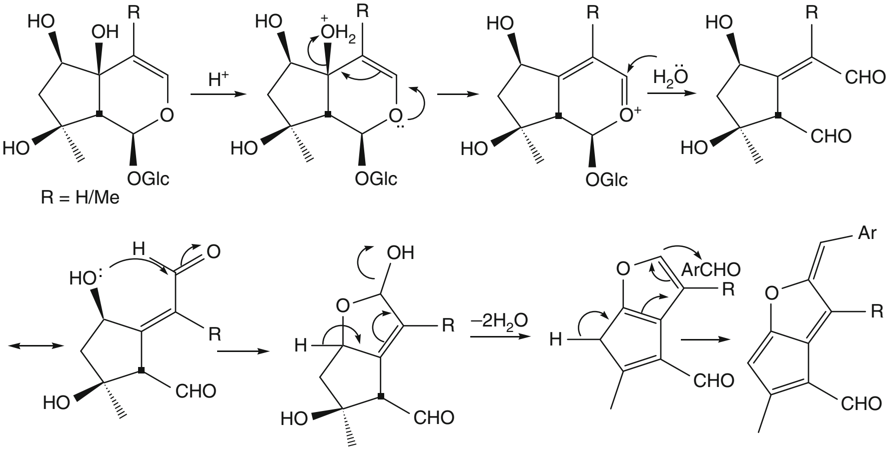
2k

4 Isolation, Chemistry, and Structural Elucidation

Isolation of individual iridoids by traditional silica gel/alumina/charcoal/dianion/Sephadex column chromatography is tedious because of their close R_f values and polar nature. Application of HPLC techniques using octadecylsilanized (ODS)SiO_2/C_{18}Si gel column or HPLC coupled with a diode-array detector and a mass spectrometer (HPLC-DAD-MS) has achieved efficient separation


R
R = H, Me
OGlc
H+
H
+
H2Ö, –H+
O+
–Glc
CHO
CHO

Scheme 97.1 Acid hydrolysis of iridoids


HO
OH
R
R = H/Me
OGlc
H+
OH2
O+
H2Ö
CHO
–2H2O
ArCHO
Ar

Scheme 97.2 Formation of furan iridoids

and isolation of pure iridoids [4–6]. Spots of individual components in thin layer chromatography (TLC) on glass plates were visualized by spraying the plates with methanolic-vanillin-HCl/H_2SO_4 or methanolic-anisaldehyde reagent followed by heating the plates at 100°C for 3–5 min.

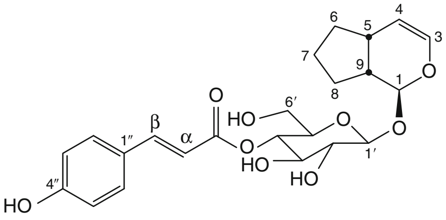
Iridoids are very sensible to acids and are hydrolyzed in mild acidic conditions (Scheme 97.1) [7].

The presence of hydroxyl group at C-5 and C-6 positions results the formation of furan derivatives of iridoids in acidic solution (Scheme 97.2) [8].

Various spectroscopic techniques, namely, UV, IR, NMR, and MS, are frequently used for structure elucidation of iridoids. Especially NMR, spectroscopic techniques including 1D ^{1}H- and ^{13}C-NMR, 2D-HSQC, HMBC, NOESY/ROESY NMR, and 3D-3D-HSQC-TOCSY (heteronuclear single-quantum coherence-total correlation spectroscopy) NMR spectroscopy are very useful for structural elucidation of iridoids. The attachment of sugars or acyl moiety to the aglucone of the iridoid glucosides may be assigned by the study of cross peaks in ^{1}H-^{1}H NOESY or ^{1}H-^{1}H ROESY NMR spectrum [2, 3]. For determination of the absolute configuration of chiral acyl moiety, namely, 2-methyl butanoyl moiety, HPLC method may be used using chiral column (Daicel Chiral OD) [4, 9]. Sometimes X-ray crystallographic study is useful to assign the stereochemical structure of iridoids. For instance, the stereostructure of triohima A and jatamanin A was established by X-ray crystallographic study [10, 11]. Several review articles on the physical constants of iridoids are available in the literature [12–18]. However, the physical constants and plant source of some monomeric iridoids of different subgroups are listed in Table 97.1 to highlight their characteristic substitution and spectral patterns and natural occurrence in various genera of several plant families.

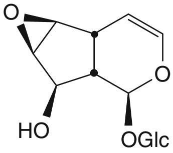
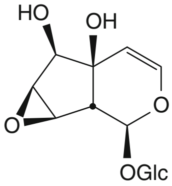
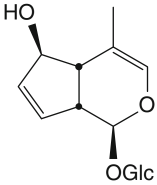
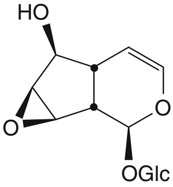
5 Examples of Different Types with Physical Constants and Plant Source

Table 97.1 Some monomeric iridoid and secoiridoid glucosides, aglucones, and derivatives with their physical constants and plant sources

Compound	Physical constants: MF (MW); mp; [α]$_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation [α]$_D$ (deg)	Plant sources with references
Iridoid glucosides having C_8-aglucone skeleton		
Undulatin (**3**) $C_{23}H_{28}O_9$: 448.17  6 5 4 3 7 9 1 O 8 O 6′ HO O O β α 1″ O HO 1′ HO 4″ HO	227–228 −177.7 (MeOH)	*Tecomella undulata* (Bignoniaceae) [19]

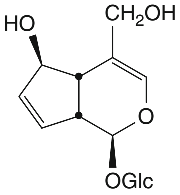
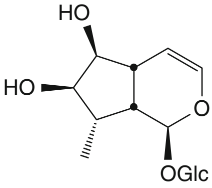
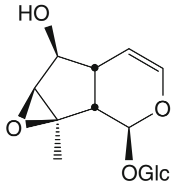
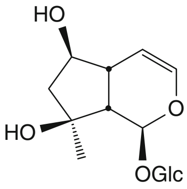
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; [α]$_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation [α]$_D$ (deg)	Plant sources with references
Isounedoside (**4**) $C_{14}H_{20}O_9$: 332.11 	— −52.0 (MeOH)	*Thunbergia grandiflora* (Acanthaceae) [20]
Stilbericoside (**5**) $C_{14}H_{20}O_{10}$: 348.11 	144–146 −61.5 (H_2O)	*Stilbe ericoides* (Verbenaceae) [21], *Thunbergia alata, T. grandiflora, T. fragrans* (Acanthaceae) [22]
Iridoid glucosides having C_9-aglucone skeleton (C-9 in C-4)		
Loasaside (**6**) $C_{15}H_{22}O_8$: 330.13 	216–220 −150.0 (H_2O)	*Mentzelia decapetala* (Loasaceae) [23]
Mentzeloside (Deutzioside) (**7**) $C_{15}H_{22}O_9$: 346.13 	266–270 −101.0 (H_2O)	*Mentzelia decapetala* (Loasaceae) [24], *Deutzia scabra* (Saxifragaceae) [25]

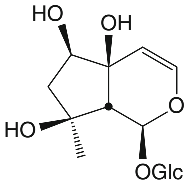
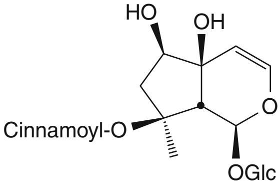
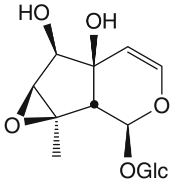
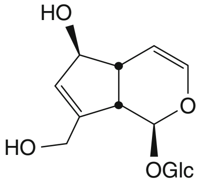
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; $[\alpha]_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant sources with references
Decaloside (**8**) $C_{15}H_{22}O_9$: 346.13 	193 −137.9 (MeOH)	*Mentzelia decapetala* (Loasaceae) [24]
Iridoid glucosides having C_9-aglucone skeleton (C-9 in C-8)		
Angeloside (**9**) $C_{15}H_{24}O_9$: 348.14 	— −37.0 (MeOH)	*Angelonia integerrima* (Scrophulariaceae) [26]
5-Deoxyantirrinoside (**10**) $C_{15}H_{22}O_9$: 346.13 	— −48.0 (MeOH)	*Linaria arcusangeli, L. flava* ssp. *sardoa* (Scrophulariaceae) [27]
Ajugol (**11**) $C_{15}H_{24}O_9$: 348.35 	— −172.1 (MeOH)	*Ajuga reptans* (Labiatae) [28–30]

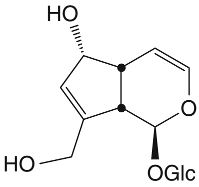
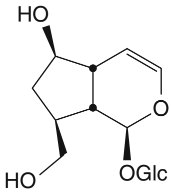
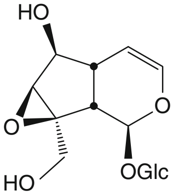
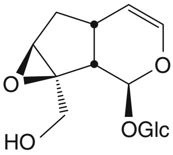
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; [α]$_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation [α]$_D$ (deg)	Plant sources with references
Harpagide (**12**) $C_{15}H_{24}O_{10}$: 364.14 	— −154.0 (MeOH)	*Harpagophytum procumbens* (Scrophulariaceae) [31], *Scrophularia ningpoensis* (Scrophulariaceae) [32]
Harpagoside (**13**) $C_{24}H_{30}O_{11}$: 494.18 	— −42.6 (MeOH)	*Harpagophytum procumbens* [31, 32]
Antirrinoside (**14**) $C_{15}H_{22}O_{10}$: 362.12 	— −78.0 (dioxane)	*Antirrhinum majus, A. speciosum, A. siculum* [33, 34], *Linaria clementei* [35]
Aucubin (aucuboside, rhinanthin) (**15**) $C_{15}H_{22}O_9$: 346.13 	180–182 −162.0 (H_2O)	*Verbascum spinosum* (Verbenaceae), *Clerodendrum* spp. (Verbenaceae) [36, 37]

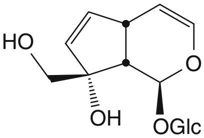
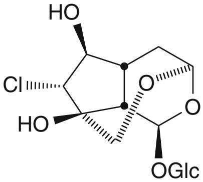
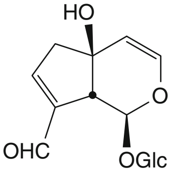
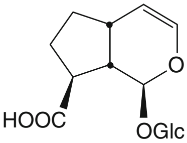
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; $[\alpha]_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant sources with references
6-*epi*-Aucubin (**16**) $C_{15}H_{22}O_9$: 346.13  	— −58.9 (MeOH)	*Tecoma chrysantha* (Bignoniaceae) [38]
(8S)-7,8-Dihydroaucubin (**17**) $C_{15}H_{24}O_9$: 348.35  	— —	*Phaulopsis imbricata* (Acanthaceae) [39]
Catalpol (**18**) $C_{15}H_{22}O_{10}$: 362.12  	203–205 −102.0 (90% EtOH)	Several plants of Bignoniaceae, Globulariaceae, Plantaginaceae, Scrophulariaceae [40–42]
6-Deoxycatalpol (**19**) $C_{15}H_{22}O_9$: 346.33  	212.5–214 −34.0 (EtOH) −47.0 (MeOH)	*Castilleja rhexifolia* ssp. *miniata* (Scrophulariaceae), *Utricularia australis* (Lentibulariaceae) [43, 44]

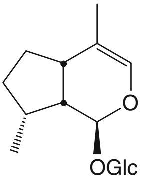
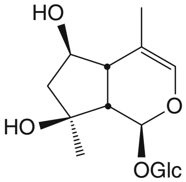
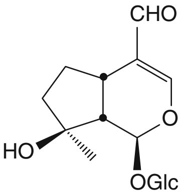
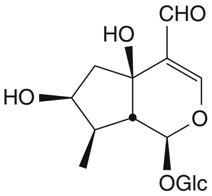
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; $[\alpha]_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant sources with references
Eranthemoside (**20**) $C_{15}H_{22}O_9$: 346.33  	— −98.0 (EtOH)	*Eranthemum pulchellum* (Acanthaceae) [45]
Glutinoside (**21**) $C_{15}H_{23}ClO_{10}$: 398.10  	185–186 (pentaacetate) −79.2 (MeOH)	*Rehmannia glutinosa* (Scrophulariaceae) [46]
Hygrophiloside (**22**) $C_{15}H_{20}O_9$: 344.32  	— —	*Hygrophila difformis* (Acanthaceae) [47]
Grandifloric acid (**23**) $C_{15}H_{22}O_9$: 346.13  	— −84.0 (MeOH)	*Thunbergia grandiflora, T. alata* (Acanthaceae). [20], *T. laurifolia* [48]

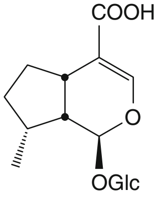
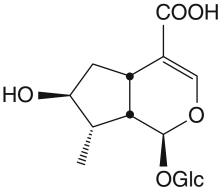
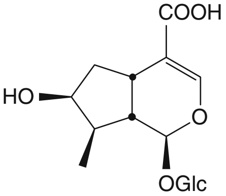
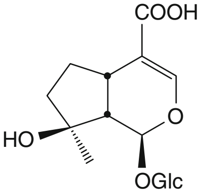
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; [α]$_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation [α]$_D$ (deg)	Plant sources with references
General type iridoid glucosides having C_{10}-aglucone skeleton		
Boschnaside (8-*epi*-iridodial glucoside) (**24**) $C_{16}H_{26}O_7$: 330.17  	131–132 (tetraacetate) −140.8 (CHCl$_3$)	*Boschniakia rossica* (Orobanchaceae) [49]
5-Deoxylamiol (**25**) $C_{16}H_{26}O_9$: 362.16  	— —	*Satureja vulgaris* (Labiatae), also prepared from shanzhiside methyl ester [22, 50]
Ixoroside (**26**) $C_{16}H_{24}O_9$: 360.14  	— +102.6 (MeOH)	*Ixora chinensis* [51], *Nepeta heliotropifolia* (Lamiaceae) [52]
Tecomoside (**27**) $C_{16}H_{24}O_{10}$: 376.14  	124–125 −123.5 (MeOH)	*Campsis chinensis (Bignoniaceae)* [53, 54]

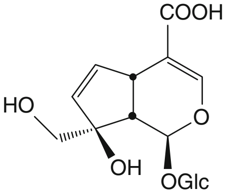
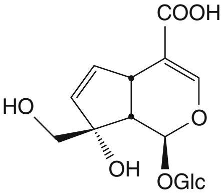
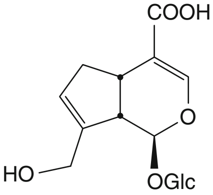
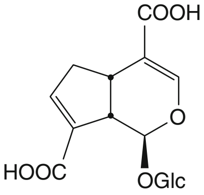
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; $[\alpha]_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant sources with references
7-Deoxy-8-*epi*-loganic acid (**28**) $C_{16}H_{24}O_9$: 360.14 	210–213 −117.0 (MeOH)	*Argylia radiata* (Bignoniaceae) [55]
8-*epi*-Loganic acid (**29**) $C_{16}H_{24}O_{10}$: 376.14 	138–139 −57.4 (MeOH	*Linaria cymbalaria* (Scrophulariaceae) [29, 56, 57]
Loganic acid (**30**) $C_{16}H_{24}O_{10}$: 376.14 	— —	*Lonicera periclymenum, Swertia caroliniensis, Gentiana pedicellata* (Gentianaceae) [58–60]
Mussaenosidic acid (**31**) $C_{16}H_{24}O_{10}$: 376.14 	— −118.0 (MeOH)	*Melampyrum cristatum* (Scrophulariaceae) [61]

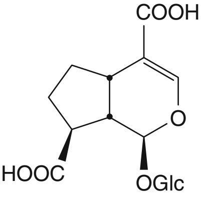
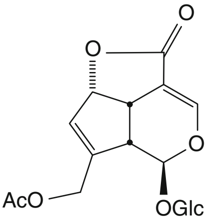
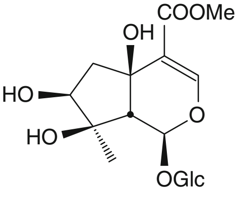
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; [α]$_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant sources with references
Gardenosidic acid (**32**) $C_{16}H_{22}O_{11}$: 390.12 	66–68 (Me ester hexaacetate) −70.7 (MeOH)	*Galium mollugo* (Rubiaceae) [62, 63], *Xeromphis nilotica* (Rubiaceae) [64]
Monotropein (**33**) $C_{16}H_{22}O_{11}$: 390.12 	161–163 −130.0 (H_2O)	*Arbutus unedo* (Ericaceae) [65, 66]
Geniposidic acid (**34**) $C_{16}H_{22}O_{10}$: 374.12 	155 +19.3 (MeOH)	*Wendlandia formosana*, *W. tinctoria* [67, 68], *Ixora chinensis* (Rubiaceae) [51]
Ixoside (**35**) $C_{16}H_{20}O_{11}$: 388.10 	— +33.6 (H_2O)	*Ixora chinensis* [51], *Wendlandia tinctoria* (Rubiaceae) [69]

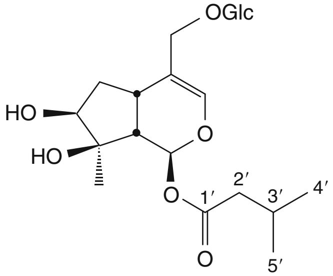
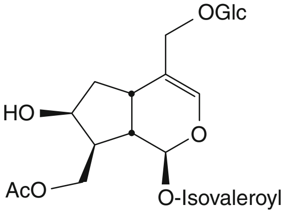
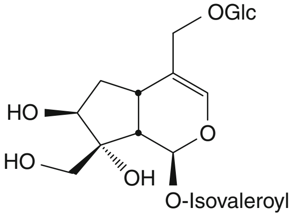
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; [α]$_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation [α]$_D$ (deg)	Plant sources with references
Forsythide (**36**) $C_{16}H_{22}O_{11}$: 390.12 	— −64.7 (MeOH)	*Forsythia viridissima* (Oleaceae) [70, 71]
Asperuloside (**37**) $C_{18}H_{22}O_{11}$: 414.12 	131–132 −200.0 (H_2O)	*Daphniphyllum macropodum*, *Rubia tinctorum*, *Asperula odorata*, *Galium aparine* [72, 73].
Lamiide (**38**) $C_{17}H_{26}O_{12}$: 422.39 	186–188 −127.0 (MeOH)	*Penstemon strictus* (Scrophulariaceae) [74, 75]

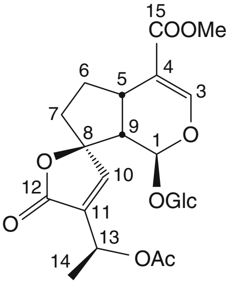
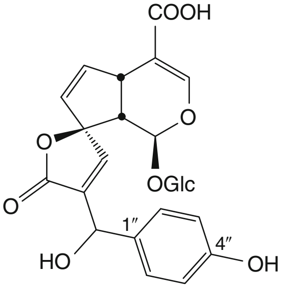
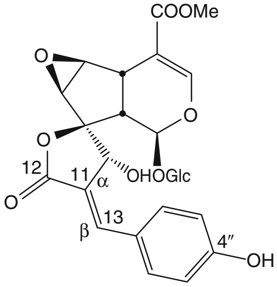
(*continued*)

Table 97.1 (continued)

Compound Name, structure	Physical constants: MF (MW); mp; $[\alpha]_{D;}$ MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant source [Reference] Plant sources with references
Valeriana-Type Iridoid Glucosides Having C_{10}-Aglucone Skeleton		
8-*epi*-Valerosidate (**39**) $C_{21}H_{34}O_{11}$: 462.21  OGlc HO HO O O 1′ 2′ 3′ 4′ O 5′	60 —	*Penstemon serrulatus* (Scrophulariaceae) [76]
10-Acetylpatrinoside (**40**) $C_{23}H_{36}O_{12}$: 504.22  OGlc HO O AcO O-Isovaleroyl	— −51.6 (MeOH)	*Viburnum rhytidophyllum* (Caprifoliaceae) [77]
Isosuspensolide F (**41**) $C_{21}H_{34}O_{12}$: 478.20  OGlc HO O HO OH O-Isovaleroyl	— −19.6 (MeOH)	*Viburnum ayavacense* (Caprifoliaceae) [78]

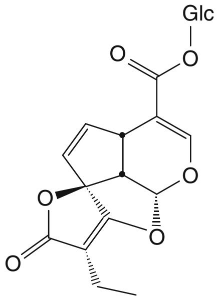
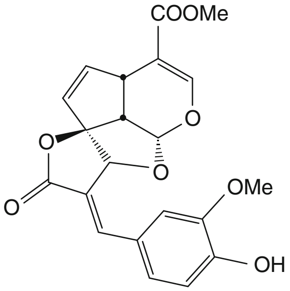
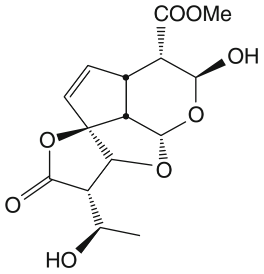
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; $[\alpha]_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant sources with references
Plumeria-type iridoid glucosides having C_{14}-aglucone skeleton		
13-*O*-Acetylplumieride (**42**) $C_{23}H_{28}O_{13}$: 512.15  15 COOMe 4 3 6 5 7 9 8 1 O O 12 10 OGlc O 11 13 14 OAc	— −37.8 (MeOH)	*Allamanda neriifolia* (Apocynaceae) [79]
Gaertneric acid (**43**) $C_{25}H_{26}O_{13}$: 534.14  COOH O O OGlc O 1″ 4″ HO OH	— +48.8 (MeOH)	*Morinda morindoides* syn. *Gaertnera morindoides* (Rubiaceae) [80]
Citrifolinoside A (**44**) $C_{26}H_{28}O_{14}$: 564.15  COOMe O O O 12 11 α OHOGlc O 13 β 4″ OH	— +65.2 (MeOH)	*Morinda citrifolia* (Rubiaceae) [81]

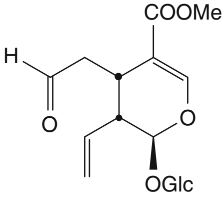
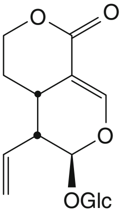
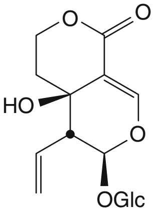
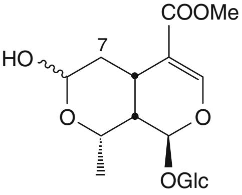
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; $[\alpha]_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant sources with references
Plumericin-Type Iridoids Having C_{14}-Aglucone Skeleton		
Plumenoside (**45**) $C_{20}H_{24}O_{11}$: 440.13 	— +117.3 (MeOH)	*Plumeria acutifolia* (Apocynaceae) [82]
Oruwacin (**46**) $C_{21}H_{18}O_8$: 398.10 	223 +193.0 ($CHCl_3$)	*Morinda lucida* (Rubiaceae) [83]
Allamancin (**47**) $C_{15}H_{18}O_8$: 326.10 	— +74.5 ($CHCl_3$)	*Allamanda neriifolia* (Apocynaceae) [79]

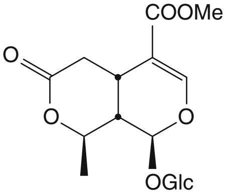
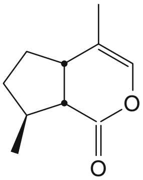
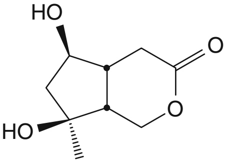
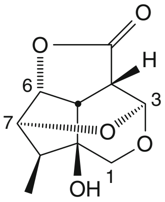
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; $[\alpha]_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant sources with references
Secoiridoid Glucosides Having C_{10}-Aglucone Skeleton		
Secologanin (loniceroside) (**48**) $C_{17}H_{24}O_{10}$: 388.14 	— −105.0 (MeOH)	*Lonicera morrowii* (Caprifoliaceae) [84]
Sweroside (**49**) $C_{16}H_{22}O_9$: 358.13 	— −236.0 (H_2O) −187.2 (MeOH)	*Swertia japonica* (Gentianaceae) [85], *Triplostegia grandiflora* (Dipsacaceae) [86]
Swertiamarin (**50**) $C_{16}H_{22}O_{11}$: 390.12 	— —	*Swertia japonica* (Gentianaceae) [87, 88]
Morroniside (isolated as 7α/7β mixture) (**51**) $C_{17}H_{26}O_{11}$: 406.15 	— −94.2 (MeOH)	*Sambucus ebulus* (Caprifoliaceae) [89]

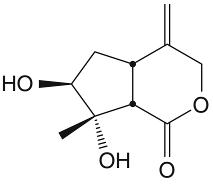
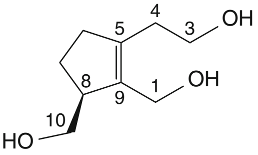
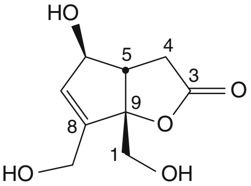
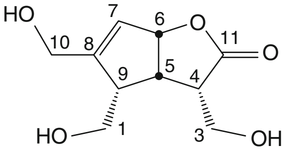
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; $[\alpha]_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant sources with references
8-*epi*-Kingiside (**52**) $C_{17}H_{24}O_{11}$: 404.13 	— −45.9 (MeOH)	*Ligustrum japonicum* (Oleaceae) [90]
Iridoid and secoiridoid aglycones and derivatives		
cis-Nepetalactone (**53**) $C_{10}H_{14}O_2$: 166.10 	— +3.7 ($CHCl_3$)	*Nepeta cataria* (Labiatae) [91]
Jioglutolide (**54**) $C_9H_{14}O_4$: 186.09 	141–142 −8.4 (MeOH)	*Rehmannia glutinosa* (Scrophulariaceae) [92]
GEIR-1 (**55**) $C_{10}H_{12}O_5$: 212.08 	119–120 +41.9 (MeOH)	*Gelsemium elegans* (Loganiaceae) [93]

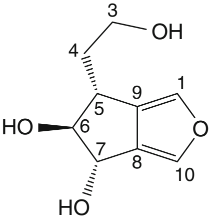
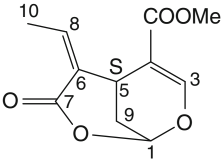
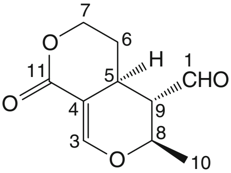
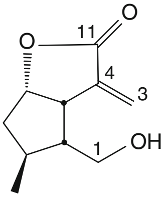
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; [α]$_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation [α]$_D$ (deg)	Plant sources with references
Jatamanin A (**56**) $C_{10}H_{14}O_4$: 198.09 	177–178 +139.0 (MeOH)	*Valeriana jatamansi* (Valerianaceae) [11]
Cerberidol (**57**) $C_9H_{16}O_5$: 172.22  4 3 OH 5 8 1 OH 9 10 HO	— +11.0 (MeOH)	*Cerbera manghas* (Apocynaceae) [94]
Rehmaglutin C (**58**) $C_9H_{12}O_5$: 200.19  HO 5 4 3 O 9 O 8 1 HO OH	— −51.4 (MeOH)	*Rehmannia glutinosa* (Scrophulariaceae) [46]
4-*epi*-Borreriagenin (**59**) $C_{10}H_{14}O_5$: 236.06  HO 7 6 O 11 O 10 8 9 5 4 HO 1 3 OH	— −5.0 (MeOH)	*Morinda citrifolia* (Rubiaceae) [95]

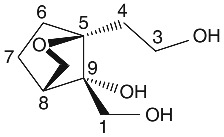
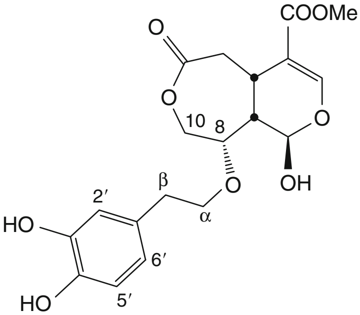
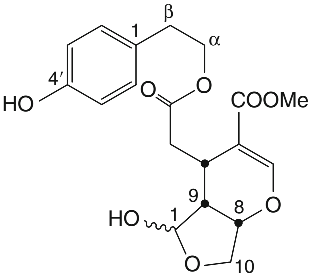
(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; $[\alpha]_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation $[\alpha]_D$ (deg)	Plant sources with references
Jiofuran (**60**) $C_9H_{12}O_4$: 184.19  3 OH 4 9 1 5 HO 6 O 7 8 10 HO	— −30.4 (MeOH)	*Rehmannia glutinosa* (Scrophulariaceae) [92]
Triohima A (**61**) $C_{11}H_{12}O_5$: 224.06  10 8 COOMe S 6 5 3 O 7 9 O O 1	143–145 +37.0 ($CHCl_3$)	*Triosteum himalayanum*, *T. pinnatifidum* (Caprifoliaceae) [10, 96]
Naucledal (**62**) $C_{10}H_{12}O_4$: 196.07  7 O 6 H 1 11 5 CHO O 4 9 8 3 O 10	115–116 −1.9 ($CHCl_3$)	*Nauclea diderrichii* (Rubiaceae) [97], *Triosteum pinnatifidum* (Caprifoliaceae) [96]
GSIR-1 (**63**) $C_{10}H_{16}O_3$: 184.11 	— —	*Gelsemium sempervirens* (Loganiaceae) [98]

(*continued*)

Table 97.1 (continued)

Compound	Physical constants: MF (MW); mp; [α]$_{D;}$	Plant source [Reference]
Name, structure	MP (°C) Optical rotation [α]$_D$ (deg)	Plant sources with references
Cyclocerberidol (**64**) $C_9H_{16}O_4$: 188.22  6 5 4 3 OH 7 O 9 OH 8 1 OH	— −16.9 (MeOH)	*Cerbera manghas* (Apocynaceae) [94]
Jasmolactone B (**65**) $C_{19}H_{22}O_9$: 394.38  O COOMe O 10 8 O β O OH 2′ α HO 6′ HO 5′	— +100.1 (MeOH)	*Jasminum multiflorum* (Oleaceae) [99]
Ligstrohemiacetal B (**66**) (revised structure of jasmolactone A). (Jaspolyanoside) $C_{19}H_{22}O_8$: 378.13  β 1 α 4′ O O COOMe HO 9 8 O HO 1 O 10	— +179 (MeOH)	*Ligustrum vulgare* (Oleaceae) [100], *Jasminum multiflorum* [99]

6 Synthesis

The diversity of biological activity exhibited by several iridoids has generated much interest for their synthesis. Due to difficulties of glycosidation of iridoid aglycones, only a few synthetic approaches in this area have been reported. Some of these synthetic routes for stereoselective synthesis of natural iridoids are discussed here briefly to make it inspiring among the synthetic chemists for their future plans.

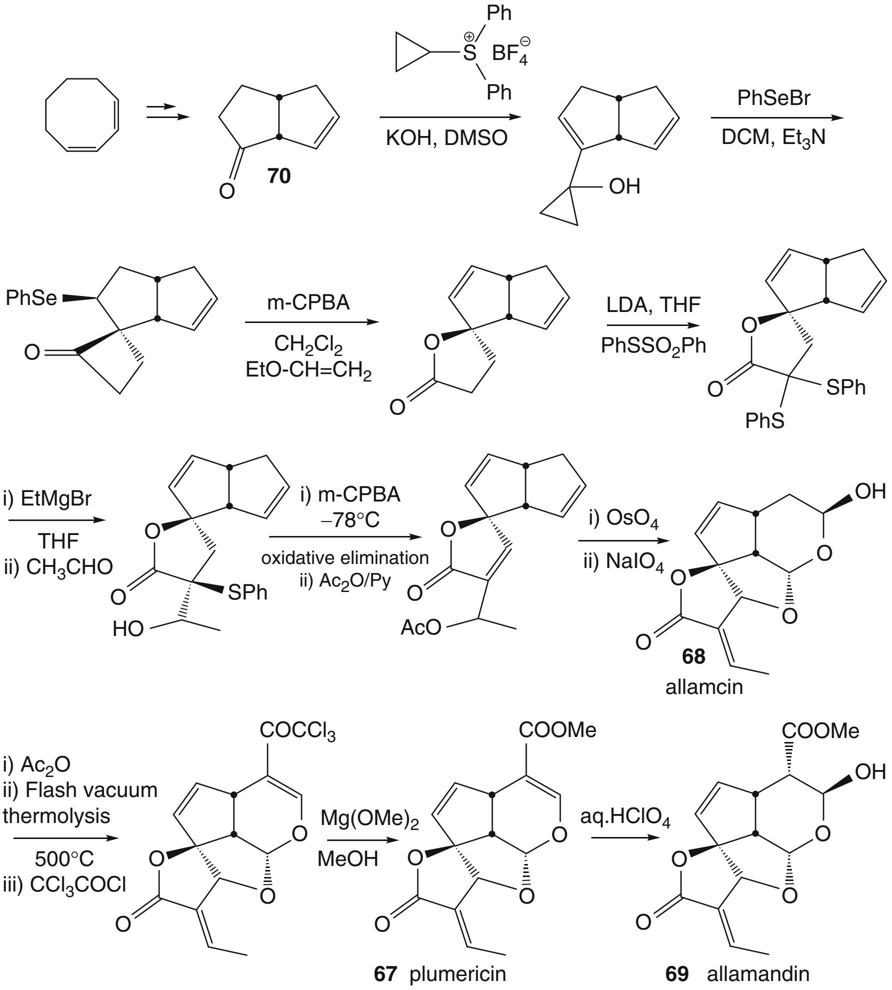
Trost et al. reported an efficient method for synthesis of plumericin (**67**), allamcin (**68**), and antileukemic allamandin (**69**), applying a biomimetic strategy. The requisite key intermediates were synthesized using the concept of spiro-annulation and stereocontrolled geminal alkylation. Their strategy required 9, 13, and 14 steps from bicyclic ketone (**70**) for synthesis of allamcin, plumericin, and allamandin, respectively. The bicyclic ketone was synthesized from cycloocta-1,3-diene in three steps. The synthetic route is outlined in Scheme 97.3 [101].

MacMillan et al. reported total synthesis of iridoids, brasoside (**71**), and littoralisone (**72**), in 13 steps in 13% overall yield [102]. Both these iridoids were isolated earlier from *Verbena littoralis*, and littoralisone (**72**) was demonstrated to be the active agent for increased NGF-induced neurite outgrowth in PC12D cells [103]. They synthesized the iridolactone aglucone (**73**) using (–) citronellol (**74**) as starting material via the intermediate, formyl enal (**74a**). Proline-catalyzed intra-molecular Michael addition of the formyl enal was the key step of their synthesis (Scheme 97.4).

Recently, Vidari et al. synthesized 9-deoxygelsemide (**75**), which was isolated earlier from *Gelsemium elegans* by Takayama et al. [104, 105]. The key synthetic steps were the variant Woodward-Prevost reaction for installation of characteristic *cis*-α-1,2-dioxygenated system at C-6 and C-7, and construction of the dihydropyran ring via formylation of γ-lactone. The total synthesis of the iridoid was achieved in 11 steps and 6.6% overall yield from enantiomerically pure lactone (**76**) (Scheme 97.5).

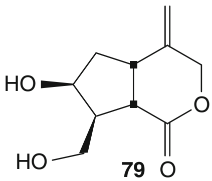
Krische et al. reported an asymmetric synthesis of (+) geniposide (**77**) in 14 steps [106]. It may be noted that (+) geniposide showed significant antitumor [107] and anti-inflammatory activity [108]. Its aglycone, (+) genipin (**145**), was found to be effective for treatment of type II diabetes [109]. The key feature of this synthesis is the construction of *cis*-fused cyclopenta [c]-pyran ring of iridoid basic skeleton by a phosphine-catalyzed intermolecular [3 + 2] cycloaddition reaction of (*S*)-enone (**78**) and ethyl-2,3-butadienoate. One of the starting materials, (S)-enone (**78**), was synthesized from furfuryl alcohol in three steps. The steps of total synthesis of (+) geniposide are outlined (Scheme 97.6).

Lee et al. also reported a total synthesis of iridolactone, 7-hydroxy-8-(hydroxymethyl)-4-methylenehexahydrocyclopenta[c]-pyran-1-(3 H)-one (**79**) via intramolecular Pd(0)-catalyzed allylic alkylation [110]. This iridolactone was isolated from the roots and rhizomes of *Nardostachys chinensis* (Valerianaceae) [111]. The plant exhibited a variety of biological activities such as sedative, antimalarials, antinociceptive, cytotoxic, and enhancement of nerve growth factor activities [112].

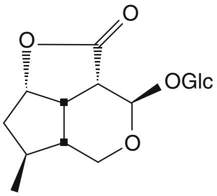

70
Ph
BF$_4^{\ominus}$
Ph
KOH, DMSO
OH
PhSeBr
DCM, Et$_3$N
PhSe
O
m-CPBA
CH$_2$Cl$_2$
EtO-CH=CH$_2$
LDA, THF
PhSSO$_2$Ph
SPh
PhS
i) EtMgBr
THF
ii) CH$_3$CHO
HO
i) m-CPBA
−78°C
oxidative elimination
ii) Ac$_2$O/Py
AcO
i) OsO$_4$
ii) NaIO$_4$
68
allamcin
i) Ac$_2$O
ii) Flash vacuum thermolysis
500°C
iii) CCl$_3$COCl
COCCl$_3$
Mg(OMe)$_2$
MeOH
COOMe
aq.HClO$_4$
67 plumericin
69 allamandin

Scheme 97.3 Biomimetic synthesis of allamcin, plumericin, and allamandin

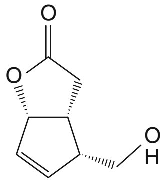
Vidari et al. reported enantioselective total synthesis of semperoside A (**80**) using hydroxymethyl γ-lactone (**81**) as a starting material in 10 steps and 17% overall yield [113].



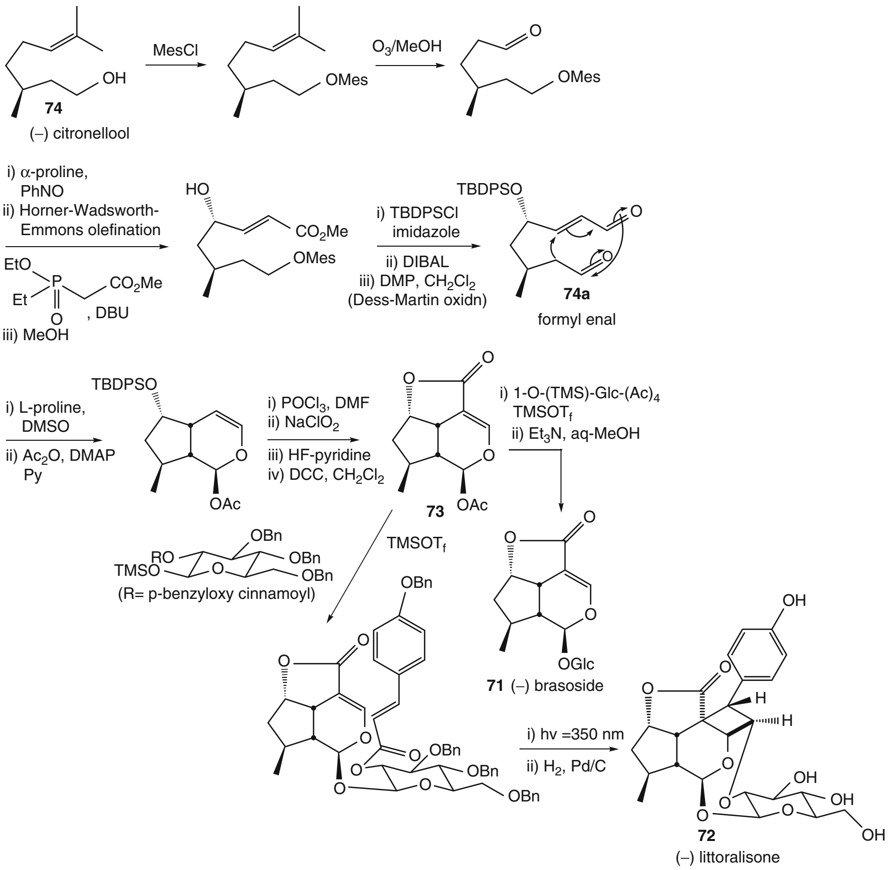
79



80



81


OH
74
(–) citronellool
MesCl
OMes
O3/MeOH
O
OMes
i) α-proline, PhNO
ii) Horner-Wadsworth-Emmons olefination
EtO
Et
P
O
CO2Me
, DBU
iii) MeOH
HO
CO2Me
OMes
i) TBDPSCl imidazole
ii) DIBAL
iii) DMP, CH2Cl2 (Dess-Martin oxidn)
TBDPSO
O
O
74a
formyl enal
i) L-proline, DMSO
ii) Ac2O, DMAP Py
TBDPSO
O
OAc
i) POCl3, DMF
ii) NaClO2
iii) HF-pyridine
iv) DCC, CH2Cl2
O
O
O
73
OAc
i) 1-O-(TMS)-Glc-(Ac)4 TMSOTf
ii) Et3N, aq-MeOH
O
O
O
OGlc
71 (–) brasoside
OBn
RO
OBn
TMSO
O
OBn
(R= p-benzyloxy cinnamoyl)
TMSOTf
OBn
O
O
O
O
O
OBn
OBn
OBn
i) hν =350 nm
ii) H2, Pd/C
OH
O
O
H
H
O
O
O
OH
OH
OH
72
(–) littoralisone

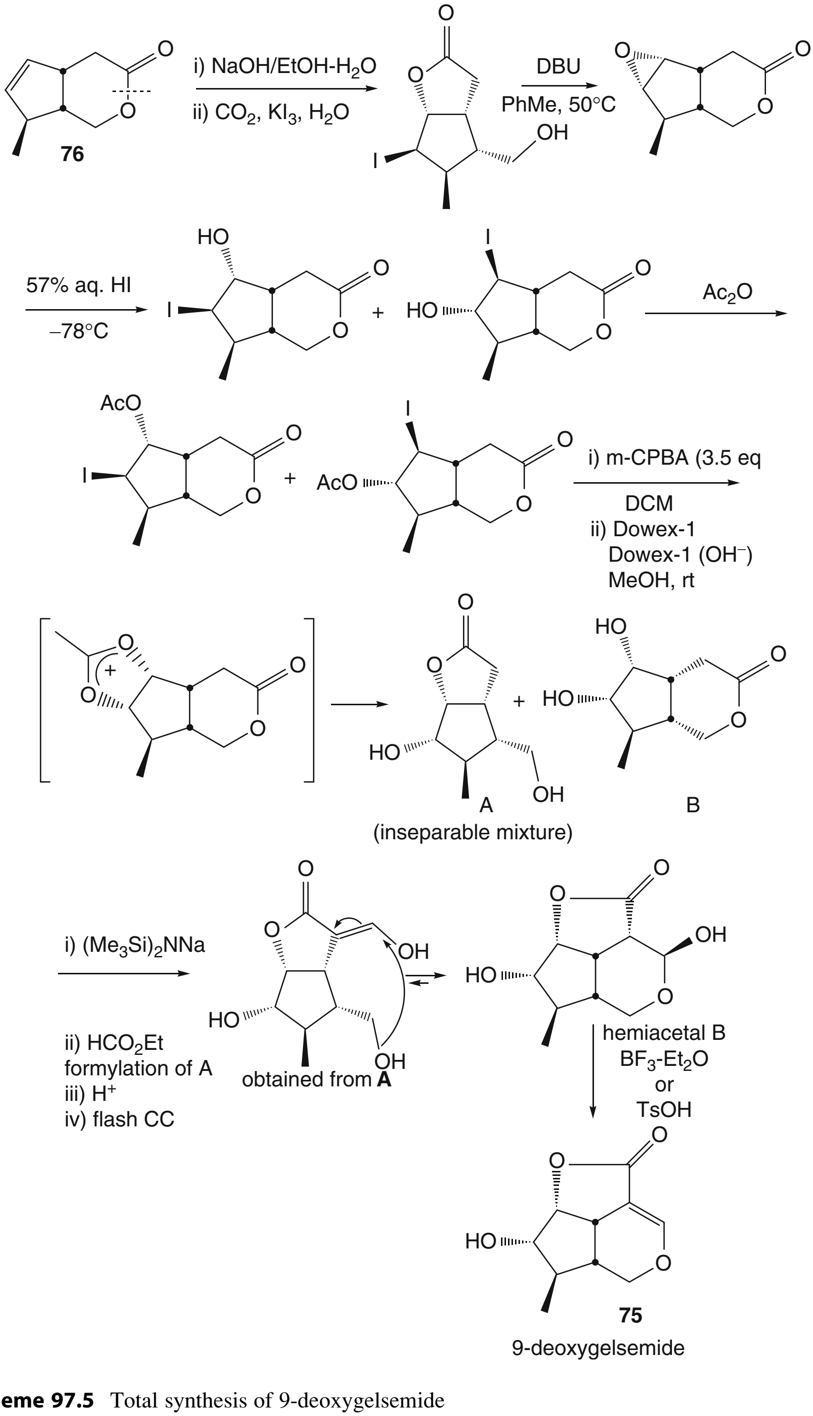
Scheme 97.4 Total synthesis of (–) brasoside and (–) littoralisone

Lupton et al. reported the total synthesis of (–) 7-deoxyloganin (**82**) in 18 steps from 2,5-dimethoxytetrahydrofuran in overall 0.8% yield. The key step of their synthetic strategy was the N-heterocyclic nucleophilic carbene-catalyzed rearrangement of α,β-unsaturated enol ester (**83**) to dihydropyranone (**84**) (Scheme 97.7) [114].

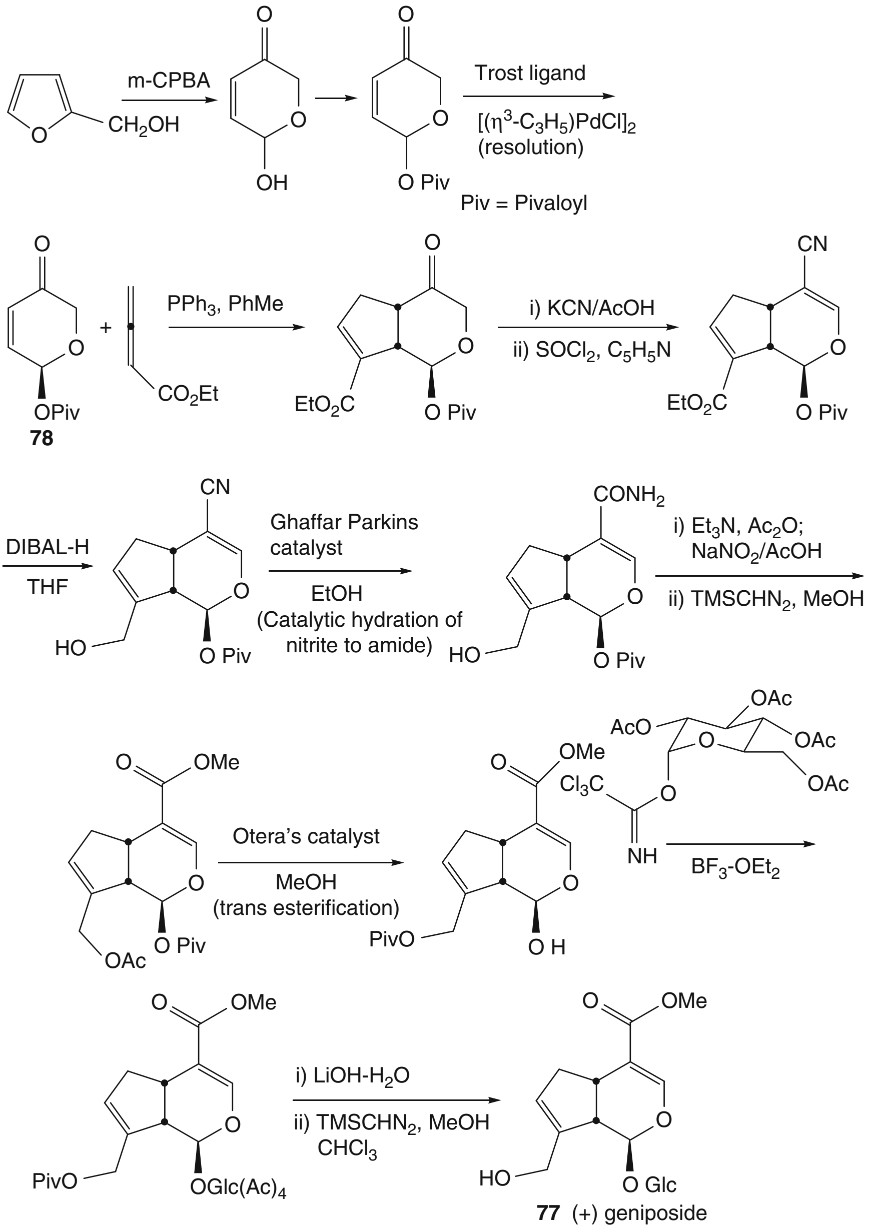
Hofferberth et al. reported diastereoselective synthesis of several ant-associated iridoids, namely, nepetalactol (**85**), dolichodial (**86**), dihydronepetalactone (**87**), and isoiridomyrmecin (**88**), from citronellal (**89**) using common laboratory reagents (Scheme 97.8) [115].

7 Biosynthesis

Several groups carried out tracer studies for the understanding of the biosynthesis of iridoid glycosides. Now we have reached to a point from which a fairly complete

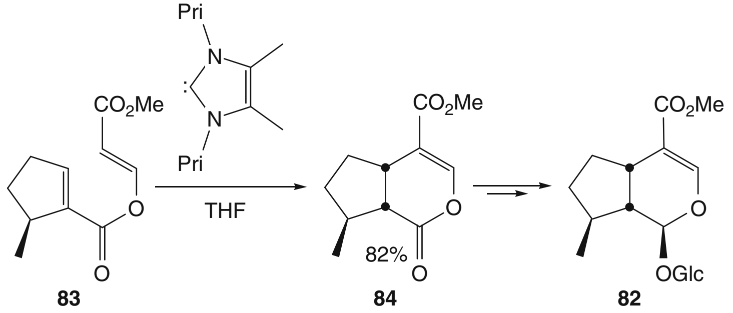

76
i) NaOH/EtOH-H2O
ii) CO2, KI3, H2O
DBU
PhMe, 50°C
57% aq. HI
−78°C
Ac2O
i) m-CPBA (3.5 eq
DCM
ii) Dowex-1
Dowex-1 (OH−)
MeOH, rt
A
B
(inseparable mixture)
i) (Me3Si)2NNa
ii) HCO2Et
formylation of A
iii) H+
iv) flash CC
obtained from A
hemiacetal B
BF3-Et2O
or
TsOH
75
9-deoxygelsemide

Scheme 97.5 Total synthesis of 9-deoxygelsemide

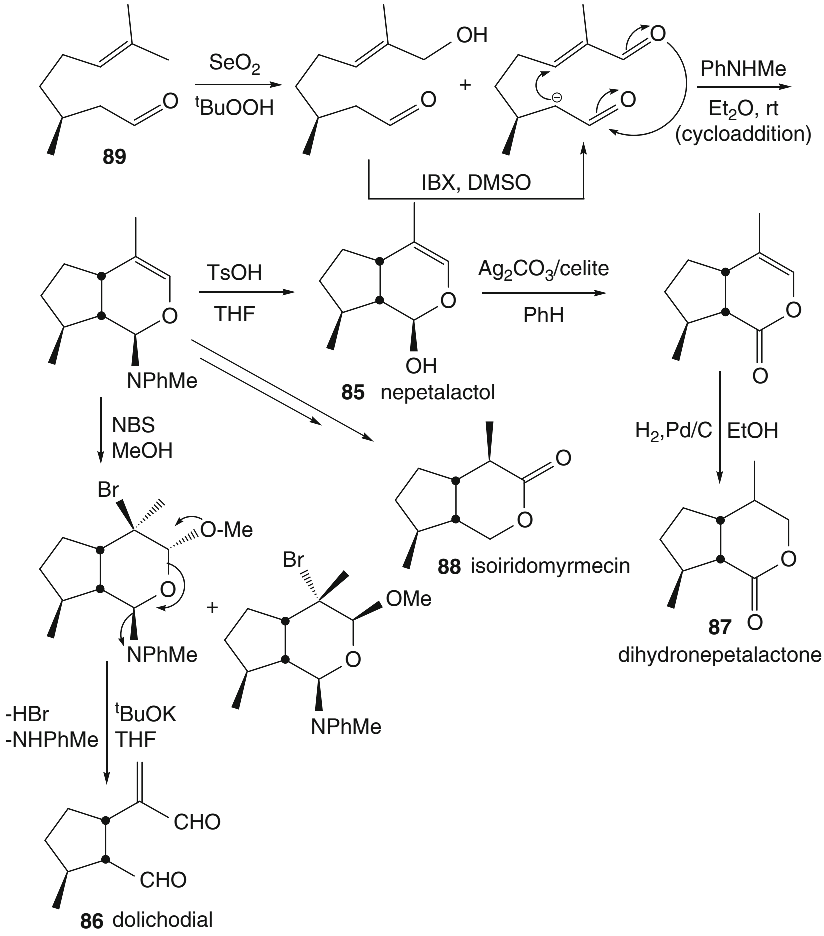

m-CPBA
CH2OH
OH
O Piv
Trost ligand
[(η3-C3H5)PdCl]2
(resolution)
Piv = Pivaloyl
OPiv
78
CO2Et
PPh3, PhMe
EtO2C
i) KCN/AcOH
ii) SOCl2, C5H5N
CN
DIBAL-H
THF
HO
Ghaffar Parkins catalyst
EtOH
(Catalytic hydration of nitrite to amide)
CONH2
i) Et3N, Ac2O; NaNO2/AcOH
ii) TMSCHN2, MeOH
OMe
OAc
Otera's catalyst
MeOH
(trans esterification)
PivO
O H
AcO
Cl3C
NH
BF3-OEt2
OGlc(Ac)4
i) LiOH-H2O
ii) TMSCHN2, MeOH CHCl3
O Glc
77 (+) geniposide

Scheme 97.6 Total synthesis of (+) geniposide

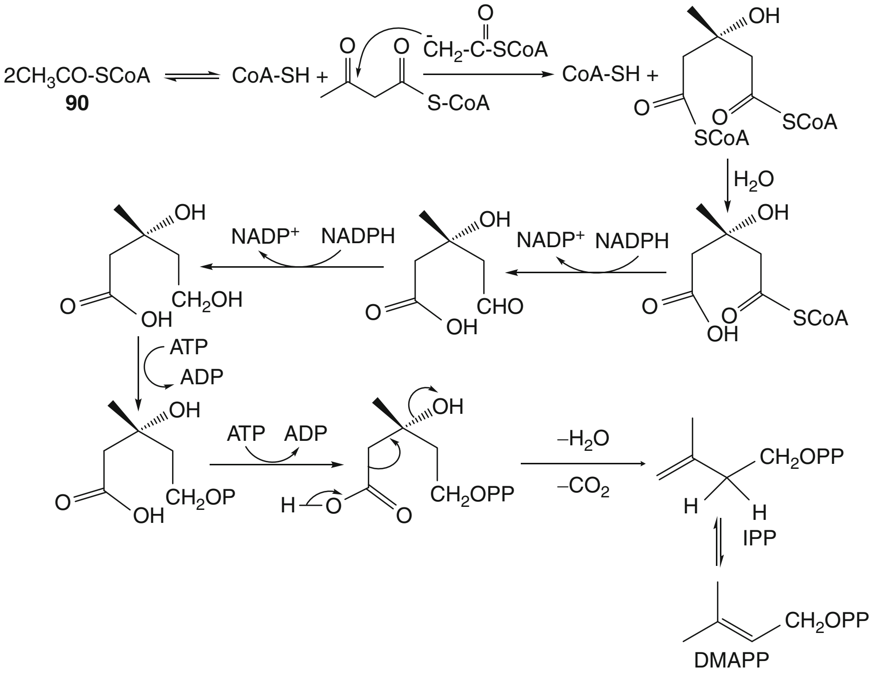
picture can be drawn. Very early, Spurgeon and Porter suggested that isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are the universal building blocks for all terpenoids [116]. IPP and DMAPP are biosynthesized in two pathways – namely, the classical mevalonic acid (MVA) pathway and the novel


Pri
N
:
N
Pri
CO2Me
O
O
83
THF
CO2Me
O
82%
O
84
CO2Me
O
OGlc
82

Scheme 97.7 Synthesis of (–) 7-deoxyloganin


O
89
SeO2
tBuOOH
OH
O
+
O
O
PhNHMe
Et2O, rt
(cycloaddition)
IBX, DMSO
O
NPhMe
TsOH
THF
O
OH
85 nepetalactol
Ag2CO3/celite
PhH
O
O
H2,Pd/C EtOH
NBS
MeOH
Br
O-Me
O
NPhMe
+
Br
OMe
O
NPhMe
O
O
88 isoiridomyrmecin
O
O
87
dihydronepetalactone
-HBr
-NHPhMe
tBuOK
THF
CHO
CHO
86 dolichodial

Scheme 97.8 Synthesis of ant-associated iridoids

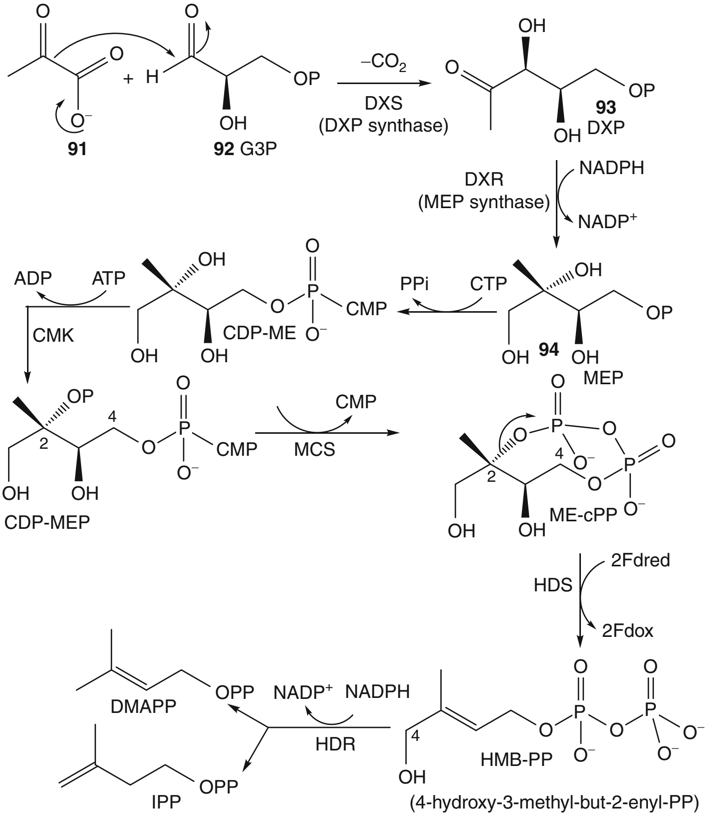


90

Scheme 97.9 MVA pathway for isoprenoid biosynthesis

2-methyl-D-erythritol 4-phosphate pathway. The MVA pathway was suggested to proceed from three molecules of acetyl CoA (**90**) in presence of nicotinamide-adenine-dinucleotide phosphate (NADPH) to form mevalonic acid as key intermediate, which in presence of adenosine triphosphate (ATP) was converted into isopentenyl pyrophosphate (IPP). IPP in presence of the appropriate enzyme was isomerized to β,β-dimethylallyl pyrophosphate (DMAPP) [117] (Scheme 97.9). The MEP pathway started from pyruvic acid (**91**) and D-glyceraldehyde 3-phosphate (**92**) via the formation of key intermediates 1-deoxy-D-xylulose 5-phosphate (DXP, **93**) and 2-methyl-D-erythritol 4-phosphate (MEP, **94**), which was converted into isopentenyl pyrophosphate (IPP) by a series of steps (Scheme 97.10) [118, 119]. Detailed tracer experiments have shown that these two pathways exist side by side in higher plants [120, 121].

In the investigation of the biosynthesis of secologanin in *Catharanthus roseus* cell culture using ^{13}C-labeled D-glucose as upstream precursor, Contin et al. established that the MEP pathway played a major role in its biosynthesis, whereas the MVA pathway was only involved in a minor way [122]. Eichinger et al. predicted a comparable result in their retro-biosynthetic ^{13}C-NMR study of loganin in *Rauwolfia serpentina* cells where they observed that the minor incorporation of mevalonate into loganin resulted from metabolic exchange between the two isoprenoid pathways [123].


91
92 G3P
–CO2
DXS
(DXP synthase)
93
DXP
DXR
(MEP synthase)
NADPH
NADP+
94
MEP
PPi
CTP
CDP-ME
ADP
ATP
CMK
CDP-MEP
CMP
MCS
ME-cPP
HDS
2Fdred
2Fdox
HMB-PP
(4-hydroxy-3-methyl-but-2-enyl-PP)
HDR
NADP+
NADPH
DMAPP
IPP
OPP

Scheme 97.10 MEP pathway for isoprenoid biosynthesis

Further studies on the search of iridoid precursors by Inouye et al. and other groups it was univocally established that monoterpene geraniol was the true precursor. In the first committed step, geraniol derived from IPP is hydroxylated by geraniol-10-hydroxylase to 10-hydroxygeraniol followed by formation of iridodial and iridotrial intermediates in subsequent steps in biosynthesis of iridoids [124–127]. Oxidation of iridotrial followed by glucosylation gave deoxyloganic acid, which on hydroxylation and esterification produced loganin (**30a**). Loganin on oxidative cleavage by enzyme secologanin synthase yielded secologanin (**101**) in *C. roseus* cell culture [128].

Damtoft et al. studied the biosynthesis of iridoids of genus *Fraxinus* by incorporation of labeled [6,7,8,10–^{2}H]-iridodial, iridotrial, and deoxyloganic acid aglucone in *F. excelsior*. They observed that both iridodial and iridotrial gave a detectable incorporation (4.5%) into 7-ketologanin (**100**), and iridotrial in addition gave low incorporations (each 1%) into 7-glucosyl 11-methyl oleoside (**102**) and excelsioside (**103**). Feeding of labeled deoxyloganic acid aglucone gave only an incorporation of 2% into excelsioside (**103**) [129].

In another set of experiment, Damtoft et al. incorporated deuterium-labeled deoxyloganic acid, 7-*epi*-loganin, loganin, and 7-ketologanin in *F. excelsior* and observed that loganin was not incorporated whereas the other three compounds gave significant incorporations into the oleosides. They also administered deuterium-labeled deoxyloganic acid and 7-ketologanic acid into *F. excelsior* and found that both the compounds gave 7–8% incorporations into excelsioside but only 7-ketologanic acid gave significant incorporations into oleoside 11-methyl ester (**102a**) and ligstroside (**104**). On the basis of these results, they concluded that in *Fraxinus* oleosides were formed from iridodial and iridotrial via the formation of 7-*epi*-loganin and 7-ketologanin or their corresponding acids (**103**) [129].

Damtoft also studied the biosynthesis of oleosides in *Syringa* by administration of deuterium-labeled loganin, 7-*epi*-loganin, and 7-ketologanin in *S. josikaea* and found that except loganin, the other two compounds were both efficiently incorporated into oleosides. In another set of experiment, they administered deuterium-labeled deoxyloganic acid and 7-ketologanin into *S. vulgaris* and found that deoxyloganic acid gave incorporations into all iridoids except for secologanoside (**105**), whereas 7-ketologanin was mainly incorporated (>16%) into oleosides (e.g., oleoside 11-methyl ester). They repeated the experiment with deuterium-labeled deoxyloganic acid and 7-ketologanic acid and observed that both were incorporated into 8-*epi* kingisidic acid (**106**). Based on these results, they concluded that in *Syringa* oleosides were biosynthesized via deoxyloganic acid and 7-ketologanin. The deoxyloganic acid on hydroxylation gave 7-*epi*-loganic acid which on oxidation gave 7-ketologanic acid and on subsequent methylation gave 7-ketologanin. 7-Ketologanic acid or its Me ester could be the intermediate precursor of 8-*epi*-kingisidic acid and its Me ester [129].

Damtoft et al. carried out feeding experiments in *Forsythia viridissima* and *F. europaea* using deuterium-labeled deoxyloganic acid and found incorporation into forsythide (**36**). On the basis of the results, they concluded that deoxyloganic acid was oxidized at C-10 to adoxosidic acid followed by further oxidation at C-10 to forsythide in *Forsythia* spp. [71].

Damtoft et al. carried out feeding experiments with deuterium-labeled secologanin and oleoside 11-methyl ester in *Fraxinus excelsior* and observed that secologanin gave only a small incorporation (0.5%) into 7-glucosyl 11-methyl oleoside whereas oleoside 11-methyl ester gave incorporations into all iridoids except secologanoside (**105**) and 8-*epi*-kingiside. Based on this result, they concluded that oleoside 11-methyl ester was formed from 7 ketologanin as immediate precursor for all other secoiridoids in *Fraxinus* spp. They rationalized the formation of oleoside 11-methyl ester from 7-ketologanin via the formation of peroxide linkage at C-7 and its rupture due to cleavage of 7,8-bond and abstraction of H-9. The formation of 8-*epi*-kingiside may occur from the peroxide intermediate via the hydroxylation at C-8 followed by and cleavage of 7,8-bond and cyclization [130].

Based on these observations, it was believed that geraniol produced from IPP mostly by MEP pathway was the true precursor of iridoids. Geraniol via the formation of intermediates, iridodial, and iridotrial produced 7-deoxyloganic acid, 7-epiloganic acid, and 7-ketologanic acid or their methyl ester as intermediate precursors of other iridoids and secoiridoids in *Catharanthus*, *Fraxinus*, *Forsythia*, and *Syringa* species (Scheme 97.11). It was also established that although secologanin was not the effective precursor of other secoiridoids, it was the key intermediate for biosynthesis of indole alkaloids in *Catharanthus* species [131].

Recently, Li et al. also studied the biosynthesis of iridoid glucoside lamalbid (**106**) in *Lamium barbatum* using ^{13}C-labeled intermediates of MEP and MVA pathways, namely, [3,4,5-$^{13}C_3$] 1-deoxy D-xylulose 5-phosphate and [2-$^{13}C_1$] mevalonolactone (MVL), and observed that only the former was incorporated into lamalbid (Scheme 97.12) [132].

Biosynthetic studies on plumieride-type iridoids in *Allamanda* spp. by Schmid et al. and Inouye et al. demonstrated that these iridoids namely plumieride (**108**) and allamandin could be biosynthesized from 10-dehydrogardenoside (**107**) (Scheme 97.13) [125, 133, 134].

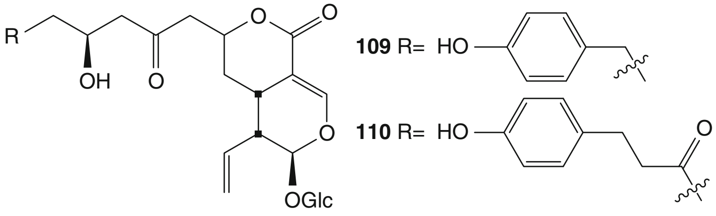
Therefore, on the basis of biosynthetic studies on iridoids by several groups, it may be inferred that both MVA and MEP routes are involved for the synthesis of acyclic monoterpene, geraniol, the key biosynthetic precursor of iridoids, but MEP route is the main route in most of the higher plants for synthesis of iridoid glucosides.

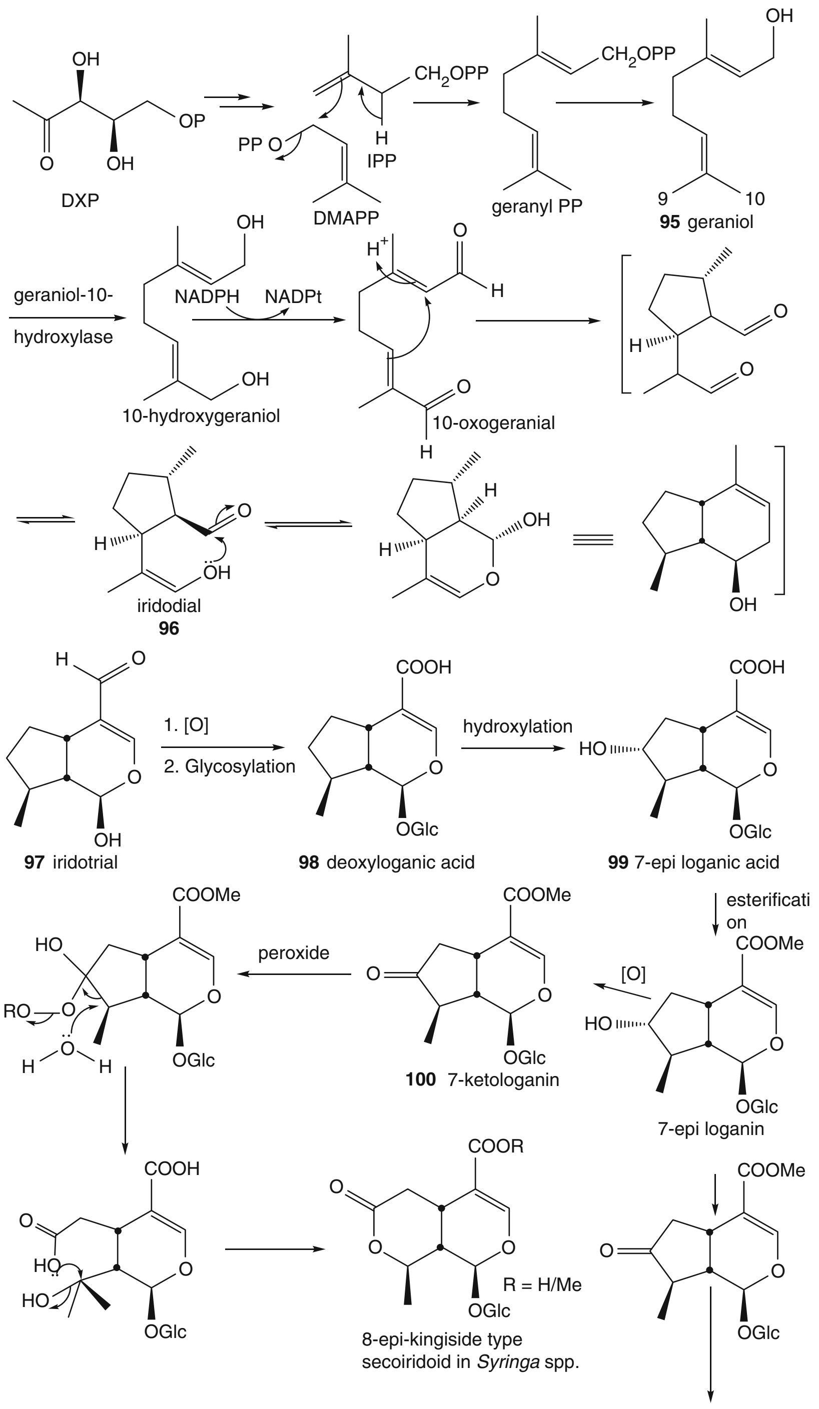
8 Pharmacology

Iridoids have been isolated from several folk medicinal plants which are used as bitter tonics, sedatives, antipyretic, cough medicine, remedies for arthritis, lumbago and muscular pains, wounds and skin disorders, etc. A good number of research papers on the bioactive iridoids isolated from several folk medicinal plants have been published in the last few decades. We have summarized here only some of the fundamental pharmacological activities of iridoids to create an interest on the importance of this class of natural products and for their wide applications in the formulations of several prospective herbal drugs.

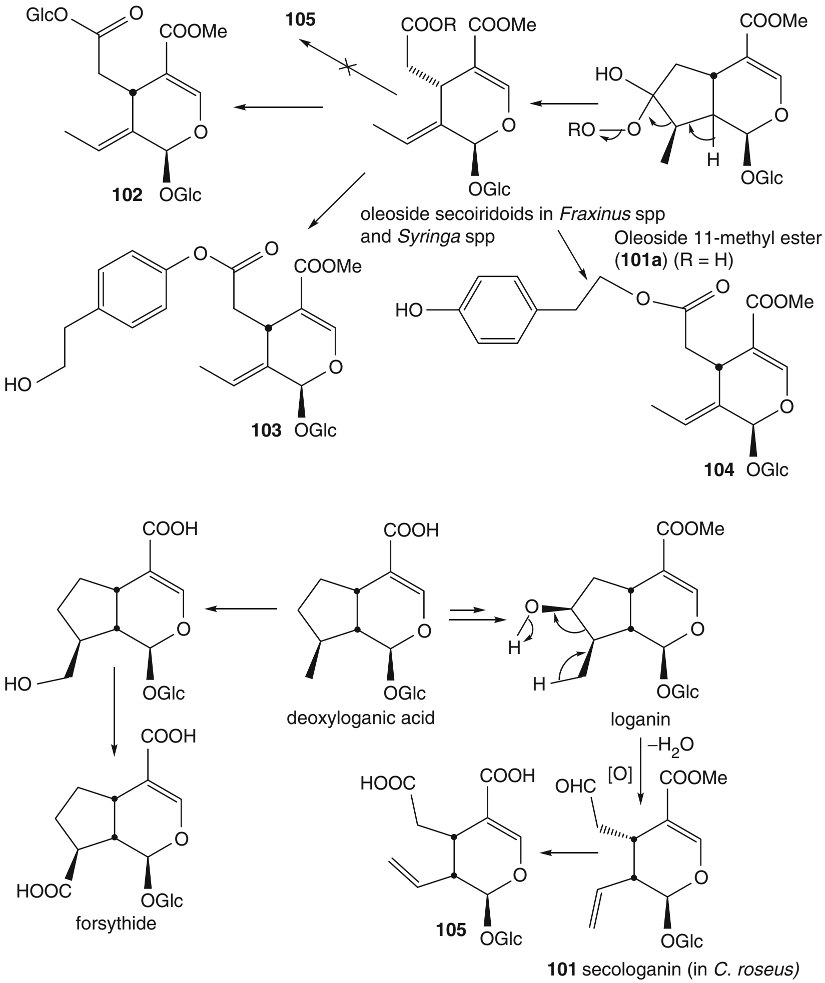
8.1 Antiallergic Activity

Two secoiridoids, hydramacrosides A (**109**) and B (**110**), isolated from the leaves of *Hydrangea macrophylla var. thunbergii* (Saxifragaceae) showed inhibitory effect on the histamine release from rat mast cell induced by antigen-antibody reaction with inhibition of 9.1% and 21.3%, respectively, at a concentration of 10^{-5} M [135].




OH
OP
O
OH
DXP
CH2OPP
PP O
H
IPP
DMAPP
CH2OPP
geranyl PP
OH
9
10
95 geraniol
geraniol-10-
hydroxylase
OH
OH
10-hydroxygeraniol
NADPH
NADPt
H+
O
H
O
H
10-oxogeranial
O
H
O
H
O
OH
iridodial
96
H
H
OH
O
OH
H
O
1. [O]
2. Glycosylation
O
OH
97 iridotrial
COOH
O
OGlc
98 deoxyloganic acid
hydroxylation
COOH
HO
O
OGlc
99 7-epi loganic acid
esterificati
on
COOMe
HO
O
OGlc
7-epi loganin
[O]
COOMe
O
O
OGlc
100 7-ketologanin
peroxide
COOMe
HO
RO
O
O
H
H
O
OGlc
COOH
O
HO
HO
O
OGlc
COOR
O
O
O
R = H/Me
OGlc
8-epi-kingiside type
secoiridoid in Syringa spp.
COOMe
O
O
OGlc

Scheme 97.11 (continued)

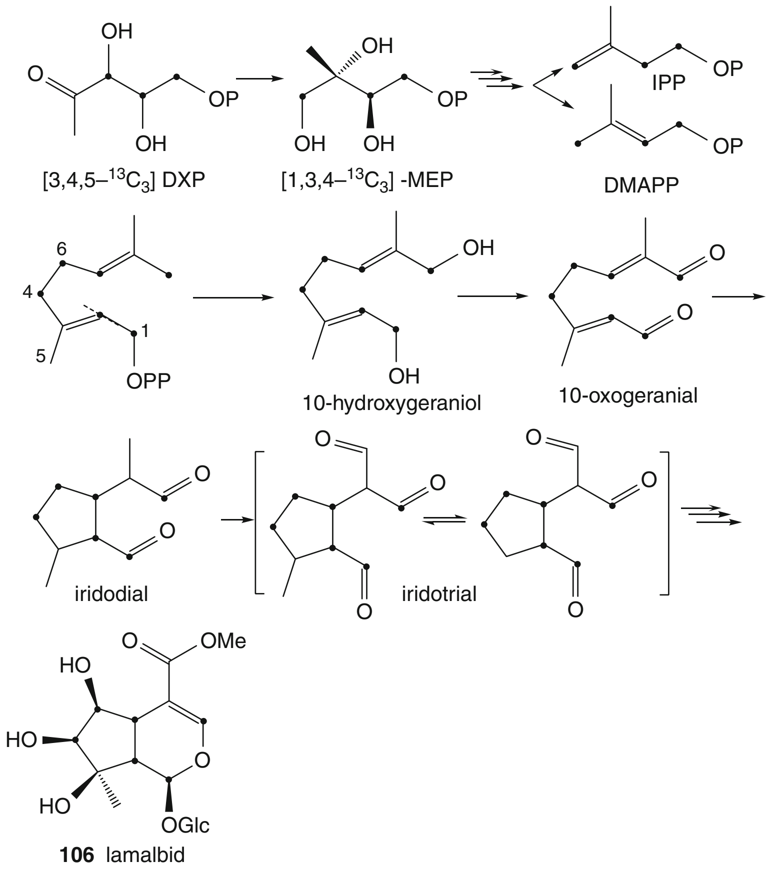

GlcO
O
COOMe
102 OGlc
105
COOR COOMe
O
OGlc
oleoside secoiridoids in *Fraxinus* spp
and *Syringa* spp
COOMe
HO
RO
O
H
O
OGlc
Oleoside 11-methyl ester
(**101a**) (R = H)
O
O
COOMe
HO
O
103 OGlc
HO
O
O
COOMe
O
104 OGlc
COOH
O
HO
OGlc
COOH
O
OGlc
forsythide
HOOC
COOH
O
OGlc
deoxyloganic acid
COOMe
O
H
H
O
OGlc
loganin
–H$_2$O
[O]
HOOC
COOH
O
105 OGlc
OHC
COOMe
O
OGlc
101 secologanin (in *C. roseus)*

Scheme 97.11 Proposed sequences for biosynthesis of iridoids in *Fraxinus*, *Syringa*, *Forsythia*, and *Catharanthus* species

8.2 Antiarthritic Activity

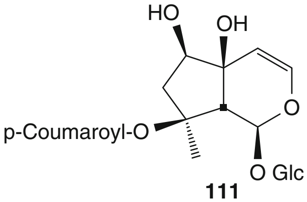
The roots of Devil's claw, *Harpagophytum procumbens*, are widely used in European countries for supportive treatment of inflammatory and degenerative diseases such as osteoarthritis, low back pain, and rheumatic complaints [136].

Boje et al. isolated a few iridoids and other class of compounds and studied their activity against human neutrophile elastase, the enzyme responsible for damage of cartilage tissue and other matrix proteins causing arthritis and rheumatism. Only two iridoids, 8-p-coumaroylharpagide (**111**) and pagoside (**112**), significantly inhibited in vitro elastase activity with an IC$_{50}$ value of 179 μgml^{-1} (331 μM)

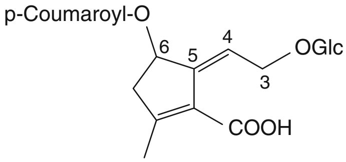

OH
O
OP
OH
[3,4,5–13C3] DXP
OH
OP
OH
OH
[1,3,4–13C3] -MEP
IPP
OP
OP
DMAPP
6
4
1
5
OPP
OH
OH
10-hydroxygeraniol
O
O
10-oxogeranial
O
O
iridodial
O
O
O
iridotrial
O
O
O
O
OMe
HO
HO
O
HO
OGlc
106 lamalbid

Scheme 97.12 Proposed biosynthesis of lamalbid with labeling patterns after administration with [3,1,5-$^{13}C_3$] DXP

and 154 μgml^{-1} (260 μM), respectively, compared to positive control caffeic acid having IC$_{50}$ of 86 μgml^{-1} (475 μM) [137].


HO
OH
O
p-Coumaroyl-O
O Glc

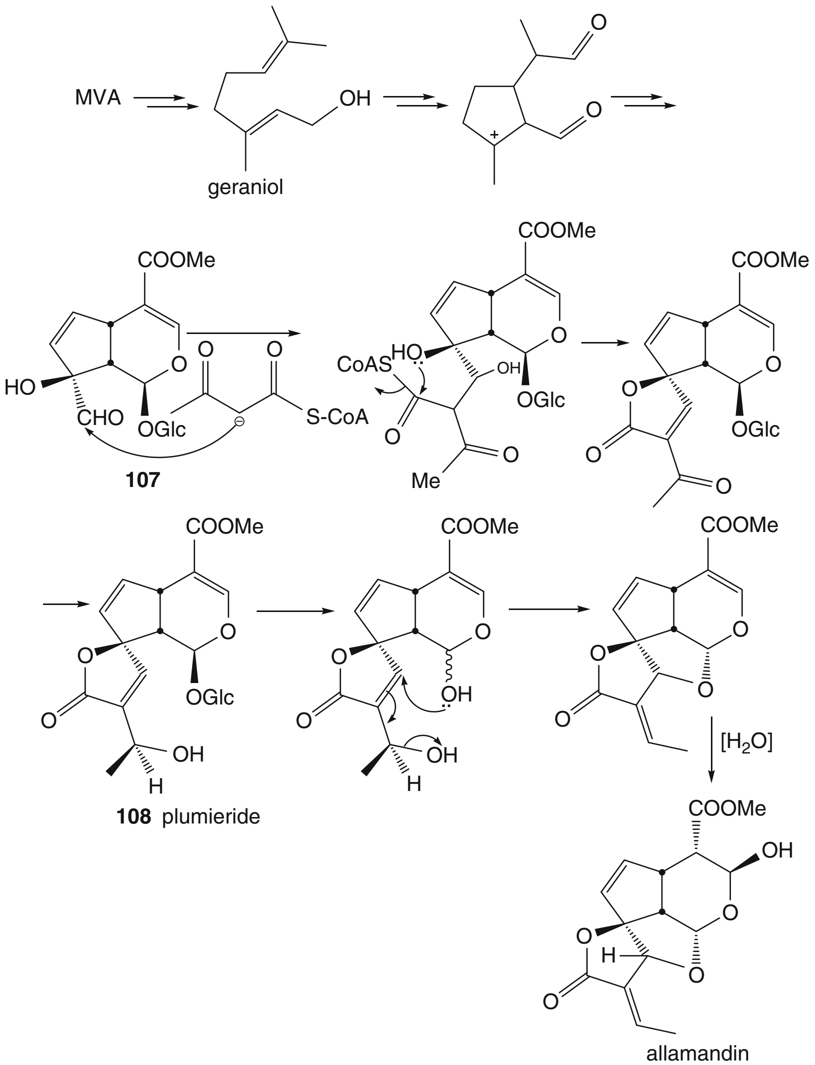
111


p-Coumaroyl-O
6
5
4
OGlc
3
COOH

112

8.3 Antibacterial Activity

Three iridoids, phloyoside 1 (**113**), phlomiol (**114**), and pulchelloside 1 (**115**), isolated from the rhizome of antiallergic Iranian flora, *Eremostachys laciniata*, exhibited


MVA
geraniol
OH
O
O
COOMe
HO
CHO
OGlc
O
O
O
S-CoA
107
CoAS
HO
OH
OGlc
Me
108 plumieride
OH
H
[H2O]
allamandin

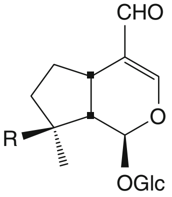
Scheme 97.13 Probable Biosynthetic pathway of plumieride and plumericin in *Allamanda* spp

moderate level antibacterial activity against five bacterial strains, *Bacillus cereus*, *Citrobacter freundii*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, with MIC value in the range of 0.05–0.50 mgml^{-1} and also displayed activity against penicillin-resistant *E. coli* with an MIC value of 0.05 mgml^{-1} [138].

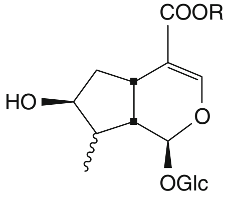
Non-glucosidic iridoid 1β-methoxymussaenin A (**116**) isolated from Chinese plant, *Cymbaria mongolica*, showed significant antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus* with an inhibition zone of 14.5, 13.9, and

14.5 mm in diameter, respectively, at a concentration of 10 μg/disk in paper-disk-assay model. The standard antibiotic, chloramphenicol, at the same concentration showed inhibition zone of 14.5, 14.9, and 15.1 mm in diameter against the respective strain [139].

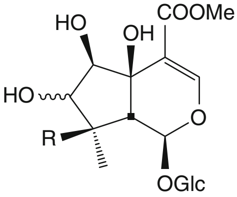
Ten iridoids were isolated by Yuan et al. from a Chinese plant, *Pedicularis kansuensis*, which has been used in folk medicine as cardiac tonic for treatment of collapse, exhaustion, and senility. Among the isolated iridoids, ixoroside (**26**), boschnaloside (**117**), 8-*epi*-loganic acid (**29**), and aucubin (**15**) at a concentration of 20 μg/disk exhibited strong antibacterial activity against *E. coli* and *S. aureus* by cup-plate method, having growth inhibition zone of 13–20 mm in diameter [140].



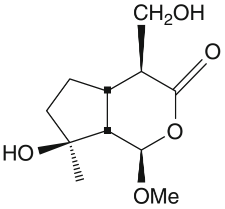
26 R = OH
117 R = H



29 α-Me, R = H
30a β-Me, R = Me
30 β-Me, R = H

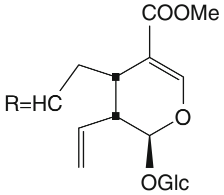


113 α-OH, R = OH
114 β-OH, R = OH
115 β-OH, R = OH

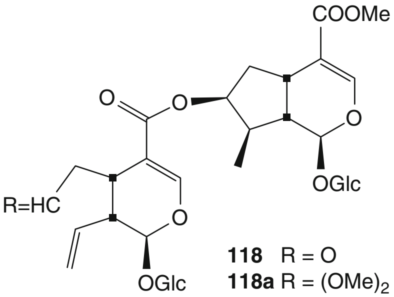


116

Loganin (**30a**), loganic acid (**30**), secologanin (**48**) and its dimethyl acetal (**48a**), and canteyoside (**118**) and its dimethyl acetal (**118a**) isolated from *Pterocephalus perennis* ssp. *perennis* (Dipsacaceae) of Greek peninsula also exhibited antibacterial activity against *Staphylococcus aureus*, *S. epidermidis*, *E. coli*, *Enterobacter cloacae*, *Klebsiella preumoniae*, and *Pseudomonas aeruginosa* in disk-diffusion method [141].



48 R = O
48a R = (OMe)$_2$

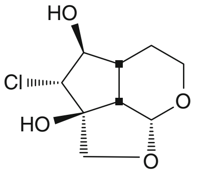


118 R = O
118a R = (OMe)$_2$

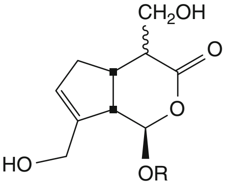
8.4 Anticancer Activity

Rehmaglutin D (**119**), 1β-hydroxy-4-epigardendiol (**120**), and 1β-methoxygardendiol (**121**) from *Cymbaria mongolica* acetone extract exhibited significant cytotoxic effect against human hepatoma (SMMC-7721), human uterine cervix carcinoma (HeLa), and mouse melanotic carcinoma (B16) cells in MTT assay model using vincristine as a positive control. The IC$_{50}$ values of compound

119 against SMMC-7721, HeLa, and B16 cells were 45.4, 60.7, and 71.2 μgml^{-1}, respectively, which were comparable to IC$_{50}$ of 63.2, 70.7, and 67.2 μgml^{-1}, respectively, of the control, vincristine [139].



119

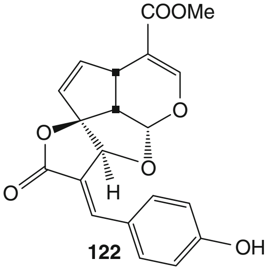


120 R = Me, C-4 β-CH$_2$OH
121 R = H, C-4 α-CH$_2$OH

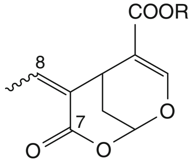
Several iridoids have been isolated from the leaves of Bangladeshi evergreen shrub, *Prismatomeris tetrandra*, having folklore use in the treatment of stomachaches and fresh wounds. Among the isolated iridoids, prismatomerin (**122**) showed remarkable in vitro antitumor activity against four mammalian cancer L-929 (murine connective tissue), KB-3-1 (cervix carcinoma), A-549 (lung carcinoma), and SW-480 (colon adenocarcinoma) cells with IC$_{50}$ values of 0.21, 0.41, 1.41, and 0.06 μM, respectively, in sulforhodamine B (SRB) protein assay. This compound also showed killing effect of solid tumor A498 (renal), PC-3 (prostrate), and MCF-7 (breast) cells with LC$_{50}$ values in the range of 100–0.6 μM [142].

Aucubin (**15**) isolated from the leaves of *Aucuba japonica* exhibited cytotoxic effect against cancer A-549 cells by blocking the cell cycle progression in the G(0)/G(1) phase and inducing apoptosis. An enzyme-linked immunosorbent assay (ELISA) showed that the G(0)/G(1) phase arrest was possibly due to p53-mediated induction of p21[143].

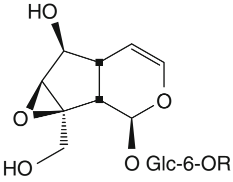
Iridolactones – triohimas A (**61**), B (**123**) and C (**124**) – isolated from Chinese diuretic herb *Triosteum himalayanum* exhibited in vitro cytotoxicity against L1210 (mouse lymphocytic leukemia) cell by inhibiting proliferation about 13%, 11%, and 18%, respectively, in MTT assay at 50 μM concentration [10].



122



61 R = Me, 8Me: *Z*
123 R = Me, 8Me: *E*
124 R = H, 8Me: *E*

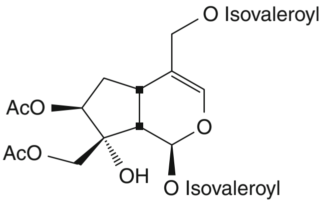


125 R = Cinnamoyl
126 R = Vinilloyl

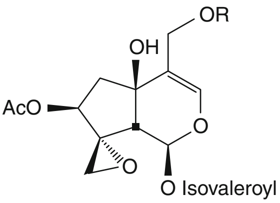
Picroliv, a mixture of iridoid glycosides, picroside 1 (**125**) and kutkoside (**126**) (1.0:1.5, w/w), isolated from the rhizomes of Indian plant, *Picrorhiza kurroa* (locally known as kutki), is a potential hepatoprotective and antitumor drug. Anand et al. studied the mechanism of therapeutic action of this herbal drug in

KBM-5, H1244, and A293 cancer cells at concentration range of 15–150 μgml^{-1} and found that it inhibited the nuclear factor–κB (NF-κB) activation pathway by modification of cysteine residue in p65 subunit in NF-κB and thus suppressed cell survival proteins and enhanced apoptosis. Their experimental results revealed that picroliv canceled tumor necrosis factor (TNF)-induced activation of NF-kB by mutation of Cys^{38} in p65 subunit and thereby inhibiting the binding of p65 to DNA. As a result, it led to suppression of NF-kB-regulated proteins including those linked with cell survival (inhibitor of apoptosis protein 1, Bel-2, Bcl-xL, survivin, TNF-receptor-associated factor 2), proliferation (cyclin D1 and Cox-2), angiogenesis (vascular endothelial factor), and invasion (IAP-1, MMP-9). Suppression of these proteins enhanced apoptosis of cancer cells [144].

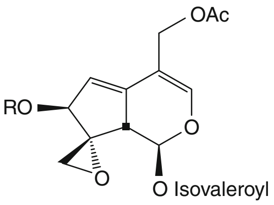
Valeriana jatamansi syn. *V. wallichii*, native to Mainland China and India, has been used for treatment of sleep problems, obesity, nervous disorders, epilepsy, snake poisoning, eye trouble, and skin diseases [145]. Lin et al. chemically investigated the plant and isolated 22 iridoids and examined their in vitro cytotoxicity against four cancer cells on MTT assay model using paclitaxel as positive control. Only five iridoids, namely, didrovaltrate acetoxy hydrin (**127**), IVHD-valtrate (**128**), 5-hydroxydidrovaltrate (**129**), valtrate (**130**), and acevaltrate (**131**), showed significant cytotoxicity against four tested A549, PC-3 M, HCT-8 (colon), and Bel 7402 (hepatoma) cancer cells with IC$_{50}$ values ranging from 1.0 to 7.4 μM. The most active compound was acevaltrate (**131**) which had IC$_{50}$ values of 2.9, 1.4, 1.0, and 1.7 μM against the A549, PC-3 M, HCT-8, and Bel 7402 cells, respectively [146].



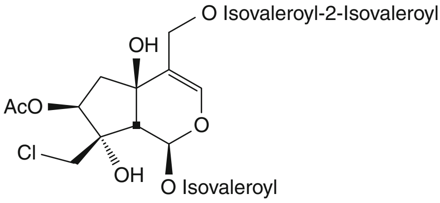
127



128 R = Isovaleroyl-2-O-Isovaleroyl
129 R = Isovaleroyl



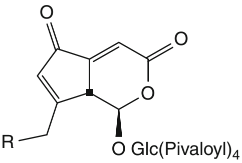
130 R = Isovaleroyl
131 R = Isovaleroyl-3-OAc



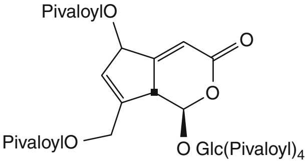
132

Volvaltrate B (**132**) isolated from both *Valeriana jatamansi* and *V. officinalis* also showed significant in vitro cytotoxic activity against A549, PC-3 M, HCT-8, and Bel 7402 cells with IC$_{50}$ values of 8.5, 2.0, 3.2, and 6.1 μM, respectively, in the MTT assay [147].

Rakotondramasy et al. and Mouries et al. prepared six unsaturated iridolactones from natural aucubin and studied their cytotoxicity against cancer L1210 and KB-3-1 cells. Among these lactones, three perpivaloyl glucosides (**133-135**) showed significant activity against L1210 cells with IC_{50} values of 3.0, 4.1, and 4.0 μM. The glucosides **135** and **136** also exhibited cytotoxicity against KB-3-1 cells of 0.56 and 0.42 μM, respectively, in MTT assay [148, 149].



133 R = OPivaloyl
134 R = H



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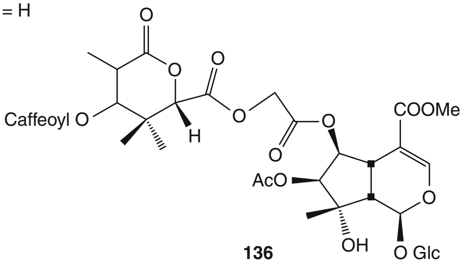
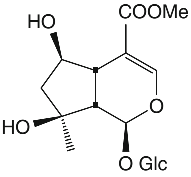


136

Sanshiside D (**136**) isolated from the sepals of *Mussaenda dona-aurora* showed significant cytotoxicity against the growth of vero, HeLa, and SMMC-7721 cancer cells with IC_{50} values of 1.99, 0.12, and 1.53 μMml^{-1}, respectively. Sanshiside methyl ester (**137**) and lamalbid (**106**) showed weak cytotoxic effect against HeLa and SMMC-7721 cells with IC_{50} values of 6.29 and 16.21, 18.31 and 41.82 μMml^{-1}, respectively. Taxol used as positive control had IC_{50} of 2.05, 0.05, and 0.74 μMml^{-1}, respectively. Possibly the presence of caffeoyl and lactone moieties in sanshiside D could be accounted for its enhanced cytotoxicity [150].

Luzonoids A-D (**138-141**) and luzonoid glycosides (**142**, **143**) isolated from *Viburnum luzonicum* showed moderate cytotoxicity against HeLa S_3, (epithelial) cancer cell with IC_{50} values of 3–7 μM [151].



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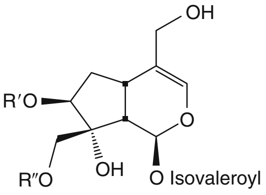
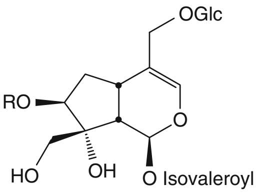


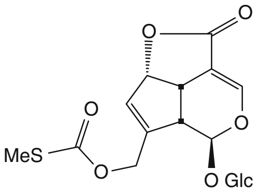
138 R′ = *E*-p-Coumaroyl, R″ = H
139 R′ = *Z*-p-Coumaroyl, R″ = H
140 R′ = H, R″ = *E*-p-Coumaroyl
141 R′ = H, R″ = *Z*-p-Coumaroyl



142 R = *E*-p-Coumaroyl
143 R = *Z*-p-Coumaroyl

Paederoside (**144**) isolated from *Paederia scandens* and genipin (**145**) and kutkoside (**126**) from *Picrorhiza kurroa* displayed significant antitumor-promoting activity by inhibiting 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-EA) induction in a nonproducer Raji cell line by a short-term in vitro assay at different molar concentrations [152].

The water extract of *Buddleja officinalis* (Buddlejaceae) inhibited vascular inflammation and atherosclerotic disorders. Recently, Tai et al. isolated several compounds from the flowers of the plant and tested their in vitro effects on the proliferation of rat aortic vascular smooth muscle cells (VSMCs). Among the tested compounds, two iridoids, namely, methylscutelloside (**146**) and methylcatalpol (**147**), showed significant inhibitory effects on homodimer platelet-derived growth factor beta polypeptide (PDGF-BB)-induced proliferation in rat aortic VSMCs. Methylscutelloside (**146**) decreased cell proliferation at 10, 30, and 50 μM, with inhibition of 39.1%, 42.0%, and 79.6%, respectively, while methylcatalpol showed significant inhibition (80%) only at 50 μM concentration. Their inhibitory effect on rat aortic VSMC proliferation may be useful for the treatment of atherosclerosis [153].



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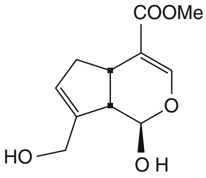

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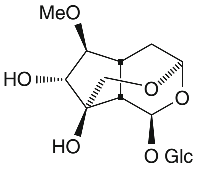

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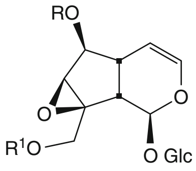
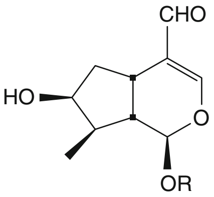


126 R = H, R′ = Vanilloyl
147 R = Me, R′ = H

8.5 Anticoagulant Activity

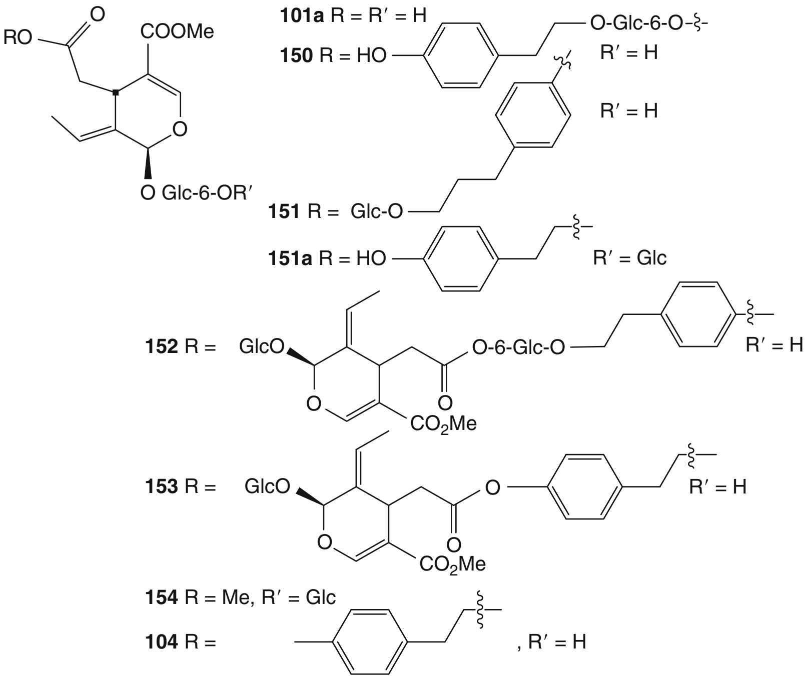
Non-glycosidic iridoids cachinol (**148**) and 1-O-methylcachinol (**149**) isolated from *Campsis grandiflora* (Bignoniaceae) leaves exhibited inhibitory activity on rat platelet aggregation with IC_{50} values of 43.2 and 38.4 μM, respectively, which were about 2-fold more inhibitory effect than that of acetylsalicylic acid (IC_{50}:75.2 μM) [154]. The leaves of the plant have been used as Chinese traditional drug for treatment of diseases caused by blood stagnation.



148 R = H
149 R = Me

8.6 Antidiabetic Activity

The aqueous seed extract of the ash tree, *Fraxinus excelsior* (FE) (Oleaceae), of temperate regions across Europe and Asia is recognized as an effective hypoglycemic and antidiabetic agent without significantly affecting insulin level [155]. Moreover, ash seed extract has hypoglycemic and antidiabetic effects in normal and streptozotocin-induced diabetic rats [156, 157]. To find out the potential active principle (s) of FE and evaluate their biological activity in adipocyte (3 T3-L1 cells) differentiation and peroxisome proliferator-activated receptor alpha (PPARα) activation, Bai et al. isolated a phenolic compound and nine secoiridoid glucosides and tested their activity. Their experimental results showed that eight iridoid glucosides, oleoside 11-methyl ester (**101a**), nuzhenide (**150**), $1'''$-O-β-O-glucosyl formoside (**151**), excelside B (**151a**), G13 (**152**), G15 (**153**), excelside A (**154**), and ligstroside (**104**), inhibited in vitro adipocyte differentiation in 3 T2-L1 cells as well as compounds excelside B, nuzhenide, G1-3, and GI-5 activated PPAR-α reporter cell system in the concentration of 10^{-4} M, which was comparable with the concentration 10^{-8} M of synthetic PPAR-α agonist, WY14.643. PPAR-α is the main target of fibrate drugs for treatment of hyperlipidemia and hyperglycemia. Possibly the inhibition of adipocyte differentiation and PPAR-α-mediated mechanism be the relevant pathways for antidiabetic activity of FE and its iridoids [158].

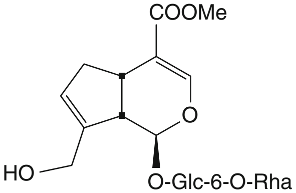


Later on, Ibarra et al. studied the health benefits of FE seed extract in obese mice that were fed a high-fat diet (HFD) over 16 weeks and observed the reduced gains in body weight by 32.3%, omental fat by 17.92%, and retroperitoneal fat by 17.78% as well as lowered fasting blood glucose level by 76.52% ($p < 0.001$) and plasma insulin level by 53.43% ($p < 0.005$) after 16 weeks. The positive effects on body weight and blood glucose level might be through the enhancement of fat metabolism through β-oxidation and inhibition of fat accumulation by the secoiridoids during the growth of the animals through PPAR-α activation in vivo. The HPLC analysis revealed that the major constituents of aqueous seed extract were nuzhenide and G13 [159].

8.7 Anti-inflammatory Activity

Genipin diglycoside (**155**) isolated from the bark of *Adina polycephala* showed in vitro inhibitory rate of 44.8% against the release of β-glucuronidase in rat polymorphonuclear (PMN) leukocytes induced by platelet-activating factor (PAF) at a concentration of 10^{-5} M; the positive control ginkgolide B showed an inhibition rate of 78.8% at the same concentration [160].

Secoiridoid, lonijaposide C (**156**) isolated from *Lonicera japonica* also showed inhibitory activity against the release of glucuronidase in rat PMN leukocytes induced by PAF with an inhibition rate of 69.5% at 10 μM. Possibly the presence of pyridinium ion at C-7 position was responsible for this inhibitory activity [161]. These iridoids may be used as anti-inflammatory drugs.



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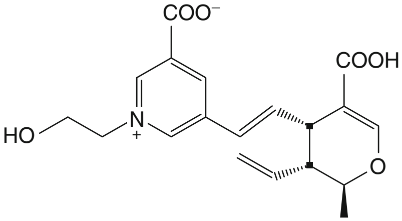
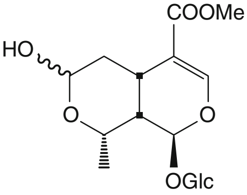


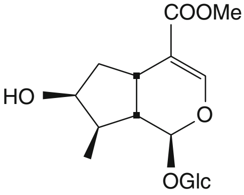
156

8.8 Anti-osteoporosis Activity

Chinese drug Liuweidihuang Wan (LW) containing the herb *Fructus corni* is used for clinical treatment of postmenopausal osteoporosis in women or elderly men. Li et al. carried out HPLC analysis of the MeOH extract of the drug LW and found morroniside (**51**) and loganin (**30a**) as major constituents. They isolated these iridoids from *Macrocarpium officinale* and evaluated their activity on clonal MC3T2-E1 cell line. This cell has the key role for bone formation and maintenance of bone mass. Its gradual loss results osteoporosis, a bone disease symptomized by reduction of bone mass, damage to the bone tissue microstructure, and consequence of the risk of bone fracture [162]. Both morroniside (**51**) and loganin (**30a**) at different concentrations (1, 10, 100 μgml^{-1}) had no effect on the proliferation of MC3T3-E1 cells, but improved the activity of alkaline phosphatase (ALP) and increased the contents of collagen type 1 and osteocalcin for the increase of the differentiation of osteoblasts. Simultaneously, the m-RNA expression of caspase 3, caspase 9, and RANKL (receptor activator of nuclear factor kappa–β ligand) was downregulated to inhibit apoptosis of osteoblasts, and that of bel-2 was upregulated to raise the survival rate of osteoblasts. All these sequences partly explained the anti-osteoporosis mechanism in MC3T3-E1 cells. Therefore, these two iridoids alone or in combination may be promising herbal drugs for treatment of osteoporosis [163].



51

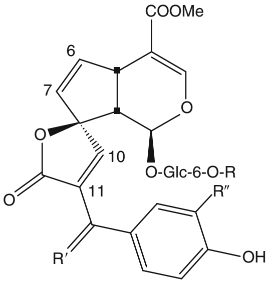


30a

8.9 Antiprotozoal Activity

In some African countries, the leaves of *Morinda morindoides* have been used for treatment of malaria. Tamura et al. isolated a plumeria-type iridoid (**157**) from the leaves of this plant and evaluated its antimalarial efficacy. It showed potent antimalarial activity against *Plasmodium falciparum* with IC_{50} value of 0.1 μM. To improve the antimalarial efficacy, they prepared four analogues, and one (**158**) of them was found to be more active against *P. falciparum* with IC_{50} of 0.04 μM. Both the parent iridoid (**157**) and its congener (**158**) were little cytotoxic against human epidermoid carcinoma kB (KB-3-1) cells (9.3% and 6.1%, respectively) at a concentration of 150 μM. Possibly the presence of ketonic functionality at C-13 position enhanced the antimalarial potency in the congener [164].

Cimanga et al. isolated five iridoids from the leaves of *M. morindoides* and evaluated their antiamoebic efficacy against *Entamoeba histolytica*. All these iridoids, namely, epoxygaertneroside (**159**), methoxygaertneroside (**160**), gaertneroside (**161**), acetylgaertneroside (**162**), and gaertneric acid (**43**), possessed significant activity with IC_{50} values of 1.3, 2.3, 4.3, 5.4, and 7.1 μgml^{-1}, respectively. All these iridoids were not toxic against MT-4 cells at their highest test concentration of 250 μgml^{-1} [165].



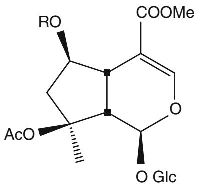
157 R = Ac, R′= H, α-OH, R″= OMe
158 R = H, R′= O,R″= OMe
159 6,7-Epoxy, R = H, R′ = H, OH, R″ = H
160 R = H, R′ =O, R″= OMe
161 R = H, R′ = H, OH, R″= H
162 R = Ac, R′ = H, OH, R″ = H
43 R = H, R′ = H, OH, R″ = H, C-4-COOH

8.10 Antiviral Activity

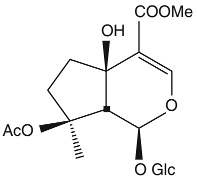
Viral infection is one of the major global health problems. Several millions of people are suffering from viral infections in every year. *Barleria prionitis* (Acanthaceae) is a traditional herbal drug in India and other Asian countries. A decoction of leaves and flowers of this plant is used to treat fever, while the green shoots are taken orally to treat whooping cough and asthma in infants and children [166, 167], as well as hot-water extract of the dried leaves and roots is also taken orally to treat bronchitis and coughs [168]. The most common cause of asthma-like symptom of infants is respiratory syncytial virus (RSV) infection in lungs [169]. Chen et al. isolated a few iridoids and other compounds and studied their activity against RSV. An inseparable *E*/*Z* mixture of 6-*O*-coumaroylbarlerin (**163** and **164**) in 3:1 M ratio exhibited anti-RSV (A2 strain) activity in a cell culture-based cytopathic effect (CPE) assay with EC_{50} of 2.46 μgml^{-1}. Under this assay condition, the mixture was moderately cytotoxic against Hep-2 cells (IC_{50}: 42 μgml^{-1}). *Barlerin* (**165**) was found to be inactive in the same assay. Therefore, the presence of coumaroyl unit at C-6 position was possibly responsible for this activity [170].

From another species of *Barleria*, *B. lupulina*, Suksamrarn et al. isolated nine iridoids from the flowers and tested their anti-HSV-1 activity. Only one iridoid ipolamiidoside (**166**) having C-5 hydroxy group was active with an IC_{50} of 41.1 μgml^{-1} [171].

8-O-acetylharpagide (**12a**), harpagoside (**13**), and scorodioside (**167**) isolated from *Scrophularia scorodonia* showed antiviral activity against vesicular stomatitis virus (VSV). The cell viability was 32.1% for 8-O-acetylharpagide at 500 μgml^{-1}, 43.3% for harpagoside at 450 μgml^{-1}, and 47.80% for scorodioside at 500 μgml^{-1}. Scorodioside also showed moderate in vitro anti-HSV-1 activity (30.6% cell viability at 500 μgml^{-1}) [172].



163 R = *E*-p-Coumaroyl
164 R = *E*-*Z*-Coumaroyl
165 R = R = H, 3-O-Ac, Cinnamoyl-O-2-Rha



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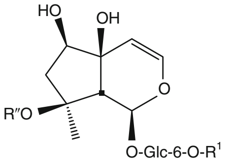
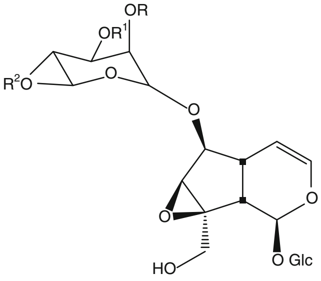


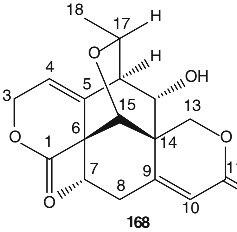
179 R′ = H, R″ = *E*-p-MeO-Cinnamoyl
180 R′ = *E*-p-MeO-Cinnamoyl, R″ = H
12a R′ = H, R″ = Ac
13 R′ = H, R″ = *E*-Cinnamoyl

Chinese drug "Qing-Ye-Dan" containing aqueous extract of *Swertia mileensis* has been used to treat viral hepatitis with high alanine transaminase (ALT) and aspartate transaminase (AST) levels by the Yi and Hani minority groups of Mile and Kaiyuan countries. Geng et al. based on this fact carried out anti-HBV (hepatitis B virus) screening using both ethanol (50% and 90%) and aqueous extracts of the plant and found that ethanol extracts showed higher inhibitory activities against HBsAg secretion (IC_{50} of 0.61 and 0.33 $mgml^{-1}$) and HBeAg secretion (IC_{50} of 1.06 and 0.55 $mgml^{-1}$, respectively) than that of H_2O extract. From these ethanol extracts, they isolated two swerilactones A and B. Among these, swerilactone A (**168**) showed significant in vitro anti-HBV activity on HepG2.2.15 cell line by inhibition of HBsAg and HBeAg secretions with IC_{50} value of 3.66 and 3.58 mM, respectively. The antiviral agent 3TC (lamivudine) used as positive control showed inhibitory activity against HBsAg and HBeAg with IC_{50} values of 17.6 and 36.2 mM, respectively [173].

The aqueous extract of the flowering tops of Spanish plant *Lamium album* along with other herbs has been traditionally used for treatment of HCV (hepatitis C virus) infections [174]. Zhang et al. evaluated the anti-HCV activity of both aqueous and methanol extracts of the flowering tops of the plant and found their activity. Working on the methanol extract, they isolated iridoid glucoside lamalbid (**106**) as major constituent. Although this compound was inactive, its aglycone, inseparable epimers named lamiridosins A and B (**169**, **170**) present as major constituents in the aqueous extract showed significant in vitro inhibition in HCV assay with IC_{50} value of 2.31 μM and were found to be nontoxic to the HepG2, 2.2 cells at a concentration of 50 μgml^{-1}. The aqueous extract containing these iridoids showed ca. 50% inhibition at 100 μgml^{-1}. They also carried out anti-HCV screening on other 14 iridoids including free aglycone genipin (**145**) and found that only genipin showed significant inhibition at a concentration of 20 μgml^{-1}. Further study on anti-HCV screening of the enzymatic hydrolysis (with β-glucosidase) products of the tested iridoids revealed that only hydrolysis products of five iridoids, namely, shanzhiside methyl ester (**137**), loganin (**30a**), loganic acid (**30**), eurostoside (**170a**), and picroside-1 (**125**), showed significant inhibition activity after a 4-h enzyme incubation process. Possibly iridoid aglucones are the active pharmacophore for anti-HCV activity [175].



167 R = Cinnamoyl, R^1 = Ac, R^2 = H
181 R^1 = p-MeO-Cinnamoyl, R^2 = Ac, R = Ac
182 R^1 = R^2 = p-MeO-Cinnamoyl, R = Ac
183 R^1 = Ac,R^2 = p-MeO-Cinnamoyl, R = Ac



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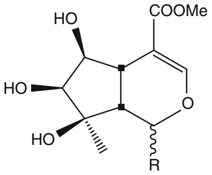
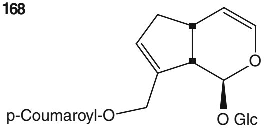


106 R= β-OGlc
169 R= β-OH
170 R= α-OH



170a

8.11 Immunomodulatory Activity

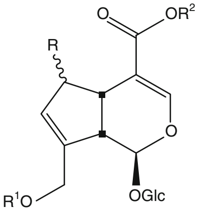
Aucubin (**15**) isolated from *Plantago major* showed immunomodulatory activity at a concentration less than 5 μM by stimulating human peripheral blood mononuclear cells (PBMC) and enhancing the secretion of interferon gamma (IFN-γ) in ELISA test [176].

8.12 Insecticidal Activity

Catalpol (**18**) isolated from *Buddleja cordobensis* showed a series of allelochemical effects related to DNA synthesis in the insect *Tribolium castaneum* by inhibition of tag DNA polymerase activity with an IC_{50} value of 47.8 μM. Thus, it exhibited post-ingestive toxic effects showing a reduction in the efficiency of conversion of injected food [177].

8.13 Melanogenesis Inhibitory Activity

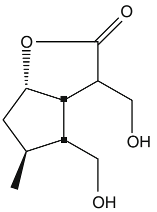
Four iridoids, 9-*epi*-6α–methoxy geniposidic acid (**171**), asperulosidic acid (**172**), deacetyl asperulosidic acid (**173**), and scandoside methyl ester (**174**), isolated from noni fruits exhibited anti-melanogenesis activity in the B16 melanoma cells induced by α-melanocyte-stimulating hormone (α-MSH) with 40–50% reduction of melanin content at 100 μM. These were almost nontoxic to the cells at this tested concentration. Their inhibitory activity may be comparable to a known melanogenesis inhibitor, arbutin (with 29% reduction of melanin content at the same concentration), which has been recognized as a useful depigmentation agent for skin whitening in the cosmetic industry [178].



171 R = α-OMe, R^1 = H, R^2 = H
172 R = α-OH, R^1 = Ac, R^2 = H
173 R = α-OH, R^1 = H, R^2 = H
174 R = β-OH, R^1 = H, R^2 = Me

8.14 Nerve Growth Factor-Potentiating Activity

Nerve growth factor (NGF) is one of the neurotrophic factors for neurodegenerative disorders. *Verbena littoralis* (Verbenaceae) was useful for treatment of dementia. To find out the bioactive chemicals, Li et al. isolated some iridoids from this plant and evaluated their NGF-enhancing activity in PC12D cells. Among the isolated iridoids, gelsemiol (**175**) and 7α-hydroxy semperoside aglucone (**176**) showed significant activity at the concentration of 100 μM, while littoralisone (**72**) exhibited moderate activity [179].



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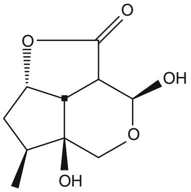
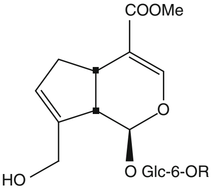


176

8.15 Neuroprotective Activity

Catalpol (**18**) showed neuroprotective efficacy in memory damage of mice induced by subcutaneous injection of D-galactose (150 mg/kg body weight) for 6 weeks and application of catalpol in the dose range of 2.5–10 mg/kg body weight for last 2 weeks [180]. It also exhibited neuroprotective effect on 1-methyl 4-phenyl pyridinium ion (MPP$^+$)-induced oxidative stress in cultured mesencephalic neurons, especially dopaminergic neurons in a dose-dependent manner [181]. Catalpol also protected the H_2O_2-injured astrocytes which have key role in neurodegenerative disorder in brain [182]. It was also effective for treatment of global cerebral ischemia in Mongolian gerbils at the dose range 1–10 mg/kg body weight [183]. Therefore, catalpol or its suitable congeners may be useful for the treatment of neurodegenerative disorder such as Alzheimer's disease and Parkinson's disease.

Geniposides, 6′-O-*trans*-p-Coumaroylgeniposide (**177**) and 6′-O-acetylgeniposide (**178**) isolated from the fruits of *Gardenia jasminoides* showed significant improvement of short-term memory capacity on an Ab42 transgenic drosophila model assay at a concentration of 7.6 μM [184].



177 R = *E*-p-Coumaroyl
178 R = Ac

Both 8-O–*E*-p-Methoxycinnmoyl harpagide (**179**) and 6′-O-*E*-p-methoxycinnamoyl harpagide (**180**) showed significant protective effect at a concentration of 1–10 μM against glutamate-induced neurodegeneration in primary cultures of rat cortical neurons. Their protective activity was comparable to that of MK-801, a noncompetitive antagonist of NMDA receptor [185].

8.16 Wound-Healing Activity

Scrophularia nodosa has been used in different parts of Ireland for the treatment of wounds [186]. Three catalpol derivatives, scopolioside A (**181**), scrophuloside A_4 (**182**), and scrovalentinoside (**183**), isolated from the dried seed pods of this plant were found to stimulate in vitro the growth of human dermal fibroblasts (HDF). Scopolioside A stimulated HDF growth over the full range (100–0.78 μgml^{-1}) of concentration tested, while the rest two compounds showed a negative dose-dependent response; the stimulation was highest at lower concentration (0.78 μgml^{-1}). It may be noted that HDF is involved in different phases of wound-healing, and hence these iridoids may be useful in formulation of wound-healing drugs [187].

9 Conclusion

The extensive isolation of iridoid glycosides from the plants of various families and exploration of their pharmacological potentials provide us new insights for utilization of these wild resources either the pure chemicals or their crude extracts for development of new natural drugs. Recent biosynthetic studies on iridoids also reveal us a new concept for synthesis of iridoid monoterpenoids via MEP pathway rather than well-known MVA pathway for terpenoid and steroid biosynthesis in higher plants. The traditional use of iridoid-rich plants as food, nutraceutical, cosmetic, and pharmaceutical industries is well justified, and their wide-scale use and cultivation may be popularized for maintenance of good health and as potential source of raw materials for pharmaceutical industries.

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Guaianolide Sesquiterpenoids: Pharmacology and Biosynthesis

98

Henrik Toft Simonsen, Corinna Weitzel
and Søren Brøgger Christensen

Contents

1 Introduction 3070
2 Definition 3071
2.1 Occurrence and Use 3071
2.2 Configuration 3072
3 Chemistry of the Guaianolides 3075
3.1 Azulene Formation 3075
3.2 Hetero-Michael Addition 3075
3.3 Selective Replacement of Ester Groups 3077
3.4 Synthesis of the Guaianolide Nucleus 3079
4 Biosynthesis of Guaianolides in Plants 3081
4.1 Guaianolide Biosynthesis in Asteraceae 3082
4.2 Guaianolide Biosynthesis in Apiaceae 3085
4.3 Stereochemical Differences Between Guaianolides from Apiaceae and Asteraceae 3086
4.4 Guaianolide Biosynthesis in Other Plant Families 3087
5 Biological Activity 3087
5.1 General Toxicity of Guaianolides 3088
5.2 Anti-inflammatory Activity of Guaianolides 3089
5.3 Thapsigargins: A Well-Studied Group of Guaianolides from Apiaceae 3090
5.4 Antiprotozoal Activity of Guaianolides 3093

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5.5 Allergic Contact Dermatitis 3094
5.6 Aromatase Inhibitors 3094
6 Conclusion 3094
References 3095

Abstract

Guaianolides are a large group of sesquiterpene lactones of chemotaxonomic as well as biological importance. The ability of the thapsigargins isolated from the genus *Thapsia*(Apiaceae) to inhibit an intracellular calcium pump and thereby inducing apoptosis has encouraged intensive research in order to develop a new drug, which at present is in clinical trial toward cancer diseases. Several other guaianolides have been investigated as chemotaxonomic markers. In particular, α-methylene guaianolides and guaianolides containing an α,β-unsaturated carbonyl moiety show biological activities.

Keywords

Guaianolide • SERCA inhibition • sesquiterpene lactones • thapsigargin

Abbreviations

DIBAL	Diisobutylaluminium hydride
DMAP	4-Dimethylaminopyridine
DMAPP	Dimethylallyl pyrophosphate
DXP	1-Deoxy-D-xylulose-5-phosphate
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
GPP	Geranyl pyrophosphate
HMG-CoA	(*S*)-3-hydroxy-3-methylglutaryl-CoA
HPMA	N-(2-hydroxypropyl) methacrylamide
IPP	Isopentenyl pyrophosphate
LDA	Lithium diisopropylamide
MEP	2-C-methyl-D-erythritol-4-phosphate
MVA	Mevalonate
PCC	Pyridinium chlorochromate
PMSA	Prostate membrane specific antigen
SERCA	Sarco/endoplasmic reticulum calcium ATPase
Ts	Paratoluenesulfonyl (tosyl)

1 Introduction

Biological properties and chemotaxonomic value of guaianolides have encouraged intensive studies. The best studied example is thapsigargin from *Thapsia garganica* L.

The skin irritating properties of the resin from this plant initiated studies that revealed the guaianolides thapsigargin and thapsigargicin as the active principles. The skin irritating effects might be related to the ability of these compounds to release histamine from mast cells [1]. In addition to thapsigargins, in particular, α-methyleneguaianolides and guaianolides possessing an α,β-unsaturated carbonyl moiety have been the focus of intensive studies as will be discussed in this chapter.

2 Definition

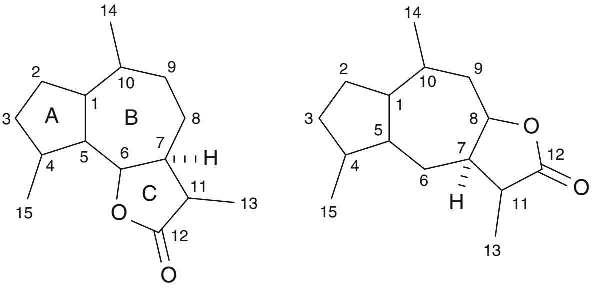
The guaianolide nucleus is defined as 3,6,9-trimethyldecahydroazuleno[4,5-b] furan-2(9bH)-one (**1**) or 3,5,8-trimethyldecahydroazuleno[6,5-b]furan-2(3H)-one (**2**) [2].

By definition, the tricyclic skeleton is depicted as shown in Fig. 98.1 with the A ring up to the left. This enables unambiguous definition of the stereochemistry of the compounds. The systematic names are seldom used; instead, the compounds are generally mentioned as 6,12-guaianolides (**1**) or 8,12-guaianolides (**2**) (Fig. 98.1). If C7, as shown, is substituted with hydrogen, this is α-disposed (Fig. 98.1). Pseudoguaianolides differ from guaianolides by the location of $CH_3$15, which in pseudoguaianolides is positioned at C5 instead of C4.

2.1 Occurrence and Use

A substructure search on all atoms in the database Reaxys using the above nuclei with zero, one, two, or three double bonds revealed that in mid-November 2011, more than 1,400 6,12-guaianolides and more than 170 8,12-guaianolides had been isolated from natural sources. The majority has been found within Asteraceae (composites) followed by Apiaceae (umbelliferous plants), but in addition, guaianolides have been reported from Lamiaceae, Porellaceae (liverwort), Magnoliaceae, and Thymelaeaceae. Furthermore, guaianolides have also been reported from marine organisms like *Pseudopterogorgia americana* (sea plume) and a *Placogorgia* species (a gorgonian). In conclusion, guaianolides are found in a diverse number of plants and other organisms, but mainly in Asteraceae and Apiaceae.

An interesting observation was made by Serkerov, suggesting that not all isolated guaianolides are produced by the organism itself but can be produced either by endophytic organisms or decay of the molding organism. Serkerov noticed that the two 8,12-guaianolides ferulidin and ferulin only occur in roots of *Ferula oopoda* (Apiaceae) that had become moldy by storage. These results could indicate that the 6,12-lactone ring is the endogenic isomer, whereas the 8,12-isomer ring in Apiaceae is formed as a result of molding. Thus, by either plant breakdown enzymes or enzymes of bacterial and/or fungal origin, badkhysin is transformed into ferulin, which is then hydroxylated into ferulidin [3]. This observation has not



1 2

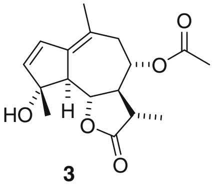
Fig. 98.1 The structure of the two guaianolides. Annotations are illustrating numbering and labeling of carbons and rings

been examined further by others, and the observation that other 8,12-lactones have been found in other plant families as shown in Fig. 98.3 contradicts this suggestion.

Many of the guaianolides are reported to exhibit high antitumor, antischistosomal, anthelmintic, antimicrobial, contraceptive, root-growth stimulatory, root-growth, antifeedant, and germination inhibitory activities, just to mention a few of the reported properties [4, 5]. These diverse biological activities make guaianolides attractive compounds in the search for new drug leads, but due to their toxicity, no drug has come to the market at present [6, 7]. Lately though, the guaianolide thapsigargin (**11**) and prodrugs thereof have given new leads in the drug discovery process [8]. This again makes guaianolides interesting synthetic and biosynthetic targets since the natural source is often limited, preventing its use as a sustainable source for obtaining the compound in kilogram scale.

2.2 Configuration

Typical substituents on the nuclei of naturally occurring guaianolides are hydroxyl groups and esterified hydroxyl groups (Fig. 98.2). Often, one, two, three, or rarely more double bonds are found in the core skeleton of guaianolides, and all carbon atoms in the nuclei might be substituted. Apart from the general rule that if a C7 is substituted with hydrogen, it is α-disposed, no further simple rules for the stereochemistry exist. A Δ11,13 double bond (**4–9**) enables the molecule to undergo hetero-Michael additions, which might be of importance for the biological activity. Some guaianolides, in addition, possess α,β-unsaturated carbonyl groups at other positions in the molecule (**5**, **6, 12**). The substituents are not restricted to hydroxyl groups and esters of these. Epoxy groups occur frequently (**7**), chloride has been found (Fig. 98.3, **14**), and examples of guaianolides substituted with hydroperoxyl groups (**18**) or endoperoxide bridges (**13**) have been characterized along with glycosylations (**16**). The amino acid group in **15** can be explained by a hetero-Michael addition of proline to the α-methylene guaianolide, and the substituent with two thiophene rings in **17** probably has a polyacetylenic fatty acid as precursor. Compound **19** is an example of a guaianolide pigment found in deep sea gorgonian,



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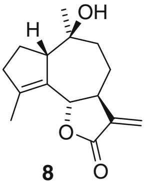

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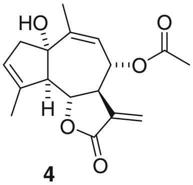

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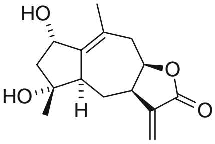

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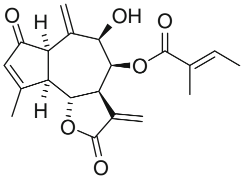

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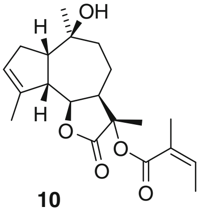

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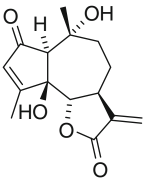

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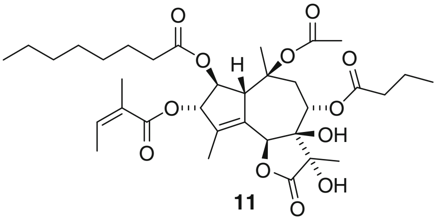
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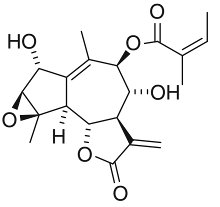
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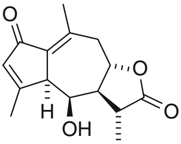

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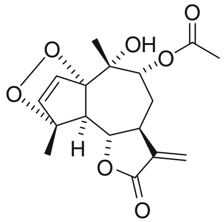
Fig. 98.2 Guaianolides isolated from plants belonging to Asteraceae (**3**–**8**, **12**) and Apiaceae (**9**–**12**). Compound **3**: matricin from *Matricaria chamomilla* [2], **4**: rupicolin A 8-*O*-acetate from *Artemisia tripartita* [2], **5**: from *Lasiolaena morii* [9], **6**: from *Artemisia xanthochroa* [10], **7**: bacchariolide from *Baccharis salicina* [11], **8**: cichopumolide from *Cichorium pumilum* [12], **9**: from *Hymenoxys scaposa* var. *villosa* [13], **10**: from *Laser trilobum* [14], **11**: thapsigargin from *Thapsia garganica* [15], and **12**: carmenin from *Ferula* sp. (Apiaceae) [16, 17] and from *Artemisia lanata* (Asteraceae) [18]



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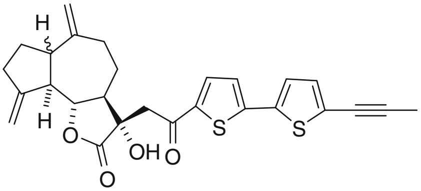

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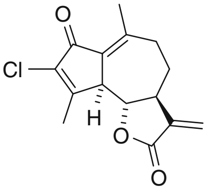

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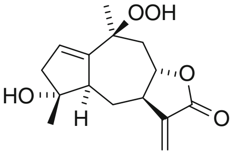

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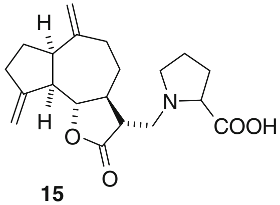

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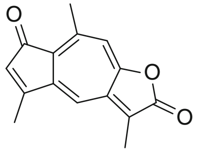

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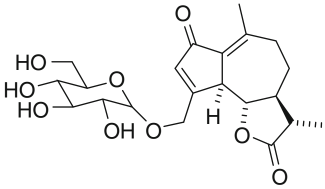

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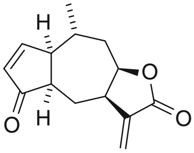


20

Fig. 98.3 Compound **13**: apressin has been isolated from *Achillea depressa* [19], **14**: 3-chlorodehydroleucodin from *Kaunia lasiophthalma* [20], **15**: saussureamine from *Saussurea lappa* [21], **16**: crepidiaside B from *Cichorium intybus* and other species [22], **17**: lappaphen A from *Arctium lappa* [23], **18**: from *Apalochlamys spectabilis* [24], **19**: from deep sea gorgonian (*Placogoria* sp.) [25] but also from terrestrial plants *Taraxacum wallichii* [26] and *Artemisia gilvescens* [27], and the norguaianolide (**20**) mexicanin E from *Helenium microcephalum* [28]

and **20** is an example of a norguaianolide (a guaianolide that has lost one methyl group, C15). In addition to monomeric guaianolides, some dimers like absintholide (**21**) and isoabsinthin (**22**) from *Artemisia absinthium* have been found (Fig. 98.4) [9].

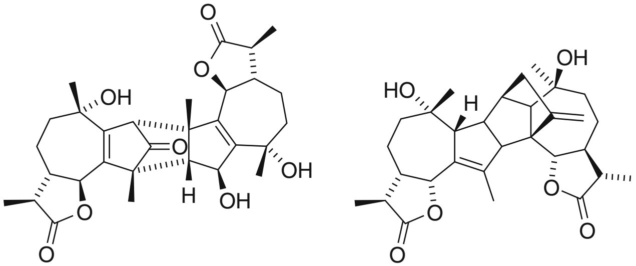


Fig. 98.4 Absintholide (**21**) and isoabsinthin (**22**) from *A. absinthium* [29]

3 Chemistry of the Guaianolides

3.1 Azulene Formation

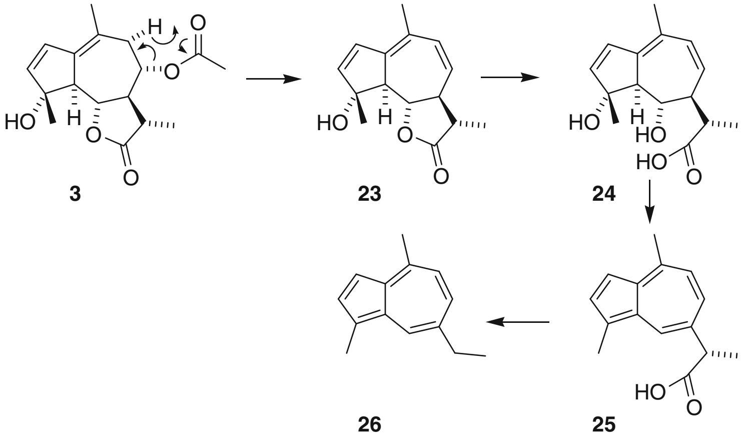
A characteristic of the essential oil of German chamomile is its blue color. During distillation, matricin (**3**) loses two molecules of water and eliminates acetic acid to give chamazulene carboxylic acid (Scheme 98.1, **25**), which decarboxylates into chamazulene (**26**). Chamazulene carboxylic acid (**25**) has been found in plasma after oral administration of **3** to healthy patients [10]. The structural similarities between **25** and ibuprofen and naproxen, both heavily used as anti-inflammatory, analgesic, and antipyretic drugs, have led to the hypothesis that the traditional use of German chamomile tea for treatment of inflammation might be rationalized by assuming that **3** is a prodrug for **25** [10]. Surprisingly, this fascinating hypothesis has not been investigated further.

In addition to **3**, also slovanolides like **27** and **28** can thermally be converted into chamazulenes. However, the presence of a hydroxyl group or an esterified hydroxyl group at C11 makes the reaction take different routes (Scheme 98.2).

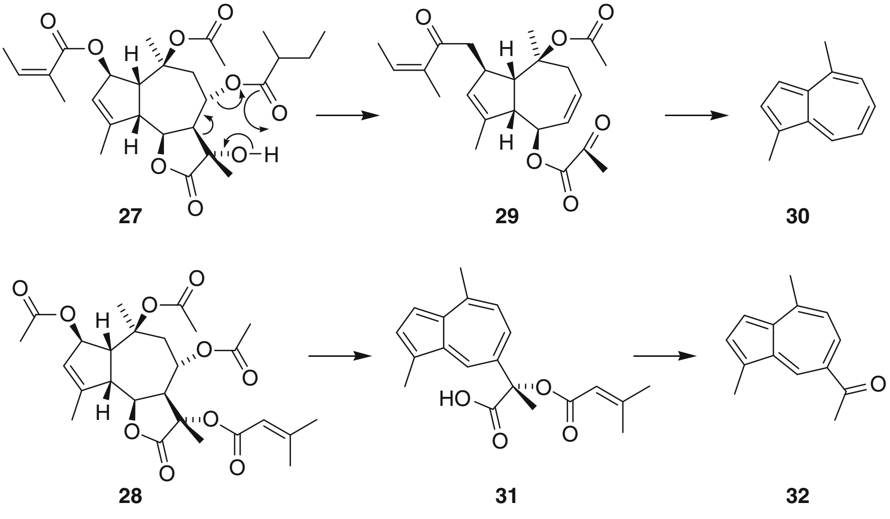
As appears from the reaction mechanism depicted in Scheme 98.2, the hydroxyl group at C11 of **27** is essential for the retro-Prince reaction affording **29**, which by elimination is converted into 1,4-dimethylazulene (**30**) [11]. The absence of the hydroxyl group in **28** prohibits the retro-Prince reaction, and instead, eliminations followed by decarbonylation afford 1,4-dimethyl-7-acetylazulene (**32**) [12].

3.2 Hetero-Michael Addition

α,β-Unsaturated carbonyl groups react by 1,4-addition with nucleophiles like thiol groups. The reaction is called Michael addition if the nucleophile is a carbanion and hetero-Michael addition if the nucleophile is a heteroatom. This type of reaction is illustrated in Scheme 98.3 using eupatundin (**33**) and cysteine as examples.

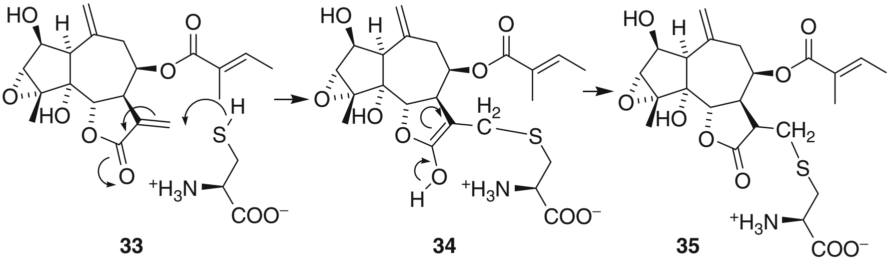


Scheme 98.1 Chamazulene (**26**) formation from matricin (**3**). Thermally, the reaction runs to **26**, whereas a reaction induced in artificial gastric or intestinal juice stops at **25**. Intermediates **23** and **24** are hypothetical.



Scheme 98.2 Formation of **30** from **27** occurs via a retro-Prince reaction that forms **29**, which eliminates three carboxylic acids to give **30**. In contrast, the absence of a hydroxyl group at C11 in **28** forces this reaction to proceed to 1,4-dimethyl-7-acetylazulene

Initially, this reaction was studied by Kupchan et al. to explain the cytotoxic activities of α-methylene sesquiterpene lactones [13]. By reaction of the guaianolide with an essential enzyme or receptor containing cysteine, a malfunctioning product might be formed that could kill the cell. In spite of several later studies, no drug based on this reaction has been registered until now,

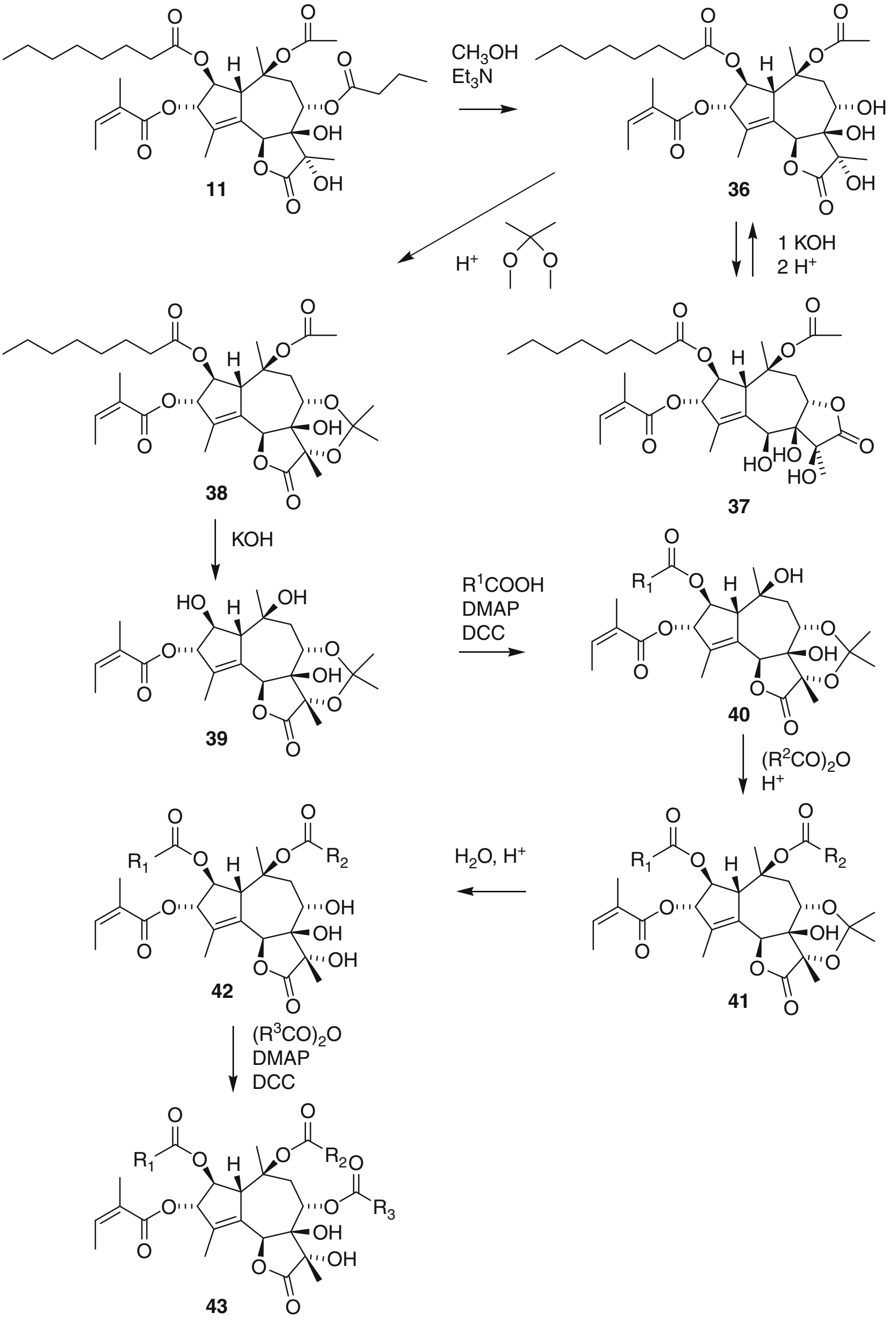


Scheme 98.3 Hetero-Michael addition of cystein to eupatundin (**33**)

but a similar principle of action lies behind the molecular mechanism of a derivative of the α-methylene sesquiterpene lactone, dimethylaminoparthenolide, which at present is in clinical trials [14]. If two α,β-unsaturated carbonyl groups are present (e.g., **5** and **6**), a product in which two molecules of cysteine have reacted with the guaianolide can be formed. In general, however, α,β-unsaturated esters esterified with the sesquiterpene nucleus do not undergo hetero-Michael additions.

3.3 Selective Replacement of Ester Groups

The potent biological activity of thapsigargin (**11**) has led to intensive studies of the binding site in the sarco/endoplasmatic calcium ATPase (SERCA) [15]. An essential requirement for these studies was selective replacement of the acyl groups esterified to four of the oxygen atoms. This possibility was first obtained by removal of the butanoyl group by treatment with mild base like triethylamine in methanol (Scheme 98.4). An additional advantage of this procedure is that under these conditions no opening of the lactone ring occurs. The use of a stronger base affords ring opening, which again after addition of acid yields a mixture of **36** and **37**. The opening of the lactone ring is prevented by masking of O8 and O11 by forming the isopropylidene acetal (**38**). Treatment with strong base converts **38** into **39**. Reaction of **40** with an acid anhydride in the presence of an acid like paratoluenesulfonic acid under controlled conditions affords selective acylation of the secondary alcohol group to give **41**. In case R^2 is a methyl, acetate is formed; this reaction might advantageously be performed with 2-propylenyl acetate. Demasking the two hydroxyl groups again affords a secondary alcohol (**42**), which again can be selectively acylated at the secondary alcohol, whereby **43** is obtained. The use of appropriate acid anhydrides or acids enables selective replacement of the three acyl groups at O2, O8, and O10. The angeloyl group at O3 might be removed after treatment with osmium tetraoxide, permanganate, and periodate, whereby the angeloyl group is converted into a pyruvate, which selectively is removed after treatment with pyridine in methanol. The mentioned reaction sequence has been used for preparation of a prodrug of thapsigargin (**11**), which, at the present, is in clinical trial.


11
CH3OH
Et3N
36
H+
1 KOH
2 H+
38
37
KOH
39
R1COOH
DMAP
DCC
40
(R2CO)2O
H+
42
H2O, H+
41
(R3CO)2O
DMAP
DCC
43

Scheme 98.4 Selective substitutions of the acyl groups of thapsigargin. Acylation of **36** with an acid anhydride catalyzed with 4-dimethylaminopyridine (DMAP) enables replacement of the butanoyl group with another acyl group

3.4 Synthesis of the Guaianolide Nucleus

Different procedures for the synthesis of guaianolides have been reviewed [16]. Some of these will be discussed in the following paragraph.

A early partial synthesis of the guaianolide nucleus was based on photochemical rearrangement of α-santonin (**44**) to give isophotosantonic lactone (**45**) [17] (Scheme 98.5). The stereochemistry of this reaction is remarkable. Irradiation of the santonin isomer, in which the C6–O6 bond is β disposed gives the C6–O6, C7–C11 *cis* isomer of **45** [18].

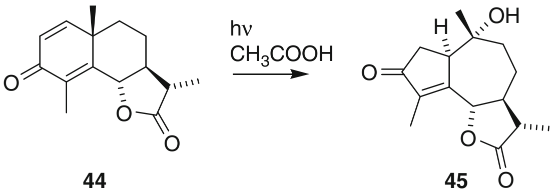
A total synthesis of a racemic guaianolide nucleus using the diketone **46** as starting material and compressenolide (**54**) as target molecule is described in Scheme 98.6. By taking advantage of the poorer reactivity of the α,β-unsaturated ketone, selective ketalization of the isolated carbonyl group is performed (**47**). Epoxidation affords a mixture of the two possible epoxides (**48a** and **48b**), which are separated by column chromatography. α−Alkylation of the ketone with isopentenyl bromide affords a mixture of the two epimers **49a** and **49b**, which can be separated using chromatography. The β-isomer is reduced with lithium in liquid ammonia to give selectively the α-diol **50**. The ketone group is then demasked by treatment with acetone and acid followed by a Wittig reaction that introduces the methylene group at C10 (**53**). Finally, the $CH_3$13 is introduced after masking of the two hydroxyl groups with trimethylsilyl chloride by alkylation using methyl iodide. Again, a mixture of epimers (**54a**, **54b**) is formed. Compressenolide is the isomer in which $CH_3$13 is α-disposed [19].

For the synthesis of **54**, the starting diketone **46** was obtained by photochemical addition of cyclopentenone (**55**) to 1,2-bis(trimethylsilyloxy)cyclopentene (**56**) as illustrated in Scheme 98.7.

A number of stereoselective syntheses of guaianolides have been based on the masked 3-hydroxycyclopentanecarboxylic acid (**64**) prepared from (*S*)-carvone (**59**) as illustrated in Scheme 98.8 [20].

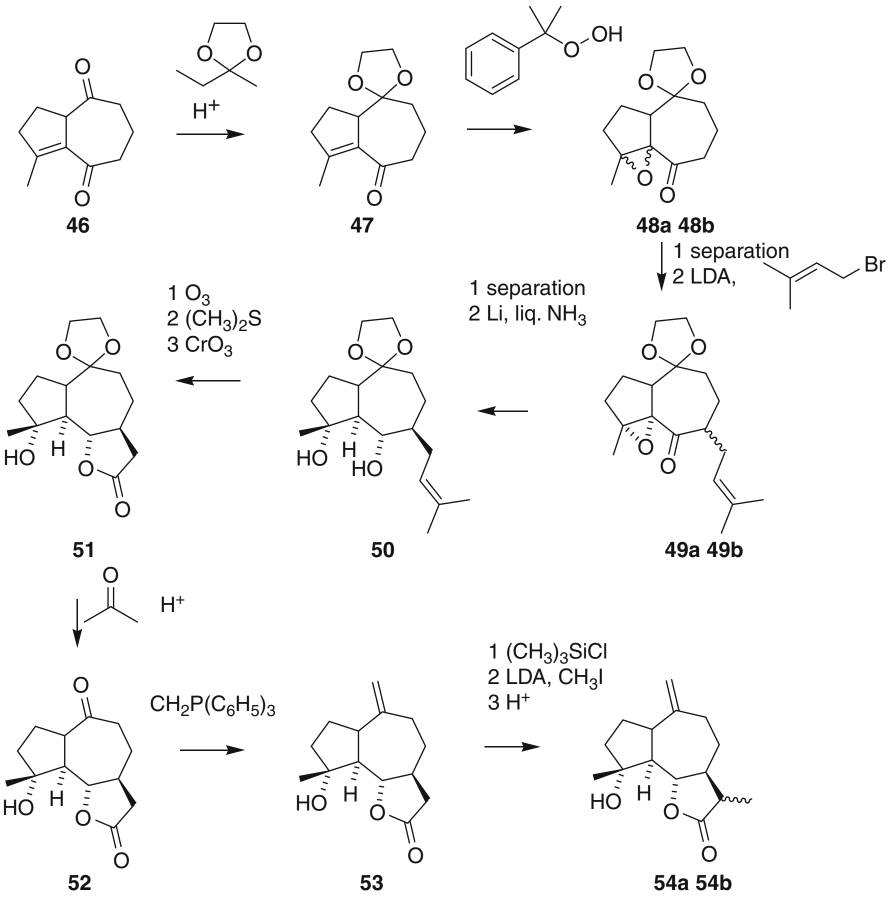
The key step in this synthesis is the Favorskii ring contraction of the α-chlorocyclohexanone (**61**) to the cyclopentane via the intermediate cyclopropanone **63**.

The hydroxycyclopentanecarboxylic acid **64** has been used as starting material for enantiomeric pure guaianolides like (−)-estafiatin, thapsigargin (**11**) [20], and (+)-cladantholide (**65**, Scheme 98.9) [21]. Formation of the tricyclic ring

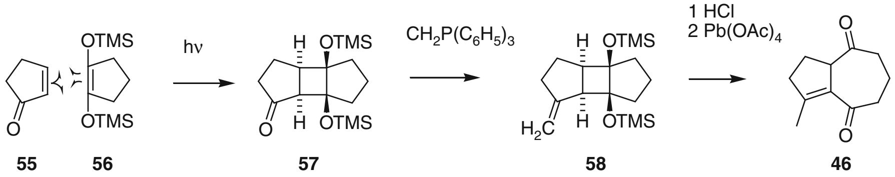


44 **45**

Scheme 98.5 Photochemical rearrangement of α-santonin (**44**) into isophotosantonic lactone (**45**)

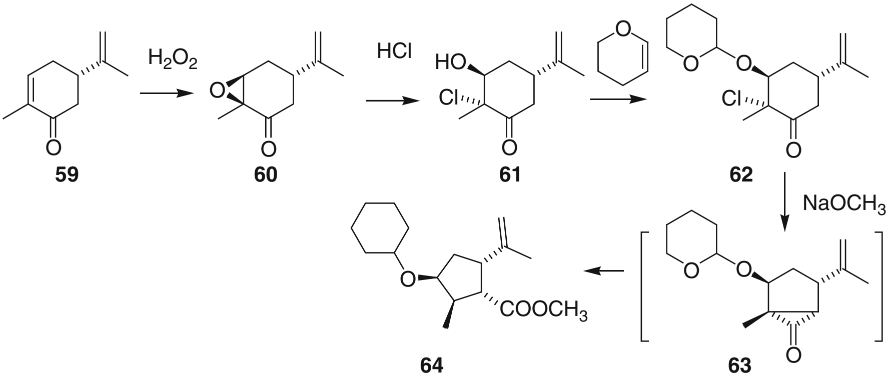

46
H+
47
48a 48b
1 separation
2 LDA,
Br
49a 49b
1 separation
2 Li, liq. NH$_3$
50
1 O$_3$
2 (CH$_3$)$_2$S
3 CrO$_3$
51
H+
52
CH$_2$P(C$_6$H$_5$)$_3$
53
1 (CH$_3$)$_3$SiCl
2 LDA, CH$_3$I
3 H+
54a 54b

Scheme 98.6 Total synthesis of racemic compressanolide (**54**)


55 56
hν
57
CH$_2$P(C$_6$H$_5$)$_3$
58
1 HCl
2 Pb(OAc)$_4$
46

Scheme 98.7 Synthesis of the starting material **46** for preparation of guaianolides

system characteristic for the guaianolide nucleus is obtained by a tandem radical cyclization of **69**. The stereoselectivity of the reaction is ensured by the preferred conformation of the intermediate radical **70**. α-Oxidation of the ketone **72** is achieved through epoxidation of the trimethylsilyl enol ether **73**.



Scheme 98.8 Construction of 3-hydroxycyclopentanecarboxylic acid using (S)-carvone (**59**) as starting material

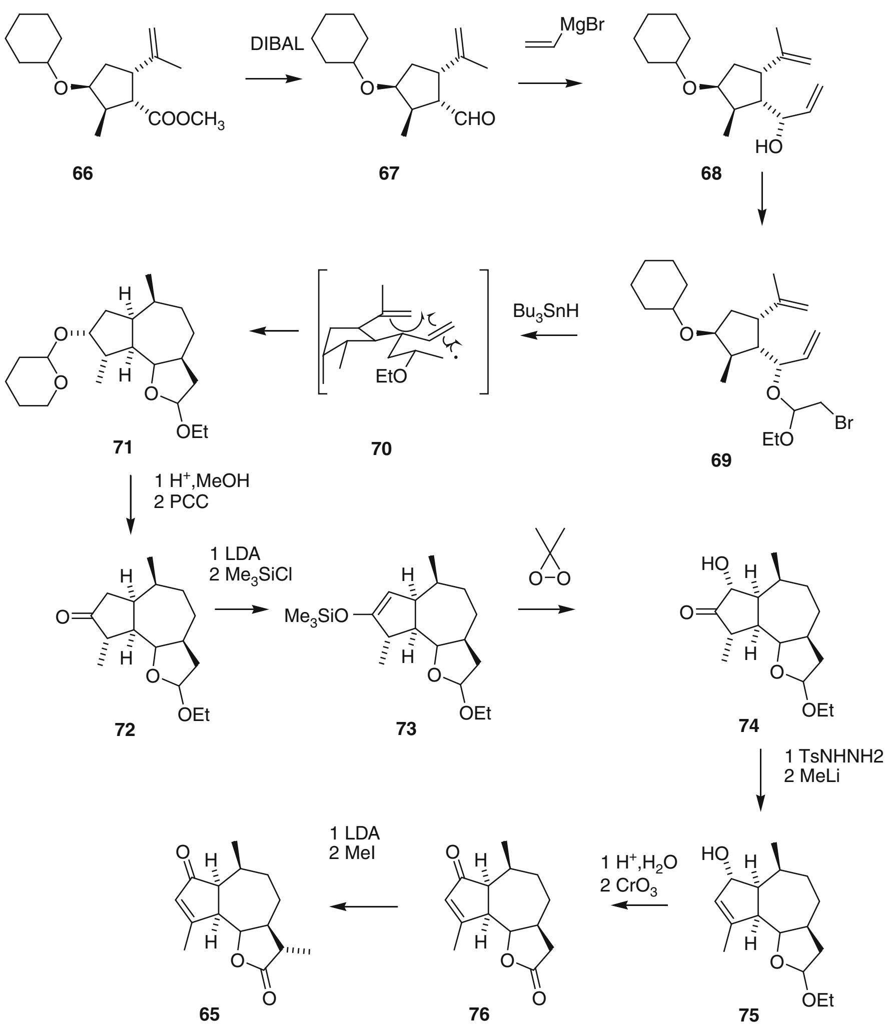
A Shapiro reaction reduces the ketone **74** into the allylic alcohol **75**. After Jones oxidation, the γ-lactone **76** is alkylated stereoselectively at C11 to give the target molecule **65**.

4 Biosynthesis of Guaianolides in Plants

In all living organisms, farnesyl synthases convert isopentenyl pyrophosphate (IPP) into farnesyl pyrophosphate (FPP) (**77**), the precursor of all sesquiterpene structures. The enzyme generating FPP, FPP synthase, has been extensively studied, and it was shown that the *E,E* conformation of FPP is the predominant product of farnesyl synthases and generally is the substrate for sesquiterpene synthases in eukaryotes [22].

From FPP, several possible cyclization reactions can occur, all of which generate substances that act as starting materials for the more than 300 cyclic sesquiterpene skeletons known. These cyclizations commence with an isomerization of *trans*-FPP (Scheme 98.10). This isomerization and the following ionization-dependent cyclization lead to a wide variety of cyclohexanoid, cyclodecanoid, cycloundecanoid, or bicyclohexanoid ring systems. Internal additions to the remaining double bonds of the initially formed carbocations also occur. Methyl migrations, Wagner-Meerwein rearrangements, and hydride shifts lead to the generation of the great variety of structures found in nature, among which sesquiterpene lactones, including the guaianolides, are found [23].

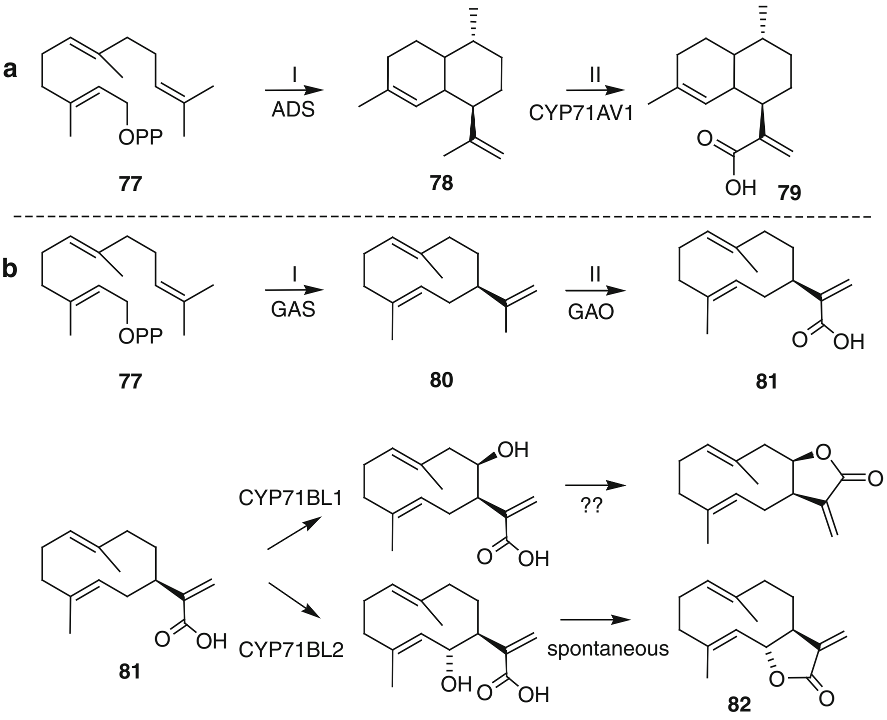
Of the sesquiterpene lactones identified so far, the one with the best described biosynthesis is the drug artemisinin, which is used in the treatment of malaria. A second well-studied sesquiterpene lactone is the germacranolide costunolide, which exhibits various cytotoxic effects. Both artemisinin and costunolide were identified in Asteraceae, and although these are not guaianolides, many of the reaction sequences generating these molecules are believed to be similar to those generating guaianolides.


DIBAL
MgBr
Bu$_3$SnH
1 H$^+$,MeOH
2 PCC
1 LDA
2 Me$_3$SiCl
1 TsNHNH2
2 MeLi
1 H$^+$,H$_2$O
2 CrO$_3$
1 LDA
2 MeI
COOCH$_3$
CHO
HO
EtO
Br
OEt
Me$_3$SiO
66
67
68
69
70
71
72
73
74
75
76
65

Scheme 98.9 Stereoselective total synthesis of (+)-cladantholide (**65**) using **66** as starting material

4.1 Guaianolide Biosynthesis in Asteraceae

The initial step of artemisinin biosynthesis is the formation of amorpha-4,11-diene (**78**) from FPP (**77**), a reaction catalyzed by amorphadiene synthase (Scheme 98.10, A). Germacrene A (**80**) was expected as an intermediate, but a single enzyme is able to facilitate the complete reaction to **78** [24]. Germacrene A synthases have been isolated from *Artemisia annua* and other asters [25]. From **78**, a multifunctional sesquiterpene oxidase, CYP71AV1, catalyzes the production of artemisinic acid (**79**) in yeast, whereas the *in planta* pathway goes to artemisinic aldehyde and

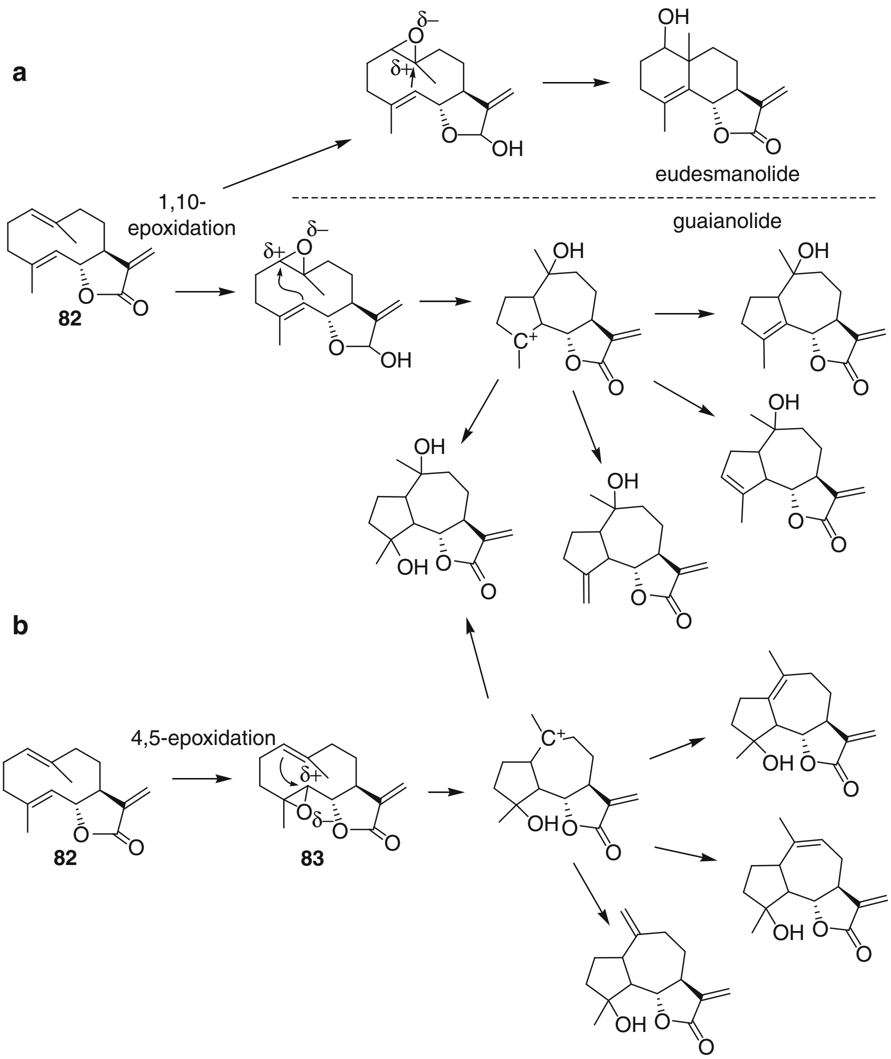


Scheme 98.10 Biosynthesis route to costunolide (**82**) in Asteraceae compared with artemisinic acid (**79**) that is spontaneously transformed in artemisinin [44, 48, 49]. *ADS* amorphadiene synthase, *GAS* germacrene A synthase, GAO germacrene A oxidase

through dihydroartemisinic acid to artemisin. The last steps in the biosynthesis responsible for converting dihydroartemisinic acid to artemisinin are less clear and are believed to occur spontaneously [24, 26].

In a series of articles from 1998 to 2002, Harro Bouwmeester and coworkers isolated several enzymes from chicory that are involved in the biosynthesis of the sesquiterpene lactone costunolide (**82**). This group recently described the formation of **80** from **77** and the subsequent conversion of **80–82** in chicory following a similar regime to that of artemisinin. This was confirmed in 2011 by Dae-Kyun Ro and coworkers with the isolation and characterization of the two enzymes CYP71BL1 and CYP71BL2 that hydroxylate C8 and C6, respectively (Scheme 98.10) [27, 28]. The formation of **82** is interesting in the context of guaianolide biosynthesis since the lactone ring has been formed before the 5- and 7-membered carbocyclic rings.

From **82**, it is hypothesized that guaianolides in chicory are generated via 4,5 epoxidation (Scheme 98.11, B) to give, e.g., parthenolide (**83**), which through opening of the epoxide by an intramolecular attack of the double bond affords the three-cyclic skeleton [27, 29, 30]. This reaction mechanism was already proposed


a
1,10-
epoxidation
δ−
δ+
OH
eudesmanolide
guaianolide
82
C+
b
4,5-epoxidation
83
δ−
δ+
O
OH

Scheme 98.11 Formations of guaianolides and eudesmanolide in Asteraceae through 1,10 and 4,5 epoxidation of costunolide (**82**) [51]

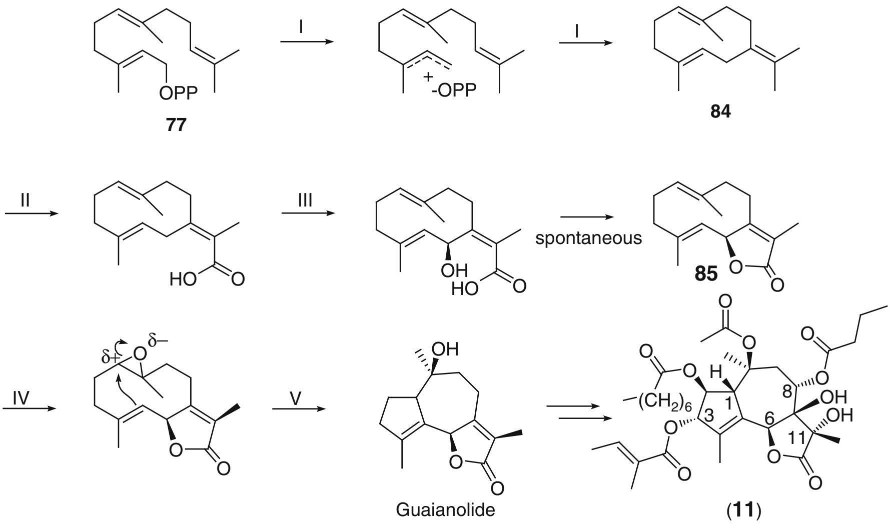
in 1995 [31]. The double bonds are formed by abstraction of a hydrogen ion attached to carbon atom neighboring the intermediate C10 carbocation. This abstraction will afford either Δ1-10, Δ9-10, or Δ10-14 double bonds. Alternatively, the carbocation might be quenched by reaction with a water molecule to give an alcohol at C10 (Scheme 98.11, B). Formation of other sesquiterpenes found in Asteraceae and Apiaceae is best explained by assuming a 1,10 epoxide as intermediate. A similar intramolecular attack as described above would afford a C4 carbocation from which isomers of Δ4-5, Δ3-4, or Δ4-15 double bonds or the equivalent alcohol at C4 may be formed (Scheme 98.11, A). Unfortunately, the enzymes involved in the last steps of guaianolide biosynthesis in Asteraceae have not been identified yet. However, studies in *Lactuca floridana* (Asteraceae) support the theory that **83** is the intermediate in guaianolide biosynthesis [32], and the isolation and characterization of the 6-hydroxylase (CYP71BL2) followed by spontaneous formation of **82** in *L. floridana* support this pathway in Asteraceae.

Notice that the suggested intermediate carbocations are tertiary carbocations but that the epoxides during the formations of guaianolides are attacked at the less substituted carbon atoms.

4.2 Guaianolide Biosynthesis in Apiaceae

No studies have yet been able to elucidate the biosynthetic pathway of guaianolides in Apiaceae. Even the biosynthesis of the otherwise well-studied compound thapsigargin (**11**) is unknown. Given the promising results against prostate cancer [33], an annual demand of one ton of thapsigargin might be expected, encouraging development of production protocols. One possibility would be biotechnological production in a genetically modified organism, an approach which, however, requires a detailed knowledge of the biosynthesis [34]. A biosynthetic pathway has been proposed (Scheme 98.12) [5]. The first step, **I**, is mediated by a germacrene B synthase. Germacrene B (**84**) has been identified in several Apiaceae species. This biosynthetic pathway could explain the stereochemistry at C1, which differs from that generally observed in guaianolides from Asteraceae [35, 36]. In steps **II** and **III**, the lactone ring is formed by enzymes similar to the Cytochromes P450 described in Scheme 98.10 followed by a spontaneous reaction, that has been shown to generate **82** (Scheme 98.10, B), and form the germacrenolide (**85**) shown in Scheme 98.12. The 1,10-epoxidation in step **IV** initiates the last step of the guaianolide formation, step **V**, in which an enzyme closes the guaianolide structure. This should, in the biosynthesis of thapsigargin (**11**), preferably proceed via 1,10-epoxidation in order to retain the Δ4-5 double bond that is present in thapsigargin, but it is likely that both 1,10 and 4,5 epoxidations can occur within plants belonging to the Apiaceae family, since the Δ4-5 double bond is not conserved within the family [5]. The final step also insures the conformations found in most guaianolides in Apiaceae, namely, 1βH, 10αCH_3, and 11βCH_3. Hydroxylation of C7 is only rarely found in guaianolides, and the 7β-hydroxyl group is unique to the thapsigargins. A likely explanation for the unique 7β-hydroxyl group is that a precursor possessing a Δ7-11 double bond is converted into an epoxide that is then opened into a trans-glycol [37].

The finding of guaiol and guaienes hydroxylated at positions C11 and C8 could suggest that the guaiene structure is formed prior to any secondary modifications. It has been observed in pine that sesquiterpene cyclases also perform these secondary modifications on the germacrene skeleton via hydride shifts, methyl migrations, and Wagner-Meerwein rearrangements [23]. It still needs to be seen whether this is also



Scheme 98.12 Proposed biosynthesis of guaianolides in Apiaceae, here depicted to afford thapsigargin (**11**) [5]. A 1,10-epoxide is suggested as an intermediate

true for enzymes found in Apiaceae, but this should be taken into consideration when elucidating the biosynthesis.

Since most of the enzymes involved in this pathway are cytochrome P450, sufficient oxygen and NADPH are crucial for the efficiency of the biosynthesis. Although the reactions catalyzed in Asteraceae by the proposed enzymes might seem similar to those observed in Apiaceae, differences in the stereochemistry of substrates and desired products might result in difficulties generating the pathway described. Attention has been called to the possibility that slight alterations in the substrate processed may alter the mechanism of the individual enzymes [38].

4.3 Stereochemical Differences Between Guaianolides from Apiaceae and Asteraceae

As observed with thapsigargin and other guaianolides, the presence of secondary modifications enhances the complexity of the stereochemistry, especially around the lactone ring. This is of particular interest with regard to the similarities and differences between Apiaceae and Asteraceae. Numerous sesquiterpene lactones have been isolated from both families [5, 39–41], but the guaianolides exhibit a peculiar difference between the two. In Apiaceae, the relative configuration of all 6,12 guaianolides identified so far differs from that of 6,12 guaianolides that have been identified in Asteraceae. In Apiaceae, the lactone ring is either 6β,7α or 6β,7β, whereas only examples of 6α,7β disposed lactones are known in Asteraceae. The difference in guaianolide structure between these two families might not seem to appear critical, but it causes dramatical changes in the conformation of the ring systems. Holub and Budesinsky proposed that these differences appear because of different substrate specificity of the enzymes involved. A possibility could be that the enzymes cyclize the *E*,*E*-FPP in different conformations [40]. Recently, an alternative route was presented based on the observation that (*E,E*)-germacradienes adopt several conformations of which ββ, βα, αβ, and αα are the most stable ones, where α and β refer to the orientation of the C14 and C15 methyl groups [42]. Thus, it was hypothesized that it is possible to transform a germacrene in the dominant αα-conformation (as observed in Asteraceae) into a ββ-conformation germacrene (as observed in Apiaceae) by adding a β-hydroxyl group at carbon C6. This would lead to steric hindrance of the αα-conformation and induce the flipping to the ββ-conformation. The conformation of either of these hypotheses would explain the biogenetic origin of guaianolides. The addition of the β-hydroxyl at C6 is likely to happen during the cyclization of FPP and would therefore lead to a germacrene-6-ol structure as the product of the terpene synthase.

4.4 Guaianolide Biosynthesis in Other Plant Families

In the plant family Lamiaceae, 8,12-lactones with a distinctive stereochemistry and lack of hydroxylation of the 11-12 bond have been isolated. Again, this indicates that different conformations of the substrates are favored by the involved enzymes.

5 Biological Activity

The presence of sesquiterpene lactones in a spectrum of plants suggests an ecological effect. A large number of sesquiterpene lactones, including some guaianolides, have been shown to be antifeedants to insects [43], and guaianolides often serve as defense compounds in the plants from which they are isolated. Conservations in structures in many parts of biomolecules occur all over the animal and plant kingdoms (privileged structures), thereby indicating that potent activity toward one kind of organism implies that other living organisms are affected as well. Plants containing guaianolides are often used to treat inflammatory disorders and related diseases [44]. Indeed, guaianolides possess a broad range of activities including cytotoxicity, antiprotozoal, antimicrobial, anti-inflammatory, and apoptotic activity [5]. In addition, some induce allergic contact dermatitis [45].

A common functional group in many guaianolides and other sesquiterpene lactones is the α-methylene-γ-lactone group and/or α,β-unsaturated cyclopentenone. Both residues react with thiol present in peptides and proteins and are of importance for the mechanism of action of guaianolides. Due to this feature, some guaianolides affect cell signaling, proliferation, apoptosis, and mitochondrial respiration [44]. It is generally thought that biological activity of sesquiterpene lactones possessing these functional residues is mediated by alkylation of nucleophiles (confer Sect. 5.2). The reaction of α,β-unsaturated carbonyl groups with peptides and enzymes has been studied by Schmidt et al. [44] and Kupchan et al. [7].

In many guaianolides, both alkylating groups are present (see Figs. 98.1, 98.2). Potential molecular targets for alkylation include 5-lipoxygenase and leukotriene C_4-synthase, both being enzymes of the leukotriene biosynthesis, glutathione, coenzyme A, papain, and nuclear transcription factors. An IC_{50} of 12 μM of helenalin on LT_4 synthase was determined to be borderline small to be of pharmacological importance for the anti-inflammatory effects [45].

In other cases, the activity is related to the presence of a pharmacophore in the molecule enabling complex formation with proteins without covalent binding. Examples of such guaianolides are the thapsigargins, which block a calcium pump in the cell and thereby induce apoptosis.

5.1 General Toxicity of Guaianolides

Guaianolides that have demonstrated potent cytotoxic activity against human leukemia cell lines have primarily been found in cell proliferation assay, and the inhibitory effects on cell aggregation are then probably caused by general toxic effects. It has been stated in several publications that the α-methylene-γ-lactone moiety of the guaianolides is crucial for the cytotoxic activity in agreement with the hypothesis of a cysteine alkylation [6, 46]. Cytotoxicity of sesquiterpene lactones, with two α,β-unsaturated carbonyl structural elements, has so far not been correlated with lipophilicity, in contrast to monofunctional sesquiterpene lactones whose activity increased with increasing log *P* [7]. A neighboring OH or O-acyl group has been found to enhance the rate of cysteine addition to the lactone. By using pseudoguaianolides as model compounds, it was observed that the cytotoxicity strongly depends on the number and type of alkylating centers. The conformation of the molecule and the number of H-bond acceptors, but not their position, are also deciding factors. The latter parameter indicates that noncovalent interactions of sesquiterpene lactones with proteins might bring the molecule in a position facilitating alkylation [47]. These studies underline that reactivity toward cysteine as well as interactions with further amino acids is of importance for their cytotoxic activity. Studies on the ability of the pseudoguaianolide helenalin to alkylate enzymes and peptides have revealed that a cyclopentenone residue in vitro is more reactive toward the thiol groups of cysteine than is the α-methylene lactone [44].

Also, synthetic derivatives of natural guaianolides have been found to be active against tumor cells. An example is the mono- and dihalohydrin analogues of repin showing significant antitumor potency. Repin itself was found to be potent against seven cancer cell lines. However, the monohalohydrins were generally less active than the parent diepoxide repin, an indication of a disruption of the active sites, namely, the α-methylene-γ-lactone moiety and possibly α,β-unsaturated carbonyls [7, 48]. It is known that the C13 side chain of the diterpenoid paclitaxel is crucial for its strong antitumor activity [49], and it was shown that esterification of repin with the side chain of paclitaxel led to increased potency in all tested cell lines. On the contrary, the presence of a diol rather than an epoxide on the cyclic skeleton of repin abolished activity. Likewise, guaianolides with an allyl hydroxy or diol in the side chain lost activity [48]. Again, these observations are in line with several other publications that have shown that decreasing the number of H-bond donors in the molecules also increased their potency [7, 47, 50–53]. The described cytotoxicity does not only apply to human cells but most likely to all eukaryotes.

5.2 Anti-inflammatory Activity of Guaianolides

In 1999, Irmgard Merfort and her group published a thorough study of sesquiterpene lactones and their NF-κB inhibitory activity [53, 54]. The term nuclear transcription factor (NF) applies to a family of highly homologous protein occurring ubiquitously in mammalian cells. NF-κB consists of two subunits of M_r 50 and 65 kDa. NF-κB is a genetic switch for many different genes encoding cytokines such as interleukins as well as inducible enzymes such as cyclooxygenase II and nitric oxide synthase. The most potent compounds in Merfort's study were pseudoguaianolides, guaianolides, germacrolides, heliangolides, melampolides, and 4,5-dihydrogermacranolides. In addition to an α-methylene-γ-lactone group, all the potent inhibitors had at least one further α,β- or α,β,γ,δ-unsaturated carbonyl group. The latter was concluded to serve as a bifunctional Michael acceptor (alkylant), with some of the structures having an additional α,β-unsaturated acyl side chain serving as a trifunctional Michael acceptor.

Additionally, Merfort concluded that sesquiterpene lactones that only have either a methylene lactone or a conjugated keto group are much less potent since they require inhibitory concentrations of about 100–200 μM. This lack of activity cannot be compensated by the presence of an α,β-unsaturated ester group. The guaianolide cumambrin A is an exception from this statement by showing a strong NF-κB inhibitory effect even significantly more active than the corresponding alcohol cumambrin B, which might be due to *O*-acetylation [54].

The mechanism of action is based on two cysteine residues, Cys37 and Cys120, in the p65 subunit of NF-κB with their free thiol groups located in a distance of 8 Å. This distance corresponds to the distance between C2 and C13 of helenalin, indicating that the pseudoguaianolide might bridge these two residues and thereby prevents the transcription factor from binding to the DNA-binding domain. A similar mechanism of action can be predicted for other bis-alkylating sesquiterpene lactones.

The same correlation between the esterified guaianolide and the free alcohol described for cumambrin has also been observed for 3β-O-(2-methylbutyryl)-moroccolide A, which showed a significantly higher inhibition of pro-inflammatory gene transcription than moroccolide A [55]. This effect may be related to acceptation of a hydrogen bond in the vicinity of the alkylating site. Such interactions, however, cannot sufficiently be explained with the present knowledge. Moreover, a more precise measurement of activity differences is needed to elucidate the structural factors that modulate the activity apart from alkylant functionality.

It has not yet been shown that the NF-κB inhibition by guaianolides is caused by alkylation at the mentioned cysteines in p65, but in many instances, the underlying chemical reaction has been shown to be correlated to the presences of low-molecular-weight thiols [47, 52, 56]. It is known that sesquiterpene lactone enzyme inhibitory activity is usually associated with the presence of free cysteine residues. Likewise, NF-κB inhibition by an alkylant of completely different structure has been demonstrated [57].

Guaianolides, unlike some other sesquiterpene lactones, have a rigid skeleton. Accordingly, the NF-κB inhibitory activity is not only correlated to the number of unsaturated carbonyl structures in the molecule but also to parameters that describe how the molecule fits into an active site. Additionally, it has been observed, as described, that an increasing number of free hydroxyl groups diminish the inhibitory activity [55]. A lower number of hydroxyl groups correlates with a higher lipophilicity followed by a better penetration through cell membranes. Whether lipophilicity, expressed as log *P*, significantly contributes to the inhibitory activity is still doubtful. Therefore, rather the position than the number of hydroxyl groups may be important for NF-κB inhibition.

Guaianolides have been shown to be favorable as starting materials for synthesis of NF-κB-inhibiting agents compared to other sesquiterpene lactones because of easier synthetic protocols [52]. The compound should possess two α,β-unsaturated carbonyl groups and an acyl moiety near the exocyclic methylene group. Moreover, it has to be considered that the QSAR studies published correlate nicely with the structure – activity requirements for cytotoxicity, making it unlikely to separate the wanted therapeutic effects from the unwanted side effects [52]. To avoid this problem, guaianolides could be either applied only externally or they could be designed as prodrugs that would be liberated at the location of inflammation for the treatment of chronic inflammatory diseases as seen with thapsigargin (Sect. 5.3).

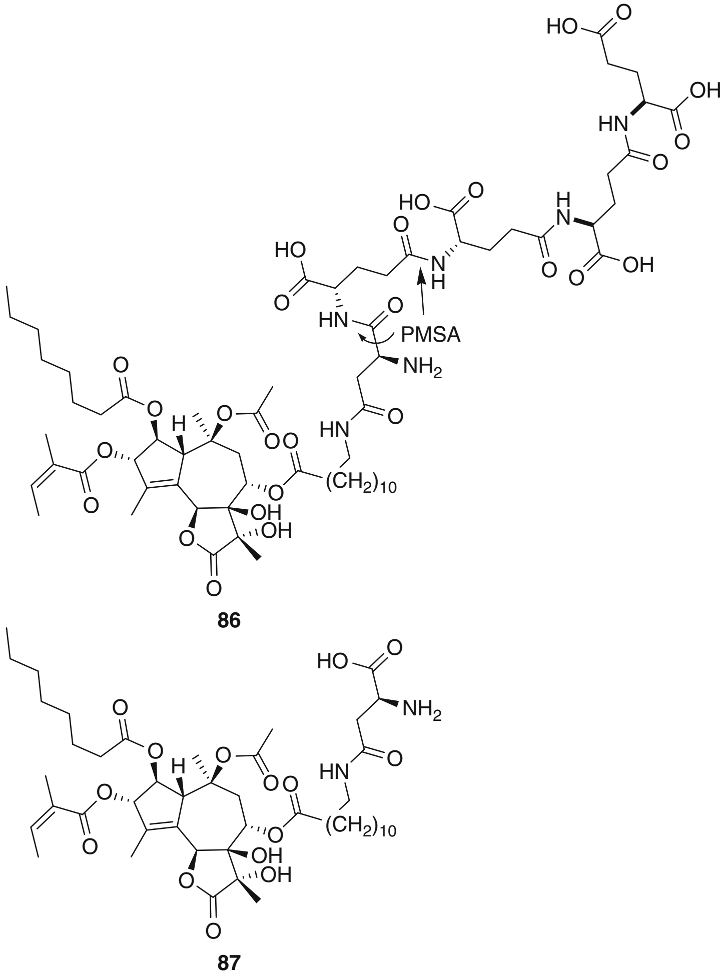
5.3 Thapsigargins: A Well-Studied Group of Guaianolides from Apiaceae

Thapsigargins are a structurally unique type of guaianolides: they are trans hydroxylated at C7 and C11 and possess a trans-annelated C7α-C6β γ-lactone ring (Fig. 98.2, **11**). Thapsigargins are known for their biological activity, despite the fact that they are lacking an unsaturated carbonyl moiety. Thapsigargin (**11**) originally was isolated as the skin irritant principle of the Mediterranean medicinal plant *Thapsia garganica*. The compound was found to be an extremely potent liberator of histamine from mast cells. Later studies revealed that the compound inhibited the calcium pump in the endo- and sarcoplasmic reticulum (SERCA) in subnanomolar concentrations. Prolonged inhibition of this pump results in an elevated cytoplasmic Ca^{2+} level, which eventually leads to the death of the cell. Thus, the anticancer activity of thapsigargins is mediated by a different mechanism than the one mentioned above. An X-ray structure of the thapsigargin-SERCA complex has enabled detailed studies of the binding site and the structural conformation of the binding complex, thereby also providing some clues used for later design of a targeted prodrug with thapsigargin as the active part [8]. Major contributions to the affinity of **11** to SERCA are lipophilic interactions of the angeloyl, the butanoyl, and the acetyl moieties with the backbone of the protein. In addition, the $CH_3$15 further assists in interaction with the pump. The importance of the different side chains has been confirmed by preparation of several analogues in which these side chains have been either removed or stereocenters had been inverted in order to prevent interaction of the appropriate side chain with SERCA. These studies revealed the importance of several of the residues present in thapsigargin (**11**). An interesting discovery was the dramatic effect caused as soon as stereochemistry at C8 changed. When changing the ester group from α to β configuration, the Ca^{2+}-ATPase inhibiting properties of the molecule decreased 3,000-fold [37]. X-ray analysis revealed that this residue was situated in a cavity between helices when bound to the SERCA protein, in which it would not fit with the alternative configuration. Equally, the results showed that removing the angeloyl group or simply changing the stereochemistry of C3 resulted in a significant decrease of the Ca^{2+}-ATPase inhibitory effect [8].

Replacement of the C10 acetoxy with a hydroxy moiety also decreased the biological activity of thapsigargin. Similarly, reversal of the stereochemistry at C3 severely decreased the affinity for the SERCA pump. These findings reveal that the localization of the side chain is of far more importance than the structure of the side chain, within certain limits [58]. The structure of the acyl group linked to the C8–O could be varied extensively if flexibility of the residue is maintained. These investigations laid the foundation of the subsequent design of thapsigargin analogues used for specific cell targeting (Fig. 98.5) [8].

A remarkable feature of thapsigargin is its ability to induce apoptosis in both slowly and rapidly dividing cells [59]. This contrasts usual chemotherapeutic agents, which mainly nonselectively target rapidly dividing cells and thus are unsuccessful in treatment of slowly developing cancer diseases. Per se, thapsigargin is unfit as a drug since it will kill all kinds of mammalian cells, irrespective of being benign or malignant. This drawback has been overcome by preparation of a prodrug. Prostate cancer cells in general proliferate slowly and are therefore not effectively treated with traditional chemotherapy. The neovascular tissue of prostate cancer tumors and many other solid tumors overexpresses the proteolytic enzyme prostate membrane specific antigen (PMSA). This enzyme is a serine protease that cleaves at specific amino acid motifs. Replacement of the 8-butanoyl-moiety in **11** with a specific peptide, including both a flexible region and the amino acid sequence recognized by PMSA, would result in a prodrug that would be activated in the proximity of cancer cells. The flexible region was determined by varying the length of the C8-positioned residue, and a candidate was chosen based on its preserved activity against prostate cancer cells (see Fig. 98.5) [60].

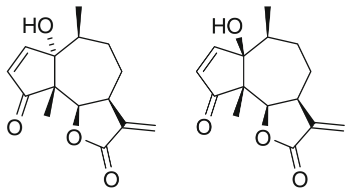
Preliminary trials showed that the prodrug (**86**) itself did not have any cytotoxic activity at 10 μM. However, the same dosage was able to completely inhibit the growth of a human prostate cancer cell line. Also, when tested in vivo in nude mice with xenografts from a human prostate cancer cell line, the level of cleaved active



86

87

Fig. 98.5 The two *arrows* indicate the cleavage site for the enzyme PMSA. Compound **86** is at the time of writing in clinical trial 1 as a drug against a broad spectrum of cancer diseases. Compound **87** is the major drug formed by cleavage of the prodrug **86** in neovascular tissues in tumors [35]



88 **89**

Fig. 98.6 The pseudoguaianolides parthenin (**88**) and hymenin (**89**)

drug was much higher in tumor tissue than in the plasma or skeletal muscle of the mice, confirming effective drug targeting [61]. A prodrug based on thapsigargin is currently being developed by GenSpera, Inc. Further results have shown that the C2-positioned octanoyloxy moiety of thapsigargin does not bind to the SERCA pump. However, the residue has affinity for the inhibition of the pump, probably through binding to the lipids in the cell membrane.

5.3.1 SERCA Activity of Other Guaianolides in Apiaceae

Of the remaining guaianolides isolated from the plant family Apiaceae, most have only been isolated for elucidation of their structures. Additionally, many of them are only present in very small amount in the plants, making the biological examination very difficult.

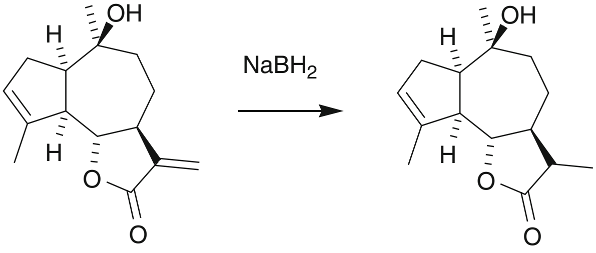
A few of the identified compounds have, however, been tested in different assays. Among those were fegolide and three other structurally related guaianolides, but none of them showed significant activity as SERCA inhibitors [62]. The lack of inhibitory effect might be explained by the absence of a O8 residue in these compounds. Two of the tested structures have an acetoxy group at C3, but this is apparently insufficient to establish an effect. Anyhow, this suggests that several of the other guaianolides isolated from Apiaceae might have SERCA-inhibiting properties since many have an ester-bound residue at O8. However, quite a few also have substituents at C11, a substituent that will interfere with SERCA binding because of its proximity to one of the helices comprising the binding site. As mentioned, publications concerning the activity of sesquiterpene lactones focus on compounds with a methylene group at C11 on the lactone ring [5].

5.4 Antiprotozoal Activity of Guaianolides

Guaianolides have also been intensively examined toward protozoa like members of the genera *Trypanosoma* (sleeping sickness), *Leishmania* (leishmaniasis), and *Plasmodium* (malaria); only studies that also tested cytotoxic activity are considered here. In all cases, an antiprotozoal activity correlates positively with cytotoxicity, and the major determinants for activity are α,β-unsaturated carbonyl residues. Certain compounds are considerably more toxic against protozoa than against mammalian cells and vice versa. A comparative QSAR analysis has been undertaken, and both activities were found to depend mainly on the same structural elements and molecular properties. The observed variance in the biological data can maybe be explained by the positioning of the various molecules in the active site [63–65].

It appears therefore difficult to exploit the relative subtle structural differences responsible for differential activity with respect to lead structure optimization. While further QSAR models might reveal a clearer and more detailed relationship, the prodrug strategy chosen for thapsigargin might be useful for protozoa as well, especially with the several unique protozoal drug targets published in the recent years.

Scheme 98.13 10-Epi-8-deoxycumambrin B (**90**) and the dihydroderivative (**91**)



90 **91**

5.5 Allergic Contact Dermatitis

Allergy provoked by contact to plants belonging to Asteraceae is in most cases caused by sesquiterpene lactones. The type of immune response involved is delayed hypersensitivity, also termed type IV allergy. Also in this case, the sensitizing potential depends on the presence of alkylating groups. It is assumed that the allergy evolves because alkylation of proteins creates proteins unknown to the organism and therefore activates the immune response. The presence of an α-methylene-γ-lactone is the main requisite; however, the presence of this group is not sufficient since some sesquiterpene lactones possessing this structural unit are not sensitizing. The pseudoguaianolide parthenin (Fig. 98.6, **88**) is the allergenic principle of the plant *Parthenium hysterophorus*, also known as the scourge of India.

No simple correlation has been found in the structure-cross-sensitivity relationships, and hymenin (**89**) shows no cross sensitivity with the diastereomer parthenin.

5.6 Aromatase Inhibitors

Aromatases are key enzymes in the biosynthesis of estrogens, and inhibitors are used for treatment of hormone-dependent breast cancer. The aromatase inhibitor aminoglutethimide (Cytadren®) is presently used as a drug. The sesquiterpene lactone 10-epi-8-deoxycumambrin B (Scheme 98.13, **90**) has been shown to be a potent inhibitor of this enzyme, but hydrogenation of the 11,13-double bond afforded an even more potent inhibitor (**90**) inhibiting the aromatase in the same concentration range as aminoglutethimide [45, 66]. The guaianolides are believed to compete with testosterone for the binding site. A further advantage of removing the Δ11-13double bond was the significant lowering of the general cytotoxicity.

6 Conclusion

This chapter gives an overview of the chemistry, biochemistry, and pharmacology of guaianolides, mainly from the two plant families Asteraceae and Apiaceae. At present, the guaianolide most likely to become drugs are derivatives of thapsigargin, which is a very selective inhibitor of the SERCA pump. Nonetheless, the data presented here show the diverse range of guaianolides that are generated by different plants. Considering the number of guaianolides, the possibility that some of these might interact with new potential pharmacological targets enabling new principles of treatments, as did thapsigargin, should not be overlooked. With the increasing knowledge of the human genome, new and attractive targets might be revealed. In light of the interesting biological activities revealed by many of the guaianolides, a compelling argument is that the rest should also be investigated in depth.

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Biosynthesis and Production of Artemisinin: A Novel Class of Molecule

99

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Contents

1 Introduction 3100
2 Biosynthetic Pathway of Artemisinin in *Artemisia annua* L. Plant 3101
3 Strategies to Enhance Artemisinin Production 3105
3.1 Biotechnological Approaches to Enhance Artemisinin Production in *Artemisia annua* L. Plant 3105
3.2 Plant Tissue Culture 3109
4 Conclusion 3114
References 3115

Abstract

Artemisinin, a sesquiterpene lactone endoperoxide and isolated from aerial parts of *Artemisia annua* L. plants (family Asteraceae; commonly known as sweet wormwood), is popular as a potent, promising, highly effective, safe, and best therapeutic agent against drug-resistant strains of *Plasmodium* sp. The low yield of artemisinin content, is a serious limitation to its ability and affordablity to the most malaria sufferers. The chemically synthesized artemisinin is also costly due to low yield of the process. The World Health Organization (WHO) recommends the use of artemisinin-based combination therapies (ACTs), for the first-line treatment of malaria. To date, *A. annua* L.

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is the only commercial source of artemisinin. Due to the urgent demand and short supply of this compound for the manufacture new antimalarial drugs (ACTs), efforts are being made worldwide to enhance its production by employing plant tissues culture and metabolic engineering of *A. annua* L. plants as well as microbes. So, in this chapter, we have discussed the various aspects of biotechnological approaches, including plant tissues culture and genetic engineering of *A. annua* L., *E. coli*, and *Saccharomyces cerevisiae*, to improve artemisinin production as reduce the cost of artemisinin based combination therapies (ACTs) to significantly below their current prices.

Keywords

Artemisia annua L. • artemisinin • malaria • metabolic engineering • plant tissue culture

Abbreviations

ACT	Artemisinin-based combination therapy
ADS	Amorpha-4, 11-diene synthase enzyme
ads	Amorpha-4, 11-diene synthase gene
Aldh1	Aldehyde dehydrogenase 1
cps	*β*-caryophyllene synthase gene
dbr2	double-bond reductase2 gene
DMAPP	Dimethylallyl diphosphate
ECS	Epi-cedrol synthase
FPP	Farnesyl diphosphate
fps	Farnesyl pyrophosphate synthase gene
fs	(E)-β-farnesene synthase gene
GPP	Geranyl diphosphate
gpps	Geranyl diphosphate synthase gene
GS	Germacrene A synthase
HMGR	Hydroxymethylglutaryl coenzyme A reductase
hmgr	hydroxymethylglutaryl coenzyme A gene
IPP	Isopentenyl diphosphate
RT-PCR	Reverse transcriptase polymerase chain reaction
sqs	squalene synthase gene

1 Introduction

Artemisinin (Qinghaosu) is a sesquiterpene lactone containing an endoperoxide bridge. It is popular as a potent, promising, safe, and best therapeutic agent against both drug-resistant and cerebral malaria causing strains of Plasmodium sp. [1]. It is a highly oxygenated sesquiterpene, containing a unique 1, 2, 4- trioxane ring structure, which is responsible for the antimalarial activity. Besides being its commercial use as antimalarial drug, it is also found to be effective for other infectious diseases such as schistosomiasis, HIV, hepatitis B, anti-leishmaniasis, and herbicidal [2–7]. Artemisinin has shown effectiveness against a variety of cancer cell lines, including breast cancer, human leukemia, colon cancer, and small cell–lung carcinomas [8, 9]. It was first isolated from the aerial parts of Traditional Chinese Medicinal herb *A. annua* L. plants by the Chinese Scientists [10]. The WHO in the year 2005 recommended the use of artemisinin derivatives in combination with other effective schizontocidal drugs, a treatment known as artemisinin-based combination therapy (ACT), for the first-line treatment of malaria [11]. The reported artemisinin contents in *A. annua* vary from 0.5 to 0.9 % (in India) and from 0.9 to 1.2 % globally [12]. There is a wide gap between the demand of artemisinin (40–50 t in India) and its supply (2 t in India). The cost of ACTs per dose at government public health clinics is around USD 1.00 and much higher at private pharmacies [13]. Due to its current use in artemisinin-based combination therapy (ACT), the global demand of artemisinin is continuously increasing. But the relatively low yield of artemisinin in *A. annua* L. plants (0.01–1.1 %) is a serious limitation to its commercialization [14, 15]. In recent years there have been large fluctuations in the price and availability of ACTs [16], and in 2007–2008 less than 15 % of children below the 5 years of age with fever received ACTs in 11 out of 13 countries surveyed [17]. So, there is an urgent need to increase the supply of artemisinin to both stabilize the price and increase the availability of ACTs in the developing world. Therefore, significant increase in the cultivation of *A. annua* L. plants would be needed to satisfy projected global demand. The chemical synthesis of artemisinin is not economically feasible, because of the complexity, low yield of the process, and lack of cost-effective, viable synthetic protocol [18]. To overcome this problem, efforts are being made worldwide to enhance its production employing various approaches such as conventional breeding and biochemical as well as physiological methods [19]. These approaches show potential for future development, but improvements delivered by them so far have not met the global demand. Therefore, the plant *A. annua* L. is still the only valid source of artemisinin.

2 Biosynthetic Pathway of Artemisinin in *Artemisia annua* L. Plant

Artemisinin biosynthesis takes place in the glandular trichomes of the plant. Different kinds of terpenoids in nature shared a common precursor, isopentenyl diphosphate (IPP); of course, artemisinin recognized as a sesquiterpene lactone is not an exception [20]. In artemisinin biosynthesis, there are two independent pathways producing IPP. One is the classical cytosolic mevalonate pathway (MVA) originating from acetyl Co-A and the other is the methylerythritol phosphate pathway (MEP) or 1-deoxy-D-xylulose-5-phosphate (DXP) localized in plastids, starting from pyruvate. The mevalonate pathway (MVA) has been discovered over half a century and the molecular genetics of MVA pathway has been elucidated. The genes involved in MVA pathway have been characterized by molecular genetics and biochemistry approaches. The cytosolic MVA pathway provided major part of the carbon skeleton for artemisinin biosynthesis (80.0 %) as compared to MEP/DXP pathway (14.0 %). Mevalonate (MVA) is the primary building block for isoprenoid biosynthesis in higher plants. It serves as a common precursor for the production of a number of compounds vital to normal plant growth and development, including carotenoids, the phytol tail of chlorophylls, plastoquinone, and ubiquinone, as well as the phytohormones viz abscisic acid (ABA), cytokinins, and gibberellins. Mevalonate also contributes to the formation of a wide variety of plant secondary metabolites such as phytoalexins, rubber, terpenoids, and indole alkaloids. Terpenoids are derived from two well-known common precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP).

In the MVA pathway, the acetyl-CoA C-acetyltransferase (AACT) catalyzes the first step, the condensation of three molecules of acetyl-CoA molecules to form acetoacetyl-CoA. 3-Hydroxy-3-methyl-CoA synthase (HMGS) catalyzed the condensation of acetyl-CoA with acetoacetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and CoA. The 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is used as intermediate for biosynthesis of various terpenes, viz., mono-, di-, tri-, and sesquiterpenes including artemisinin [21–23]. HMG-CoA is then reduced by the enzyme HMG-CoA reductase (HMGR) to yield mevalonic acid (MVA). HMGR is a rate-limiting enzyme that can be inhibited specifically by lovastatin and catalyzes the NADP-dependent synthesis of mevalonate from HMG-CoA, which is the most important committed step of the MVA biosynthetic pathway. The 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), under the catalysis of mevalonate kinase, is converted to mevalonate 5-diphosphate, which is subsequently decarboxylated to yield IPP [24].

Besides the mevalonate pathway, a non-mevalonate pathway (MEP/DXP) operates in plastids. It also provides carbon for the synthesis of artemisinin. The DXP pathway was firstly discovered in plants in the year 1994 by Dr. Schwarz during his study on the biosynthesis of the ginkgolides in *Ginkgo biloba* [25]. Due to rapid development in bioinformatics and comparative genomics, that are revolutionizing the study of plant metabolism, the genes involved in the DXP pathway have been cloned and identified in different organisms. 1-Deoxy-D-xylulose-5-phosphate synthase (DXPS) catalyzes the thiamine pyrophosphate-dependent acyloin condensation between carbon atoms 2 and 3 of pyruvate and glyceraldehyde 3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate. In the plants, DXPS is the first enzyme and a rate-limiting enzyme of the DXP pathway a potential target for metabolic engineering involved in the biosynthesis of isoprenoids plastids. It is 1-Deoxy-5-phosphate reductoisomerase (DXR) catalyzes the rearrangement and subsequent reduction of DXP to form 2-C-methyl-D-erythritol 4-phosphate (MEP), it is also the rate-limiting enzyme in this pathway [23]. 2-C-methyl-D-erythritol 4-phosphate cytidylytransferase (MCT) catalyzes the third step of the DXP pathway to form CDP-ME by conjugating CDP and MEP. 4-(Cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (CMK) catalyzes the phosphorylation of CDP-ME to form CDP-MEP. 2-C-Methylerythritol 2, 4-cyclodiphosphate synthase (MECPS) catalyzes the conversion of CDP-MEP to 2-C methylerythritol 2, 4-cyclodiphosphate (ME-cPP). The final two steps on the DXP pathway include the formation of hydroxymethylbutenyl 4-diphosphate (HMBPP) from ME-cPP catalyzed by hydroxymethylbutenyl 4-diphosphate synthase (HDS) and the direct conversion of HMBPP in to a 5:1 mixture of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by IPP/DMAPP synthase (IDS).

Upon the synthesis of IPP (C_5) and its isomer DMAPP (C_5) by either MVA or DXP pathways, the next step is chain elongation. The carbonium ion is a potent alkylating agent that can react with IPP (three molecules of IPP converted into one molecule of geranyl diphosphate (GPP) by condensation reaction catalyzed by enzyme geranyl diphosphate synthase), to yield geranyl diphosphate (GPP). Farnesyl pyrophosphate synthase (FPS) is the prenyltransferase that catalyzes 1′-4 condensation reactions of IPP with the allylic diphosphates, dimethylallyl diphosphate (DMAPP), to produce farnesyl pyrophosphate (FPP).

After FPP synthesis, the biochemical reactions and enzymes involved have not been fully understood and well characterized. Akhila et al. [21] proposed a complete biosynthetic pathway for artemisinin, starting from mevalonic acid and IPP. The pathway branches at FPP. FPP is converted in to squalene by the enzyme squalene synthase (SQS) and subsequently into sterol. SQS is the key enzyme catalyzing the first step of the sterol biosynthetic pathway, a pathway in competition with that of artemisinin biosynthesis [26].

The first committed step in artemisinin biosynthesis in *A. annua* L. is the conversion or cyclization of FPP to amorpha-4, 11-diene by the enzyme amorpha-4, 11-diene synthase (ADS) [27–30]. Amorpha-4, 11-diene is the direct precursor of artemisinin biosynthesis. In the following step, amorpha-4, 11-diene is hydroxylated to yield artemisinic alcohol. This reaction is catalyzed by a multifunctional cytochrome P450-dependent hydroxylase (*CYP71AV1*) capable of multiple oxidation of amorpha-4, 11-diene. This is followed by oxidation at C-11 of amorpha-4, 11-diene by the cytochrome P450-dependent hydroxylase (*CYP71AV1*) to give artemisinic alcohol. This enzyme can also oxidize the artemisinic alcohol to artemisinic aldehyde and then further on to artemisinic acid [31, 32]. It has long been assumed that artemisinic acid is a direct precursor of artemisinin. The pathway also branches at artemisinic aldehyde to give artemisinic acid by the action of cytochrome P450-dependent hydroxylase (*CYP71AV1*) [31, 32] or to give dihydroartemisinic aldehyde by the action of artemisinic aldehyde Δ11 reductase (Dbr2) [33] in which C11–C12 double bond of artemisinic aldehyde is reduced and subsequently oxidized into dihydroartemisinic acid catalyzed by aldehyde dehydrogenase 1 (Aldh1) [34]. The dihydroartemisinic aldehyde is reduced into dihydroartemisinic alcohol. This reaction is catalyzed by the enzyme dihydroartemisinic aldehyde reductase (*Red1*), but this enzyme does not convert artemisinic aldehyde into dihydroartemisinic alcohol despite its close similarity with grater chemical reactivity than dihydroartemisinic aldehyde [35]. Dihydroartemisinic alcohol appears to be a “dead-end product” that may be affecting the yield of artemisinin in a negative way (Fig. 99.1).

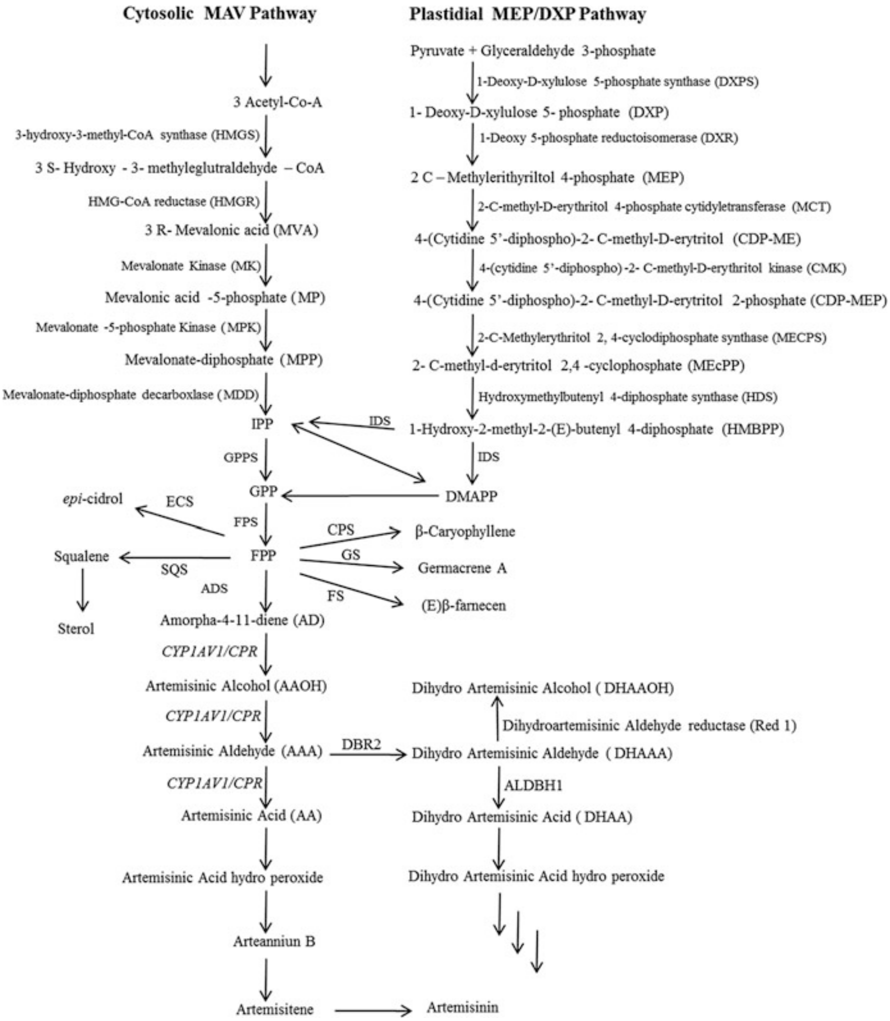
Cytosolic MAV Pathway
Plastidial MEP/DXP Pathway
3 Acetyl-Co-A
3-hydroxy-3-methyl-CoA synthase (HMGS)
3 S- Hydroxy - 3- methyleglutraldehyde – CoA
HMG-CoA reductase (HMGR)
3 R- Mevalonic acid (MVA)
Mevalonate Kinase (MK)
Mevalonic acid -5-phosphate (MP)
Mevalonate -5-phosphate Kinase (MPK)
Mevalonate-diphosphate (MPP)
Mevalonate-diphosphate decarboxlase (MDD)
IPP
GPPS
GPP
FPS
FPP
Pyruvate + Glyceraldehyde 3-phosphate
1-Deoxy-D-xylulose 5-phosphate synthase (DXPS)
1- Deoxy-D-xylulose 5- phosphate (DXP)
1-Deoxy 5-phosphate reductoisomerase (DXR)
2 C – Methylerithyriltol 4-phosphate (MEP)
2-C-methyl-D-erythritol 4-phosphate cytidyletransferase (MCT)
4-(Cytidine 5'-diphospho)-2- C-methyl-D-erytritol (CDP-ME)
4-(cytidine 5'-diphospho)-2- C-methyl-D-erythritol kinase (CMK)
4-(Cytidine 5'-diphospho)-2- C-methyl-D-erytritol 2-phosphate (CDP-MEP)
2-C-Methylerythritol 2, 4-cyclodiphosphate synthase (MECPS)
2- C-methyl-d-erytritol 2,4 -cyclophosphate (MEcPP)
Hydroxymethylbutenyl 4-diphosphate synthase (HDS)
1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP)
IDS
IDS
DMAPP
epi-cidrol
ECS
Squalene
SQS
Sterol
ADS
CPS
β-Caryophyllene
GS
Germacrene A
FS
(E)β-farnecen
Amorpha-4-11-diene (AD)
CYP1AV1/CPR
Artemisinic Alcohol (AAOH)
CYP1AV1/CPR
Artemisinic Aldehyde (AAA)
DBR2
CYP1AV1/CPR
Artemisinic Acid (AA)
Artemisinic Acid hydro peroxide
Arteanniun B
Artemisitene
Artemisinin
Dihydro Artemisinic Alcohol (DHAAOH)
Dihydroartemisinic Aldehyde reductase (Red 1)
Dihydro Artemisinic Aldehyde (DHAAA)
ALDBH1
Dihydro Artemisinic Acid (DHAA)
Dihydro Artemisinic Acid hydro peroxide

Fig. 99.1 Proposed biosynthetic pathway of artemisinin in *Artemisia annua* L. plant. *SQS* Squalene synthase, *ADS* amorpha-4, 11-diene synthase, *FPP* Farnesyl diphosphate, *FPS* Farnesyl pyrophosphate synthase, *GPP* Geranyl diphosphate, *GPPS* Geranyle diphosphate synthase, *GS* germacrene A synthase, *DMAPP* Dimethylallyl diphosphate, *IPP* isopentenyl diphosphate, *CPS* β-caryophyllene synthase, *DBR2* double bond reductase 2, *ECS* epi-cedrol synthase, *FPP* fanesyl diphosphate, *FS* (E)-β-farnesene synthase (Drawn based on information published by Abdin et al., [18] and Zhang et al., 2005)

The conversion of dihydroartemisinic acid to artemisinin is believed to be a nonenzymatic spontaneous photooxidation or autoxidation reaction. The mechanism of this complex transformation is shown to involve four steps: first, initial reaction of the C11–C12 double bond of dihydroartemisinic acid with single molecular oxygen; second is the Hock cleavage of the resulting tertiary allylic hydroperoxide; third is the oxygenation of the enol product from Hock cleavage; and fourth is the cyclization of the resulting vicinal hydroperoxyl-aldehyde to the 1, 2, 4-trioxane system of artemisinin. The other pathway includes artemisinic acid converted to arteannuin B. Based on labeling studies, artemisinic acid and its derivatives, such as artemisinic acid hydroperoxide, arteannuin B, and artemisitene, have been suggested as precursors to artemisinin. Clearly, a more detailed knowledge of the biochemistry of C11 double-bond reduction during artemisinin formation is important in differentiating the proposed routes, which can be considered "early" and "late" reduction pathways [36].

3 Strategies to Enhance Artemisinin Production

There are a number of limitations to obtain plant-derived compounds. They may be restricted to one species or genus and might be formed only during a particular stage of growth or development or under specific seasonal, stress, or nutrient availability conditions [37, 38]. Chemists are trying to synthesize plant-derived compounds via organic chemistry. This is often hampered by the chemical complexity, the specific stereochemistry, and the economic feasibility. Metabolic engineering may offer prospects to overcome the lack of availability of such compounds, through the advancement of the molecular biology techniques, including cloning, recombinant DNA, and knowledge of the plant biosynthetic pathways.

3.1 Biotechnological Approaches to Enhance Artemisinin Production in *Artemisia annua* L. Plant

To date, cultivated *A. annua* L. plants are the only natural source of this antimalarial drug worldwide. However, the low yield of artemisinin in *A. annua* L. (0.30–1.00 % on dry weight) is a serious limitation to the commercialization in the form of ACTs. Chemical synthesis is economically not feasible and also not affordable to developing countries for treatment of malaria. In order to enhance artemisinin content in *A. annua* L., various efforts have been attempted globally. In last two decades, much progress has been made to elucidate the biosynthetic pathway of artemisinin in *A. annua* L. and cloning as well as characterization of the rate-limiting genes (enzymes) and a number of other genes that are involved in the biosynthesis of artemisinin in *A. annua* L. plants. This information offers the genetic manipulation of *A. annua* L. to increase the artemisinin content by overexpression of endogenous and heterologous rate-limiting genes and suppression of genes which encode the first enzymes of the competing branched pathways.

3.1.1 Metabolic Engineering

Metabolic engineering in plants involves the modification of biosynthetic pathways to increase the carbon flux toward, particular desired metabolites. It aims at three goals: overproduction of the desired compound, less production of the unwanted compounds, and the production of a novel compound (one which is produced in nature, but not in the host plant) [39]. Strategies, that are involved to increase the level of valuable target molecules, could be either increasing flux to the target molecule, overcoming rate limiting steps, reducing flux through competing pathways, overexpressing regulatory gene(S), or transcription factors to induce the pathways, inhibiting or limiting catabolism of the desired molecule. However, it needs to understand the regulation of the metabolic pathways involved at the levels of product formation. Specific enzymes and genes involved with other aspects such as intracellular transport and compartmentation of the desired molecule should also be taken into account to enhance its biosynthesis. Considerable work on engineering single step in the metabolic pathway has already been performed in *A. annua* L. plants. In our laboratory, we have however, engineered two steps that are very crucial in artemisinin biosynthesis [42, 43, 44].

3.1.2 Upregulating of Pathways (Overexpression of Endogenous and Heterologous Genes)

Overexpression of single or multiple genes through genetic engineering approach may lead to the increase in some rate-limiting enzymes involved in metabolism and consequently results in the accumulation of the target products. The cytokinin gene, isopentenyl transferase (IPT), catalyzes the condensation of isopentenyl diphosphate (IPP) with adenosine monophosphate to give isopentenyl AMP (iAMP) [40]. Sa et al. transferred the ipt gene isolated from A. *tumefaciens* into *A. annua*. In the transgenic plants obtained, the cytokinin (iPA and iP) content was elevated two- to threefold, the chlorophyll content increased by 20–60 %, and artemisinin content increased by 30–70 % compared to the control plants [41]. The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the conversion of HMG-CoA to mevalonate, the first committed step in isoprenoid biosynthesis pathway in plants. In our laboratory, HMG-CoA reductase (*hmgr*) gene isolated from *Catharanthus roseus* L. was overexpressed in *A. annua* L. by using *Agrobacterium*-mediated gene transfer technology. Artemisinin content in one of the *A. annua* L. transgenic lines obtained was 38.9 % higher than that in non-transgenic plants, and HMGR enzyme activity in transgenic *A. annua* L. was also higher than that in the non-transgenic lines [42, 43] (Fig. 99.1). Recently, in our lab, HMGR gene from *C. roseus* L. along with amorpha-4, 11-dienesynthase (*ads*) from A. *annua* L. was also overexpressed in *A. annua* L. plants. Significant enhancement of artemisinin content (7.65-fold) was observed in one of the transgenic lines than non-transgenic plants [44]. Chen et al. previously reported that FPS (farnesyl diphosphate synthase) is a key regulatory enzyme in sesquiterpene biosynthesis [45]. *fps* gene was first cloned from *A. annua* by Matsushita et al. in the year 1996 [46] and 34.4 % artemisinin content and two- to threefold FPS activity were noticed in the transgenic lines than the transgenic plants developed by overexpressing the endogenous fps gene [2, 47]. Banyai et al. have overexpressed the endogenous *fps* gene in *A. annua* L. and studied its effect on biosynthesis of artemisinin content in transgenic A. *annua* L. plants. They showed 2.5–3.6-fold higher artemisinin content in transgenic lines than that of wild-type plants [48, 49]. Recently, 1.8-fold enhancement of artemisinin content was also reported in transgenic plants by co-overexpressing of two key regulatory genes (*hmgr* and *fps*) isolated from *A. annua* L. plants [49]. Further, overexpression of two key genes together, cytochrome P450 monooxygenase (*CYP71AV1*) and cytochrome P450 reductase (*CPR*), of artemisinin biosynthetic pathway had resulted in 2.4-fold higher artemisinin content than that in the control plant [50]. WRKY transcription factor study illustrates that WRKY proteins often act as repressors as well as activators and that members of the family play key roles in both the repression and repression of important plant processes. Majority of the artemisinin biosynthetic genes expressions were activated by transient expression of *AaWRKY1* cDNA. These results strongly suggest that *AaWRKY1* transcription factor participates in the regulation of artemisinin biosynthesis [51]. Besides engineering of isoprenoid synthetic pathway and artemisinin biosynthetic pathway, the overexpression of cyrptochrome 1 (CRY1) gene isolated from *Arabidopsis thaliana* was also led to achieve high artemisinin content (30–40 % higher) in transgenic *A. annua* L. plants when compared with the non-transgenic plants [52] (Fig. 99.1).

3.1.3 Downregulating the competing Pathways (Suppression of Squalene and Caryophyllene Synthase Genes)

In isoprenoid biosynthesis, FPP plays a central role as an intermediate precursor for synthesis of various sesquiterpene including β-caryophyllene [53], germacrene A [54], E-β-farmescene, *epi*-cedrol [55], squalene (first committed precursor of sterol biosynthesis) [26], and amorpha-4,11-diene (first committed precursor of artemisinin biosynthesis) [27–30]. All genes (full length cDNAs) responsible for encoding the enzymes associated with the synthesis of above sesquiterpene have been cloned and sequenced. Among the six above-mentioned sesquiterpene synthases, except *ADS*, all the other five sesquiterpene synthases compete for FPP with artemisinin biosynthesis. So, if the expression of gene encoding these sesquiterpene synthase(s) could be blocked by antisense technology or RNA interference, then the FPP contribution to artemisinin biosynthesis could possibly be increased and the biosynthesis of artemisinin could be enhanced. The antisense fragment of β-caryophyllene synthase cDNA (*asCPS*) was introduced into *A. annua* L. plants by an *Agrobacterium*-mediated transformation approach to assess the effects of inhibiting the competitive pathway on artemisinin biosynthesis by Chen et al. The artemisinin content of one of the transgenic lines was increased up to 54.9 % compared with the wild-type plants [56]. Similarly, sterol biosynthesis was also downregulated by suppressing the expression of squalene synthase gene (sqs) (Fig. 99.1) using hpRNA-mediated RNAi technology through *Agrobacterium*-mediated transformation method in *A. annua* L. plants. It resulted into increased artemisinin content (3.4-fold) rather than sterol production [57].

In one of the recent studies, five plant- and yeast-derived genes, involved in the mevalonate and artemisinin biosynthetic pathways, were cloned into a single mega-expression vector and introduced into *Nicotiana tabacum* resulting into the biosynthesis of artemisinin. Though the artemisinin levels in the transgenic tobacco plants were lower than that in *A. annua* L. plants naturally, the experimental platform developed may, in future, lead to the biosynthesis of various natural products in other heterologous plant systems [58].

3.1.4 Synthetic Biology

Due to the urgent demand and short supply of this antimalarial compound and till date, *A. annua* L. plants still the only source of artemisinin and its supply for the commercial production of ACTs [18] is a serious limitation to meet the global demand. Remarkable efforts have been made in the past towards structure modification of artemisinin and its analogue production. But, the chemical synthesis of artemisinin is not economically feasible due to the complexity and low yield of the process [59]. The semisynthesis of artemisinin following microbial production of a precursor molecule may however, be feasible [60]. So, microbial transformation serves as a valuable tool in the modification. Elucidation of biosynthetic pathway, including successful cloning, characterization, and expression of sesquiterpene synthase genes, provides an opportunity to manipulate the pathway in microbes other than plants. The reconstruction of the complete biosynthetic pathway towards artemisinin biosynthesis in transgenic yeast and bacteria has, however, not been achieved. The synthetic biology, therefore may be an alternative way to develop more potent antimalarial agents by production of artemisinin precursor with improved in vivo stability. Progress in production of artemisinin through metabolic engineering of genetically modified microbes is now being explored. The engineered microbial systems producing artemisinin or its precursor, which chemically can be converted to artemisinin, should facilitate the production of this by providing a cheap and environmentally benign alternative to extraction from the plant. For the production of appropriate precursors for artemisinin biosynthesis, the mevalonate pathway producing IPP along with synthetic amorpha-4, 11-diene synthase gene has been mobilized from yeast to *E. coli*, to produce amorphadiene [61] (Fig. 99.1). In recent years, efforts were also done to improve the production of amorphadiene through heterologous overexpression of mevalonate and, artemisinin biosynthetic genes from *A. annua* L. plants in yeast and yeast-conform variant to yield >40 g/L product. A chemical process was developed to convert amorpha-4, 11-diene to dihydroartemisinic acid, which could subsequently be converted to artemisinin [62, 63]. Similarly, the metabolic engineering of *S. cerevisiae* using engineered mevalonate pathway, and heterologous overexpression of amorphadiene synthase, as well as a novel cytochrome P450 monooxygenase (*CYP71AV1*) from *A. annua* that performs a three-step oxidation of amorpha-4, 11-diene, to produce high titers (up to 100 mg/l) of artemisinic acid is the next step hoping to semisynthesis of artemisinin [32, 64]. Artemisinic acid can also be reduced to dihydroartemisinic acid, which is auto-oxidized to artemisinin [65]. Artemisinic acid available from these transgenic microbes facilitates the subsequent partial synthesis of artemisinin by either chemical or biotransformational process, thereby providing an attractive strategy alternative to the direct extraction of artemisinin from *A. annua* L. The engineered microbes, capable of producing artemisinic acid at a significantly higher specific productivity than *A. annua* L. plants and, yield optimization as well as industrial scale-up, hence, will be required to raise artemisinic acid production to a level high enough to reduce the cost of artemisinin based combination therapies (ACTs).

3.2 Plant Tissue Culture

In principle, each plant cell, tissue, organ, or any part of the plant have the ability to divide and differentiate in to whole plants or organism in vitro under aseptic condition through a method known as micropropagation. This excellent whole process is known as "plant tissue culture." Plant cell culturing was initiated in the 1930s [66] and would offer alternatives to improve the production of the secondary metabolites, since natural harvest is sometimes bulky and not feasible from an economic point of view. The whole plants, plant organs, and even single cells can be used for the production of artemisinin and other secondary metabolites or pharmaceuticals. Various physiochemical factors such as quality of light [67, 68], temperature [68], carbon sources, nature of the nitrogen source and their relative amounts [69], water and salt stress [70] phosphate concentration [71], pH, ions concentrations, composition of various phytohormones of medium, and type of the explants influence the regeneration efficiency of plants, plant growth, as well as secondary metabolites including artemisinin [72, 73]. The main advantages of plant cell culturing are easy up-scaling, simple purification schemes due to product secretion, environmental friendliness, and amenability to strict control with regards to the FDA manufacturing standards [74]. Plant cell cultures are also not subjected to the changes in environmental conditions, thus the production of the desired compounds could take place at any location and season [75]. In order to enhance the artemisinin production in in vitro culture of *A. annua* L., the chemical composition of media was altered through addition of gibberellic acid or casein hydrolystate; by omitting plant growth regulators; by precursor feeding (mevalonic acid); by influencing the biosynthesis routing through inhibition of the sterol synthesis using miconazole, naftifine, or terbinafine; by altering the gene expression with 5-azacytidine or colchicine; and by elicitation, using cellulase, chitosan, glutathione, or nigeran. Casein hydrolysate, a source of amino acids and oligopeptides, at low concentration enhances artemisinin production in *A. annua* shoot cultures [76]. A combination of BA and kinetin was found to increase the yields of artemisinin in cultured shoots by 3.6- and 2.6-fold [77]. GA3, a plant hormone that can induce blooming, has been reported to improve growth and artemisinin biosynthesis in shoot cultures, root cultures, and plantlets of *A. annua* [78–81]. Recently, Banyai et al. [82] showed significant enhancement in artemisinin content at flower initiation stage in A. *annua* L. plants after exogenous GA_3-treatment plants in pot culture experiments as compared to the non-treated plants. Abscisic acid (ABA), defined as a stress hormone, plays a central role in responses to biotic and abiotic stresses [83]. It was also reported that ABA could stimulate the accumulation of some secondary metabolites [84]. Various concentrations of ABA evaluated to enhance artemisinin tested by treating *A. annua* L. plants. Significant enhancement of artemisinin content (65 % higher than that of control plant at 10-mM ABA) was observed in ABA-treated *A. annua* L. plants as compared to the non-treated plants [85]. Effects of various ratios and combinations of sugars on artemisinin biosynthesis were investigated in *A. annua* L. plants.

200 % enhancements of artemisinin content were observed in glucose-fed seedlings, while 80 % reduction were noticed in the seedling fed with 27 g/l sucrose + 3 g/l Palatinose [86, 87]. Most groups did not find artemisinin in root part of *A. annua* plant. However, artemisinin content in the shoot part of cultured plantlets was higher than that in the cultured shoots without roots [88, 89]. Enhancing the artemisinin production by precursor feeding was also investigated. Addition of artemisinin precursors to the tissue culture media of *A. annua* L. had resulted in a fourfold increase of artemisinin in the tissue and an 11-fold increase of artemisinin in the spent medium [90]. The feeding of mevalonic acid alone, however, did not induce in enhancement of artemisinin production [76]. But the addition of some compounds such as naphtiphine (an inhibitor of the enzyme squalene epoxidase) to the medium improved the artemisinin production. Other additions, such as 5-azacytidine (a gene regulator), colchicine (a gene regulator), miconazole (an inhibitor of sterol demethylase), and terbinaphine (an inhibitor of the enzyme squalene epoxidase), were too toxic for the cultures to induce an enhancement in the artemisinin production [76]. Kudakasseril et al., however, reported a concentration-dependent increase in the levels of artemisinin and growth of shoot cultures with miconazole [22]. Other sterol inhibitors, such as chlorocholine chloride, 2-isopropyl-4-(trimethylammonium chloride)-5-methylphenylpiperidine carboxylate, and 4- chloro-2-(2–diethylaminoethoxyphenyl)-2-(4-methylphenyl) benzene ethanol, increased both the incorporation of $_{14}$C-IPP into artemisinin by cell-free extracts and the production of artemisinin in shoot cultures of *A. annua*. Effect of triacontanol and chlormequat on growth, plant hormones, and artemisinin yield in *A. annua* L. plants was studied by Shuka et al. [91]. They found that statistically significant positive effect on artemisinin level as well as on plant height, leaf, and herbage was observed by applying triacontanol at 1.0 and 1.5 mg/l^{-1} on *A. annua* L. plants over control plants. Similarly, artemisinin level was also increased but the plant height was decreased at higher concentration of chlormequat (1,000 and 1,500 mg/l^{-1}) over non-treated plants.

3.2.1 Hairy Root and Cell Suspension Cultures

The word “hairy root” (HR) was initially given by Stewards et al. as far back as 1900 [92]. The unique symptom of HR disease is the formation of a hairlike mass of roots. A large number of small unlimited proliferative multibranched adventitious roots overhang as fine hairs directly from the infection site in response to *Agrobacterium rhizogenes* infection, a phenomenon that gave rise to the term “hairy root” [93]. The distinctiveness of the HR-causing organism remained uncertain for a long time. Riker et al. [94] later described and named the hairy-root-causing microorganism as *Phytomonas rhizogenes*, which was later renamed *Agrobacterium rhizogenes* [94]. *A. rhizogenes* is a Gram-negative soil pathogenic bacterium. They classified the genus *Agrobacterium* into two clusters: cluster 2 includes *A. rhizogenes* strains together with some *A. tumefaciens* strains. This conclusion has been accepted and given recognition by other authors [95]. Ackermann first prepared the hairy roots by transformation of higher plants with response to *A. rhizogenes* in the year 1973 [96]. Porter reported that more than 450 species of many different genera and families are known to be susceptible to the infection by *A. rhizogenes* [97] since then many more additions have been made to the list. The use of *A. rhizogenes* has been receiving attention in the past four decades in secondary metabolism research.

The employment of hairy root culture technology offers new opportunities for in vitro production of plant secondary metabolites. For the past two decades, most of the research efforts that use differentiated cultures instead of cell suspension cultures have focused on transformed (hairy) roots. The fast growth, genetic, and biochemical stability; low doubling time; ease of maintenance; and ability to synthesize a range of chemical compounds offer an additional advantage over other undifferentiated plant cell cultures. Hairy roots can synthesize more than a single metabolite and so prove economical for commercial production purpose [98]. It is true that some biosynthetic pathways are not expressed in roots, but in green plant parts such as leaves and shoots. However, hairy root cultures have been shown to accumulate those metabolites also that normally accumulate only in the aerial parts of an intact plant. Artemisinin was thought to accumulate only in the aerial part of the *A. annua* L. plants [99], but several reports have shown that hairy roots can also produce artemisinin [90, 100–102]. *A. annua* L. cell and tissue cultures have been explored for the production of artemisinin, although the yields obtained are not commercially attractive [103, 104]. Hairy root culture exhibited fast growth and high lateral branching on growth regulator-free MS medium. A comparative study in terms of yields from cell suspension culture and hairy root culture had been investigated by Sauerwein et al. [105].

Attempts were made to improve the artemisinin production by optimizing chemical and physical environmental factors. Wang and Tan [106] reported the influence of the ratio of NO_3/NH_4 and total initial nitrogen concentration on the artemisinin yield in hairy roots. With the ratio of NO_3/NH_4 at 5:1(w/w), the optimum concentration of total nitrogen for artemisinin production was 20 mM. Under this concentration, artemisinin production was 57 % higher than that in the standard MS medium [106]. Weathers' research group investigated the effects of media sterilization method and types of sugar on growth and artemisinin accumulation of *A. annua* hairy roots. They found that biomass from filter-sterilized medium was greater than that from autoclaved medium, but artemisinin accumulation from filter-sterilized medium was less than that from autoclaved medium. Growth of hairy roots in the medium with sucrose (3.99 g DW/l) was equivalent to the growth in the medium with fructose (3.75 g DW/l) and significantly better than in the medium with glucose (2.16 g DW/l), while the roots that grew in glucose showed a dramatic stimulation in artemisinin content, which is three- and twofold higher than that in medium with sucrose and fructose [107]. The effects of light irradiation on growth and production of artemisinin were studied in hairy root cultures of *A. annua* L. by Liu et al. [108]. They found that when the hairy roots were cultured under illumination of 3,000 lx for 16 h using several cool-white fluorescent lamps, the dry weight and artemisinin concentration reached 13.8 g/l and 244.5 mg/l, respectively [108]. Wang et al. [67]

investigated the dependence of biomass of hairy roots and artemisinin content on the light spectrum. They found that the highest biomass (5.73 g DW/l) and artemisinin content (31 mg/g) were obtained under red light at 660 nm which were 17 and 67 % higher than those obtained under white light, respectively [67]. Temperature in the range of 15–35 °C also affected growth and artemisinin biosynthesis in the cultured *A. annua* hairy roots. The maximum hairy root growth was found at 25 °C. However, the highest artemisinin content in the root cultures was observed at 30 °C [68]. Wang et al. studies the synergistic rolls of cerebrosides and nitric oxide on stimulation of artemisinin synthesis by applying cerebrosides and nitric oxide on *A. annua* L. hairy root culture. 2.3-fold increase of artemisinin was achieved at 20-day-old treated hairy roots as compared to the control [109]. Recently, Caretto et al. have established *A. annua* cell cultures that are able to produce artemisinin and to respond to the elicitor effect of methyl jasmonate (MeJA) [110]. Some artemisinin produced by these cultures was also observed in the culture medium. In order to improve the artemisinin biosynthesis in *A. annua* cell suspension culture, they investigate the roll of cyclodextrins on artemisinin biosynthesis. Cyclodextrins are nonreducing cyclic oligomers of 1, 4-α-D-linked glucose units, derived from starch by the action of microbial enzyme cyclodextrin glycosyltransferase. The most common CDs are α-, β-, and γ-CDs, which are formed by six, seven, and eight glucose units, respectively. Among the natural CDs, β-CDs, in particular, that are widely used in plant cell cultures to improve the production of various secondary metabolites have been described [111, 112]. The capability of cyclodextrins to form host–guest inclusion complexes with artemisinin has already been reported in several studies using pure artemisinin and various β-CDs. A synergistic effect of β-CDs and MeJA on resveratrol biosynthesis has also been reported. Native β-cyclodextrins, as well as the chemically modified heptakis (2, 6-di-O-methyl)-β-cyclodextrin (DIMEB) and 2-hydroxypropyl-β-cyclodextrins, were added to the culture medium of *A. annua* suspension cultures, and their effects on artemisinin production were analyzed. The synergistic effects of a cyclodextrin and methyl jasmonate treatment were also investigated. 55-mM DIMEB, as well as a combination of 50-mM DIMEB and 100-μM methyl jasmonate, was highly effective in increasing the artemisinin levels in the culture medium. The observed artemisinin level (27 μmol g^{-1} dry weight) was about 300-fold higher than that observed in untreated suspensions [113].

Further attempts were made to enhance yield of artemisinin content through development of hairy roots of high yielding transgenic strains of *A. annua* L. plants by *Agrobacterium rhizogenes*-mediated transformation [114, 107]. Several key genes involved in the biosynthesis of artemisinin have been introduced and overexpressed in *A. annua*. These genes encode enzymes involved in the biosynthesis, artemisinin viz farnesyl diphosphate synthase (FPS) [46], amorpha-4, 11-diene synthase (ADS) [27–30], and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) [42–44, 47, 49, 115]. Hairy root cultures of *A. annua* L. that had also established genetic transformation by it via *A. rhizogenes* were transformed with fps from *G. aboreum*. The artemisinin content in the transgenic plants, which were regenerated from the hairy root cultures, was significantly higher than in the nontransgenic plant [116].

3.2.2 Environmental Regulation and Altered Physiology of Hairy Roots

Factors such as light, carbon source and its concentration, the ionic concentration of the medium, the pH of the medium, phytohormones, temperature and light quality [117], inoculum size, nature of the nitrogen source and their relative amounts [69], and phosphate concentration [71] are known to influence growth and secondary metabolism [72, 118]. Light, temperature, CO_2 and O_2 stress, as physical factors, play important role in controlling greening, growth, and secondary product formation in hairy root cultures.

3.2.3 Elicitation and Artemisinin Production

Plants and in vitro cultured plant cells show physiological and morphological responses to microbial, physical or chemical factors, which are known as "elicitors." They could also be defined as chemical substance which, when introduced in small concentration to a living cell system, initiate or improve the biosynthesis of specific compounds. Elicitation is induced or increased synthesis of secondary metabolites by the plants to ensure their survival, persistence, and competitiveness [119]. Elicitation at right stage of culture, concentration of elicitors, and appropriate combination of the medium and elicitor are also the regulating factors responsible for enhancement of secondary metabolites. Elicitor treatment at late log phase results in higher biomass yield as well as secondary metabolite production, whereas during early phase, if cultures are elicited, that leads to the immediate increase in secondary metabolite production only and lowering the biomass yield [120]. In order to choose the proper elicitor, it is also necessary to understand the signals involved in the process of elicitation. There is evidence that the use of elicitors has been an important strategy for improving the production of secondary metabolites in hairy roots. Elicitation is an interesting metabolic process, which has been used to investigate cell signaling pathways in secondary metabolite biosynthesis.

Oligogalacturonides (OGAs), the pectic fragments released from the plant cell walls, are the well-known oligosaccharides triggering a variety of defense responses in plants. OGA elicitor also exhibits highly specific activity of inducing secondary metabolite production. In early reports, pathogenic and endophyt fungal mycelia have been found to induce for artemisinin biosynthesis. OGA has also induced artemisinin biosynthesis in *A. annua* hairy root cultures. The different fractions of oligogalacturonides (OGA) from polygalacturonic acid hydrolysate had been partially purified using column chromatography. The isolated fraction, OGA2 was found to stimulate the accumulation of artemisinin in *A. annua* L. hairy roots. The isolated fraction, OGA2, was found to stimulate accumulation of artemisinin in *A. annua* L. hairy roots. The 16-day-old hairy root cultures, when exposed to the OGA elicitor (60 lg/mL) for 4 days, accumulated up to 11.3 mgL^{-1} artemisinin, which was 55.2 % higher than control plants. This increase in artemisinin production by hairy roots was due to OGA-induced reactive oxygen species (ROS) – in elicitation of artemisinin biosynthesis. Hydrogen peroxide and oxygen free radicals, which constitute ROS, could be the second messengers in accelerating the production of salicylic acid, jasmonate, and ethylene. These signaling molecules then switch on the expression of defense response genes leading to induced biosynthesis of artemisinin in hairy root cultures. Similar results were obtained by Putalun et al. [122], where they found that artemisinin production by hairy roots of *A. annua* L was increased to sixfold with the addition of 150 mgL^{-1} chitosan alone with MJ, 1.5-fold with the addition of chitosan and YE. On the other hand, Wang et al. studied the accumulation of artemisinin induced by fungal-derived cerebrosides. They observed that cerebrosides induced nitric oxide (NO) burst leading to enhanced artemisinin biosynthesis in the hairy roots [109, 122].

Jasmonic acid (JA), methyl jasmonate (MJ), and salicylic acid (SA) are naturally occurring phytohormones and known as "secondary messengers." They are capable of transducing normal developmental signals or adverse environmental stimuli to plant cells for initiating protective responses against oxidative stress through antioxidant defense enzymes and secondary metabolites [110, 125–135]. Baldi and Dixit have reported enhanced artemisinin production by the cell suspension culture of *A. annua* after the addition of MJ in the cell medium [138]. In previous studies, jasmonate and SA were reported to enhance artemisinin content in *A. annua* L. plants and in cell suspension culture [136, 137]. Further, the allene oxide synthase (AOS) was reported to catalyze the first committed step in jasmonate biosynthesis [138]. Very recently, a full-length cDNA of AOS gene named as AaAOS was cloned from *A. annua* L. and the magnitude of its expression was strongly correlated with the artemisinin content in *A. annua* L. plants treated with MeJ, ABA, and ethylene [139].

4 Conclusion

Malaria is a global health problem that affects more than three million peoples specially children annually. It is currently treated with artemisinin-based combination therapies (ACTs). Artemisinin is an endoperoxide sesquiterpene lactone, synthesized in aerial parts of *A. annua* L plants. Due to relatively low content of this compound (0.3–1.0 %) in *A. annua* L. [44] and the nonavailability of economically viable conventional synthetic processes, the ACTs become quite expensive and unaffordable, especially for economically disadvantaged people in developing countries, where malaria frequently occurs. In order to meet high demands and reducing the cost of ACTs, there is now an urgent need to improve the methods for artemisinin production. Various approaches are being explored worldwide to enhance the production of artemisinin both in vivo as well as in vitro. Metabolic engineering offers promising perspectives to improve artemisinin yields by either overexpressing genes encoding rate-limiting enzymes of isoprenoid and artemisinin biosynthesis pathways or suppressing the expression of genes encoding the rate-limiting enzymes of competing pathways. These include hmgr, ads, fps, CYP7AV, CRY1, red1, asCPS, and sqs [42–45, 47–52, 54, 56–58]. Recently, one yeast- and five plant-derived genes involved in MVA and artemisinin biosynthesis were overexpressed in tobacco to generate artemisinin [60] (Fig. 99.1). However, this novel work has not resulted in increased artemisinin accumulation in transgenic tobacco so as to meet the future demand. This approach had, however, opened a new window to genetically engineer the entire or partial secondary metabolic pathways in heterologous plants/organism for the production of valuable secondary metabolites including artemisinin. In addition to *A. annua* L., genetically modified microbes were also developed to produce intermediates of artemisinin biosynthesis [32] for semisynthesis of artemisinin. Similarly, the production of artemisinin in hairy roots and cell suspension cultures was demonstrated. These processes, however, need optimization and scaling up to produce artemisinin at commercial scale using bioreactors. Applied genomics, proteomics, and metabolomics are continuing to expand our knowledge of metabolic pathways, while advances in systems biology would help us to model the impact of different modifications more accurately. These, finally, will help in improving the yield of artemisinin and reducing the cost of ACTs [121, 123, 124].

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Nivalenol (Fungal Sesquiterpenes) 100

Yoshiko Sugita-Konishi and Makoto Kimura

Contents

1 Introduction 3124
2 General Biology 3125
2.1 Common/Systematic Name 3125
2.2 Molecular Formula/Molecular Weight 3126
2.3 General Characteristics 3126
2.4 Spectral Data 3126
3 Mycology 3126
4 Biosynthesis 3129
4.1 History of Trichothecene Biosynthesis Studies 3129
4.2 Formation of 12, 13-Epoxytrichothec-9-ene Skeleton (Trichothecene Skeleton): Early Stage of Biosynthesis 3129
4.3 Molecular and Genetic Studies of *F. sporotrichioides* as a Basis to Understand NIV Biosynthesis 3130
4.4 Biosynthesis of NIV 3133
5 Toxicology 3134
5.1 Absorption, Distribution, Metabolites, and Excretion 3134
5.2 Acute Toxicity 3136
5.3 Short-Term Studies of Toxicity 3136
5.4 Chronic Studies and Carcinogenesis 3139
5.5 Genotoxicity 3141
5.6 Immunotoxicity 3142
6 Other Toxicities 3144
7 Outbreaks Associated with NIV 3144
8 Analytical Methods 3144

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9 Exposure Assessment of NIV and Regulation 3145
9.1 Occurrence of NIV in Food and Feed 3147
9.2 NIV Intake 3148
9.3 International Evaluation 3149
9.4 Regulation 3150
10 Conclusion 3150
References 3150

Abstract

Nivalenol (NIV) belongs to the B-type trichothecene mycotoxins produced by *Fusarium* species. The occurrence of NIV contamination is limited to certain areas around the world, such as Japan, Korea, New Zealand, and a part of Europe, where it has had adverse effects on human and animal health. This chapter focuses on the mycology, occurrence, biosynthesis, toxicology, methods of analysis, and risk assessment of NIV.

Keywords

Analysis method • biosynthesis • exposure assessment • mycology • nivalenol • toxicity

Abbreviations

bw	Body weight
DON	Deoxynivalenol
ECEU	European Commission Union
EFSA	European Food Safety Authority
FHB	Head blight
FX	Fusarenon-X (4-acetyl NIV)
JECFA	Joint expert committee of food additives
$LD_{50:}5$	50% lethal dose
LOAEL	Low-observed-adverse-effect level
LPS	Lipopolysaccharide
NIV	Nivalenol
S9	S9 liver microsomal enzymes
SCF	Scientific Committee on Food
ZEN	Zearalenone

1 Introduction

Nivalenol (NIV) was discovered by Japanese scientists from the culture medium of *Fusarium nivale* strain Fn-2B obtained from *Fusarium* head blight-infected wheat, in Japan [1]. Subsequently, Fn-2B was reclassified as a new species, *F. kyushuense*

O'Donnell & T. Aoki [2] from molecular phylogenetic analyses. Other Japanese researchers identified the chemical structure of NIV, fusarenon-X (4-acetyl NIV), and deoxynivalenol (DON) [3–6].

Trichothecene mycotoxins are the main mycotoxins produced by *Fusarium* species. Although there are macrocyclic and non-macrocyclic mycotoxins, the latter primarily contaminate wheat, barley, and maize and are classified into two types: Type A, including T2 toxin and HT2 toxin, diacetoxyscirpenol, and neosolaniol and type B, including DON, NIV, and 4-acetyl NIV. T2 toxin is reportedly the causative agent of outbreaks of foodborne diseases that occurred in the Orenburg region of the USSR during the 1930s–1940s. This outbreak was termed alimentary toxic aleukia (ATA), and symptoms of this disease include nausea, emesis, diarrhea, leukopenia, hemorrhages, and shock-mediated death.

Although the outbreak caused by type B trichothecenes is less severe than ATA, acute human illnesses caused by the consumption of *Fusarium*-infected wheat and barley have been reported in Japan, India, and China [7–9]. In these outbreaks, DON, NIV, and zearalenone were commonly detected in food.

Concerning type B trichothecenes, chronic and acute adverse health effects are of considerable concern. DON and NIV have been reported to suppress the immune system, depending on the dose and frequency of exposure [10].

Compared to DON, the distribution of NIV-producing fungi is limited and lacks toxicological and exposure data. DON has been evaluated by the FAO/WHO Joint Expert Committee of Food Additives while NIV has not. In countries where NIV contaminates cereals, the adverse health effect induced by NIV is a serious problem, and NIV is considered to be one of the mycotoxins whose risk needs to be assessed and regulated. In Europe, a large-scale surveillance of trichothecenes in food from 2000 to 2002 revealed the occurrence of NIV contamination [11]. In Japan, as NIV-producing fungi also occur, the occurrence of DON and NIV were surveyed in food. Risk assessment has been completed by the Food Safety Commission (FSC) of Japan. The European Commission's (EC) (now the European Union, or EU) Scientific Committee on Food (SCF) evaluated and determined a provisional daily tolerable intake as 0.7 μg/kg of body weight (bw) [12], but the FSC of Japan concluded that this level should be 0.4 μg/kg of bw.

This chapter reviews the latest findings on the mycology, biosynthesis, occurrence, toxicology, analytical methods of analysis, and risk assessment of NIV.

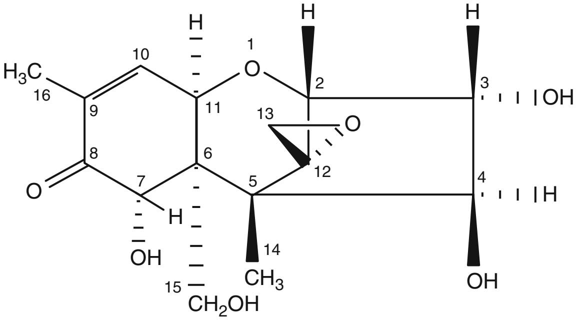
2 General Biology

2.1 Common/Systematic Name

CAS (No.23282-20-4)
3α, 4β, 7α, 15-tetrahydroxy-12, 13-epoxytrichothec-9-en-8-one, IUPAC
12, 13-epoxy-3α, 4β, 7α, 15-tetrahydroxytrichothec-9-en-8-one

2.2 Molecular Formula/Molecular Weight

$C_{15}H_{20}O_7$: 312. 32



Nivalenol

2.3 General Characteristics

Crystals from methanol; mp., 80–90 °C; dried in presence of P_2O_5 in reduced pressure; mp., 222–223 °C, tetraacetate; mp., 168–170 °C, $[\alpha]_D^{24}$+ 21.54° (c = 1.3, in EtOH)

2.4 Spectral Data

λ↓Max ↑MeOH 218 Nm (Ɛ = 7,500), Tetraacetate, 227 Nm (Ɛ = 7,900)

3 Mycology

Fusarium head blight (FHB) is a harmful disease that infects wheat, barley, and other cereals. This disease not only reduces grain yield and quality but also causes the contamination of trichothecene mycotoxins such as DON and NIV and other mycotoxins in the grain. The pathogens of FHB, *Fusarium* species, can be classified into two chemotaxonomic groups, the DON chemotype and the NIV chemotype [13–15]. Lee et al. [14] clarified that a single gene (*Tri13*) is responsible for the differential ability to produce DON or NIV. *Fusarium* DON chemotypes are found worldwide, while NIV chemotypes are found in more restricted regions, namely, limited areas of Asia, Africa, Europe, and North America [16, 17]. *F. kyushuense* and *F. graminearum* complex species (*F. asiaticum*, *F. culmorum*, *F. crookwellense*, *F. equiseti*, and *F. poae*) are also reported to produce NIV (Table 100.1).

The *F. graminearum* species complex [*F. graminearum* Schwabe; teleomorph: *Gibberella zeae* (Schwein.) Petch] is a species complex consisting of at least nine

Table 100.1 NIV-producing fungi

	Production of mycotoxins			
Fusarium species	DON	NIV	Commodity	Country/region
F. graminearum complex species	+	+	Wheat variety, rice, maize	Whole world
F. graminearum	+	-	Wheat variety, rice, maize	Temperate zone (cold zone of the northern hemisphere, especially): Japan (all), Korea, China
F. asiaticum	-	+	Wheat variety, rice	Temperate zone (especially warm temperature regions): Japan (Honshu and the south), Korea, China
F. vorosii	+	-	Wheat	Japan (Hokkaido), Hungary
F. culmorum	+	+	Wheat variety, maize	Temperate zone (especially cold regions): Europe, Asia, Africa, North and South America, Oceania
F. crookwellense	-	+	Wheat variety, maize	Temperate zone (especially cold region): Japan (Hokkaido)
F. equiseti	-	+	Wheat variety, maize	Subtropics, temperate zone
F. kyushuense	-	+	Wheat variety, rice	Japan (western Japan), China
F. poae	-	+	Wheat variety, maize	Temperate zone (especially cold regions): Japan (Hokkaido)
F. pseudograminearum	+	-	Wheat variety	Chiefly Australia

biogeographically structured lineages based on molecular phylogenetic analyses using worldwide collections. The lineages are as follows: lineage 1 is *F. austroamericanum*, lineage 2 is *F. meridionale*, lineage 3 is *F. boothii*, lineage 4 is *F. mesoamericanum*, lineage 5 is *F. acacia-mearnsii*, lineage 6 is *F. asiaticum*, lineage 7 is *F. graminearum* s. str., lineage 8 is *F. cortaderia*, while lineage 9 is *F. brasilicum*. On potato dextran agar (PDA), the *F. graminearum* species complex produces abundant white mycelia that become yellow to brownish or rose-colored as the cultures age (for 2 weeks); the color of the bottom surface of the colony is usually deep red. The undersurface is usually carmine red (Fig. 100.1). Macroconidia are usually long, slender, and slightly curved to straight, with five to six septa and a well-developed foot cell. Microconidia are absent; chlamydospores are rare but may form in macroconidia [18].

Trichothecene chemotypes of the *F. graminearum* species complex consist of three production groups according to strain differences: DON and 3-acetyl deoxynivalenol (3ADON), DON and 15-acetyl deoxynivalenol (15ADON), and NIV [13–15]. In the *F. graminearum* species complex, lineage classification is not well correlated with the trichothecene chemotype [19, 20].

Regional mycological studies discovered that geographic differences exist among these trichothecene chemotypes [21, 22]. The 3ADON productive group

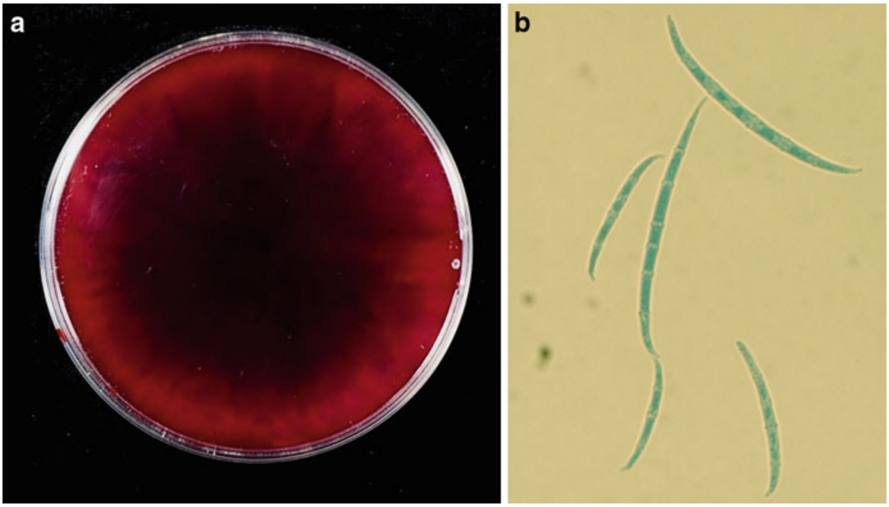


Fig. 100.1 *Fusarium graminearum.* (**a**): giant colony on PDA (*reverse side*) (**b**) Macroconidia

was not detected in 15 strains of *F. graminearum* s. str., and all 13 strains of *F. cortaderia* in New Zealand were of the NIV production group [23]. The NIV production group in *F. asiaticum* has been identified and is likely to represent about 25 % of the population of the *F. graminearum* species complex in Louisiana, USA [24]. In Japan, *F. graminearum* s. str. is predominant in the northernmost island (Hokkaido), while in southern areas, *F. asiaticum* is predominant [25].

In terms of pathogenicity, DON-producing strains are more aggressive and virulent than NIV producers [26, 27]. However, the NIV production group in *F. asiaticum* isolated from the western part of Japan was significantly more virulent than the most virulent DON chemotype *F. graminearum* s. str. strains [28].

Fusarium culmorum (W. G. Smith) Sacc. is the second most important FHB pathogen in wheat and strains of both DON and NIV producers exist. *F. culmorum* inhabits cooler areas such as North, Central, and Western Europe; North America; and Eastern Australia. Strains of both the DON and NIV production groups were isolated from England and Wales: in the south and west of England and Wales, NIV chemotypes are predominant, whereas in the north and east, DON chemotypes are predominant [29].

Fusarium poae (Peck) Wollenw. exists in Europe, North America, and Japan. It is one of the causative fungi causing FHB in small-grain cereals. In the northernmost area of Japan, the NIV production group in *F. poae* plays a role in the contamination of grains with NIV [30]. The NIV production group of *F. poae* was frequently found in Sweden, and NIV contamination has become a considerable concern in Scandinavia [31].

Fusarium crookwellense L.W. Burgess, P. E. Nelson & Toussoun was first isolated in Australia in 1971 as a new species [32]. It is another FHB pathogen found in small-grain cereals in Poland, New Zealand, China, Canada, Japan, and other countries [33]. *F. crookwellense* isolated from scabby wheat in the northernmost area of Japan produced NIV, 4-acety NIV, and zearalenone (ZEN) when cultured on rice-based medium [30].

4 Biosynthesis

4.1 History of Trichothecene Biosynthesis Studies

In earlier days, biosynthesis studies of trichothecenes were initiated using *Trichothecium roseum* as a model [34]. Having elucidated the scheme of biosynthesis from farnesyl pyrophosphate (FPP; **1**) (see Fig. 100.2), via a cyclized product trichodiene (TDN; **2**), to trichothecin [35, 36], researchers have moved to study *Fusarium* species that produce agriculturally important trichothecenes, such as T-2 toxin, DON, and NIV. Feeding experiments with TDN demonstrated its precursor role in DON biosynthesis [37], as was the case of *T. roseum*. Other postulated intermediates in the biosynthetic pathway were thereafter proven by blocked mutant analysis, precursor feeding experiments, and molecular genetic approaches using *Fusarium* species, including *F. culmorum*, *F. graminearum*, and/or *F. sporotrichioides* [38–40]. Compared to trichothecenes of other fungal genera, *Fusarium* trichothecenes are distinguished by the presence of a hydroxyl or *O*-acetyl at C-3.

4.2 Formation of 12, 13-Epoxytrichothec-9-ene Skeleton (Trichothecene Skeleton): Early Stage of Biosynthesis

In the biosynthesis of *Fusarium* trichothecenes, TDN is oxygenated by a cytochrome P450 monooxygenase (CYP) in the following order: 2α-hydroxylation, 12, 13-epoxidation, 11α-hydroxylation, and 3α-hydroxylation (Fig.100.2). The last oxygenation step proceeds only in *Fusarium* species, which makes *Fusarium* trichothecenes unique among all others (lacking a C-3 substituent) of non-*Fusarium* origin. Under acidic conditions, the resulting fully oxygenated intermediate, isotrichotriol (**3**), appears to undergo second cyclization nonenzymatically to give isotrichodermol (**4**) [41], the first trichothecene intermediate with a toxic 12,13-epoxytrichothec-9-ene skeleton (for a comprehensive review, see ref. [42]). Although isotrichodermol was not isolated as a natural product from wild-type *Fusarium* strains in the biosynthesis studies of many research groups, this may not be unreasonable in view of the importance of 3-*O*-acetylation in trichothecene biosynthesis; perhaps, isotrichodermol must readily be converted to isotrichodermin (ITD; **5**) (see Fig. 100.2) for self-protection of the trichothecene-producing fusaria [43]. ITD, a natural product isolated from a wild-type strain, is further metabolized to various important *Fusarium* trichothecenes [44].

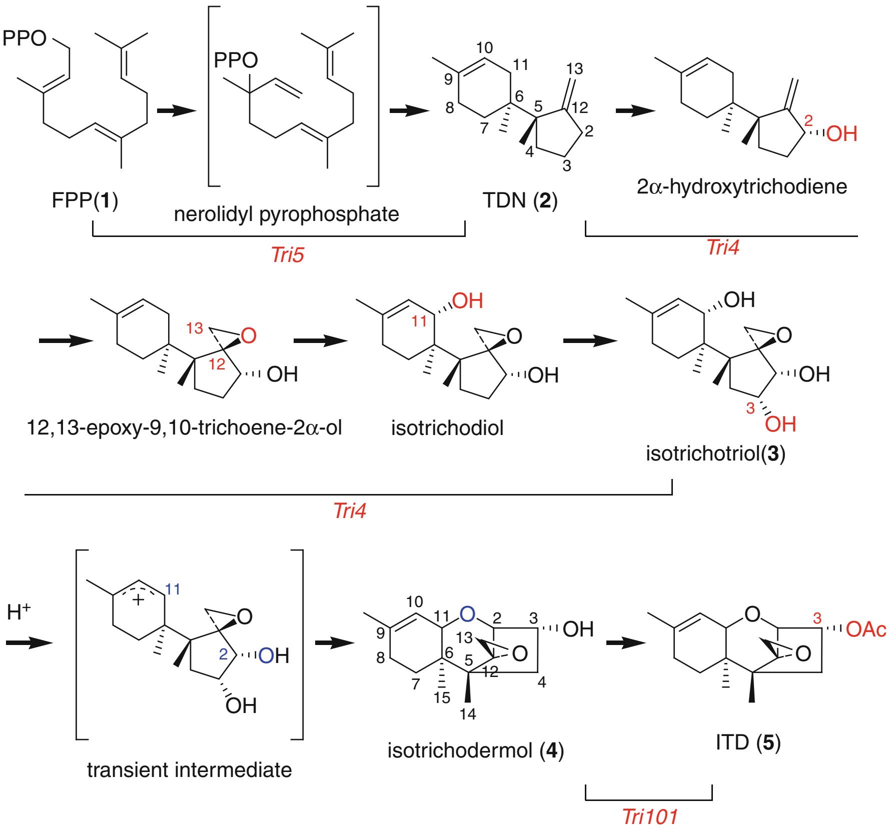

PPO
FPP(1)
PPO
nerolidyl pyrophosphate
Tri5
10
11
9
8
7
6
5
4
13
12
2
3
TDN (2)
2
OH
2α-hydroxytrichodiene
Tri4
13
O
12
OH
12,13-epoxy-9,10-trichoene-2α-ol
OH
11
O
OH
isotrichodiol
OH
O
OH
3
OH
isotrichotriol(3)
Tri4
H+
+
11
O
2
OH
OH
transient intermediate
10
9
8
7
11
O
2
3
OH
13
6
5
12
O
4
15
14
isotrichodermol (4)
O
3
OAc
O
ITD (5)
Tri101

Fig. 100.2 A common pathway to ITD in the biosynthesis of *Fusarium* trichothecenes. These early steps are conserved between type A (e.g., T-2 toxin) and type B (e.g., NIV) trichothecene producers. *Tri* genes that are responsible for these steps are indicated below the half-brackets in red letters. Functional groups highlighted in red indicate that they appeared as the results of expression of these *Tri* genes. Oxygen atom involved in intramolecular attack to C-11 in the nonenzymatic cyclization is shown in *blue*

4.3 Molecular and Genetic Studies of *F. sporotrichioides* as a Basis to Understand NIV Biosynthesis

In 1989, the first isolation and characterization of trichothecene biosynthetic gene was reported for T-2 toxin-producing *F. sporotrichioides* using an antibody raised against a purified enzyme [45]; this gene, designated *Tri5* (formerly *Tox5* in the literature), is responsible for the first committed step in the biosynthesis. Since cosmid clones containing *Tri5* complemented the *tri3*$^{-}$ and *tri4*$^{-}$ blocked mutants, but not the *tri1*$^{-}$ mutant, at least some of the trichothecene biosynthetic genes (*Tri* genes) proved to be clustered around *Tri5* [46]. On the basis of this finding, other *Tri* genes, including *Tri3* and *Tri4*, were identified on this core gene cluster. Three additional *Tri* genes necessary for T-2 toxin biosynthesis, *Tri101* alone and

Tri1 and *Tri16* adjacent to each other, occur separated from the core gene cluster (for reviews, see ref. [42, 47]). Roles of the *Tri* genes in T-2 toxin biosynthesis were examined by molecular genetic approaches, including targeted gene disruption and heterologous gene expression. The functions of *F. sporotrichioides* pathway *Tri* genes (*FsTri* genes) are summarized as follows (see Figs. 100.2 and 100.3):

1. *Tri5* (encoding trichodiene synthase). TRI5 catalyzes cyclization of all-*trans*-FPP via nerolidyl pyrophosphate to TDN [36, 45].
2. *Tri4* (encoding a multifunctional CYP responsible for conversion of TDN to isotrichotriol). *Fusarium* TRI4 [48], which is grouped into a CYP58 family, catalyzes four consecutive oxygenation steps from TDN to isotrichotriol as follows: TDN → 2α-hydroxytrichodiene → 12,13-epoxy-9,10-trichoene-2α-ol → isotrichodiol → isotrichotriol [49].
3. *Tri101* (encoding trichothecene 3-*O*-acetyltransferase). TRI101 catalyzes conversion of isotrichodermol to ITD [50]. Different from other pathway *Tri* genes, *Tri101* was first cloned from *F. graminearum* as a gene that confers resistance to T-2 toxin; subsequent analysis with *F. sporotrichioides* revealed its orthologue in the region of synteny [51]. In addition to isotrichodermol, many trichothecenes, including DON, T-2 toxin, and NIV, serve as good substrates of TRI101 [42]. In the amino acid sequence of TRI101, consensus sequences of acetyltransferases, HXXXDG and DFGWGKP, are found [50]. Apart from its role of self-protection against trichothecenes, C-3 acetyl is essential to serve as substrates of the enzymes in later steps in the biosynthesis.
4. *Tri11* (encoding ITD C-15 hydroxylase). TRI11, the first member of a new CYP family CYP65A1, catalyzes hydroxylation of ITD to give 15-deacetylcalonectrin (15-deCAL; **6**) [52].
5. *Tri3* (encoding 15-deCAL 15-*O*-acetyltransferase). TRI3 catalyzes 15-*O*-acetylation of 15-deCAL to give calonectrin (CAL; **7**) [53]. Other trichothecenes also serve as good substrates of TRI3. However, compared to 3-acetyltrichothecenes, 3-hydroxytrichothecenes are rather poor substrates [54]. The two consensus sequences of acetyltransferases described in the above section (3) were conserved in TRI3 as was the case with TRI101.
6. *FsTri13* (encoding 3-acetyltrichothecene C-4 hydroxylase). In T-2 toxin biosynthesis, FsTRI13 catalyzes hydroxylation at C-4 of CAL [55]. In addition to CAL, other trichothecenes with functional groups at C-8 (and also at C-7) serve as good substrates of FsTRI13.
7. *Tri7* (hypothesized to encode 3-acetyltrichothecene 4-*O*-acetyltransferase). TRI7 is involved in 4-*O*-acetylation of 3, 15-diacetoxyscirpenol (3, 15-DAS; **8**) and its derivatives in T-2 toxin biosynthesis [56]. However, different from TRI101 and TRI3, TRI7 does not possess the consensus sequences conserved among acetyltransferase family; TRI7 shows no amino acid sequence similarity to any other proteins reported so far. Attempts to prepare recombinant TRI7 were not successful, and its enzymatic function has not yet been rigorously proven.
8. *FsTri1* (encoding 3-acetyltrichothecene C-8 hydroxylase). FsTRI1 mainly catalyzes hydroxylation of 3, 4, 15-triacetoxyscirpenol (3, 4, 15-TAS; **9**)

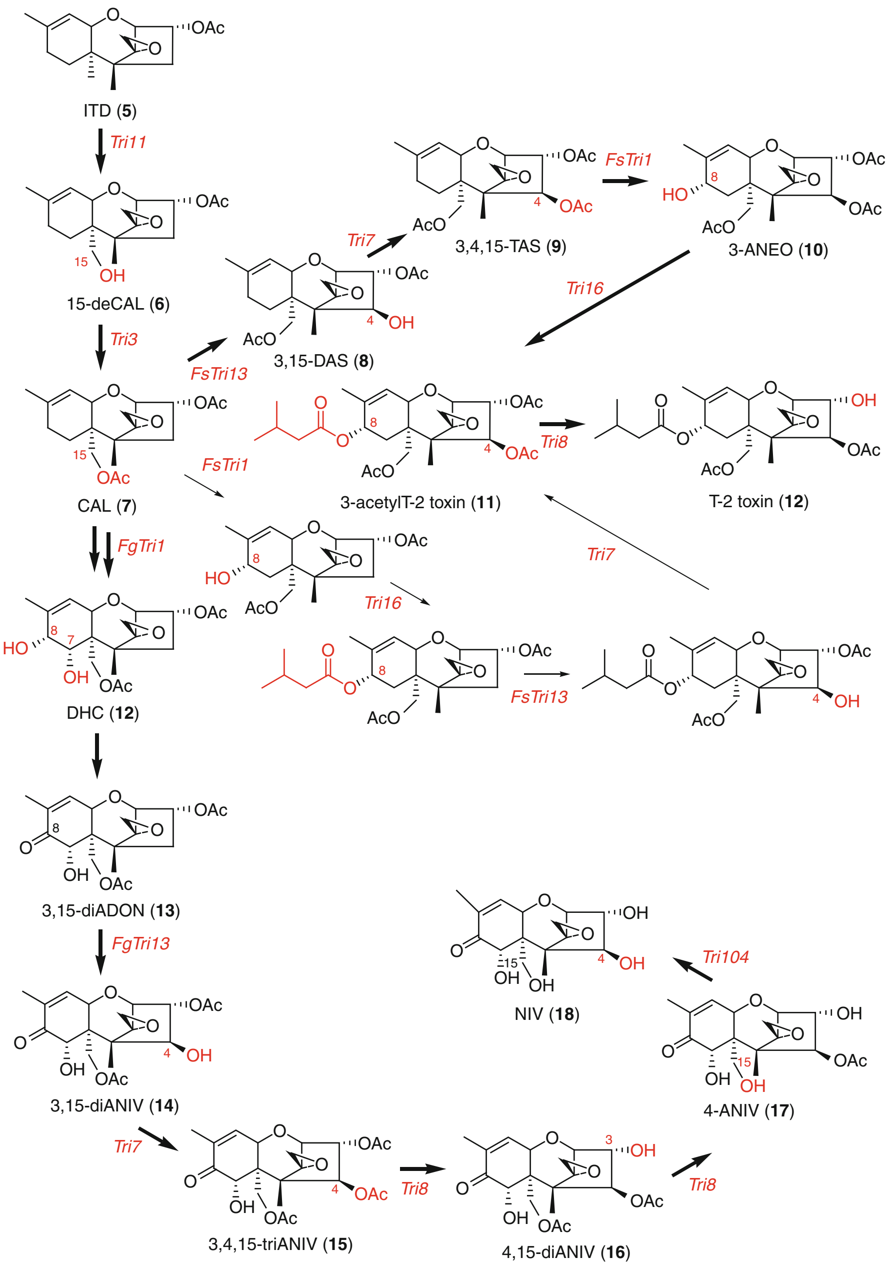

ITD (5)
Tri11
15-deCAL (6)
Tri3
FsTri13
3,15-DAS (8)
Tri7
3,4,15-TAS (9)
FsTri1
3-ANEO (10)
Tri16
CAL (7)
FsTri1
3-acetylT-2 toxin (11)
Tri8
T-2 toxin (12)
FgTri1
DHC (12)
Tri7
Tri16
FsTri13
3,15-diADON (13)
FgTri13
3,15-diANIV (14)
NIV (18)
Tri104
4-ANIV (17)
Tri7
3,4,15-triANIV (15)
Tri8
4,15-diANIV (16)
Tri8

Fig. 100.3 Biosynthetic steps to *F. sporotrichioides* T-2 toxin and *F. graminearum* NIV. Only major pathways to each trichothecene molecule are indicated, and minor pathways are not illustrated. *Tri* genes that are responsible for these steps are indicated in red letters. Functional groups highlighted in *red* indicate that they appeared as the results of expression of these *Tri* genes

(and to a lesser extent CAL) to give 3-acetylneosolaniol (3-ANEO; **10**) (and 8-hydroxycalonectrin) in T-2 toxin biosynthesis [57]. FsTRI1 shows broad substrate specificities and also accept ITD (**5**) and 3, 15-DAS as substrates. The broad substrate specificity of FsTRI1 and FsTRI13 allow branching of the biosynthetic pathway after CAL (**7**) in T-2 toxin biosynthesis (Fig. 100.3).

9. *FsTri16* (encoding an acyltransferase necessary for formation of ester side chain groups at C-8). FsTRI16 mainly catalyzes esterification at C-8 of 3-ANEO to give 3-acetyl T-2 toxin (**11**) [58].
10. *Tri8* (encoding trichothecene deacetylase). TRI8 removes an acetyl from C-8 of the trichothecene skeleton in T-2 toxin biosynthesis [59].

In addition to the pathway *Tri* genes, the core gene cluster contained two regulatory *Tri* genes and one transporter *Tri* gene, which are important for T-2 toxin production: *Tri6* encoding a zinc fingerlike transcription factor [60], *Tri10* encoding a novel protein with a role of other *Tri* gene activation [61, 62], and *FsTri12* encoding a trichothecene efflux pump [63]. *FsTri12* is indispensable for a high level production of T-2 toxin.

The structure of the core gene cluster and the function of the *Tri* genes were conserved between *F. sporotrichioides* and *F. graminearum* [14, 55, 56, 64, 65]. In this way, the identification of *FsTri* genes served as a useful tool for molecular biological analysis of *F. graminearum* that produce type B trichothecenes [66].

4.4 Biosynthesis of NIV

Compared to the structure of T-2 toxin, NIV is characterized by the presence of a keto at C-8 and a hydroxyl at C-7; also, C-4 and C-15 are not acetylated, and instead, hydroxyls are attached to these positions (see Fig. 100.3). To elucidate the biosynthetic pathway of NIV, *FgTri* genes in the core gene cluster were isolated on the basis of nucleotide sequence similarity, and their functions were characterized by the molecular approaches. As to the homologues of *FsTri1* and *FsTri16* that occur outside the core genes cluster, their sequences shared similarity only at the amino acid sequence level; *Tri1* is much more divergent between these *Fusarium* species (viz., 59 % identity between FsTRI1 and FgTRI1), and *Tri16* was found as a pseudogene adjacent to *FgTri1* [67]. The results of targeted gene disruption and heterologous gene expression suggested that most of the enzymes encoded by the *FgTri* genes show the same features as those of *F. sporotrichioides*. However, the following two enzymes exhibit significant differences in their activities depending on their origin:

1. Compared to FsTRI13, the substrates of FgTRI13 appear to be limited to a group of trichothecenes that have a hydroxyl at C-7 and/or a keto at C-8.
2. FgTRI1, but not FsTRI1, catalyzes oxygenation at both C-7 and C-8 of 4-deoxytrichothecenes [68].

Together with the analyses of other *FgTri* genes, a major biosynthetic pathway to NIV is hypothesized to proceed as illustrated in Fig. 100.3: ITD → 15-deCAL → CAL → 7,8-dihydroxycalonectrin (DHC, **12**) → 3,15-diacetyldeoxynivalenol

(3,15-diADON, **13**) → 3,15-diacetylnivalenol (3,15-diANIV, **14**) → 3,4,15-triacetylnivalenol (3,4,15-triANIV, **15**) → 4,15-diacetylnivalenol (4,15-diANIV, **16**) → 4-acetylnivalenol (4-ANIV, **17**) → NIV (**18**). In this scheme, all the biologically acetylatable position, namely, C-3, C-15, and C-4, are once acetylated in this order in the biosynthesis and then deacetylated at a later stage. Recently, a gene responsible for deacetylation at C-4 of 4-ANIV was identified and characterized. This gene, designated *Tri104*, is separated from all other known *Tri* genes in the genome of *F. graminearum* (our unpublished results).

5 Toxicology

5.1 Absorption, Distribution, Metabolites, and Excretion

NIV is mainly absorbed from the intestine, and 11–48 % of administered NIV remains in human bodies until 7.5 h post administration [69]. Acetyl NIV is immediately converted into NIV in serum after intravenous and oral administration. The bioavailability of acetyl NIV was 9.8 % and 19.5 % in broiler chickens and ducks, respectively [70].

In vivo examination using healthy swine showed that NIV was mainly absorbed from the ileum [71]. In vitro examination using a Caco-2 cell line (human intestinal epithelial cell line) showed that apical to basal transportation of NIV was simple diffusion depending on energy [72]. The maximum concentration of ^{3}H-labeled NIV and acetyl NIV was detected in serum after 60 min and 30 min in female ICR mice. The area under the curve of acetyl NIV was 10-fold higher than that of NIV. Acetyl NIV is metabolized in the liver and kidney [72].

NIV is converted by intestinal flora bacteria into less toxic de-epoxy NIV in the intestine as is DON. When NIV was anaerobically cultured with swine feces in vitro, NIV was converted into epoxy NIV. Swine that lacked de-epoxidation ability acquired this ability after their faeces with known de-epoxidation ability (containing as yet unclassified intestinal flora bacteria with de-epoxidation ability) were spread out in pens [73]. When NIV and stomach juice of bovine lumen were anaerobically cocultured in vitro, about 80 % of NIV was converted into de-epoxy NIV [73]. NIV and acetyl NIV are distributed in the serum, liver, kidney, and placenta [74]. Eighty percent of administered NIV was excreted in feces as de-epoxy NIV, while 1 % was excreted in urine as NIV in Wistar rats [75]. In chickens, trace levels of NIV were detected in liver and bile, while NIV and de-epoxy NIV were excreted through the feces in more than 10 % of administered NIV [76]. NIV and acetyl NIV were transmitted to milk in lactating mice [74].

As shown in Fig. 100.4, NIV is metabolized by intestinal flora into de-epoxy NIV and then excreted to urine and feces, while acetyl NIV is converted into NIV in serum and organs.

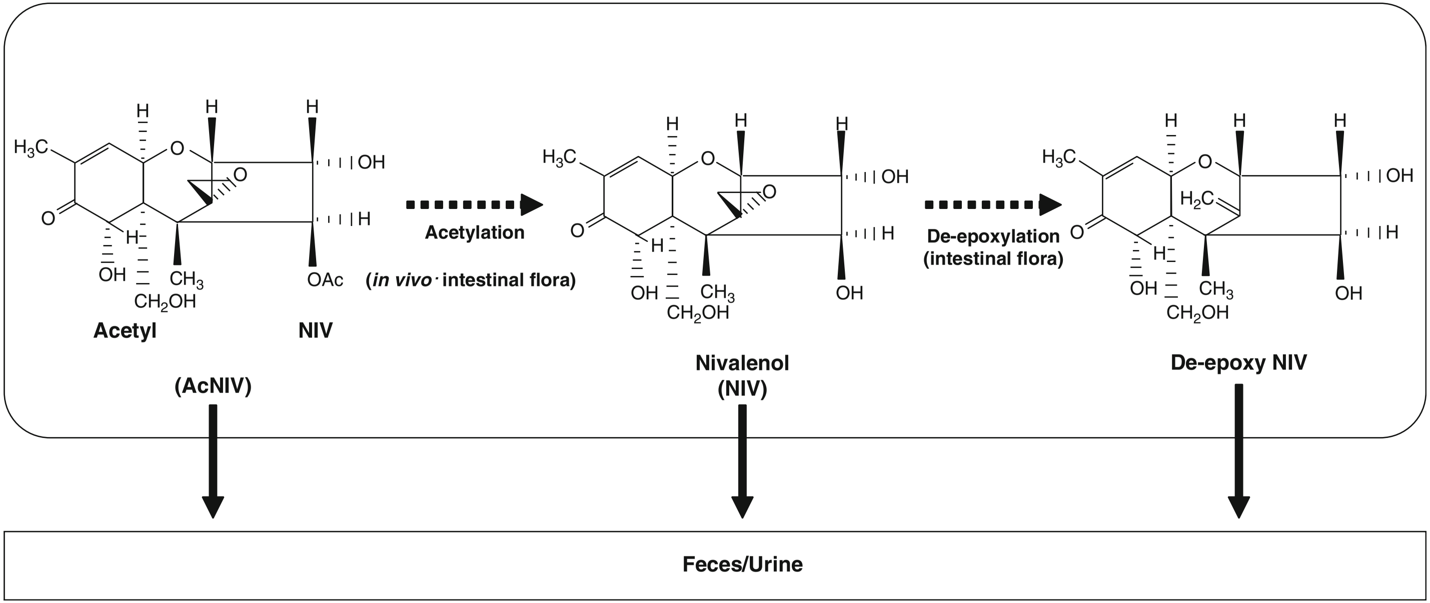

Acetyl NIV
(AcNIV)
Acetylation
(*in vivo*· intestinal flora)
Nivalenol
(NIV)
De-epoxylation
(intestinal flora)
De-epoxy NIV
Feces/Urine
H_3C
O
H
OH
CH_3
CH_2OH
OAc
H_2C

Fig. 100.4 Metabolite pathway of NIV

Table 100.2 LD_{50} of NIV after oral administration

Animal and strains	LD_{50} (mg/kg bw)	References
Mouse, ddY, male, 6 weeks old	38.9	[77]
Rat, F344, male, female, 5 weeks old	19.5	[78]

5.2 Acute Toxicity

Acute toxicity of NIV is shown in Table 100.2.

In 6-week-old male ddY mice, the 50 % lethal dose (LD_{50}) of NIV was 38.9 mg/kg of bw after oral administration, 7.4 mg/kg of bw after intraperitoneal injection, 7.2 mg/kg of bw after subcutaneous injection, and 7.3 mg/kg of bw after intravenous injection. Mice died within 3 days due to the effusion of blood and congestion in the intestine. In 5-week-old F344 rats, the LD_{50} of NIV was 19.5 mg/kg of bw/os and 0.9 mg/kg of bw after subcutaneous injection. Diarrhea and congestion in the gastrointestine were observed. In duck, vomiting was observed after 1.0 mg/kg of bw of NIV, and 0.4 mg/kg of bw of acetyl NIV was administered by subcutaneous injection [79].

5.3 Short-Term Studies of Toxicity

Short-term studies of toxicity were performed in mice, rats, pigs, and chickens, as summarized in Table 100.3.

5.3.1 Mice

C57BL/6 mice were given moldy rice containing at 0, 5, 10, and 30 mg/kg of NIV for 24 days. At 30 mg/kg of NIV, a significant reduction in red blood cell number was observed, but organ weight and the rate of weight gain did not change [80]. When using a diet containing NIV-contaminated rice at 0, 6, 12, and 30 mg/kg for 4 or 12 weeks, weight gain was inhibited in a dose-dependent manner. From these results, LOAEL was estimated to be 0.7 mg/kg of bw [82].

5.3.2 Rats

When Sprague-Dawley rats were treated with diets containing NIV at 0, 6, and 12 mg/kg for 2 or 4 weeks, a significant reduction in feed intake was observed after 1–2 weeks in the group given 0.6 mg/kg of NIV. However, rats recovered after 4 weeks. The low-observed-adverse-effect level (LOAEL) was estimated at 0.6 mg/kg of bw based on the reduction in organ weight [83]. Toxicity studies of single oral administrations using male and female F344 rats showed sedation, eyelid closure, staggering gait, diarrhea, and congestion of the lung and digestive tract. The oral LD_{50} value was estimated at 19.5 mg/kg in both sexes in a repeat oral test, purified NIV was given orally at daily doses of 0.4 and 2.0 mg/kg of bw for 30 days. Takahashi et al. reported subchronic toxicity using F344 rats fed

Table 100.3 Short-term studies of NIV

Study, strain, sex, age, group	Route of administration period	Dose		Critical effect	LOAEL (mg/kg bw/day)	NOAEL (mg/kg bw/day)	Notes	References
		(mg/kg of diet)	(mg/kg bw/day)					
Mouse, C57BL/6, 6 weeks	Diet, contam. 24 days	0, 5, 10, 30	0, 0.6, 1.2, 3.5[a]	Decreasing trend of erythrocytes and leukocytes in a feed of 30 mg/kg Polyribosome damage of bone marrow cells in a feed of 30 mg/kg	3.5[a]	1.2[a]	Used rice mold	[80]
Mouse, C54B16, 7 weeks	Forced oral dosage 3 times per week, 28 days		0, 0.014, 0.071, 0.355, 1.774, 8.870 mg/kg bw, 3 times/week	Increase in plasma phosphate, decrease of urea, alkaline phosphatase activity, and increase of IgG in 8.870 mg/kg bw/day	3.8[b]	0.76[b]		[81]
Mouse, C57BL/6, 7 weeks	Diet, contam. 4 or 12 weeks	0, 6, 12, 30	0, 0.7, 1.4, 3.5[a]	Reduced bw gain and feed intake; dose-dependent increase in serum alkaline phosphatase activity; decrease in adipose tissue	0.7[a]		Used rice mold	[82]
Rat, Sprague-Dawley, 6 weeks	Diet, contam. 14 or 28 days	0, 6, 12	0, 0.6, 1.2[c]	Reduced feed intake (early administration), organ weight changes, increase in CYP2B1/2 of liver microsomal, slight guidance of CYP1A2 in the feed more than 1.5 kg/ml bw	0.6[c]	–	–	[83]
Rat, F344, 5 weeks	Forced oral dosage 30 days		0, 0.4, 2.0	No abnormalities in serum (biochemical and hematological assays) The weight of liver and spleen increased intentionally; however, it did not change after a histopathological inspection using 2.0 mg/kg bw	2.0	0.4	–	[78]
Rat, F344, 6 weeks	Diet, contam. 90 days	0, 6.25, 25, 100	0, 0.4, 1.5, 6.9	Reduced bw in more than 1.5 kg/ml bw	1.5	0.4	–	[84]
Rat, F344, 6 weeks	Diet, contam. 90 days	0, 6.25, 25, 100	0, 0.4, 1.5, 6.9	Reduced bw, loose passage, thymic atrophy, decrease in the number of bone marrow cells, diffuse hypertrophy of basophilic cells with	0.4	–	–	[85]

(*continued*)

Table 100.3 (continued)

Study, strain, sex, age, group	Route of administration period	Dose		Critical effect	LOAEL (mg/kg bw/day)	NOAEL (mg/kg bw/day)	Notes	References
		(mg/kg of diet)	(mg/kg bw/day)					
				an increase of castration cells in the anterior pituitary, increase in the atretic follicle in feed with more than 1.5 kg/ml Male bw was reduced by a diet of more than 25 mg/kg Female white blood cell count was reduced by a diet of more than 6.25 mg/kg				
Pig, 51 days (6 males/ group)	Diet, contam. 21 days	0, 2.5, 5		Gastrointestinal erosion and renal disease in some Decrease in spleen cells in the feed of 5 mg/kg Time-dependent increase in the amount of IgA-producing feed more than 2.5 mg/kg	–	–	–	[86]
Chicken, 7 days (6 males/ group)	Diet, contam. 20 days	Experiment I: 0, 0.5, 2.5, 5, Experiment II: 0, 3, 6, 12		Experiment I: Increase in plasma uric acid concentration in the feed containing 5 and 2.5 mg/kg Experiment II: Feed efficiency and rate of weight gain and food consumption were reduced in the feed containing 6 and 12 mg/kg	–	–	–	[87]
Chicken, white leghorn, layer, 55 weeks	Diet, contam. 50 days	0, 1, 3, 5		Decreases in alkali phosphatase, total protein glucose in plasma in feed with 5 mg/kg Gastrointestinal erosion, duodenum internal bleeding, swollen cloaca, and oviducts with immature eggs in feed with 3 and 5 mg/kg Light, enlarged, and fragile livers at 1 mg/kg feed	–	–	–	[76]

[a] Corresponding value of EU Scientific Committee for Food
[b] Value corresponding to dose/day, three times a week
[c] Estimated intake amount by corresponding value of EU Scientific Committee for Food

a diet containing 0, 6.25, 25, or 100 ppm of pure NIV for 90 days. A decrease in bw and loose stools were observed at 100 ppm. In both sexes, bw was also reduced at 25 ppm in males from 6 weeks. In a hematological observation, the white blood cell count decreased after exposure to 100 ppm in males and to 6.25 ppm in females. Based on hematological data, the LOAEL of NIV was determined to be less than 6.25 ppm (corresponding to 0.4 mg/kg of bw/day for both males and females) [86].

5.3.3 Pigs

After pigs were exposed to purified NIV, no feed refusal, vomiting, or change in clinical appearance occurred, but a macroscopic examination showed gastrointestinal erosion and signs of nephropathy at low doses. Exposure to a high dose of NIV decreased the number of spleen cells. Histological data indicated that exposure of pigs to NIV in the diet caused pathological changes in the kidneys and gastrointestinal tract and reduced the number of splenocytes [87].

5.3.4 Chickens

When male broiler chickens were exposed to feed containing NIV, there was an increase in the uric acid concentration in serum, reduction in the rate of weight gain, gastrointestinal erosion, duodenum internal bleeding, swollen cloaca, and oviducts with immature eggs [76].

5.4 Chronic Studies and Carcinogenesis

Two long-term studies have been reported by the same Japanese group. In both studies, moldy rice powder containing NIV was used as the diet. This moldy rice was estimated to contain 3,147 mg/kg of NIV but no fusarenon-X. The first was a 1-year feeding study in which female C57BL/6CrSlc (SPF, 7-week-old mice) were given diets containing 0, 6, 12, and 30 mg/kg NIV; bw gain and feed efficiency showed a dose-dependent correlation. No changes were observed in the liver, thymus, spleen, kidneys, stomach, adrenal glands, pituitary gland, ovaries, sternum, bone marrow, lymph node, brain, and small intestines with or without Peyer's patch portion. Leukopenia was observed in the group administered 30 mg/kg NIV after 6 months and in all NIV-treated groups after 1 year. The LOAEL was determined to be 6 mg/kg of diet (corresponding to 0.68 mg/kg of bw) [77]. The other report was a 2-year feeding study in which the feeding conditions were identical to the 1-year feeding study. A reduction in bw gain was observed in all treated groups of animals. In the group given 30 mg/kg of NIV, leukopenia was observed, but it was not statistically significant. No tumors were found in any of the treated groups. Compared with the ratio of naturally occurring tumors, there was no difference between the treatment group and the control group. The LOAEL was 6 mg/kg feed (corresponding to 0.66 mg/kg of bw) [89] (Table 100.4).

Table 100.4 Long-term studies of toxicity

Study, strain, sex, age, group	Route of administration (solvent), period	Dose		Critical effect	LOAEL (mg/kg bw/day)	NOAEL (mg/kg bw/day)	Notes	References
		(mg/kg of diet)	(mg/kg bw/day)					
Mouse, C57BL/6CrS1c	Diet, contam. 1 year	0, 6, 12, 30	0, 0.68, 1.51, 3.84	Diet group after 6 months at 30 mg/kg, 1 year after NIV in all treatment groups, significant white corpuscle reduction, dose-dependent decrease in absolute weight, and increase in relative weight of liver, kidney, thymus	0.7		Used rice mold	[77]
				Histological abnormality was observed				
Mouse, C57BL/6CrS1c	Diet, contam. 2 years	0, 6, 12, 30	0, 0.66, 1.38, 3.49	Reduced bw gain in all treatment groups	0.7		Used rice mold	[88]
				Decrease in absolute kidney weight in the diet group of 30 and 12 mg/kg				
				Decrease in kidney weight, concentration in the serum of nonesterified fatty acid and alkaline phosphatase increased in the diet group of 12 mg/kg in a dose-dependent manner				
				Tumor could have caused by NIV was not observed				

Table 100.5 Genotoxicity studies of NIV

			Results		
Endpoint	Test system	Concentrations	Without a metabolic activation system	With a metabolic activation system	References
A: In vitro studies					
Sister chromatid exchange	Chinese hamster V79-E cells	5–50 μM/plate	Slightly pos.	Slightly pos.	[89]
Chromosome aberrations	Chinese hamster V79-E cells	5–50 μM/plate	Neg.	Slightly pos.[a]	[89]
Chromosome aberrations	Chinese hamster V79 cells	0.001–0.03 μg/mL	Pos. (3-fold)	N/a	[90]
Chromosome aberrations	Chinese hamster V79 cells	0.03 μg/mL	Pos. (3-fold)	N/a	[91]
Transformation	v-Ha-ras-transfected BALB/3 T3 mouse embryo cells	0.01–0.2 μg/mL	Neg.	N/a	[96]
DNA damage (comet assay)	CHO cells	50–100 μg/mL	Pos.	N/a	[92]
B: In vivo study					
DNA damage (comet assay)	(Male) ICR mice treated with NIV (20 mg/kg bw)		Oral administration: pos. (kidneys, bone marrow, stomach, jejunum, and colon)		[92]
			Intraperitoneal administration: pos. (colon only)		

[a] All aberrations were daughter chromatid exchange
N/a Not tested

5.5 Genotoxicity

Table 100.5 shows a summary of the results of genotoxicity studies of NIV. Some in vitro studies have been reported (Table 100.5A) using V79-E cells (a Chinese hamster lung-derived cell line), NIV induced cell cycle retardation. In the presence of metabolic activation (S9 mix), slight chromosomal aberrations were seen. These effects were nonspecific, suggesting that they were caused by inhibited protein synthesis [89].

In a chromosome aberration test using V79 cells, NIV purified from contaminated corn and barley induced, at 0.001–0.03 μg/mL and 0.03 μg/mL, respectively, a two to three-fold increase in chromosomal aberrations compared with the control [91, 92]. In a short-term transformation assay using v-Ha-Ras-transfected BALB/3 T3 cells, NIV showed no initiation or promotion activity [96]. A single-cell gel electrophoresis (comet) assay of NIV was conducted using CHO cells and ICR mice (4 males/group). At 50 and 100 μg/mL, NIV damaged the DNA of CHO cells in the absence of a metabolic activation system [92].

In an in vivo comet assay (Table 100.5B), oral treatment with NIV (20 mg/kg bw) resulted in DNA damage in the kidneys, bone marrow, stomach, jejunum, and colon. After intraperitoneal administration of NIV, no DNA damage was observed except in the colon [92].

5.6 Immunotoxicity

5.6.1 Effects on Immune Responses

As many scientists have indicated, NIV stimulates or suppresses the immune system depending on the dose as well as the presence of DON. Oral administration of 10 and 15 mg of NIV induced apoptosis in CD4(+) and CD8 (+) cells in thymus, Peyer's patch, and spleen in a dose-dependent manner [93, 94]. The effect of NIV on susceptibility against infectious diseases was shown by an in vivo infection experiment using BALB/c mice in which NIV at 6 mg/kg had no effect on survival rate against *Salmonella* infection [95]. However, in an in vitro experiment using RAW 264.7 cells, NIV inhibited the transcription activity and expression of inducible NO synthase (iNOS) by lipopolysaccharide (LPS) [96] (Sugiyama 2010).

5.6.2 Changes in Serum IgA Levels and IgA Nephropathy

Increasing IgA and induction of IgA nephropathy by NIV have been reported. These effects have been observed in mice but not in rats ([84], Table 100.6).

After C57BL/6 mice (ten males/group) were treated three times/week for 4 weeks by oral gavage with NIV (solvent: 5 % gum arabic water solution), the highest dose in that experiment (8.870 mg/kg bw) group showed a significant increase in plasma IgG but no changes in IgA [81], but 0.071 mg/kg bw/day and higher dose groups (0.355 mg/kg bw/day) showed a significant increase in plasma IgA [98]. When C3H/HeN, C3H/HeJ, and BALB/c mice (9–12 females/group) were treated for 4 or 8 weeks with feed containing 0, 6, or 12 mg/kg feed (corresponding to 0, 0.9, or 1.8 mg/kg bw/day, respectively) of purified NIV, the NIV-treated groups demonstrated an increase in IgA accumulation in glomeruli and increased serum IgA, particularly in the 12 mg/kg feed dose group at 8 weeks [99]. In a single oral administration study, Peyer's patches of BALB/c mice given NIV at 0 or 15 mg/kg bw showed a significant increase in all B cell subpopulations, particularly IgA + B cells, with the numbers of IgA + and IgM + B cells remaining higher than those of the control group [100] (#649).

Interestingly, an experiment using ovalbumin-TCR Tg (OVA-specific T cell receptor transgenic) mice (4 males/group) indicated that NIV significantly inhibited total IgE production and OVA-specific IgE, IgG1, and IgA production [101]. In F344 rats, NIV increased IgM level significantly but not IgG and IgA level even in the group given an oral dose of 6.9 mg/kg bw/day [84].

Table 100.6 Effect of NIV on IgA production

Study, strain, sex, age, group	Route of administration (solvent), period	Dose		Critical effect	LOAEL (mg/kg bw/day)	NOAEL (mg/kg bw/day)	Notes	References
		(mg/kg of diet)	(mg/kg bw/day)					
Mouse, C57BL/6, 6 weeks	Forced oral dosage 4 weeks in 3 days/week		0, 0.014, 0.071, 0.355, 1.774, 8.870 mg/kg bw, 3 times/week	Increase in plasma IgG administration group of 8.870 mg/kg bw No changes in IgA		3.8[a]		[81]
Mouse, C57BL/6, 6 weeks	Forced oral dosage 4 weeks in 3 days/week		0, 0.071, 0.355 mg/kg bw, 3 times/week	Increase in the plasma of IgA	0.03[a]			[98]
Mouse, C3H/HeN, C3H/HeJ, BALB/c, 6–8 weeks	Diet, contam. 4 or 8 weeks	0, 6, 12	0, 0.9, 1.8[b]	Increase of serum IgA (with the increase) immunopathological change of the kidney similar to IgA-induced renal damage	0.9[b]		Used rice mold	[99]
Mouse, BALB/c, 5 weeks	Single, oral route (10 % DMSO)		0, 15	Increasing IgA cell in Peyer's patch cells, decreasing pan-T cell, pan-B cell in lymph node	15			[101]
Egg albumen (ovalbumin)-specific T cell receptor αβ-Tg mouse, BALB/c, 8–13 weeks,	Drinking water, 2 or 4 weeks	0, 6	0, 0.9[b]	Inhibition of anti-OVA IgE, IgG1 and IgA antibody, Inhibition of IL-4 production, induction of IL-2 production in spleen	0.9[b]			[101]
Rat, F344, 5 weeks (10 males and females/group)	Diet, contam. 90 days	0, 6.25, 25, 100	0, 0.4, 1.5, 6.9	IgM increase in the treated group 6.9 mg/kg bw/day No change in IgA and IgG		6.9		[84]
Pig, 51 days (6 males/group)	Diet, contam. 21 days	0, 2.5, 5		IgA in plasma had no significant difference compared with control				[86]

[a] Value corresponding to dose/day three times/week
[b] Estimated intake amount by corresponding value of EU Scientific Committee for Food

In pigs fed with purified NIV at 0, 2.5, or 5 mg/mg in feed for 21 days, no significant differences were observed in plasma IgA levels between the control and treatment groups [87].

6 Other Toxicities

NIV inhibits protein and DNA synthesis but not RNA synthesis [102]. NIV inhibited protein synthesis in rabbit reticulocytes with an IC_{50} value of 6 μg/mL [103]. Cytotoxicity of NIV, 4-acetyl NIV, and de-epoxy NIV on 3 T3 cell growth was IC_{50} 1.19 ± 0.06 mM (373 ± 20 ng/mL), 0.72 ± 0.04 mM (255 ± 13 ng/mL), and 64.2 ± 3.14 mM (19030 ± 930 ng/mL), respectively [104].

7 Outbreaks Associated with NIV

Outbreaks linked with *Fusarium*-contaminated cereal-based foods occurred in Japan and Korea during the 1940s–1960s [9]. The symptoms reported were nausea, diarrhea, and emesis. In China, more than 30 gastroenteritis outbreaks from 1961 to 1981 were associated with the consumption of "scabby," i.e., *Fusarium*-infected wheat, barley, or maize [8]. Trichothecenes were predicted to be the causative agent. An outbreak of trichothecenes occurred in the Kashmir Valley, India, during June to September 1987 in which 50,000 people suffered from gastrointestinal disorders [105, 106]. The cause of the outbreak was assumed to be the consumption of bread made from mold-damaged wheat, evidenced by the presence of molds such as *Fusarium* sp. and *Aspergillus* sp. and varying quantities of trichothecene mycotoxins. The concentration of DON, acetyl DON, NIV, and T-2 toxin in causative wheat was 0.34–8.4, 0.6–2.4, 0.03–0.1, and 0.55–4 mg/kg, respectively [105, 106].

8 Analytical Methods

Since NIV occurs as a co-contaminant with other trichothecene mycotoxins, it is often analyzed simultaneously with the co-contaminants rather than alone. Analytical methods developed so far include thin layer chromatography (TLC); capillary gas chromatography (GC) with electron-capture detection (ECD), flame ionization detection (FID), or mass spectrometric detection (GC/MS); high-performance liquid chromatography (HPLC) with ultraviolet (UV), fluorescence, or mass spectrometric detection; supercritical fluid chromatography (SFC); and time-of-flight mass spectrometry (LC/TOF-MS).

Usually, to analyze trichothecenes in foods and feeds, solvent extraction is essential. Aqueous methanol and acetonitrile are commonly used for extraction. For applying GC–MS, HPLC, and LC–MS/MS, a sample needs to be cleaned up with a charcoal-alumina-celite, florisil, silica gel, or solid-phase extraction column. For laboratory experiments, TL is very useful because of its low cost and simplicity.

However, for surveillance studies, trichothecene mycotoxins coexist with other trichothecenes, and a simultaneous analytical method for the determination of some trichothecenes and *Fusarium* toxins is considered to be more practical than a single method. A decade ago, GC was very popular to analyze some trichothecene mycotoxins in food, but for GC analysis, various derivatives are needed that are sometimes troublesome.

GC is typically used conventionally for simultaneous analysis for trichothecene mycotoxins and ZEN but requires trimethylsilyl derivatization before analysis. GC–FID produced good results in a validation of the EU Standards, Measurements and Testing Programme [107]. However, the GC method requires a derivatization procedure, which generally causes a loss of time and recovery.

On the other hand, LC using UV requires no derivatization procedure [108–111]. HPLC–UV (220 nm) for the GC–MS determination of DON and NIV has been developed in Japan.

Over several years, many LC–MS and LC–MS/MS methods were reported for the simultaneous analysis of trichothecene mycotoxins, including DON and NIV. These methods have been applied to the hygienic control and surveillance of mycotoxins. However, LC–MS requires the use of expensive internal standards such as isotopically substituted compounds. Meanwhile, the precision of LC–UV has been valued, although its sensitivity is lower than that of LC–MS.

A selective analytical method based on HPLC, combined with atmospheric pressure photoionization (APPI) mass spectrometry, has been developed for the simultaneous determination of NIV and DON. A liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI–MS) method based on time-of-flight MS (TOF/MS) with a real-time reference mass correction technique was also developed for the simultaneous determination of *Fusarium* mycotoxins (NIV, DON, fusarenon-X, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, HT-2 toxin, T-2 toxin, diacetoxyscirpenol, ZEN) and *Aspergillus* mycotoxins (aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2) in corn, wheat, cornflakes, and biscuits [112].

Sulyok et al. [113] reported the first validated method for the determination of 39 mycotoxins in wheat and maize by liquid chromatography with electrospray ionization–triple quadrupole mass spectrometry (LC/ESI–MS/MS) without the need for any cleanup. The 39 analytes included A and B trichothecenes (including deoxynivalenol-3-glucoside), ZEN and related derivatives, fumonisins, enniatins, ergot alkaloids, ochratoxins, aflatoxins, and moniliformin, and six trichothecene mycotoxins (NIV, DON, fusarenon-X, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, and T-2 toxin).

Taken together, these analytical methods should be chosen for any purpose. For surveillance, GC–MS and LC–MS/MS are efficient because of their sensitivity, and for enforcement of regulation, HPLC would be useful.

9 Exposure Assessment of NIV and Regulation

Exposure to mycotoxins depends on the contamination level in different foods and on the intake of those foods, which is influenced by the dietary culture of each country.

Table 100.7 Analytical methods for determination of NIV

Extraction	Cleanup column	Derivatization	Determination	Toxins analyzed	Detection limit (NIV)	Commodities	References
$CH_3CN—H_2O$ (85+15)	Charcoal-alumina-Ceite column	None	TLC $Alcl_3$ spray	DON, NIV, T-2, HT-2, DAS and two others	0.3–0.8 μg/g	Wheat, corn	[116]
$CH_3CN—H_2O$ (3+1)	Florisil colum	TMCS	GC-ECD. GC-MS	DON, NIV	10 ng/g	Wheat, barley, corn and their product	[113, 114] 8.26–&$$ $;
MeOH+H_2O (7+1)	Silica gel column	TMCS, HFBA	GC-ECD. GC-MS	DON, NIV, T-2, HT-2, DAS and two others	20 ng/g (DON, NIV), 50–200 ng/g (HT-2, DAS)	Wheat, wheat flour, barley, corn flour, rye flour	[118, 119] 23.25–&$$ $;
$CH_3CN—H_2O$ (85+15)	–(dilution only)	5	LC-MS/MS ESI	NIV, FX, T-2, DON, 3ADON, 15ADON	Not described	Rice medium	[120] Sagawa (2006)
$CH_3CN—H_2O$ (84+16)	–(dilution only)	None	LC-MS/MS APCI	NIV, DON, D3Gl, FX, ADON, HT-2, T-2, ZON	5 ug/kg (NIV barley, malt)	Barley, malt	[121] Malachova (2010)
$CH_3CN—H_2O—CH_3COOH$ (79+20+1)	–(dilution only)	None	LC-MS/MS ESI	NIV, DON, 3ADON and 36 others	20 ug/kg (NIV wheat)	Wheat, maize	[115] Sulyok (2006)
$CH_3CN—H_2O$ (85+15)	MultiSep #227	None	LC-MS APPI	NIV, DON	0.2 ug/kg (NIV wheat)	Wheat	[111] Tanaka (2009)
$CH_3CN—H_2O$ (85+15)	MultiSep #226	None	LC-TOFMS APCI	NIV, DON, 3ADON, and 13 others	2.4 ug/kg (NIV corn)	Corn, wheat, cornflasks, biscuits	[122] Tanaka (2006)

TLC Thin Layer Chromatography, *TMCS* trimethylsilylchlorosilane, *HFBA* Heptafluorobutyric anhydride, *HPLC* high-performance liquid chromatograpy, *GC-MS* gas-chromatograpy-mass spectrometry, *GC-ECD* gas chromatograpy with electron-capture detection, *LC-MS/MS* liquid chromatograpy-mass spectrometry, *LC-TOF/MS* liquid chromatography – time-of-Flight-mass spectrometry

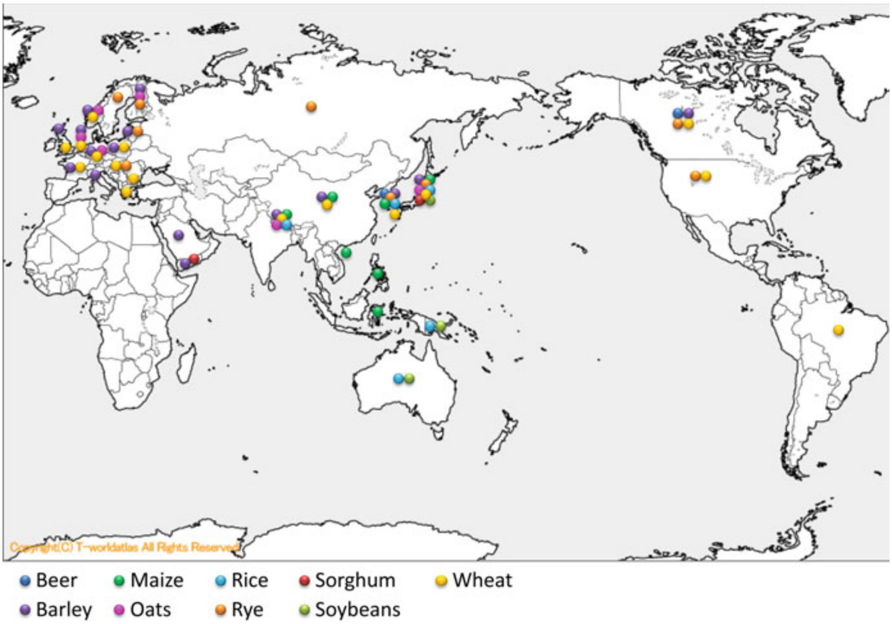


Fig. 100.5 Distribution of food contaminated with NIV (Modification cited by Ref. [120])

These data are based on established regulation levels of individuals or international institutes. For mycotoxins, it is ideal to correct the actual monitoring data over several years in raw and processed food. Especially, since exposure to trichothecenes and their toxicity are of concern in young children, it is important to assess the intake by age layers. FAO/WHO organized the Global Environment Monitoring System/Food Contamination Monitoring and Assessment Programme (GEMS/Food) and utilizes this information to assess the intake of mycotoxins (Table 100.7).

9.1 Occurrence of NIV in Food and Feed

Compared to the worldwide distribution of DON, NIV contamination is found only in limited areas. In Fig. 100.5, the commodities contaminated with NIV that have been reported are plotted [120]. Contamination of cereals (wheat, oats, barley, maize, rice, rye) has been frequently found in Far East Asia (China, Korea, Japan), Southeast Asia (the Philippines, Vietnam), Oceania (New Zealand, Australia) Europe (Germany, Poland, Norway, the Netherlands), and Eastern Europe (Lithuania). The contamination of soybeans (50 μg/kg of NIV) has also been reported in Australia [121].

From SCOOP data [11], food and food raw materials were shown to be contaminated by trichothecenes (DON, NIV, FX, T-2, and HT2 toxin, T-2 triol, diacetoxyscirpenol, neosolaniol, and verrucarol).

Table 100.8 NIV contamination in food in European Union member states

Commodities	% Positive	Mean (μg/kg)	Maximum (μg/kg)
Wheat	14	24	440
Maize	35	np	340
Barley	8	15	351
Oats	21	56	1,860
Rye	5	np	48

Adapted from Ref. [11]

Table 100.9 Occurrence of nivalenol in wheat in Eastern Asia (2002–2007)

Country	Commodities	Mean (μg/kg)	Maximum level (μg/kg)	References
Japan	Wheat	35	1,000	[122]
	Barley	172	3,000	
Korea	Maize	168	332	[123] [124]
	Barley	390	2,038	
	Rice (brown rice)	164	569	
China	Wheat	29	1,035	[125],
	Corn	18	1,400	

Table 100.8 shows NIV contamination in European countries. Among the cereals, mainly wheat, oats, wheat, barley, and rye are exposed to NIV. In Europe, the level and frequency of DON contamination are often higher than NIV contamination. Thus, it is generally believed that DON is more predominant than NIV and that their contamination levels shift in a parallel manner, i.e., when the DON level decreases, the NIV level also decreases. However, in the southern island of Japan (Kyushu), only the NIV-producing group strain exists (described in 1.3. mycology). Since commodities harvested from this area are contaminated with NIV, it has become a serious problem for human health.

Far East countries, including Japan, Korea, and China, are also suffering from NIV contamination of grain. In Japan, NIV concentration in barley was higher than that in wheat, while in Korea, barley was the most susceptible commodity contaminated with NIV. NIV contamination in rice has been reported in Korea but not in Japan. Since rice is the dietary staple in most Far East countries, even if the contamination level is low, it is of concern to human health (Table 100.9).

9.2 NIV Intake

The surveillance of NIV in food and estimated NIV intake were conducted in limited countries [22, 126]. Table 100.10 shows estimated NIV intake in the EU, UK, and Japan.

Table 100.10 Estimated NIV intake

Country	Age	Sex	NIV intake mean (95 % tile) ng/kg bw/day
Austria	Whole population		78 (274)
Denmark	Whole population		30 (72)
France	Adult		58 (99)
Finland	Adult		27 (np)
Norway	Adult	M	57 (110)
	Adult	F	50 (93)
Sweden	Adult		6 (13)
UK	Adult	M	25 (np)
	Adult	F	17 (np)
France	Children		94 (307)
Norway	1–6 years		113 (263)
UK	Infant		62 (np)
	1–4 years		64 (np)
	4–6 years		64 (np)
	7–10 years		50 (np)
	11–14 years		34 (np)
Japan	1–6 years		10 (330)
	7–14 years		10 (230)
	15–19 years		10 (180)
	>20 years		>10 (110)

Adapted from Refs. [11, 122]
np not provided

As shown in Table 100.10, viewing the entire population, the highest mean and 95th percentile of estimated NIV intake was shown in Austria. Among the exposed adults, in France, the estimated intake of NIV was highest compared to adults of other countries. However, the intake by children was higher than by adults. In young children, mean intake in the UK was 62–64 ng/kg bw/day, and these values were higher than in Japanese children.

9.3 International Evaluation

The FAO/WHO Joint Expert committee of Food Additives has not evaluated NIV contamination levels. The IARC has evaluated carcinogenesis of the toxins produced from *F. graminearum*, *F. culmorum*, and *F. crookwellense*, such as ZEN, DON, and acetyl NIV [127]. Their conclusion was that metabolites of these three *Fusarium* species should be placed in group 3, i.e., not classified as carcinogenic for humans.

The Scientific Committee for Food (EC) reported values for DON in 1999, for NIV in 2000, and for T-2 and HT-2 toxins in 2002[128]. According to their opinion, temporary tolerable daily intake (t-TDI) of NIV was estimated to be 0.7 μg/kg based on NOAEL and 0.7 mg/kg temporary bw based on the results of 1- and 2-year repeated dose studies reported by Ref. [77, 88].

In 2010, the FSC of Japan evaluated DON and NIV and concluded that the provisional maximum tolerable daily intake (PMTDI) of NIV was 0.4 μg/kg bw/day based on LOAEL (0.4 mg/kg bw/day) of subacute repeated dose toxicity studies using purified NIV [86]. As the PMTDI of DON was adopted to be 1.0 μg/kg bw, it had the same value as JECFA and EFSA.

9.4 Regulation

To date, no country has established a standard for NIV yet. However, regarding the risk assessment of NIV, Japan and EU lead the world. These countries have serious problems related to the management of NIV-producing fungi. The establishment of a regulation level for NIV is one of the effective means to prevent contamination of NIV in cereal grain.

10 Conclusion

Because NIV is a minor mycotoxin in the world, information for risk assessment is very poor. Of course, European countries and Japan have recognized the threat posed by NIV to human health and have started to evaluate this risk. In the risk assessment of NIV, it is recommended to take into account the effect of co-contamination with other trichothecene mycotoxins, namely, DON. As further studies, combination toxicology studies of NIV and DON are needed.

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Taxol and Related Compounds 101

Hojjat Sadeghi-aliabadi and Afsaneh Yegdaneh

Contents

1 Introduction 3160
2 Taxol 3161
2.1 Discovery of Taxol 3161
2.2 Sources of Taxol 3161
2.3 Structure Elucidation 3162
2.4 Isolation of Taxol 3164
2.5 Mechanism of Biological Activity 3165
2.6 Toxicology 3165
2.7 Clinical Trials 3166
2.8 Biosynthesis 3166
3 Docetaxel (Taxotere) 3167
3.1 Mechanism of Biological Activity 3167
3.2 Toxicology 3168
4 Natural Taxanes 3168
4.1 Taxanes Having an Exocyclic Double Bond Between C-4 and C-20 3169
4.2 Taxanes Containing an Extra Bond Connecting Transannular Atoms 3169
4.3 Taxanes Having a C-4 Epoxide 3169
4.4 Taxanes with an Exetane Ring at C-4 and C-5 3169
4.5 Miscellaneous Taxanes 3170
5 Conclusions 3170
References 3170

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Abstract

Paclitaxel is a mitotic inhibitor used in cancer chemotherapy. It was discovered in the US National Cancer Institute program in 1967 when Monroe E. Wall and Mansukh C. Wani isolated it from the bark of the Pacific yew tree, *Taxus brevifolia*, and named it taxol. When it was developed commercially, the generic name was changed to paclitaxel.

Paclitaxel is now used to treat patients with ovarian, breast, and advanced forms of Kaposi's sarcoma. Paclitaxel stabilizes microtubules and, as a result, interferes with the normal breakdown of microtubules during cell division. Together with docetaxel, it forms the drug category of the taxanes.

While offering substantial improvement in patient care, paclitaxel has been a relatively controversial drug. There was originally concern because of the environmental impact of its original sourcing, no longer used, from the Pacific yew. So in recent years, extensive research has been done to find a way to produce it from alternative resources including related compounds and also to lessen the side effects of paclitaxel.

Keywords

Biosynthesis • Clinical trial • Docetaxel • Paclitaxel • Taxoids • Toxicology

1 Introduction

Nature has been a source of medicinal agents for thousands of years. Compounds extracted from natural products have been used either as a new drug or a lead molecule to synthesize modern therapeutic agents in the treatment of variety of diseases for centuries. Pharmacologic activities have been found in different kinds of secondary metabolites such as alkaloids, terpenoids, coumarins, flavonoids, and lignans, all firstly isolated from plants.

During the last 60 years, unique classes of natural anticancer compounds have been isolated from plants. These plant products, represented by the *Vinca* and *Colchicum* alkaloids, as well as other plant-derived products such as paclitaxel (Taxol) and podophyllotoxin showed great promise for new drug discoveries based on nature.

The discovery of the novel diterpenoid anticancer agent taxol in 1971 by Monroe Wall and his collaborators ranks in retrospect as one of the most significant discoveries ever made in the field of naturally occurring anticancer agents. Although extensive researches have been done on natural anticancer agents, only a handful of plant-derived natural products have been found to show clinically useful activity, and taxol is clearly a member of this group.

This chapter is going to summarize the different aspects of discovery, analysis, semisynthesis, clinical application, side effects, and also related compounds of taxol as a valuable natural product in cancer therapy.

2 Taxol

2.1 Discovery of Taxol

After a strong interest about cancer in the United States in 1955, National Cancer Institute (NCI) organized a cancer drug screening program of plant extracts [1]. These studies led to a discovery that the stem bark extract of the Pacific yew tree, *Taxus brevifolia*, showed cytotoxicity in the preliminary screening against KB cells using MTT assay and also activity against carcinosarcoma in rats and leukemia in mice. At that time there were only a few methods available for separation, and of course, they were not so sophisticated. The method for taxol isolation began with ethanol extraction of 12 kg of Pacific yew bark followed by partitioning of the extract between chloroform and water. The chloroform phase was the active one, and further isolation was done to separate taxol from this fraction. It took about 2 years to isolate 0.5 g pure taxol from *T. brevifolia* bark in 1966. The molecular formula of $C_{47}H_{51}NO_{14}$ obviously indicated a complex structure. Some researchers claimed that the isolation of compounds belonged to the taxane family, but at that time only a few members had been elucidated.

The isolation of taxol was first presented at the American Chemical Society meeting in Florida in 1967 [2]. In the main paper published in 1971, the reported yield of taxol was 0.02 % from dried bark. It was also reported that taxol was present in other Taxus species including *T. baccata* and *T. cuspidate* [3].

2.2 Sources of Taxol

The only original source of taxol was the bark of the Pacific yew. Yews are small coniferous trees or shrubs in the Taxaceae family. They are relatively slow growing and can be very long-lived and reach heights of 1–40 m, with trunk diameters of up to 4 m. However, alternative sources range from the use of genetic engineering and tissue culture to partial and total syntheses. Other Taxus species such as *T. globosa, T. baccata, and T. wallichiana* also contain taxol or precursor of this compound.

Spjut reported as follows: The genus *Taxus* includes 24 species and 55 varieties distributed across the northern temperate and subtropical regions. The species are classified into three groups by differences in leaf epidermal (outer skin-like layer) and stomatal (donut-like cell) features. These are the *wallichiana* group with 11 species, occurring from central Himalayas to Indonesia; the *baccata* group with 9 species in temperate Eurasia, Northern Africa, and eastern North America; and the *sumatrana* group with 4 species overlapping in distribution with the *wallichiana* subgroup in Asia, but absent from North America. The *wallichiana* group is further divided into subgroups *wallichiana* and *chinensis*, and the *baccata* group is divided into the *baccata* and *cuspidata* species alliances [4].

From Taxaceae family, *Taxus brevifolia* has been firstly used as a good source of taxol and related taxoids.

2.3 Structure Elucidation

The structure of taxol (Figs. 101.1 and 101.2) was elucidated on the basis of the H-NMR spectrum (Fig. 101.3), in 1971 [3], but the full elucidation was done by X-ray crystallography. Although taxol was poorly soluble in various solvent systems and readily crystallized, the crystals were fine needles and unsuitable for X-ray. Today after almost 40 years, there is no any report of X-ray crystal structure of taxol. Mild methanolysis of taxol resulted in a methyl ester and a tetraol. The ester was characterized using X-ray crystallography as its p-bromobenzene derivative, and the tetraol was characterized as its bis diacetate. X-ray crystallography data, together with H-NMR data, gave the complete structure of taxol.

IUPAC recommended the numbering system for taxol and taxoids as shown in Fig. 101.1 [5].

Taxol is differentiated from most other taxane diterpenoids by its ester side chain at C-13 and by its oxetane ring D. It can be viewed as the *N*-benzoyl-β-phenylisoserine ester of baccatin III. The conventional planar representation of taxol can be misleading since its structure is best described as inverted cup shape as shown in Fig. 101.2.

The structure-activity relationship of taxol has been studied by a multitude of researchers. The molecule can divide into two parts: the side chain and the taxol skeleton. The side chain of taxol is critical for maintaining activity. The structure-activity of the side chain has revealed the following features:

- Protection of the C2′ hydroxyl group as an ester results in loss of activity in terms of microtubuline stabilization but not in cytotoxicity.
- The C3′ amide-acyl group is critical but may be aromatic or alkyl in nature.
- The C3′ bound nitrogen can be replaced by an oxygen atom without loss of activity.
- The C3′ aryl group is needed; replacement by a methyl group reduces the activity 19-fold.

Taxol consists of several rings including a four-membered ring, a six-membered ring, and an eight-membered ring and peripheral functionalities. Modification of the taxol skeleton and elucidation of its structure-activity have led to the following features:

- The oxetane ring is crucial for maintaining the activity; ring opening leads to a dramatic decrease in bioactivity.
- Contraction of the eight-membered ring to a seven-membered ring still provides the molecule with tublin depolymerization properties.
- Removal of the C-10 acetyl group does not have any effect on the activity.
- C2-*O*-benzoyl group removal causes a drastic reduction in its activity

The original discoverers of taxol reported a molecular ion at m/z 854, but later researchers only report fragment ion peaks under these conditions.

The mass spectrum of taxol shows good molecular ions at [M+H]+, [M+Na]+, and [M+K]+, and fragment ions are observed corresponding to loss of the protonated C-13 side chain and to the loss of acetic acid.

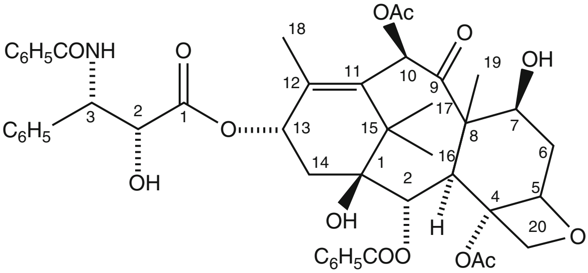
C6H5CONH
O
C6H5
3
2
1
O
OH
18
12
11
OAc
10
O
9
19
OH
17
13
15
8
7
6
14
1
16
2
5
4
OH
H
20
O
C6H5COO
OAc

Fig. 101.1 Structure of taxol and its IUPAC numbering system

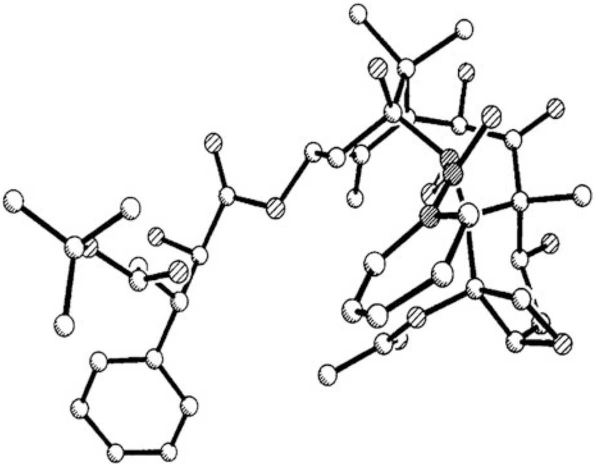

Fig. 101.2 Inverted cup shape of taxol

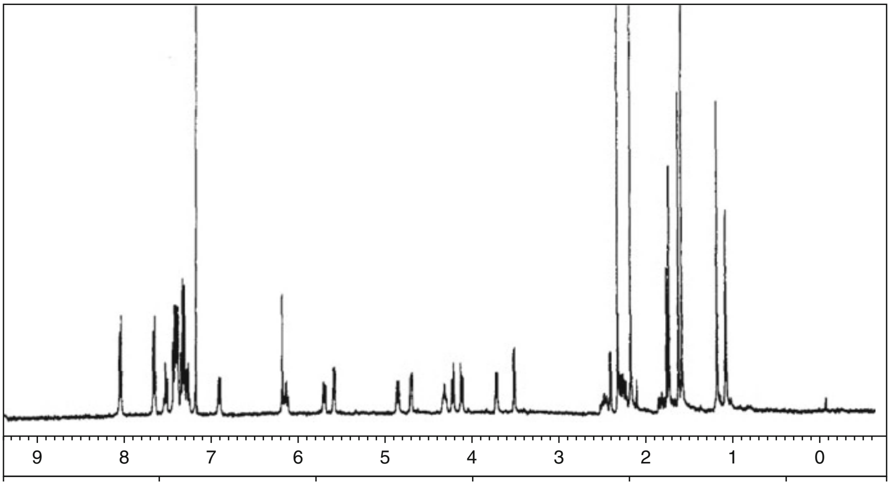

Fig. 101.3 H-NMR of taxol

2.4 Isolation of Taxol

The need to carry out extensive surveys of Taxus species for their taxol content and to monitor taxol in clinical studies has spurred efforts to develop new analytical methods for taxol purification. Solvent extraction methods are always inexpensive and easy to handle. Different solvent systems started from very nonpolar solvents (e.g., n-hexane) [6]; semipolar system (chloroform and acetone or combination of dichloromethane: methanol) [7] and very polar system (methanol or ethanol alone or with H_2O) [7] have been employed for extraction of taxoids from bark or leaves of Taxus species. To date only chromatographic methods have been reported. Sadeghi-aliabadi et al. in their studies to find an optimized solvent for taxol extraction indicated that the solvent 100 % acetone is the best solvent to extract higher amount of taxol from *Taxus baccata* needles. They confirmed their results by HPLC, ESI-LCMS, and biological cytotoxic evaluations of extracted compounds [8].

2.4.1 TLC

Two TLC methods have been developed for detection of taxol in plant extracts. In one system a cyano-modified silica gel plate was used, with two-dimensional development using both normal phase (CH_2Cl_2:hexane:AcOH, 9:10:1) and reverse phase (H_2O:MeCN:MeOH:THF, 8:5:7:0.1) development.

The other system used a diphenyl-modified silica plate, with hexane:isopropyl alchol:acetone, 15:2:3, in the normal phase dimension and MeOH:H_2O, 7:3, in the reverse phase dimension. Both systems separated taxol and taxol B adequately and also separated several other compounds from *T. brevifolia* extract [9].

2.4.2 HPLC

HPLC methods were developed to separate taxol from cephalomannine, 10-deacetylcephalomannine, baccatin III, and 10-deacetylbaccatin III. Both isocratic and gradient reverse phase methods were tested, using cyano- and phenyl-bonded silica gel columns. Both columns gave adequate separations, but the cyano column required less organic solvent and thus tended to give higher back pressures and decreased column life. The optimum mobile phase for the phenyl column was a linear gradient starting with MeOH:H2O:MeCN (20:65:15) going to MeOH:H_2O: MeCN (20:45:35) in 20 min and ending with MeOH:H_2O:MeCN (20:25:55) in 30 min.

A similar system, but with slightly different solvent ratios, was used for the analysis of taxol and 10-deacetylbaccatin III in T*axus* species previously discussed. The analysis of *T. brevifolia* for taxol content previously noted was carried out using C18 columns and MeOH:H2O, 68:32, as the solvent. A method for analysis of taxol in *T. chinensis* has also been described. Very recently another method for the separation of taxol and cephalomannine has been published. This method uses a cyanopropyl column with hexane:iPrOH (2:1) and may be more amenable to preparative work than the reverse phase systems previously described [10–11].

HPLC has also used to determine taxol and related compounds in biological fluids [13]. After evaluating C8-, cyanopropyl-, and phenyl-bonded phases, Riley and his co-workers selected a C8-bonded phase column with a mobile phase of MeOH:NaOAc buffer (0.02 M, pH 4.5, 35:65 v/v) as their system for analysis of taxol in human plasma and urine.

A reverse phase HPLC assay was also used for analysis of taxol in human plasma and urine during the phase I trials of taxol; in this case a C18 column was used with a gradient of H_2O:MeCN, 65:35 to 0:100, as the mobile phase. Similarly, a reverse phase method was used for the analysis of taxol and its decomposition products in the cell culture media and in the rat bile [10–13].

2.4.3 LCMS

LCMS and LC-MS-MS have been developed as feasible and reliable techniques for detecting paclitaxel, 10-DABIII, baccatin III, cephalomannine, and other related taxanes. ESI in positive-ion mode and an MRM mode were utilized to optimize sensitivity and accuracy [8].

2.5 Mechanism of Biological Activity

Many of the important chemotherapeutic agents such as vinca alkaloids operate by destabilizing microtubules, while taxol appeared to work by microtubule stabilization. Microtubules are involved in many areas of cell physiology but essential in the cell division process. During cell division, they are the major structural components of the mitosis and mitotic spindle (the area of cell where the 2 sets of chromosomes align for separation). Taxol is believed to act as a mitotic spindle poison by binding to polymeric tubulin forming a highly stabilized microtubule. Inhibiting the release of monomeric tubulin from the ends of the microtubule polymer causes the inherently unstable assembly and disassembly process to be hampered, and therefore, cell division is suppressed [14].

2.6 Toxicology

The toxicology studies were performed on mice, Sprague-Dawley rats, and beagle dogs were quite problematic. Large single doses were toxic to the point of death when administered to beagle dogs, but tolerable on repeated smaller doses. The major toxicities assigned to taxol were myelosuppression, lymphoid depletion, emesis, diarrhea, mucosal ulcerations, and toxicity to male reproductive organs. These toxins were cumulative over a 5-day period which indicated a single injection schedule may be preferred. However, as a single injection schedule would require problematically large vehicle volumes, the clinical plan was to try a variety of administration schedules in the clinical trials [15].

2.7 Clinical Trials

Phase I of clinical trials of taxol began in 1983 and was aimed at determining the maximum tolerated dose in humans and any dose-limiting toxicities. At first, the drug caused some serious hypersensitivity reactions, but decreasing the rate of infusion or premedicating patients with antihistamines and steroids avoided such reactions. Studies in this phase showed efficacy of taxol against ovarian cancer.

In phase II, the activity of drug was tested on a large number of patients, and these trials are still being conducted. Some of the completed trials caused great excitement and were quiet encouraging. Some of them even showed a 30 % improvement among patients with advanced ovarian cancer.

The main problem in phase III studies was the large amounts of the drugs that were needed in this step. The CRADA (Cooperative Research and Development Agreement) was won by the Bristol-Myers Company, and they did an outstanding job of marketing supplies of taxol available and conducting the studies in cooperation with the NCI that led to the successful marketing approval in refractory ovarian cancer in 1992. The next major clinical development of taxol was the report of activity of taxol in primary metastatic breast cancer with a response rate of 56 %. It seems that breast and ovarian cancer are not the only taxol-treatable cancers [15].

2.8 Biosynthesis

Taxol biosynthesis consists of three distinct aspects:

(a) *Formation and functionalization of the diterpene moiety*

Terpenes share a biosynthetic pathway that forms isopentenyl pyrophosphate (IPP) from acetic acid. All the various classes of terpenes are then generated from IPP.

Diterpenes are produced from cyclization of geranylgeranyl pyrophosphate (GGPP), and the taxane ring system formation can be proposed to go by an electrophilic cyclization of GGPP, possibly from cembrane diterpenes or verticillin intermediates. The large number of functionalities present in taxol must be introduced into the diterpenoid ring system following the generation of the taxane ring system, and functionalization includes hydroxylation, acetylation of hydroxyl groups, oxidation to a ketone, and generation of the oxetane ring system.

(b) *Formation of the phenylisoserine side chain*

This part is usually made from phenylalanine.

(c) *Formation of taxol from its components*

The assumption would be that the terpene moiety is fully elaborated to the level of baccatin III and the C-13 side chain is attached last [16].

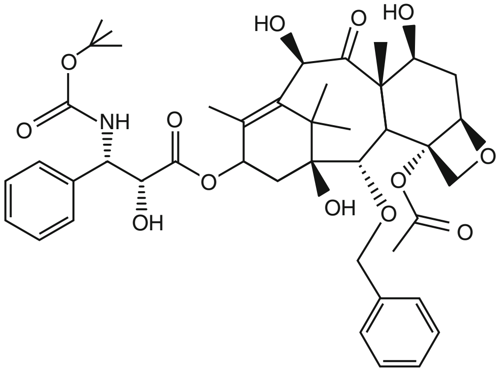

Fig. 101.4 Structure of docetaxel

3 Docetaxel (Taxotere)

Due to scarcity of paclitaxel, extensive research was carried out leading to the formulation of docetaxel (Fig. 101.4), an esterified product of 10-deacetyl baccatin III, which is extracted from the renewable and readily available leaves of European yew tree.

Docetaxel differs from paclitaxel at two positions in its chemical structure. It has a hydroxyl functional group on C-10 (see Fig. 101.1) whereas paclitaxel has an acetate ester, and a tert-butyl carbamate ester exists on the phenylpropionate side chain instead of the benzyl amide in paclitaxel. The C-10 functional group change causes docetaxel to be more water soluble than paclitaxel.

Docetaxel usually given to treat breast cancer, prostate cancer and non-small cell lung cancer either alone or incombination with other chemotherapeutic agents.

3.1 Mechanism of Biological Activity

The cytotoxic activity of docetaxel is exerted by promoting and stabilizing microtubule assembly, while preventing physiological microtubule depolymerization/disassembly in the absence of GTP. This leads to a significant decrease in free tubulin, needed for microtubule formation, and results in inhibition of mitotic cell division between metaphase and anaphase, preventing further cancer cell progeny.

Because microtubules do not disassemble in the presence of docetaxel, they accumulate inside the cell and cause initiation of apoptosis. Apoptosis is also encouraged by the blocking of apoptosis-blocking bcl-2 oncoprotein. Both in vitro and in vivo analyses show that antineoplastic activity of docetaxel against a wide range of known cancer cells would be greater than paclitaxel, possibly due to its more rapid intracellular uptake.

The main mode of therapeutic action of docetaxel is the suppression of microtubule dynamic assembly and disassembly, rather than microtubule bundling leading to apoptosis, or the blocking of bcl-2 [17].

3.2 Toxicology

Docetaxel is a chemotherapeutic agent and is a cytotoxic compound and so is effectively a biologically damaging drug. As with all chemotherapy, adverse effects are common and many varying side effects have been documented. Because docetaxel is a cell cycle specific agent, it is cytotoxic to all dividing cells in the body including tumor cells as well as hair follicles, bone marrow and other germ cells. For this reason, common chemotherapy side effects such as alopecia occur that sometimes can be permanent. However, the drug company Sanofi-Aventis claims that they do not routinely keep this data. Northwest France is conducting a survey to establish exactly how many patients are being disfigured in this way. Independent studies show it could be as high as 6.3 % which puts this ASE in the "common and frequent" classification.

Hematological adverse effects include neutropenia (95.5 %), anemia (90.4 %), febrile neutropenia (11.0 %), and thrombocytopenia (8.0 %). Deaths due to toxicity accounted for 1.7 % of the 2,045 patients, and incidence was increased (9.8 %) in patients with elevated baseline liver function tests (liver dysfunction) [18].

4 Natural Taxanes

The study of the secondary metabolites of the yew started in the nineteenth century, when a mixture of taxanes was obtained by a German pharmacist in 1856. These compounds were named as taxines, and also their structure characterization was extremely slow, due to the complexity of the structure and the need of modern spectroscopic instruments. Furthermore, the taxine obtained earlier had inconsistent physical properties (m.p.: 82–124) and later was shown to be a mixture of at least seven compounds. Seven years later, in 1963, the constitution of the taxane nucleus was elucidated for the first time as tricyclic esterified polyols with acids, such as taxinine whose stereochemistry was established 3 years later.

Systematic studies on the non-alkaloidal components of the yew tree were started in the late 1960s, and several new members of the taxane family were discovered. Incredible accomplishment shifted the attention of the scientists to paclitaxel and also attracted wide studies on various species of Taxus which led to the isolation of many new taxane family members. To date, over 100 taxanes have been isolated and structurally elucidated and divided into several structural subclasses:

4.1 Taxanes Having an Exocyclic Double Bond Between C-4 and C-20

This is the largest subclass of taxanes and including compounds with or without side chains at C-5; different oxidation states at C-1, C-13, and C-7; and ester functionalities at C-2, C-9, and C-10.

4.2 Taxanes Containing an Extra Bond Connecting Transannular Atoms

This class shares the common feature of a transannular bond, although the structure may look quite different. It is exemplified by taxinine K, taxinine L, and taxinine M. Change in the functional groups at C-1, C-2, C-5, C-7, C-9, C-10, and C-13 are also common, e.g., taxagifine.

4.3 Taxanes Having a C-4 Epoxide

This class contains relatively fewer compounds, including baccatin I, 1-hydroxy-baccatin I, C-5 isophenylalanine side chain derivatives, and some new derivatives having nicotinate substituent at C-9 [19].

4.4 Taxanes with an Exetane Ring at C-4 and C-5

This class of taxanes, such as paclitaxel as a characteristic compound, is usually found in quite small quantities in different parts of the yew tree, but they seem to be the most interesting class of taxanes having promising anticancer potential. Their key factor is a ketone group at C-9 and also a complex side chain at the C-13 position instead of C-5 position that is often observed in other classes of taxanes.

Baccatin III was the first compound of this class, isolated from the heartwood of *Taxus baccata* in 1966 by Chan et al., and its structure was elucidated [20]. Many compounds have been extracted from the bark of *Taxus baccata*, *Taxus wallichiana*, or *Taxus brevifolia* that differ only in the nature of the substituent at C-1, C-2, and C-4 positions.

An important biosynthetic precursor of paclitaxel, 10-deacetylbaccatin III, was also isolated in reasonably good yield from the leaves of *Taxus baccata* in 1981 and also from the bark of *Taxus brevifolia* in 1982. It serves as the starting material for the semisynthesis of paclitaxel through a coupling reaction with an appropriately protected side chain that can be prepared synthetically.

4.5 Miscellaneous Taxanes

Besides to the classes of taxanes discussed before, there are also some miscellaneous taxanes having a diversity of structural modifications in the tricyclic skeleton. Some examples include taxine A with a unique rearranged skeleton; brevifoliol (natural diterpene isolated from *Taxus baccata*) and taxchinine A (acetoxybrevifoliol) contain a five-membered A ring in place of the usual six-membered ring; hydroxylated analogs of the taxanes which have an exocyclic double bond between C-4 and C-20 (class A) and can also be viewed as the oxetane ring-opened compounds of the taxane class D [19].

New generation of taxoids with systematic modifications at the C-2, C-10, and C-3′positions was designed and led to the discovery of very promising candidates for future studies [21].

5 Conclusions

Paclitaxel and docetaxel, as very powerful anticancer drugs, proved to be a life saver for thousands of patients with various types of cancer. However, these drugs are expensive and difficult to synthesize and cause undesirable side effects as well as drug resistance, and their natural sources are limited. These mean that to remain taxol as an inexpensive drug in the pharmaceutical markets, separation of other biologically active compounds from renewable sources and new methods of synthesis or biotechnological cultivation techniques of plant cell and tissues to supply new generation of taxoids would be demanded.

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Diterpenes for Therapeutic Use

102

Virginia Lanzotti

Contents

1 Introduction 3174
2 Chemical Structure 3175
2.1 Biosynthetic Origin 3175
2.2 Linear Diterpenes and their Cyclization 3176
2.3 Bicyclic Diterpenes 3178
2.4 Tricyclic Diterpenes 3179
2.5 Tetracyclic Diterpenes 3179
2.6 Macrocyclic Diterpenes 3180
2.7 Biological Activity and Therapeutic Uses 3181
2.8 Bicyclic Diterpenes 3182
2.9 Tricyclic Diterpenes 3183
2.10 Tetracyclic and Pentacyclic Diterpenes 3184
2.11 Macrocyclic Diterpenes and Derivatives 3186
3 Conclusion 3189
References 3189

Abstract

Diterpenes are a structurally diverse class of C_{20} natural compounds, widely distributed in nature and originating by condensation of four isoprene units derived from mevalonate or deoxyxylulose phosphate pathways. The latter, recently discovered, originates the diterpene compounds in plants. Diterpenes can be classified as linear, bicyclic, tricyclic or tetracyclic, pentacyclic, and macrocyclic diterpenes depending on their skeletal core. In nature, they are commonly found in a polyoxygenated form with keto and hydroxyl groups, these last often esterified by small-sized aliphatic or aromatic acids.

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Diterpenes have attracted growing attention because of their interesting biological and pharmacological activities. Although thousands of diterpene compounds have been described in nature from terrestrial and marine organisms, only few of them became clinically effective. Overall, the anticancer drug taxol, used in therapy against ovarian, breast, and lung cancer, with its synthetic water-soluble analogue taxotere, is an example of unusual structure discovered from nature and used as medicine. Promising diterpenes are the ginkgolides showing potent and selective antagonistic activity toward platelet-activating factor increasing in conditions of shock, burns, ulceration, and inflammation skin diseases. Also used in therapy is the diterpene resiniferatoxin, an ultrapotent vanilloid, isolated from the *Euphorbia resinifera* latex, in clinical trials for bladder hyperiflexia and diabetic neuropathy. The diterpenes used in therapy will be described together with other promising bioactive diterpenes with particular attention to those isolated from plants.

Keywords

Bioactivity • biosynthesis • classes • diterpenes • plant • structure • therapy

Abbreviations

DMAPP	Dimethylallyl diphosphate
DXP	Deoxyxylulose
GGPP	Geranylgeranyl pyrophosphate
IPP	Isopentenyl diphosphate
MDR	Multidrug resistance
MVA	Mevalonic acid
PGP	p-Glycoprotein

1 Introduction

Diterpenes are a heterogeneous class of natural compounds based on 20 carbon atoms. They are widely distributed in nature, both in terrestrial and marine organisms, and originate by condensation of four isoprene units derived from mevalonate or deoxyxylulose phosphate [1] pathways, wherein the latter pathway was recently discovered taking place in the biosynthesis of diterpene compounds in plants [2]. As for other classes of natural compounds diterpenes have shown an importance as biomarker of certain species, thus contributing to their chemotaxonomy classification [3]. For example, macrocyclic diterpenes and their cyclization products have been isolated only from *Euphorbiae* plants of which they could be considered as biomarker [4]. Although thousands of diterpene compounds have been described in nature from terrestrial and marine organisms, only few of them became clinically effective [5]. Active compounds isolated from plants are used directly in therapy or as prototype of bioactive lead to develop more active and less toxic analogues. In this chapter, we describe the main structural type of diterpenes isolated from plants, their biosynthesis, biological activity, and therapeutic uses.

2 Chemical Structure

The most important diterpene skeletal types in terms of number and distribution are shown in Fig. 102.1, classified as bicyclic, tricyclic, tetracyclic and pentacyclic, and macrocyclic ring.

In nature, they are commonly found in a polyoxygenated form with keto and hydroxyl groups, the last free or esterified by small-sized aliphatic or aromatic acids.

2.1 Biosynthetic Origin

Diterpenes are formed by head (isopropylidene end) to tail (alcohol end) of four isoprene (C_5H_8) building blocks. The isoprene unit itself originates by the decomposition of natural cyclic hydrocarbons giving rise to the biochemically active isoprene units, identified as the pyrophosphate esters dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Scheme 102.1).

The decomposition reaction proceeds in nature by two biosynthetic routes: the mevalonate pathway, via mevalonic acid (MVA) (Scheme 102.2), and the recently discovered mevalonate-independent pathway, via deoxyxylulose phosphate (DXP) (Scheme 102.3), the latter taking place in plants. In Scheme 102.2, a schematic representation of the mevalonate pathway is shown in which three molecules of acetyl-coenzyme A derived by carbohydrate, fat, or protein catabolism yield by aldol-type reaction β-hydroxy-β-methylglutaryl-coenzyme A (HMG-CoA), which is irreversibly reduced to *R*-mevalonic acid (MVA), whose phosphorylation and elimination reaction afford IPP. This molecule is converted by an isomerase into an equilibrium mixture with DMAPP.

Scheme 102.3 reports the mevalonate-independent pathway, also named as deoxyxylulose (DXP) or methyl erythritol phosphate pathway. It starts from pyruvic acid and D-glyceraldehyde to form 1-deoxy-D-xylulose 5P (DXP) reduced to 2 C-methyl-D-erythritol 4P which by phosphorylation affords a cyclic intermediate 2 C-methyl-D-erythritol 2,4-cyclophosphate. This compound by elimination reaction followed by tautomerization gives IPP and DMAPP, the latter possibly formed either independently or by isomerization.

IPP and DMAPP react as shown in Scheme 102.4 to generate geranyl pyrophosphate (GPP), whose further reaction with IPP produce farnesyl pyrophosphate (FPP) that reacts with another molecule of IPP to afford geranylgeranyl pyrophosphate (GGPP), representing the linear diterpene C_{20} precursor.

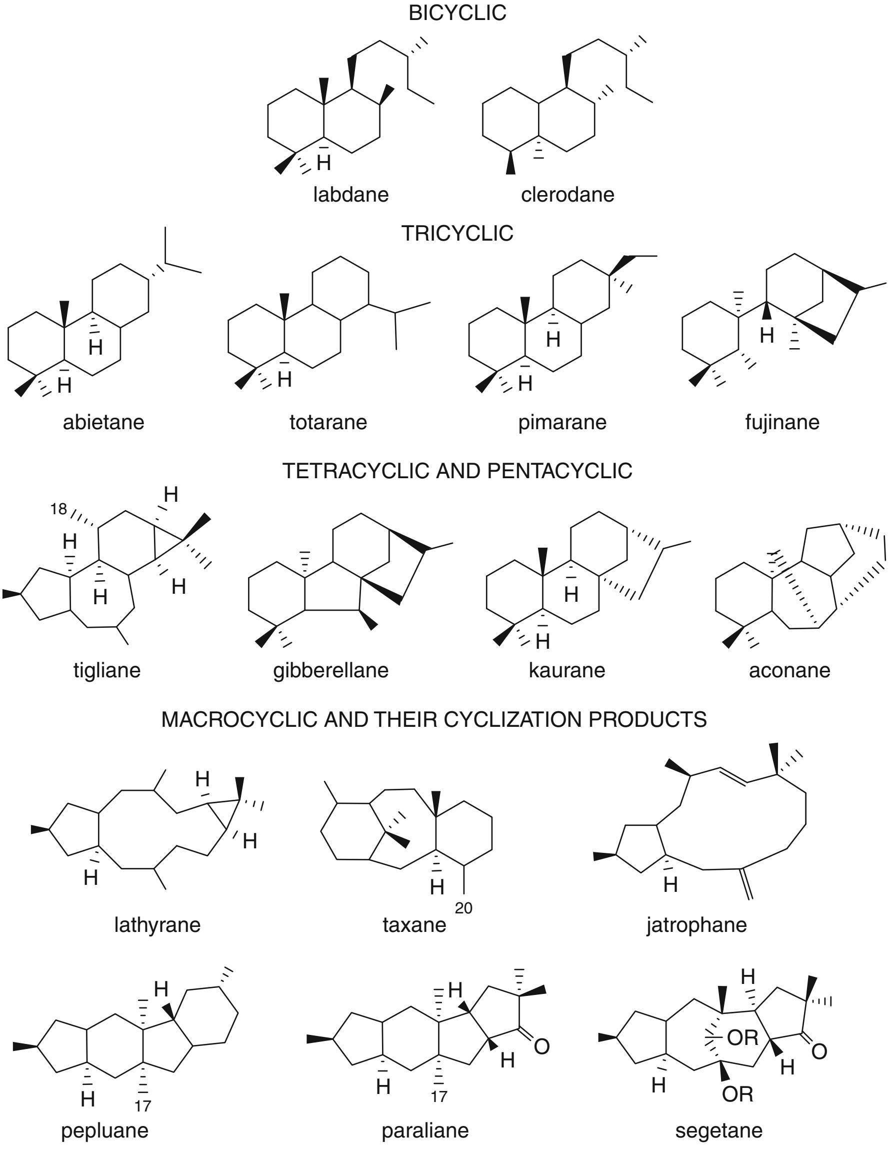
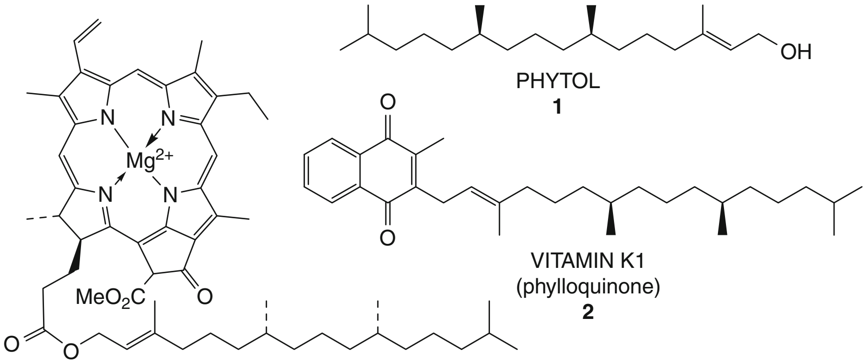

BICYCLIC
H
labdane
clerodane
TRICYCLIC
H
H
abietane
H
totarane
H
H
pimarane
H
fujinane
TETRACYCLIC AND PENTACYCLIC
18
H
H
H
H
tigliane
gibberellane
H
H
kaurane
aconane
MACROCYCLIC AND THEIR CYCLIZATION PRODUCTS
H
H
H
lathyrane
H
20
taxane
H
jatrophane
H
H
17
pepluane
H
O
H
H
17
paraliane
H
OR
O
H
H
OR
segetane

Fig. 102.1 Main diterpene skeletal types

2.2 Linear Diterpenes and their Cyclization

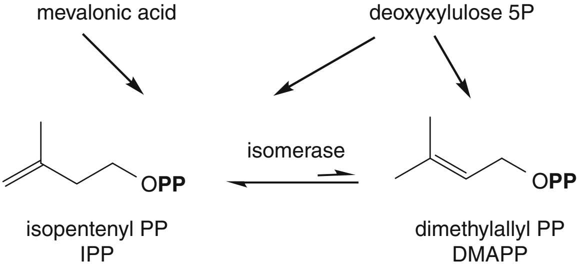
Linear diterpenes are less distributed in nature. Phytol (**1**), a reduced form of *geranylgeraniol*, is an example of linear diterpenes, constituting the side-chain of chlorophylls, for example, phylloquinone, the vitamin K$_1$ (**2**) and chlorophyll A (**3**).



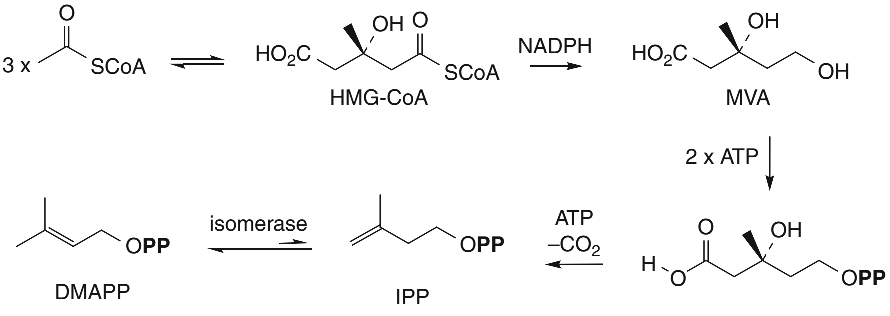
PHYTOL
1

VITAMIN K1
(phylloquinone)
2

CHLOROPHYLL A
3

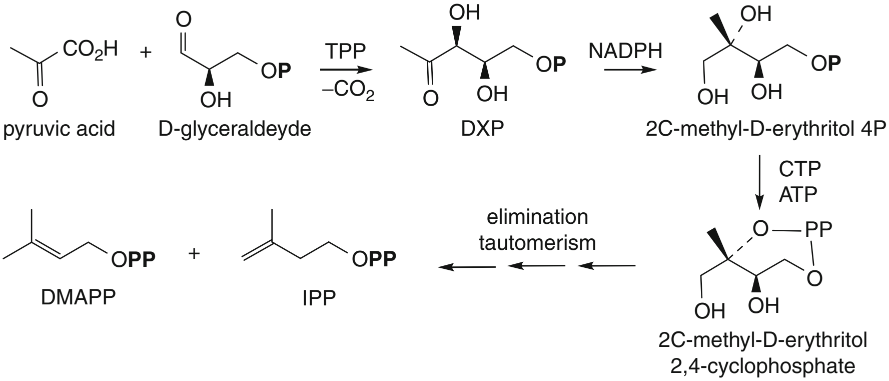

mevalonic acid
deoxyxylulose 5P
isomerase
OPP
OPP
isopentenyl PP
IPP
dimethylallyl PP
DMAPP

Scheme 102.1 The isoprene units, IPP and DMAPP obtained by mevalonic acid and deoxyxylulose 5P

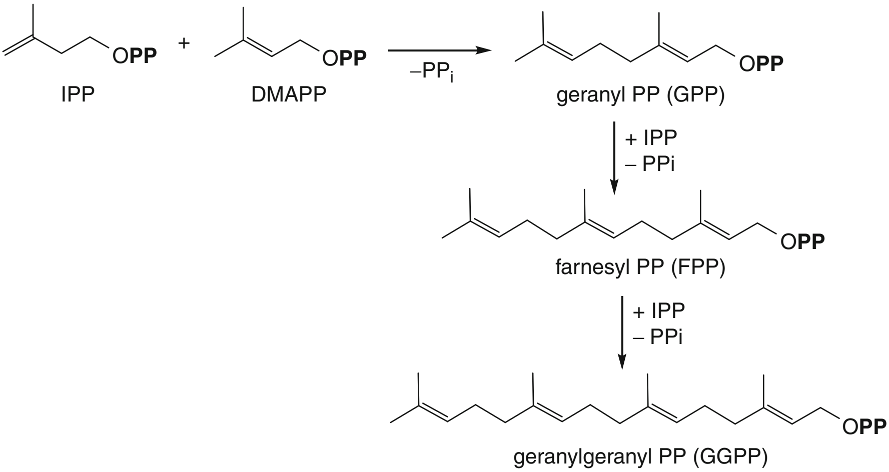

3 x
SCoA
HMG-CoA
NADPH
MVA
2 x ATP
ATP
–CO2
isomerase
DMAPP
IPP
OPP

Scheme 102.2 The mavalonate pathway

Most abundant are instead cyclic diterpenoids that originate by cyclization reactions of GGPP from both sides of the molecule: tail (alcohol end) or head (isopropylidene end). Cyclization generally involves carbocations and proceeds by a concerted addition mechanism generating different cyclic systems, due to the folding of the acyclic substrate chain on the specific enzyme and originating rich and wide array of cyclic systems [6].


pyruvic acid
D-glyceraldeyde
TPP
−CO2
DXP
NADPH
2C-methyl-D-erythritol 4P
CTP
ATP
2C-methyl-D-erythritol
2,4-cyclophosphate
elimination
tautomerism
DMAPP
IPP

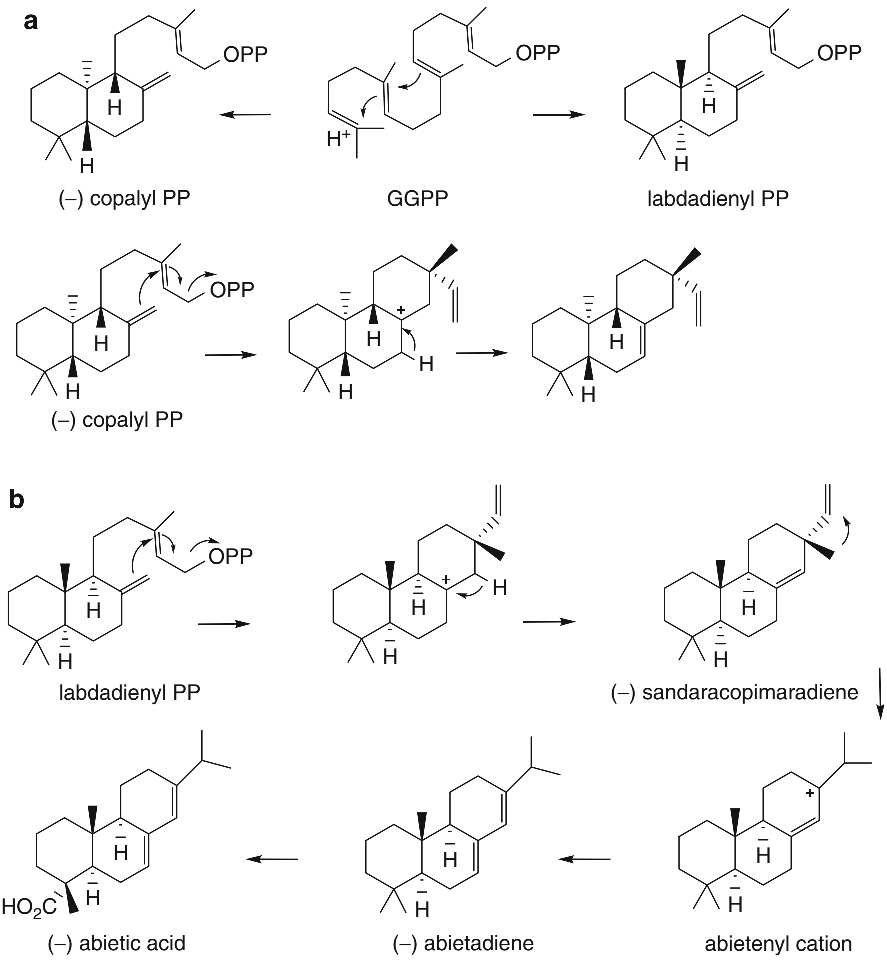
Scheme 102.3 The deoxyxylulose 5P pathway


IPP
DMAPP
−PPi
geranyl PP (GPP)
+ IPP
− PPi
farnesyl PP (FPP)
+ IPP
− PPi
geranylgeranyl PP (GGPP)

Scheme 102.4 The biosynthesis of GGPP

2.3 Bicyclic Diterpenes

The cyclization of GGPP is initiated by a proton attach, followed by a concerted cyclization sequence terminated with the loss of a proton from a methyl thus obtaining (−) copalyl PP or its enantiomer (+) copalyl, commonly known as labdadienyl PP (Scheme 102.5) depending on the enzyme surface.


a
OPP
H
H
(–) copalyl PP
H+
GGPP
labdadienyl PP
(–) copalyl PP
+
b
labdadienyl PP
(–) sandaracopimaradiene
HO2C
(–) abietic acid
(–) abietadiene
abietenyl cation

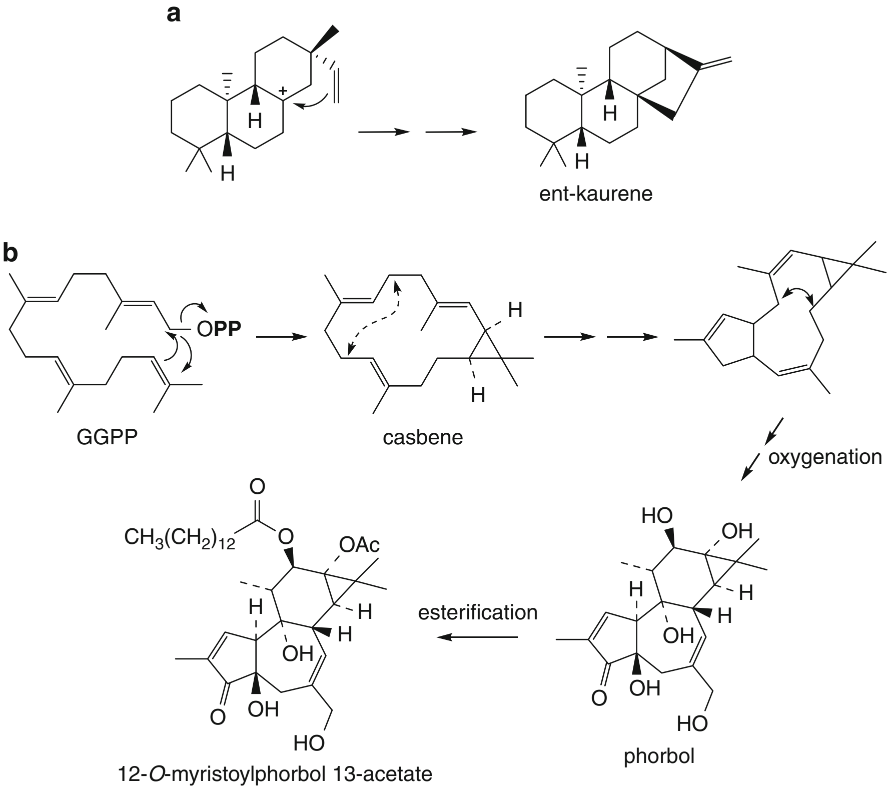
Scheme 102.5 The biosynthesis of tricyclic diterpenoids from (−)-copalyl PP (a) and labdadienyl PP (b)

2.4 Tricyclic Diterpenes

Scheme 102.5 shows the formation of tricyclic diterpenoids derived from (−)-copalyl PP (a) and from labdadienyl PP (b). These compounds give rise to further cyclization and rearrangements as shown in the following paragraph.

2.5 Tetracyclic Diterpenes

The biosynthesis of kaurane diterpenoids shown in Scheme 102.6a starts from the cyclic carbocation formed by cyclization of copalyl PP.


a
H
H
H
H
ent-kaurene
b
OPP
GGPP
H
H
casbene
oxygenation
O
$CH_3(CH_2)_{12}$
O
OAc
H
H
H
OH
O
OH
HO
12-O-myristoylphorbol 13-acetate
esterification
HO
OH
H
H
H
OH
O
OH
HO
phorbol

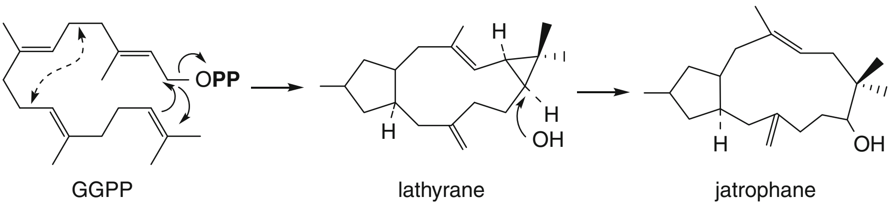
Scheme 102.6 The biosynthesis of tetracyclic diterpenoids based on: (a) ent-kaurene and (b) phorbol skeletons

In Scheme 102.6b reports the key biosynthetic steps for the formation of atisane-type diterpenoids based on a tigliane tetracyclic skeleton whose consecutive oxidation affords phorbol that is esterified in nature giving rise to phorbol ester such as that shown in the Scheme, 12-O-myristoylphorbol 13-acetate.

2.6 Macrocyclic Diterpenes

Scheme 102.7 reports the formation of lathyrane and jatrophane skeletons starting from GGPP.

They are poly-oxygenated with keto groups and alcoholic function often esterified with small-sized aliphatic (acetic, propionic, butyric, iso-butyric)



Scheme 102.7 The cyclization of GGPP affords the macrocyclic lathyrane and jatrophane diterpenes

and aromatic (benzoic and nicotinic) acids. These compounds are less distributed in nature and are *Euphorbiaceae* plants. Their further cyclization affords the derivatives reported in Fig. 102.1.

2.7 Biological Activity and Therapeutic Uses

The biological significance of diterpenoids in plants is part of the general question concerning the role of secondary metabolites in the living organisms. It is unrealistic to expect a unique function for a class as a whole, composed as they are, of compounds of such varied and often restricted distribution, or to expect a biological role for each one of its members. A number of diterpenoids with insect growth regulatory activity [7], insect antifeedant [8], or insecticidal activity [9] have been isolated from higher plants. Insect chemistry has revealed the importance of several diterpenoids as pheromone [10] or defense chemicals [11]. Furthermore, several soft corals elaborate diterpenoids as highly toxic to fish [12].

Specifically, in the area of diterpenoids, a clear physiological role is found for gibberellins based on gibberellane skeleton (Fig. 102.1) as endogenous plant growth factor [13]. These diterpenoid acids were originally isolated from the fungus *Gibberella fujikuroi*, a pathogen which causes overgrowth of rice seedlings (tall straggly plants are obtained). The gibberellins are shown to be the causative agents of this syndrome, but they have been also isolated from many healthy plants. Indeed they seem to be ubiquitous plant hormones, responsible for increased growth, and induction of flowering. Phytol (Fig. 102.1) is an integrated part of chlorophyll constituting its lipophilic side chain necessary for the biological activity [14]. Retinol (Fig. 102.2), the vitamin A, originating by beta-carotene, is essential for mammalian growth. Retinol and related compounds are involved in the visual function of not only mammals, but also insects and fish [15].

Besides the above diterpenoids, which are basic to certain life processes, a number of diterpenoids with a variety of biological activities that may be essential for the welfare of the organism producing it have been isolated. These compounds differ from the earlier mentioned diterpenes in that their natural distribution is

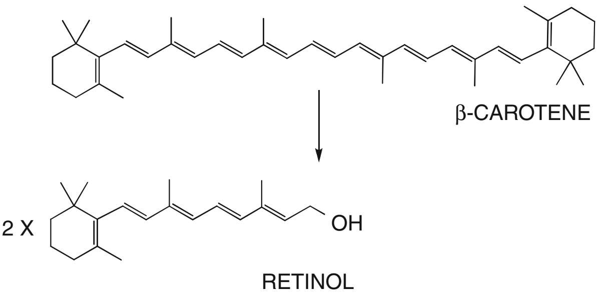


Fig. 102.2 Biosynthesis of retinol by the break of the central double bond of β-carotene

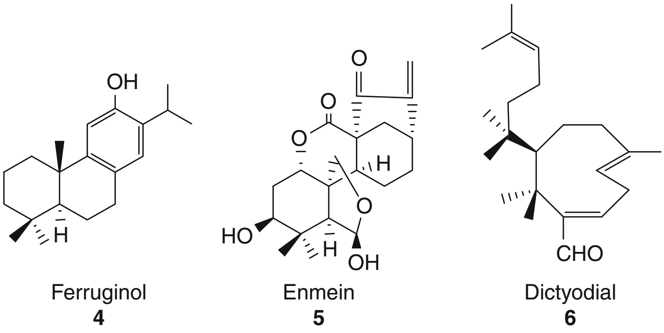
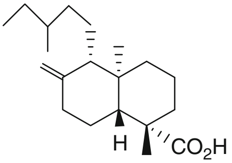


Fig. 102.3 Natural antibiotics from terrestrial and marine organisms

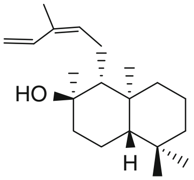
highly restricted. A number of diterpenes with plant growth regulatory activities have been described. Some kaurane (Scheme 102.6a) derivatives, for example, mimic gibberellin activity [16]. A number of diterpene antibiotics (Fig. 102.3) have been isolated from higher plants: ferruginol (**4**) [17] from *Podocarpus ferrugineus* resin and enmein (**5**) [18] from the leaves of *Isodon trichocarpus* have shown, respectively, antifungal and antibacterial activity. Likewise, several marine organisms are able to elaborate antibiotic compounds, for example, dictyodial (**6**) [19] from *Dictyota crenulata and D. flabellata.*

2.8 Bicyclic Diterpenes

Over a thousand of diterpenes based on a labdane and clerodane skeleton (Fig. 102.1) have been isolated from plants, marine organisms, fungi, and bacteria. Among them, those based on a labdane skeleton found in conifer plants (genus *Juniperus*, family Cupressaceae) and named communic acids (**7**) showed promising biological activities. In fact, antibacterial, antitumoral, hypolipidemic, relaxing smooth muscle activities have been reported and reviewed recently [20]. They were also used as building blocks for the semi-synthesis of other interesting bioactive compounds, such as quassinoids and antioxidant abietanes.



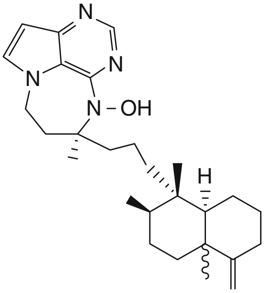
Communic acid
7



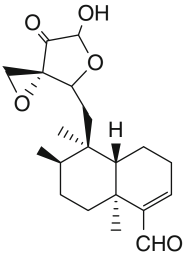
cis-abienol
8

The labdanoyl diterpene cis abienol (**8**) is a major component of the aromatic oleoresin of balsam fir, *Abies balsamea*, and it is used in the fragrance industry [21]. It is implicated in the biosynthesis of abietadiene/sandaracopimaradiene skeletons being an intermediate of the biosynthesis of these compounds in plants, used for fragrance production.

Also the clerodane class of diterpenes exhibited a wide range of biological activities. Of particular interest is asmarine A and B (**9**) [22] that showed antiproliferative activity against several human cancer cell lines and, clerocidin (**10**) [23], a naturally occurring antibiotic showing also anticancer and antimicrobial activities.



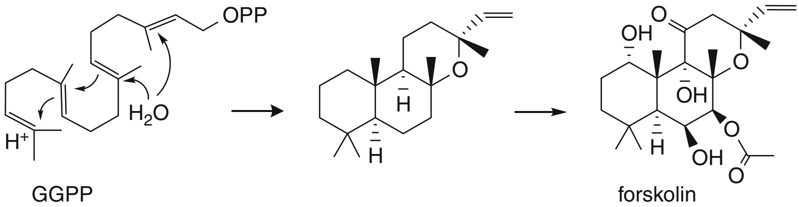
asmarine A,B
9



clerocidin
10

2.9 Tricyclic Diterpenes

Among these compounds, of particular interest is forskolin (Scheme 102.8) whose third ring is heterocyclic rather than carbocyclic. It has been isolated from *Coleus forskohlii* a plant used in Indian traditional medicine as hypothensive and in reducing spasm of the intestine. Forskolin (**11**), whose biosynthesis is



forskolin
11

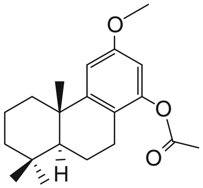
Scheme 102.8 The biosynthesis of forskolin

showed in Scheme 102.8, is present in 0.1 % yields in the extract, is able to stimulate adenyl cyclase activity having effects on cardiovascular disease and possessing broncho-spasmolytic activity [24]. Its use is limited by the poor solubility in water and for this reason the research is aiming at developing analogues with increased solubility.

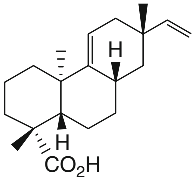
Totarol (**12**) is a compound isolated from totara tree together with several analogues. The compound was found to potentiate the antibiotic methycillin by reducing the minimum concentration against resistant *Staphylococcus aureus* [25].

Cyclization of copalyl PP affords (−)-acanthoic (**13**) acid that showed a potent anti-inflammatory property, whose synthesis was recently developed [26].

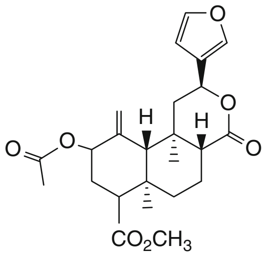
The tricyclic diterpene salvinorin A (**14**) is a trans-clerodane diterpenoid isolated from the Mexican plant *Salvia divinorum*. It acts as a kappa opioid receptor agonist and it is the first non-alkaloid compound acting on this receptor [27]. Salvinorin A is the most potent hallucinogen isolated from plants and it is also capable of inhibiting excess intestinal motility (e.g., diarrhea), through a combination of k-opioid and cannabinoid receptors.



Totarol
12



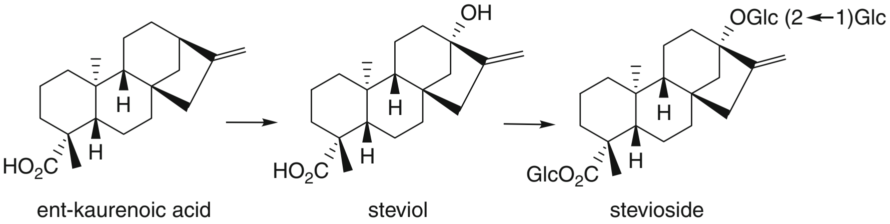
Acanthoic acid
13



Salvinorin A
14

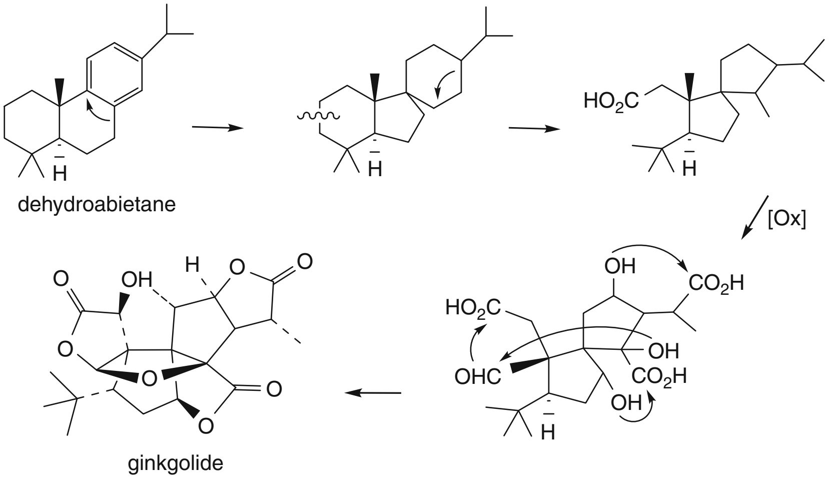
2.10 Tetracyclic and Pentacyclic Diterpenes

This is a large class of diterpenes originating by further cyclization and rearrangement reactions of the tricyclic fused rings. The therapeutic effects


OH
OGlc (2←1)Glc
H
H
HO2C
GlcO2C
ent-kaurenoic acid
steviol
stevioside

15

Scheme 102.9 The biosynthesis of stevioside

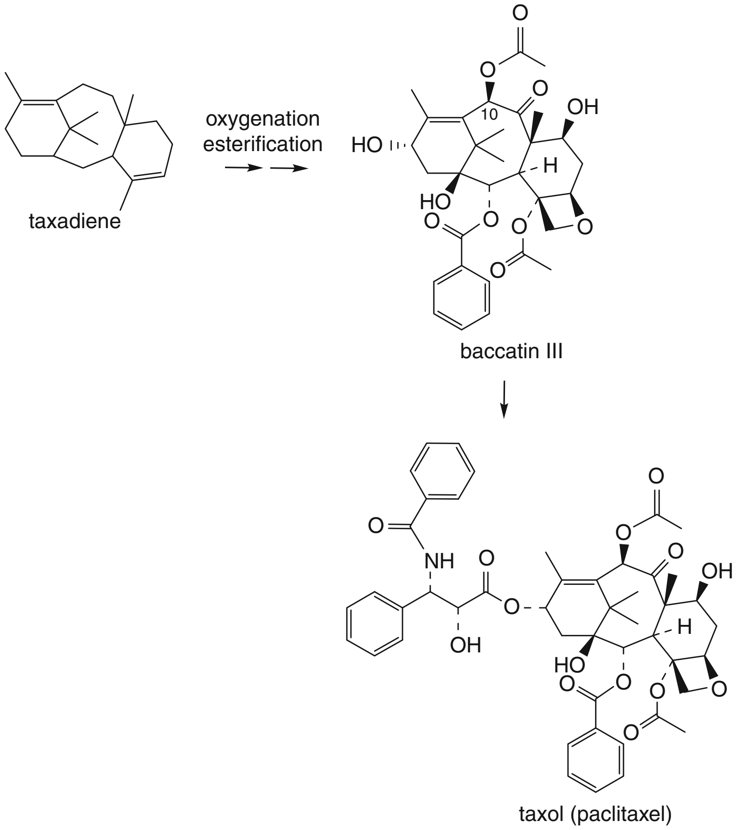

H
dehydroabietane
HO2C
[Ox]
OH
CO2H
OHC
CO2H
O
OH
H
ginkgolide

16

Scheme 102.10 The biosynthesis of ginkgolide

of members of this group of compounds are well studied. Among others, diterpenes from *Stevia* and from *Ginkgo* received particular attention from food industries. Stevioside (**15**), closely related to ent-kaurenoic acid and steviol (Scheme 102.9), is isolated from *Stevia rebaudiana* in large amounts (3–10 %) and it is 100–300 times as sweet as sucrose because of the presence of glucosyl ester and glucoside linkages in the molecule [28]. For this reason it has received increasing attention in the last years and it is now commercialized as sweetening agent.

Ginkgolides (**16**) have been isolated from *Ginkgo biloba* in high yields (0.1–0.25 %) [29]. The biosynthetic route for their formation is reported in Scheme 102.10. Extracts of the plant have been used traditionally to improve

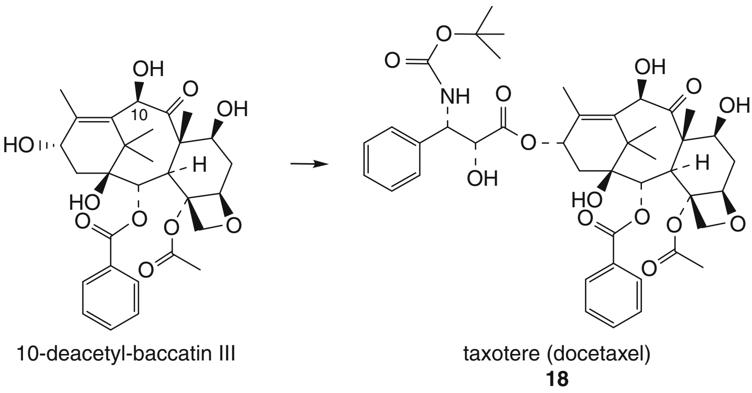


Scheme 102.11 The biosynthesis of taxol (paclitaxel)

peripheral and cerebrovascular diseases. For this reason, extracts are marketed against both cerebral and cerebrovascular circulation and the decline of cognitive function and memory processes of old age.

2.11 Macrocyclic Diterpenes and Derivatives

Among this class of compounds of great relevance is taxol (**17**), also named paclitaxel, based on taxane skeleton (Fig. 102.1), extracted on 1971 from the bark of the Pacific yew, *Taxus brevifolia* [30]. This compound has attracted growing attention because of its anticancer activity against several tumor cell lines not responding to other treatments, such as ovarian and breast cancers, non-small-cell and small-cell lung cancer, and cancers to the head and neck. Although, the drug is

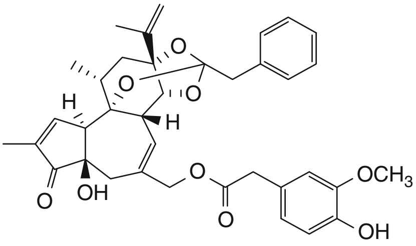


Scheme 102.12 The semi-synthesis of taxotere (docetaxel) from 10-deacetyl-baccatin III

used successfully in cancer therapy, a problem to be addressed is its supply because the 2 g necessary for one treatment is extracted from the barks of six plants which are 100 years old. Taxol derives biosynthetically from taxadiene whose oxygenation on the skeleton and esterification afford 10-deacetyl-baccalin III further esterified to taxol (Scheme 102.11). The problem of the taxol supply has been addressed by developing its semi-synthesis at increasing yields, starting from baccatin III e 10-deacetylbaccatin III, both present in *Taxus baccata*, the common yew, growing in Europe and the USA. The research in this field is also using microorganism, such as the fungi *Taxomyces adreanae* and *Pestalotiopsis microspora*, isolated from the inner bark of *T. brevifolia* and *T. wallichiana* respectively, which is able to produce taxol and taxanes at higher levels. More recently, a new taxol analogue, taxotere (docetaxel) (**18**), has been prepared by semi-synthetic route from 10-deacetyl-baccatin III (Scheme 102.12). The compound shows higher water solubility than taxol and possesses the same key pharmacophoric elements: the four-membered oxetane ring and the complex ester side chain in the structure. Taxol acts as antimitotic by binding the microtubules, promoting their assembly from tubulin, and stabilizing them against depolymerization during cell division. Thus, the abnormal microtubulin-tubule equilibrium disrupts the normal mitotic spindle apparatus and blocks cell proliferation. Taxol is also able to bind a second target, a protein which blocks the process of apoptosis, thus allowing apoptosis to proceed.

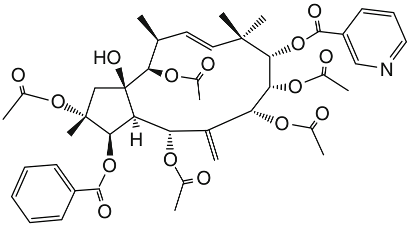
Resiniferatoxin, RTX, (**19**) is also an important drug isolated from the latex of *Euphorbia resinifera*, known since ancient times with the name *euphorbium* and used as purgatives, to remove warts and as a cathartic, in asthma and bronchial catarrh [30]. RTX is an ultrapotent vanilloid in clinical trials for bladder hyperiflexia and diabetic neuropathy [31].

The Euphorbiaceae plants are also called spurges, from the Latin word *expurgare* meaning to purge out, since these plants produce a toxic latex which can cause poisoning in humans and animals, skin dermatitis, cell proliferation, and tumor promotion (co-carcinogen activity). These severe irritant effects, especially on mucous membranes and the eye, are due to the diterpene esters, mainly esters of phorbol (Scheme 102.6) which activate protein kinase C, an important and widely distributed enzyme responsible for phosphorylating many biochemical entities. The permanent activation of protein kinase C is thought to lead to the uncontrolled cancerous growth.



resiniferatoxin
19

Recently, the discovery of interesting pharmacological properties of diterpenoids has opened new frontiers for research studies on *Euphorbia* genus. In fact, examination of other *Euphorbiaceae* yielded unusual diterpenoids, which make this plant family a prolific producer of jatrophanes [32] and of their cyclization products: lathyranes [33], pepluanes [34], paralianes [35], and segetanes [36]. These diterpenes showed antibacterial, anticancer, PGE_2-inhibitory, prolyl endopeptidase inhibitory, antifeedant, anti-HIV, analgesic, anti-inflammatory, and P-glycoprotein (PgP) inhibitory activity [37]. This protein is an interesting pharmacological target belonging to the class of ATP-binding proteins. It has been recognized as the biological factor responsible for the incoming of cross-resistance phenomena to drugs, known as multidrug resistance (MDR). MDR is particularly relevant in clinical practice: in fact some cancers (e.g., colon and kidney adenocarcinoma) are resistant to several chemiotherapeutic agents, while in others (e.g., leukemia and infantile neuroblastoma) after an initial success, a resistance to the pharmacological therapy appears. Among the tested diterpenes, pepluanin A (**20**) appears as the most promising drug having an activity two times higher than the drug cyclosporine A in reducing the resistance of cancerous cell lines to the cytotoxic drug daunomycin [38].



pepluanin A
20

3 Conclusion

The chapter describes the chemical structure and biological activities of the diterpenes isolated from nature. Particular attention is given to the biosynthetic routes affording a wide array of chemical diversity in this class of isoprenoids. The diversity in chemical structure of these compounds reflects also diversity in their distribution in the organisms, thus helping in chemotaxonomic classification. As the other classes of natural compounds, their role in the organisms has not yet been clarified [39]. However, they have attracted growing attention because of their interesting biological and pharmacological activities. Although thousands of diterpene compounds have been described from terrestrial and marine organisms, only few of them became clinically effective. Overall, the anticancer drug taxol, used in therapy against ovarian, breast, and lung cancer, with its synthetic water-soluble analogue taxotere, is an example of unusual structure discovered from nature and used as medicine. Other diterpenes used in therapy are the ginkgolides, forskolin, and resiniferatoxin.

Thus, diterpenes have shown a physiological role in the organisms (gibberellin, phytol, vitamin A, retinol), a specific taxonomic distribution (jatrophanes, lathyrane), a variety in their scaffold, inspiring chemists for the developing of parallel synthesis, and last but not least a huge array of therapeutic uses.

References

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Diterpene Sweeteners (Steviosides) 103

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Contents

1 Introduction 3194
2 Chemical Structure and Sweetness Property of Steviosides 3195
3 Analysis of Steviosides 3195
4 Stevioside Content in *Stevia rebaudiana* Bertoni 3196
5 Purification of Steviosides 3197
6 In Vitro Propagation of *Stevia rebaudiana* Bertoni 3197
7 Biosynthesis of Steviosides 3198
8 Steviosides in Food Industry 3198
9 Sensory Properties of Steviosides 3199
10 Safety Evaluation of Steviosides 3199
11 Standard Specifications of Steviosides in Various Countries 3200
12 Health Benefits of Steviosides 3200
13 Conclusion 3201
References 3201

Abstract

Stevioside is an ent-kaurene type diterpenoid glycoside isolated from leaves of *Stevia rebaudiana* Bertoni. Leaves of this plant produce zero-calorie diterpene glycosides (stevioside and rebaudioside), a non-nutritive, high-potency sweetener. Stevioside content in leaves will vary in field grown plants and is also influenced by agroclimatic conditions. *Stevia* leaves are also in use for their medicinal benefits. In this chapter, natural distribution and cultivation of *Stevia*,

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in vitro propagation, analysis of steviosides, purification of steviosides, their structural characteristics, biosynthesis, applications in food industry and health benefits are presented.

Keywords

HPLC • *Stevia* • steviosides

Abbreviations

4-PU	Cytokinin *N*-4-(Pyridyl)-*N*′-phenylurea
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
IPP	Isopentenyl-diphosphate
LC-MS-ESI	Liquid chromatography mass spectrometry electrospray ionization
MEP	2-C-methyl-D-erythritol-4-phosphate
MVA	Mevalonic acid

1 Introduction

Stevia rebaudiana Bertoni is native to tropical and subtropical regions of North America and South America. There are nearly 240 species of *Stevia*. It is grown widely in countries like Brazil, Colombia, Paraguay, and Venezuela. *Stevia rebaudiana* Bertoni is a perennial herb of Asteraceae family [1]. *S. rebaudiana* (Bertoni) was rediscovered by Dr. M.S. *Bertoni* in 1888. Natural sweeteners are mainly plant constituents with over 75 sweet compounds reported. These sweet compounds fall mainly within the terpenoid, flavonoid, and protein compounds [2]. Among sweet compounds of plant origin, the most widely studied are steviol glycosides, that is, Stevioside and Rebaudioside A. It is a natural, noncaloric sweet-tasting plant used around the world for its intense sweet taste [3, 4]. The crop was initially established in Japan, subsequently in China. Standardized extract and pure stevioside was utilized commercially in Japan for sweetening and flavoring foods and beverage as a substitute for several synthetic sweeteners. *Stevia* is distinguished by the presence of the sweet diterpene glycosides: rebaudioside A, rebaudioside C, stevioside and dulcoside in its leaf tissue [5, 6]. Due to the high concentration of such sweet principles in the leaves of Stevia plants, these are known as honey leaf of sweet chrysanthemum or “sweet herb of Paraguay.” These sweeteners impart 250 times sweetness than sucrose. *Stevia* has been recognized as a crop of economic value and is now cultivated in India, China, Japan, Taiwan, Thailand, Korea, Brazil, Malaysia [7], some European countries, and all over South America [8]. Sweet extracts of *Stevia* are being used commercially in many countries for sweetening a variety of products including pickled vegetables, sea foods, soft drinks, soy sauce, and confectionary products [9]. Reportedly, *Stevia* extract represents more than 40 % of the high-intensity sweetener market in Japan.

In India, *Stevia* has been successfully cultivated in recent years in many areas of Indian states: Rajasthan, Maharashtra, Kerela, and Orissa. The increasing demands for natural sweeteners have driven the farmers in India toward large-scale *Stevia* cultivation.

2 Chemical Structure and Sweetness Property of Steviosides

Structurally, stevioside (13-[2-*O*-β-D-glucopyranosyl-X-glucopyran-osyl) oxy] kaur-16-en-19-oic-acid β-D-glucopyranosyl ester) is a glycoside with a glucosyl and a sophorosyl residue attached to the aglycone steviol, which has a cyclopentanon hydrophenanthrene skeleton. All of these isolated diterpenoid glycosides have the same chemical backbone structure (steviol) but differ in the residues of carbohydrate at positions C13 and C19 [10]. These eight glycosides consist of a diterpene backbone with a variety of substituent groups which include glucose, rhamnose, and/or xylose. The major components of the leaves are stevioside (5–10 % of total dry weight), rebaudioside A (2–4 %), rebaudioside C (1–2 %), and dulcoside A (0.4–0.7 %) [11]. The chemical structures of stevioside and its related compounds which include steviol, steviolbioside, stevioside, dulcoside A, B and rebaudioside A,B,D,E are shown in Fig. 103.1. The fold increase in sweetness of these glycosides shown in parenthesis compared to sucrose is dulcoside A (50–120), rebaudioside A (250–450), rebaudioside C (50–120), rebaudioside B (300–350), rebaudioside D (250–450), rebaudioside E (150–300), steviolbioside (100–125), and stevioside (300) [13]. Detailed studies with stevioside revealed that elongation of 13-*O*-glucosyl moiety up to a total number of four glucosyl units, accompanied by reduction of glucosylation at 19-*O*-glucosyl moiety, is associated with the enhancement of intensity of sweetness of the compound [14].

3 Analysis of Steviosides

High-performance liquid chromatography (HPLC), being more sensitive and accurate, has been adapted for the estimation of stevioside content in the samples [15–18]. The identity of stevioside and rebaudioside A was reportedly confirmed by LC–MS detection with electrospray ionization (ESI) in the negative ion mode [12]. For separation and quantification through HPLC analysis NH_2 column [19–21], C18 column [12, 16, 18], and carbohydrate columns [22] have been used. NH_2 columns have a high selectivity for all steviol glycosides. Detection by UV, mass spectrometry [23], and amperometry [24] has been reported. The separation of all eight of the known *S. rebaudiana* sweet glycosides, namely, Stevioside, Steviolbioside, Rebaudioside A–E, and Dulcoside-A has been reported [5]. Makapuguay et al. (1984) [19] reported on HPLC separation of the same compounds, using an NH_2 column with a linear gradient.

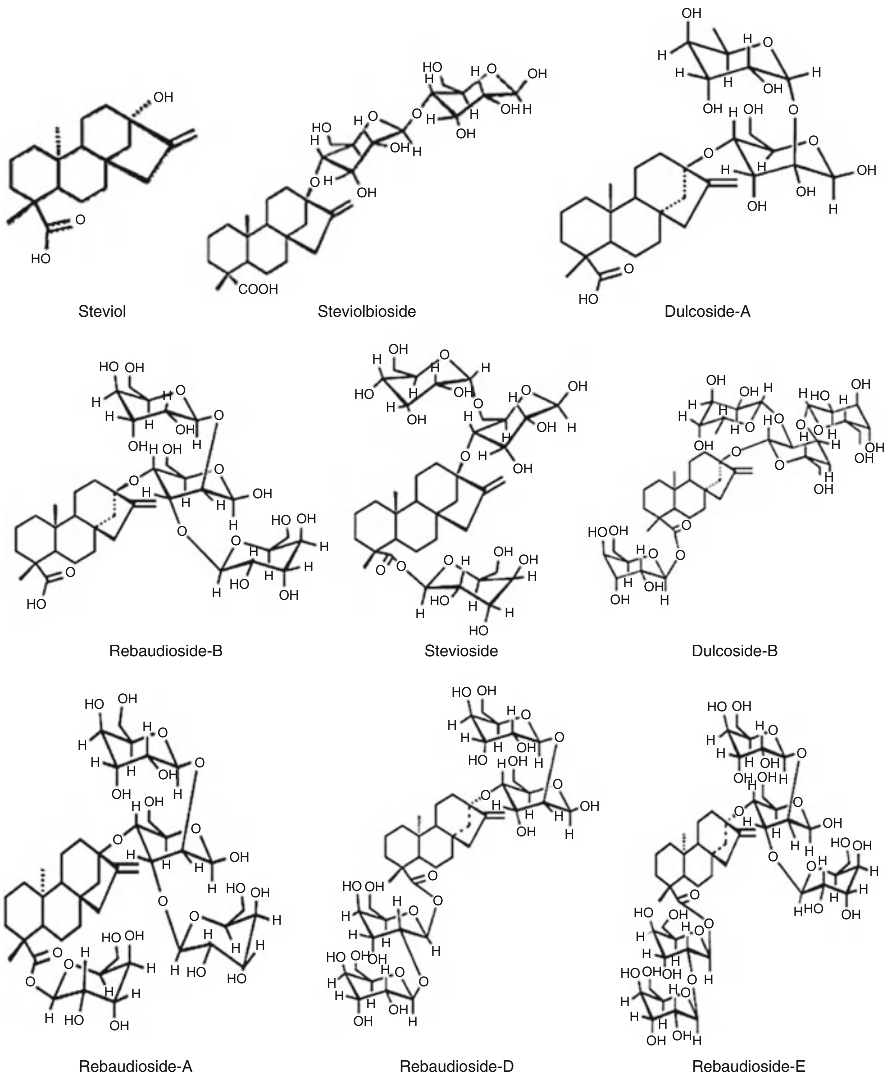
Steviol
Steviolbioside
Dulcoside-A
Rebaudioside-B
Stevioside
Dulcoside-B
Rebaudioside-A
Rebaudioside-D
Rebaudioside-E

Fig. 103.1 Major steviosides from *Stevia rebaudiana* Bertoni (Adapted from Rajasekaran et al. [12])

4 Stevioside Content in *Stevia rebaudiana* Bertoni

The leaves are found to contain a complex mixture of eight sweet diterpene glycosides, including stevioside, steviolbioside, rebaudiosides (A, B, D, E), and dulcoside A, B [25]. The most abundant among them are steviosides and rebaudiosides, depending on variety and growing conditions. Their amounts vary between 4 % and 20 %, depending on the plant cultivar and agroclimatic conditions like locations, latitude, altitude, topography, and rainfall [26–28]. The four most important steviol glycosides found in *Stevia* plant tissues are 5–10 % stevioside, 2–4 % rebaudioside A, 1–2 % rebaudioside C, and 0.5–1 % dulcoside A [29–31]. Rebaudioside B, D, and E may also be present in little quantities.

In India, the overall yield of stevioside was maximum from January harvest of second year crop (regenerated) (141.35 kg/hectare (ha)) in field grown plants and minimum in case of January harvest in the first year (27.74 kg/ha). In stem stevioside, yield was maximum in the regenerated crop harvested in September (60.71 kg/ha) followed by the regenerated crop harvested in January (48.02 kg/ha). Lowest stevioside yield in stem was obtained in the September harvest in the first year of the crop (14.75 kg/ha) [32]. Stevioside contents generally declined in the order leaves > shoots > roots > flowers. Steviosides proportion in leaves would change depending on the plant age and phase of development. The highest amount of the steviosides in leaves suggest both synthesis and primary accumulation of stevioside compounds occur in leaves [3]. Highest individual stevioside concentration in leaves from greenhouse plants was recorded for steviolbioside (64.80 g/kg dried plant).

5 Purification of Steviosides

Potential widespread use of steviosides requires an easy and effective extraction method. Enzymatic extraction of stevioside from *Stevia rebaudiana* leaves with cellulase, pectinase, and hemicellulase, using various parameters, such as concentration of enzyme, incubation time, and temperature, has been reported [33]. Purification is achieved through adsorption, column chromatography, and ion-exchange electrolytic technique for rebaudioside A, rebaudioside C, and dulcoside A [34]. An ultrafiltration (UF) process was reported for recovery of 45 % steviosides from an extract of leaves. Final purification was achieved by two consecutive mixed bed ion-exchange processes. The ion exchangers improved purity of the final product to 90 % [35].

6 In Vitro Propagation of *Stevia rebaudiana* Bertoni

The seeds of *Stevia* show a very low germination percentage [36], and propagation by seed does not allow the production of homogenous populations, resulting in great variability in important features like sweetening level and composition [37]. Micropropagation is commonly used to generate elite clonal plantlets in larger number in both economically important crops and ornamental plants. In vitro propagation of *S. rebaudiana* has been reported by a number of authors [37–39]. In vitro propagation of *Stevia* has been reported from nodal [1, 37, 40], leaf [41, 42], cell suspension cultures [43], and shoot apex [44–47]. The preferred method of developing uniform plant materials is through micropropagation adopting tissue culture methods. This provides consistent crop yield with fairly reproducible stevioside production. The induction of somatic embryogenesis from leaf explants on a medium supplemented with the cytokinin *N*-4-(Pyridyl)-*N*′-phenylurea (4-PU) has been reported [48]. Shootlets were regenerated from nodal explants of *Stevia rebaudiana* Bertoni through axillary shoot proliferation [49]. Mass propagation of shoots of *Stevia rebaudiana* using large-scale bioreactor has been reported [50]. Moreover, the influence of copper [51] and Fe-EDTA [52] in micropropagation and enhancement in biomass was reported in *Stevia rebaudina*. MS medium [53] supplemented with 3.0 mg/L Benzyl amino purine (BAP) was found to be optimum for shoot regeneration [54]. The influence of auxins and cytokinins in callus induction [55] and multiple shoot induction [56] from apical and nodal segments was also reported in S*tevia rebaudiana*. For the first time the production of stevioside in callus cultures of *S. rebaudiana* was reported [57]. Stevioside biosynthesis has been reported in callus, shoots, and rooted shoot cultures of *S. rebaudiana* [58].

7 Biosynthesis of Steviosides

Steviol glycosides are derived from the mevalonic acid pathway. The ent-kaurene skeleton of stevioside is formed *via* 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway [59, 60]. High activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in chloroplasts of *Stevia rebaudiana* has been reported [61]. Hence, the investigators anticipated mevalonic acid (MVA) as an intermediate of steviol biosynthetic route on the basis of the fact that HMG-CoA reductase is a key enzyme of the MVA route to isopentenyl-diphosphate (IPP).

8 Steviosides in Food Industry

Sweeteners are the most important ingredients in the food industry. Stevioside is an intense sweetener, and the extract of *Stevia* finds extensive use in Japan, China, Russia, Korea, Paraguay, Argentina, Indonesia, Malaysia, Australia, New Zealand, South America, and others, to sweeten local teas, medicines, food and beverages [62]. Being a low-calorie sweetener and dietary supplement for food, it was also approved decades ago and is commercially available as an alternative sweetener in foods and beverages [63]. It is estimated that about 30 million Indians are presently suffering from diabetes and by 2025 India’s contribution to the diabetic global population would be a whopping 89 million. The increasing consumption of sugar (sucrose) leads to excess calories, but also is not advisable for diabetic and obese patients. *Stevia* is likely to become a major source of high-potency sweetener for the growing natural food market in the future [64]. Therefore, low-caloric sweeteners have been investigated to substitute sugar [65]. Their organoleptic characteristics indicate that stevioside could be applied as a substitute for sucrose as sweetener in different products including pickled vegetables, seafoods, soft drinks, soy sauce, and confectionary products [66]. Stevioside levels in few foods have been shown in Table 103.1.

Table 103.1 Stevioside levels in foods

Food	Stevioside level (mg/kg)
Beverages (soft drinks, fruit drinks)	600
Breads	160
Biscuits	300
Cold confectionery	500
Desserts	500
Delicacies	1,000
Pickles	1,000
Sauces	1,000
Sweet corn	200
Yogurt	500

Adapted from The Joint Expert Committee on Food Additives (JECFA) at its 63rd, 68th, and 69th meeting [67]

At the present time, *Stevia rebaudiana* extract accounts for 40 % of the sweetener market in countries such as Japan, Korea, and Malaysia. *Stevia* extract and stevioside are officially approved as food additives in Brazil, Korea, Japan, and United States [68, 69]. Japan was the first country in Asia to market stevioside as a sweetener in food and drug industries.

9 Sensory Properties of Steviosides

Stevioside and rebaudioside A were tested for stability in carbonated beverages and found to be both heat and pH stable [22]. But, rebaudioside A was subject to degradation upon long-term exposure to sunlight. Several Japanese studies reveal that stevioside is highly stable [70]. Relative to other high-potency sweeteners, such as aspartame, bitterness tends to increase with concentration for both stevioside and rebaudioside A [71]. Both stevioside and rebaudioside A are synergistic in mixtures with other high-potency sweeteners such as aspartame and are good candidates for inclusion in blends [72].

10 Safety Evaluation of Steviosides

The toxicology and safety of stevioside used as a sweetener were studied by different investigators [73, 74]. Extensive safety and toxicological testing of stevioside have shown that it is safe for human consumption. Moreover, dental research suggests that the product may actually suppress the growth of oral microorganisms [75]. Nevertheless, in feeding experiments with rats and hamsters, stevioside was metabolized to steviol by the bacterial flora of the cecum [76]. Animal studies did not show any adverse effects or toxicity associated with stevioside consumption [77, 78]. Studies of acute toxicity, an LD_{50} of 8.2 g kg^{-1} for a refined stevioside extract, were cited by Katayama et al. [79]. The toxicology and safety of stevioside used as a sweetener were reviewed [80]. An acceptable daily intake (ADI) of 7.9 mg stevioside/kg body weight was suggested [81]. The safety of its use in food and beverages has been established and many regulatory boards around the world including the United States, Australia, New Zealand, and much of Asia and Latin America have permitted its usage for human consumption.

11 Standard Specifications of Steviosides in Various Countries

There are different views about standard specifications in different countries. In Japan and Korea its usage as a sweetener is permitted [69]. Initially the US government banned *Stevia* but in the year 2008, the extract of *Stevia* Rebaudioside-A was approved as food additive [81]. Today the number of countries permitting its usage is increasing. Governing bodies around the world have concluded that *Stevia* is safe for use as a general purpose sweetener. Specifically, in 2008 and 2009, the Food and Agriculture Organization/World Health Organization's Joint Expert Committee on Food Additives (JECFA), a global panel of food ingredient safety experts, and the United States Food and Drug Administration stated that the use of pure steviol glycosides (≥95 %) is safe for human consumption as a nonmedical ingredient up to 4 mg/kg of body weight/day. In 2011, the European Commission authorized the use of pure steviol glycosides (≥95 %) in foods and beverages across the European Union.

12 Health Benefits of Steviosides

The leaves of *Stevia* have insulin balancing properties [82]. Use of these sweetening compounds of *Stevia* has increased dramatically due to health concerns related to sucrose usage, such as dental caries, obesity, and diabetes. It is recommended for diabetes, has been extensively tested on animals, and also has been used by humans with no side effects. Several studies have reported the antihyperglycemic, insulinotropic, glucagonostatic, and antihypertensive effects of *Stevia* glycosides [83–85]. The sweet compounds pass through the digestive process without chemically breaking down, making it safe for diabetic and obese people, so there is no caloric intake [69]. These *Stevia* extracts have long been used in Southern Africa to treat diabetes [82]. Their ingestion causes a slight suppression of plasma glucose levels and significantly increased glucose tolerance in normal adult humans [82]. *Stevia* leaves are also in use for their medicinal benefits in hypertension, obesity, topical dressing for wounds, and other skin disorders [62]. Stevioside is reported to be beneficial for treating hypertension and hyperglycemia [83, 85]. Stevioside and related compounds are also reported to possess antitumor activity [86]. The anticancer efficacy of stevioside and six related compounds including the aglycones steviol and isosteviol were also evaluated [87]. Stevioside consumption and health effects have been shown in Table 103.2.

Table 103.2 Brief summary of published work on stevioside consumption and health effects

Stevioside consumption and health effects	Reference
Cardiovascular effects	[88]
Insulin balancing properties	[82]
Endocrine function	[89]
Topical dressing for wounds, and other skin disorders	[62]
Antitumor and anticancer activity	[86, 87]
Antihypertensive effects	[84]
Anti-inflammatory	[90]
Antidiarrheal activity	[91]

13 Conclusion

The demand for non-nutritive sweetener is ever increasing due to change in lifestyle, increased cases of diabetes and hypertensive patients, and enhanced longevity leading to geriatric-related problems. In this context, *Stevia* with its long history of usage as a source of sweetener is increasingly gaining attention. Already several countries have taken up its cultivation and processing for use as non-nutritive sweetener. Health benefits add value to the usage of steviosides or *Stevia* extracts. A number of different brands of *Stevia* products are available and are marketed internationally. However, regulations vary in different countries and need to be made uniform for acceptance. The global regulatory bodies have come to trust and accept *Stevia* as a safe food ingredient. Scientific validity of safety and creation of awareness is required for acceptance by populations. The available scientific evidences and extensive usage already in place make this crop and its metabolite stevioside as a potential non-nutritive sweetener which is here to study and benefit mankind.

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Triterpenoid Saponins: A Focus on Polygalaceae

104

Marie-Aleth Lacaille-Dubois, Clément Delaude, and Anne-Claire Mitaine-Offer

Contents

1 Introduction 3206
2 Chemistry of the Polygalaceae 3206
2.1 General Procedures 3206
2.2 Presenegenin Glycosides 3207
2.3 2-Oxo-olean-12-ene-3β,27-dihydroxy-23,28-dioic Acid Glycosides 3213
2.4 11-Oxo-olean-12-ene-2β,3β,27-trihydroxy-23,28-dioic Acid Glycoside 3213
2.5 Medicagenic Acid Glycosides 3213
2.6 2-Oxo-olean-12-ene-23,28-dioic Acid Glycoside 3214
2.7 Bayogenin Glycosides 3214
2.8 3β,23,27,29-Tetrahydroxy-olean-12-en-28-oic Acid Glycoside 3214
3 Pharmacological Studies on Polygalaceae 3214
3.1 Immunoadjuvant Activity 3215
3.2 Antiproliferative Activity 3216
3.3 Anti-inflammatory Activity 3217
3.4 Hypolipidemic Activity 3217
3.5 Activity on the Central Nervous System 3219
4 Conclusion 3220
References 3221

Abstract

The aim of this chapter is to give the newest results on the chemistry and pharmacological properties of Polygalaceae saponins during the period

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2005–2012. Around 60 new triterpene saponins were isolated and characterized, having mainly presenegenin, medicagenic acid, their 2-oxo or 11-oxo structural analogs, and bayogenin as aglycone. The sequence 3-O-(*β*-D-glucopyranosyl) presenegenin 28-{O-*β*-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-*β*-D-fucopyranosyl} ester often encountered in the genera *Polygala, Securidaca*, and *Atroxima* supported our previous conclusions that they may represent a chemotaxonomic marker for the Polygalaceae family. Additional glycosylations and acylations with 4-methoxy-, 3,4-dimethoxy-, or 3,4,5-trimethoxycinnamoyl groups contribute to the great chemical diversity of these saponins. The promising potential of the Polygalaceae saponins as immunoadjuvant, antiproliferative, anti-inflammatory, hypolipidemic, antidepressant, neuroprotective, and cognitive enhancer compounds has been highlighted.

Keywords

Biological activity • chemotaxonomy • Polygalaceae • presenegenin • triterpene saponins

1 Introduction

Many important reviews summarized different aspects related to triterpene and steroid saponins from a chemical and biological point of view [1–5]. Due to the enormous number of reports that appears worldwide, we have to set some limits. Interest on the Polygalaceae family, one of the four families of the Fabales order [6], has increased, specially due to its medicinal and taxonomic importance [7]. The most representative genus of Polygalaceae family is *Polygala*, comprising ca. 50 % of the 1,000 species of the family [6]. This genus consists of more than 500 species from all over the world of which 39 species are distributed in China. Some of these species have been used as traditional Chinese medicine for treating disturbances of the central nervous system (CNS) and inflammation [7]. Therefore, this review will highlight the newest phytochemical and biological achievements on the Polygalaceae saponins since our last review article dated from 2005 [8]. The main genera of Polygalaceae which have been reported in the literature during the period 2005–2012 are *Polygala*, *Carpolobia*, *Nylandtia*, *Securidaca*, and *Atroxima*, the genus *Polygala* being the most represented. They were reported to contain triterpene saponins, most of them having presenegenin as aglycone. The first part of this chapter will present the isolation and structures of the new and unusual saponins, whereas the second part is related to updated biological and pharmacological results.

2 Chemistry of the Polygalaceae

2.1 General Procedures

The extraction and isolation of the saponins from Polygalaceae is based on several methods, which were already reported and are still being used [8]. Briefly, after extraction with polar solvents (70 % aqueous MeOH or EtOH), the resulting crude extract is submitted to several chromatographic steps including column chromatography (CC), vacuum liquid chromatography (VLC), medium-pressure liquid chromatography (MPLC), and semi-preparative high-performance liquid chromatography (1/2 prep HPLC), resulting in the isolation of triterpene saponins, some of them being isolated as pairs of (*E*/*Z*) acylated isomers (Figs. 104.1–104.7). Various supports were used such as Sephadex LH-20, normal- and reversed-phase RP-18 silica gel, macroporous resin D-101, and reversed-phase YMC-Pack ODS-A. The structural determination of the saponins is based on 1D- and 2D-NMR experiments (COSY, TOCSY, NOESY, HSQC, HMBC) and mass spectrometry (ESI-HR-MS, FAB-MS) [8]. Recently, 18 saponins from *Polygala tenuifolia* were investigated by electrospray ionization ion trap multiple-stage mass spectrometry (ESI-ITMS) in positive- and negative-ion modes [9] in order to provide diagnostic key fragment ions important for the structural elucidation of saponins in this species.

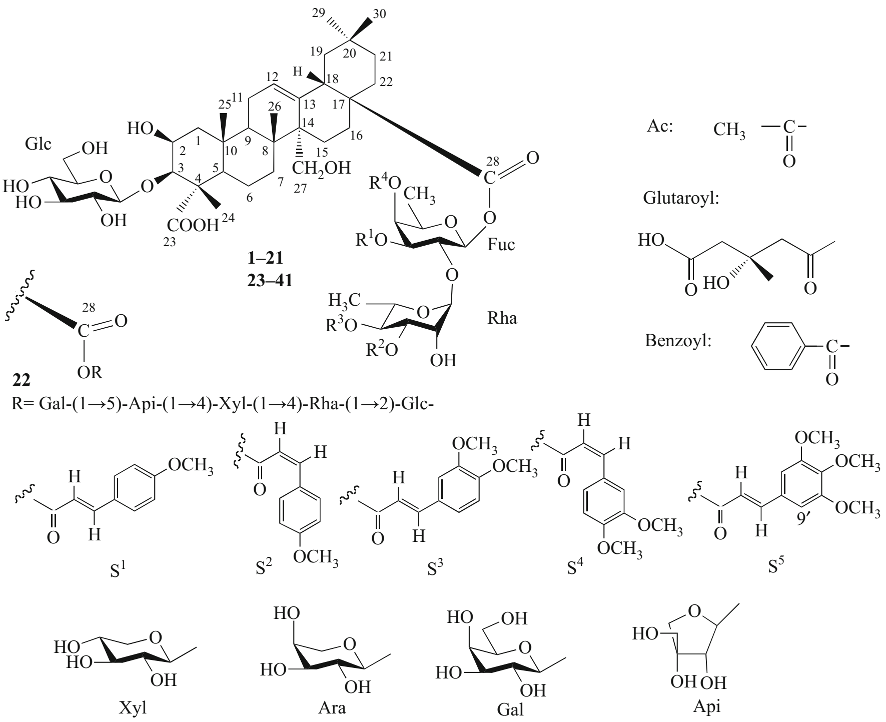
Furthermore, analytical reliable studies have been developed in order to evaluate the quality of *Polygala japonica* Houtt, a traditional Chinese herbal medicine from different locations in China. An HPLC method using an evaporative light scattering detector (ELSD) has been developed allowing the simultaneous quantification of six triterpenoid saponins in the drug [10]. The separation has been achieved on a Discovery C-18 analytical column. The authors concluded that the total content of the six saponins could be used as chemical markers to assess the quality of *P. japonica*.

During the period 2005–2012, around 60 new triterpene saponins (**1**–**57**, Figs. 104.1–104.7) were isolated and characterized in several Chinese and African species such as *P. ruwenzoriensis, P. arenaria, P. japonica, P. tenuifolia, P. crotalarioides, P. micrantha, Carpolobia alba, Nylandtia spinosa, Securidaca longepedunculata, S. welwitschii, and Atroxima liberica*. They will be presented below according to the nature of their aglycone, presenegenin (**1**–**41**, Fig. 104.1) and its keto structural analogs at C-2 (**42**,**43**, Fig. 104.2) or C-11 (**44**, Fig. 104.3), medicagenic acid (**45**–**52**, Fig. 104.4) and its keto derivative at C-2 (**53**, Fig. 104.5), bayogenin (**54**–**56**, Fig. 104.6), and 3,23,27,29-tetrahydroxy-olean-12-en-oic acid (**57**, Fig. 104.7).

2.2 Presenegenin Glycosides

The first main group of saponins is represented by some forty presenegenin glycosides (Fig. 104.1) in *Polygala, Nylandtia, Carpolobia, Securidaca*, and *Atroxima* species, half of them (**1**–**22**) being isolated from *Polygala* genus.

From *Polygala ruwenzoriensis*, five new saponins (**1**–**5**), of which **1**–**4** as two pairs of (*E*/*Z*)-isomers, were isolated by successive MPLC [11]. They share the same sequence 3-O-(*β*-D-glucopyranosyl)presenegenin 28-{O-*β*-D-xylopyranosyl-(1→4)-O-α-L-rhamnopyranosyl-(1→2)-*β*-D-fucopyranosyl} ester (3-O-Glc-presenegenin-28-O-Fuc^2-Rha^4-Xyl) with additional substitutions of the position 4 of fucose moiety by either an (*E*)- and (*Z*)-4-methoxycinnamoyl (**1**,**2**) or an (*E*)- and (*Z*)-3,4-dimethoxycinnamoyl group (**3**,**4**). The position 4 of the xylopyranosyl (Xyl)


Glc
HO
OH
COOH
23
24
H
CH2OH
27
R4O
CH3
R1O
Fuc
O
28
C
H3C
R3O
R2O
OH
Rha
1–21
23–41
22
OR
R= Gal-(1→5)-Api-(1→4)-Xyl-(1→4)-Rha-(1→2)-Glc-
Ac:
Glutaroyl:
Benzoyl:
OCH3
S1
S2
S3
S4
S5
9′
Xyl
Ara
Gal
Api

	R^1	R^2	R^3	R^4	Source	Ref
1	H	H	Ara-(1→4)-Xyl	S^1	*Polygala ruwenzoriensis*	[11]
2	H	H	Ara-(1→4)-Xyl	S^2	*P. ruwenzoriensis*	[11]
3	H	H	Ara-(1→4)-Xyl	S^3	*P. ruwenzoriensis*	[11]
4	H	H	Ara-(1→4)-Xyl	S^4	*P. ruwenzoriensis*	[11]
5	H	H	Gal-(1→4)-Xyl	H	*P. ruwenzoriensis*	[11]
6	H	H	[Glc-(1→3)]-Gal-(1→4)-Xyl	H	*P. arenaria*	[11]
7	Rha	Api	Xyl	S^1	*P. tenuifolia*	[12]
8	Rha	Api	Xyl	S^2	*P. tenuifolia*	[12]
9	S^5	Glutaroyl-(1→3)-Api	Gal-(1→4)-Xyl	H	*P. tenuifolia*	[13]
10	S^5	Glutaroyl-(1→3)-Api	Ara-(1→3)-Xyl	H	*P. tenuifolia*	[13]
11	Gal	Glutaroyl-(1→3)-Api	Ara-(1→3)-Xyl	S^5	*P. tenuifolia*	[13]
12	H	Glutaroyl-(1→3)-Api	Gal-(1→4)-Xyl	S^5	*P. tenuifolia*	[13]
13	Rha	H	Xyl	S1	*P. tenuifolia*	[13]
14	Rha	H	Xyl	S^5	*P. tenuifolia*	[13]
15	Rha	Api	Ara-(1→3)-Xyl	H	*P. tenuifolia*	[14]
16	Rha	Api	Xyl-(1→3)-Xyl	S^1	*P. tenuifolia*	[15]
17	S5	Api	Ara-(1→3)-Xyl	H	*P. tenuifolia*	[16]
18	H	H	H	H	*P. japonica*	[17]
19	Bzoyl-^{4}Rha	Api	Ara-(1→3)-Xyl	S^1	*P. japonica*	[18]

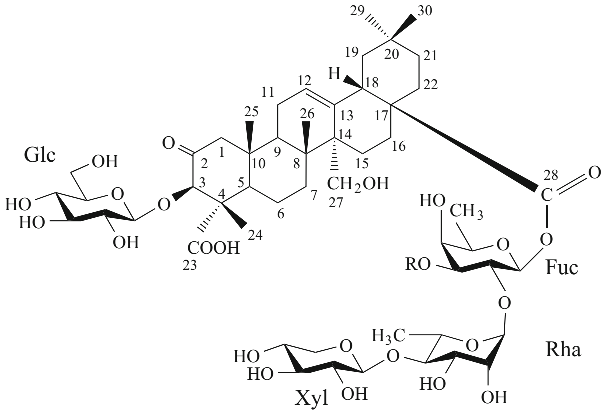
Fig. 104.1 (continued)

20	Bzoyl-^{4}Rha	H	Gal-(1 →4)-Xyl	S^1	*P. japonica*	[18]
21	Ac-^{4}Rha	Api	Ara-(1 →3)-Xyl	S^1	*P. japonica*	[18]
23	Api	H	Gal-(1 →4)-[Ara-(1 →3)]-Xyl	Ac	*Carpolobia alba*	[11]
24	H	Api	Gal-(1 →4)-[Ara-(1 →3)]-Xyl	H	*Nylandtia spinosa*	[19]
25	H	H	Gal-(1 →4)-[Ara-(1 →3)]-Xyl	H	*N. spinosa*	[19]
26	H	H	Api-(1 →4)-[Gal-(1 →2)]-Xyl	H	*N. spinosa*	[19]
27	H	H	Api-(1 →4)-Xyl	H	*N. spinosa*	[19]
28	H	Api	Api-(1 →3)-Xyl	S^5	*Securidaca longepedunculata*	[20]
29	Ac-^{6}Glc	Api	Api-(1 →3)-Xyl	S^5	*S. longepedunculata*	[20]
30	Gal	Api	Api-(1 →3)-Xyl	S^5	*S. longepedunculata*	[20]
31	H	Api	Api-(1 →3)[Ara-(1 →4)-Xyl	S^5	*S. longepedunculata*	[20]
32	Glc	H	Xyl	S^3	*S. welwitschii*	[21]
33	Glc	H	Xyl	S^4	*S. welwitschii*	[21]
34	Glc	Ac	Gal-(1 →4)-Xyl	S^3	*S. welwitschii*	[21]
35	Glc	Ac	Gal-(1 →4)-Xyl	S^4	*S. welwitschii*	[21]
36	H	H	Gal-(1 →3)-Xyl	H	*S. welwitschii*	[21]
37	H	H	Ara-(1 →3)-Xyl	S^3	*Atroxima liberica*	[22]
38	H	H	Ara-(1 →3)-Xyl	S^4	*A. liberica*	[22]
39	Xyl-(1→3)-Glc	H	Ara-(1 →4)-Xyl	S^3	*A. liberica*	[22]
40	Xyl-(1→3)-Glc	H	Ara-(1 →4)-Xyl	S^4	*A. liberica*	[22]
41	Ac-^{6}Glc	H	Xyl	S^4	*A. liberica*	[22]

Fig. 104.1 Presenegenin glycosides

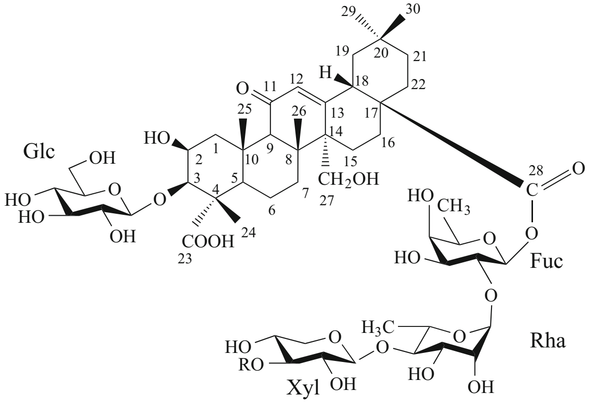
unit is substituted by a mono- or a disaccharide moiety. From *P. arenaria*, a new compound (**6**) without additional substitutions at Fuc-4 was isolated and characterized. The Xyl unit was substituted in a branched sequence by a *β*-D-glucopyranosyl (Glc) at C-3 and a *β*-D-galactopyranosyl (Gal) moiety at C-4 [11].

From *P. tenuifolia*, a plant which has been used in traditional Chinese medicine for thousands of years as an expectorant, tonic, tranquilizer, and to treat dementia, amnesia, and neurastenia, 11 saponins (**7**–**17**) were isolated. They share the same common sequence cited above (3-O-Glc-presenegenin-28-O-Fuc^2-Rha^4-Xyl) with additional substitutions at Fuc-3 either by a Rha (**7**, **13**–**16**) [12–15] or a Gal moiety (**11**) [13] or a (*Z*)-trimethoxycinnamoyl unit in the case of **9**, **10**, and **17** [13, 16]. In the compounds **7**, **8**, **11**–**14**, and **16**, an acylation was observed at Fuc-4 by either an (*E*)- or (*Z*)-4-methoxy- or an (*E*)-trimethoxycinnamoyl group [12, 13]. The originality of some compounds was observed by an unusual substitution at C-3 of Rha [13]. Namely, the presence of a *β*-D-apiofuranosyl (Api) unit which is acylated at C-5 by a glutaroyl moiety (**9**–**12**) is very rare in the family of Polygalaceae. Finally, the Xyl of the trisaccharide sequence at C-28 is free or substituted by a monosaccharide at C-3 or C-4.



	R	Source	Ref
42	H	*Polygala crotalarioides*	[23]
43	Glc	*Polygala crotalarioides*	[23]

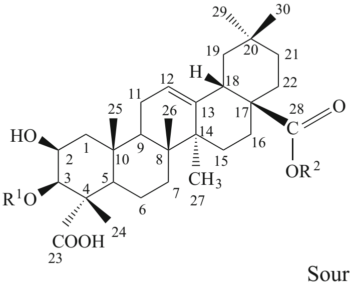
Fig. 104.2 Glycosides of 2-oxo-olean-12-ene-3*β*,27-dihydroxy-23,28-dioic acid



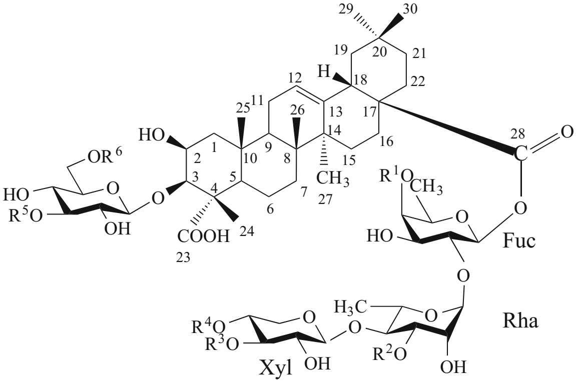
	R	Source	Ref
44	Gal-(1→5)-Api	*Polygala japonica*	[17]

Fig. 104.3 Glycoside of 11-oxo-olean-12-ene-2*β*,3*β*,27-trihydroxy-23,28-dioic acid

From the roots of *P. japonica* which have been used in Chinese folk herbal medicine as an expectorant, anti-inflammatory, antibacterial, and antidepressant agent, five presenegenin glycosides **18–22** were isolated [17, 18]. The originality of compounds **19–21** sharing the same above-mentioned common sequence at C-3 and C-28 of the presenegenin is due to the double substitution of Fuc at C-4 by an (*E*)-4-methoxycinnamoyl unit and at C-3 by an unusual substitution pattern of a Rha acylated at C-4 by a benzoyl unit (**19**, **20**) or by an acetyl group (**21**). The originality



	R^1	R^2	Source	Ref.
45	Glc	Xyl-(1→4)-[Api-(1→3)]-Rha-(1→2)-Glc	*Polygala japonica*	[24]
46	H	Xyl-(1→4)-[Api-(1→3)]-Rha-(1→2)-Glc	*P. japonica*	[25]
47	H	Xyl-(1→4)-Rha-(1→2)-Glc	*P. japonica*	[25]

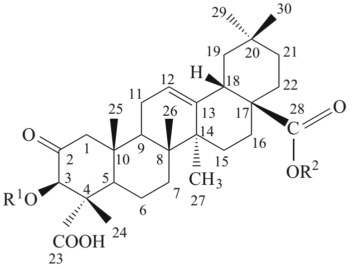


	R^1	R^2	R^3	R^4	R^5	R^6	Source	Ref.
48	H	H	H	Gal-	H	H	*Polygala micrantha*	[26]
49	H	H	H	Ac-^{6}Gal	H	H	*P. micrantha*	[26]
50	H	Api	Api	H	Glc	Glc	*P. micrantha*	[26]
51	S^1	Api	Ara	H	H	H	*Securidaca longepedunculata*	[27]
52	S^5	Api	Ara	H	H	H	*S. longepedunculata*	[27]

Fig. 104.4 Medicagenic acid glycosides

of compound **22** is due to the unusual esterification at C-28 by a Glc^2-Rha^4-Xyl oligosaccharidic chain instead of a Fuc^2-Rha^4-Xyl chain.

The last derivatives of presenegenin **23–41** isolated from other genera than *Polygala*, particularly from *Carpolobia*, *Nylandtia*, *Securidaca*, and *Atroxima*, share the same sequence 3-O-Glc-presenegenin-28-O-Fuc^2-Rha^4-Xyl. In *Carpolobia alba* [11], one compound was isolated having an acetylation at Fuc-4 (**23**), whereas this position is free in the four new compounds from *Nylandtia spinosa* (**24–27**) [19]. This Fuc-4 position is acylated by an (*E*)-3,4,5-trimethoxycinnamoyl group in *Securidaca longepedunculata* (**28–31**) [20] and by a (*Z*)- or (*E*/*Z*)-3,4-dimethoxycinnamoyl group in *S. welwitschii* (**32–35**) [21] and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16
C
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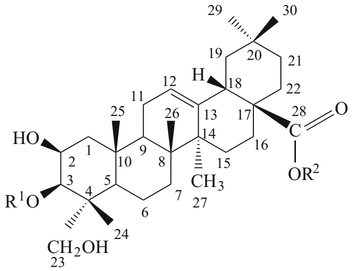
15
3
5
CH3
OR2
R1O
4
7
27
6
COOH
24
23

	R^1	R^2	source	Ref.
53	Glc	Xyl-(1→4)-[Api-(1→3)]- Rha-(1→2)-Glc	*P. japonica*	[24]

Fig. 104.5 Glycoside of 2-oxo-olean-12-ene-23, 28-dioic acid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9
14
16
2
10
8
15
OR2
3
5
CH3
R1O
4
7
27
6
24
CH2OH
23

	R^1	R^2	Source	Ref.
54	Glc	Xyl-(1→4)-Rha-(1→2)-Glc	*P. japonica*	[25]
55	H	Xyl-(1→4)-[Api-(1→3)]-Rha-(1→2)-Glc	*P. japonica*	[25]
56	H	Gal-(1→4)-Xyl-(1→4)-Rha-(1→2)-Glc	*P. japonica*	[25]

Fig. 104.6 Bayogenin glycosides

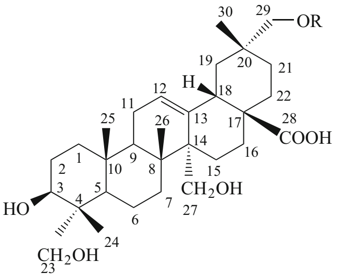

30
29
OR
19
20
21
12
H
18
11
22
25
26
13
17
28
COOH
14
16
1
9
2
10
8
15
3
5
CH2OH
HO
4
7
27
6
24
CH2OH
23

Fig. 104.7 Glycoside of 3*β*, 23,27,29-tetrahydroxy-olean-12-en-28-oic acid

	R	Source	Ref.
57	Glc-(1→2)-Glc	*P. japonica*	[17]

Atroxima liberica (**37–41**) [22]. The Fuc-3 position is either free (**24–28**, **31**, **36–38**) or glycosylated by a monosaccharide unit (Api) in *C. alba* (**23**), Gal or Glc in both *Securidaca* species (**30**, **32–35**), and by a dissacharide unit Xyl-(1→3)-Glc (**39–40**) in *A. liberica*. A branched trisaccharide at C-4 of Rha seems to be a common structural feature in *C. alba* and *N. spinosa*, whereas a linear disaccharide or a monosaccharide at this position is most common in the compounds from the two species of *Securidaca* and *A. liberica*. The C-3 position of Rha is mostly free or in some rare cases glycosylated by an Api (**24** in *N. spinosa*, **28–30** in *S. longepedunculata*) or acylated by an acetyl group (**34**, **35** in *S. welwitschii*).

2.3 2-Oxo-olean-12-ene-3*β*,27-dihydroxy-23,28-dioic Acid Glycosides

Two triterpene saponins (**42**, **43**, Fig. 104.2) having the unusual aglycone, 2-oxo-olean-12-ene-3*β*,27-dihydroxy-23,28-dioic acid, were isolated from *Polygala crotalarioides*, a well-known folk tonic medicine in Yunnan Wa (China) [23]. They possess the common substitutions at C-3 by a Glc and at C-28 by the sequence Fuc^{2}-Rha^{4}-Xyl which is substituted by a glucopyranosyl unit at Fuc-3 in the case of **43**.

2.4 11-Oxo-olean-12-ene-2*β*,3*β*,27-trihydroxy-23,28-dioic Acid Glycoside

One triterpene saponin (**44**, Fig. 104.4) having the unusual aglycone, 11-oxo-olean-12-ene-2*β*,3*β*,27-trihydroxy-23,28-dioic acid, was isolated from *Polygala japonica* [17]. On the common structural sequence found at C-28, Xyl is glycosylated by a disaccharide unit, Gal-(1→5)-Api.

2.5 Medicagenic Acid Glycosides

This aglycone is relatively rare in this family and has been encountered in few compounds (**45–52**, Fig. 104.4) isolated from three species *Polygala japonica* [24, 25], *P. micrantha* [26], and *Securidaca longepedunculata* [27]. The study of the aerial parts of *P. japonica* led to the isolation of three saponin derivatives of medicagenic acid (**45–47**) which possess the sequence Glc^{2}-Rha^{4}-Xyl at C-28 with additional substitution by an Api at Rha-3 in the case of **45** and **46**. The micranthosides A–C (**48–50**) isolated from *P. micrantha* possess the common sequence Fuc^{2}-Rha^{4}-Xyl with additional substitutions (Api, Gal, or Gal-Ac at different positions). Furthermore, the free position C-3 of the aglycone in the case of **46** and **47**, and its substitution by a branched triglucoside in the case of **50**, underlined the originality of these compounds. The study of *S. longepedunculata* led to the isolation of two medicagenic glycosides called securidacasides

A and B (**51**, **52**) having a common sequence at C-28 with an acylation at Fuc-4 by an (*E*)-4-methoxycinnamoyl (**51**) or by an (*E*)-3,4,5-trimethoxycinnamoyl (**52**) unit [27].

2.6 2-Oxo-olean-12-ene-23,28-dioic Acid Glycoside

Another new saponin (**53**, Fig. 104.5) isolated from *P. japonica* showed a very similar structure than compound **45** with the same sugar substitution pattern at C-3 and C-28, but differed only by the presence of a carbonyl group at C-2 of the aglycone instead of a secondary alcoholic function [24]. To the best of our knowledge, the 2-oxo-olean-12-ene-23,28-dioic acid as aglycone is encountered in this family for the first time.

2.7 Bayogenin Glycosides

Three derivatives of bayogenin (2*β*,3*β*,23-trihydroxyolean-12-en-28-oic acid) called polygalasaponins F, G, and J (**54**–**56**, Fig. 104.6) were isolated from *P. japonica* [25]. Two of them (**55**, **56**) were monodesmosidic C-28 oligosaccharide ester saponins. According to literature data [8] and updated reports, it seems that this plant is the unique source of bayogenin in the Polygalaceae family. As in compounds **45**–**47**, polygalasaponins F, G, and J possess the sequence Glc^2-Rha^4-Xyl at C-28 instead of the common sequence Fuc^2-Rha^4-Xyl. Furthermore, additional substitutions were observed (Api at Rha-3 in the case of **55**, Gal at Xyl-4 in the case of **56**).

2.8 3*β*,23,27,29-Tetrahydroxy-olean-12-en-28-oic Acid Glycoside

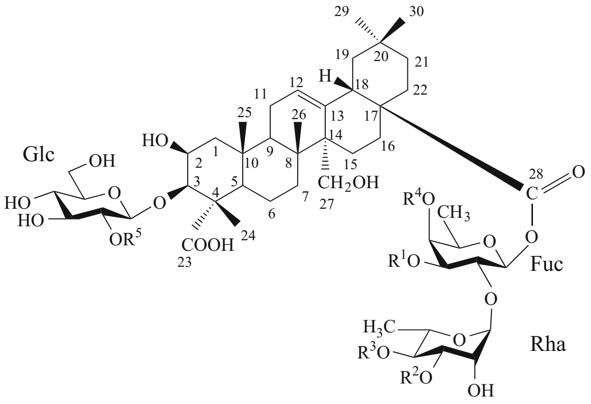
The study of *Polygala japonica* [17] led to the isolation of polygalasaponin XLVII (**57**, Fig. 104.7), having a free C-3 OH position and an unusual substitution pattern of the aglycone with the sugar chain attached to C-29 of the aglycone.

3 Pharmacological Studies on Polygalaceae

Plant saponins are a group of naturally occurring glycosides including a large number of biologically and pharmacologically active compounds [1, 28]. We will summarize below some of the recent advances on the Polygalaceae saponins with immunoadjuvant, antiproliferative, anti-inflammatory, and hypolipidemic properties as well as effects on the central nervous system (CNS). These studies will rationalize the traditional uses in folk medicine of some representative examples of Polygalaceae, belonging to the genus *Polygala*.

3.1 Immunoadjuvant Activity

If the quillaic acid derivatives and their analogs from *Quillaja saponaria* (Rosaceae) have been extensively studied for their immunoadjuvant properties and used as immunoadjuvants in commercial animal vaccines [29], few saponins from other sources have been reported to possess this property except some families including the Polygalaceae. Following encouraging results of earlier immunological studies on crude *P. senega* saponin extracts [30], eight pure triterpene saponins were isolated from this extract and their immunological activities evaluated [31]. Female CD-1 mice were immunized subcutaneously with ovalbumin (OVA) alone or mixed with *P. senega* saponins or *Quillaja* saponins as reference compounds (QS-18, QS-21). After a second immunization (day 10), the animals were killed (day 20) and serum samples were used for the measurement of anti-OVA immunoglobulin IgG and IgGa by enzyme-linked immunosorbent assay (ELISA). Onjisaponin A (**59**, Fig. 104.8) and onjisaponin B also called senegin III (**60**, Fig. 104.8) showed the most significant increase ($p < 0.01$), similar to QS-18 and QS-21 in anti-OVA IgG levels when compared with the control group of mice



	Nom	R^1	R^2	R^3	R^4	R^5	Source	Refs
58	PS1	Rha	Api	Rha-(1→4)-Gal(1→ 4)-Xyl	S^2	H	*P. senega*	[31]
59	PS3 (onjisaponin A)	Rha	Api	Gal-(1→4)-Xyl	S^2	H	*P. senega*	[31]
60	PS5 (onjisaponin B=senegin III)	Rha-	H	Gal-(1→4)-Xyl	S^2	H	*P. senega*	[31, 32]
61	senegasaponin a	H	Api	Gal-(1→4)-Xyl	S^2	H	*P. senega*	[32]
62	senegasaponin b	H	H	Gal-(1→4)-Xyl	S^2	H	*P. senega*	[32]
63	reinioside C	Ac	H	Xyl	Ac	Glc	*P. aureocauda*	[34]
64	polygalasaponin XXXII	Rha	Api	Ara-(1→3)-Xyl	S^1	H	*P. tenuifolia*	[9, 51]

Fig. 104.8 Some representative bioactive saponins from Polygalaceae

immunized with OVA alone. In the same way, three presenegenin saponins **58–60** (Fig. 104.8) were shown to enhance IgG2a antibody levels ($p < 0.05$), when compared with the control group. By using another strain of mice such as BALB/c mice, compounds **58–60** were found to promote higher level of IgG and IgG2a antibody production ($p < 0.1$ to $p < 0.01$) when compared with the control group. The effect of onjisaponin B (**60)** was similar to that observed with the reference compound QS-21 [31]. Furthermore, onjisaponin B (**60**), when administered with an antigen in BALB/c mice, significantly enhances production of interleukin-2 (Il-2) (similar to QS-21, $p < 0.05$) by spleen lymphocytes in response to OVA, thereby promoting a Th1-type immune response. Production of interferon-gamma (IFN-γ) in CD-1 and BALB/c mice did not appear to be significant for all saponins tested in comparison with the control group, although the concentration of IFN-γ was higher with some saponins than those of reference. This study confirmed that the adjuvant activity is not confined to *Q. saponaria* saponins but also characteristic of those isolated from *P. senega*. Since the presence of a dimeric fatty acid at C-4 of fucose in *Quillaja* saponins was reported to be a key factor in promoting a Th1-type immune response, the presence of a 4-methoxycinnamoyl group at C-4 of fucose in **58–60** might represent a structural feature important in the adjuvant activity. Furthermore, the carboxyl group at C-23 can form hydrogen bounds and thus be associated in adjuvant activity. In this work, it was shown that *P. senega* saponins may cause less toxic effects than *Quillaja* saponins that are presently used in commercial animal vaccine formulations.

3.2 Antiproliferative Activity

Uncontrolled angiogenesis is pathological and often associated with diseases such as atherosclerosis, arthritis, diabetic retinopathy, and cancer. Although several antiangiogenic agents have been approved for cancer chemotherapy, new lead compounds with novel action mechanisms and less side effects are still needed. Therefore, the antiangiogenic effects of senegasaponins have been recently studied [32]. The antiproliferative effect of senegasaponins from *P. senega* was evaluated against human umbilical vein endothelial cells (HUVECs) and various tumor cell lines, murine neuroblastoma cells (neuro 2A), human epidermoid carcinoma cells (KB3-1), human chronic myelogenous leukemia cells (K 562), and murine sarcoma cells (S 180) [32]. Senegin II, senegin III (**60**), senegin IV, and senegasaponins A (**61**) and B (**62**) exhibited antiproliferative activity against HUVECs with IC_{50} in the range 0.6–6.2 μM, with a selective index of 7–100-fold in comparison with those for several cancer cell lines, the most active being **60–62** (Fig. 104.8) (IC_{50} 0.59–1.1 μM). The 28-O-oligosaccharidic ester chain and the methoxycinnamoyl moiety were shown to be essential in this activity, since the deacyl mixture of senegasaponins and tenuifolin exhibited no antiproliferative activity against HUVECs and tumor cells. Some in vitro and in vivo assays were reported to see if the antiproliferative activity was based on inhibition of angiogenesis. Therefore, the effect of senegin III (**60**) was investigated on the migration and tubular formation of HUVECs stimulated by human recombinant vascular epithelium growth factor (VEGF) using a chemotaxicell chamber and an angiogenesis kit, respectively [32]. The results showed that the tubular network was prevented in a dose-dependent manner by senegin III in the 0.001–1.0 μM concentration range. These results suggested a possible antiangiogenic activity of senegin III, although its inhibitory effect for the VEGF-induced migration of HUVECs might not have a significant contribution to the antiangiogenic effect. Moreover, senegin III (2.5 mg/kg) suppressed tumor growth in the ddY mice sc-inoculated murine sarcoma S 180 cells, similar to cisplatin at 1 mg/kg. The formation of new vessels was assessed by using a Matrigel plug assay in mice [32]. The quantification of angiogenesis showed a significant decrease of 50 % and 60 % hemoglobin content in the groups treated with doses of 1.0 and 2.5 mg/kg of senegin III (**60**), respectively, proving its in vivo antiangiogenesis activity. The effects of **60** on VEGF-mediated signal transduction pathway in HUVECs showed that this compound would have a different mechanism of action from conventional antiangiogenic agents such as bevacizumab, sorafenib, and sunitinib. Another assay to elucidate the possible mechanism of antiangiogenic activity has shown that compound **60** induced pigment epithelium-derived factor (PEDF) protein expression in the cell lysate from 0.1 μM. All these observations showed that senegin III might have multiple targets to show the antiangiogenic effect.

3.3 Anti-inflammatory Activity

The use of *P. japonica* in traditional Chinese medicine (TCM) in the treatment of various inflammatory disorders suggests that active compounds from the plant might be potential anti-inflammatory agents. A bioguided fractionation led to the isolation of six pure triterpene saponins. Three of them, which are bayogenin derivatives [**54**, polygalasaponin V (3-O-(*β*-D-glucopyranosyl)bayogenin-28-{O-*β*-D-xylopyranosyl-(1→4)-[*β*-D-apiofuranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl}ester), and bayogenin-3-O-glucoside] showed significant anti-inflammatory activity in vivo [24, 33]. At the dose of 0.1 mg/kg, they significantly inhibited carrageenan-induced acute paw edema in mice. Furthermore, bayogenin-3-O-glucoside was shown to significantly inhibit the production of inflammatory mediators, nitrite oxide (NO), in LPS-stimulated RAW264.7 macrophages. These results support the wide use of *P. japonica* in TCM. It seems that the primary alcoholic function at C-4 of bayogenin and the carboxyl group at C-17 were crucial for an acute anti-inflammatory effect [24, 33].

3.4 Hypolipidemic Activity

The pathogenesis of atherosclerosis involves hyperlipidemia, endothelium dysfunction, infiltration of monocytes, activation of monocytes into macrophages, and smooth muscle cell proliferation [34]. The “oxidation theory” of atherosclerosis implies that agents that effectively inhibit low-density lipoprotein (LDL) oxidation and lesion development are potential antiatherogenic compounds. The roots of *Polygala aureocauda* Dunn (= *Polygala fallax* Hemsl) which is a Chinese medicinal herb, commonly used to treat infective inflammation and hypercholesterolemia, are reported to possess antivirus, hypolipidemic, and antioxidant activity [35]. A study examined the effects of the main constituent of the roots, a triterpene saponin named reinioside C (**63**, Fig. 104.8), on hyperlipidemic mice in vivo and on oxidative lesions induced by Ox-LDL in endothelium cells, macrophages, and smooth muscle cells in vitro. Mice were given a hyperlipidemic diet for 30 days and then administered reinioside C (4, 8, 16 mg/kg/day, *p.o.*) for 30 days. Then the serum lipid, superoxide dismutase (SOD), malonaldehyde (MDA), the total cholesterol (TC), and triglycerides (TG) in the liver extract were measured. The results showed that reinioside C (**63**) decreased serum and liver tissue lipid profiles in hyperlipidemic mice. In order to elucidate the mechanism of action, an in vitro study was achieved on HUVECs, peritoneal macrophages, and smooth muscle cells (SMCs) pre-incubated with reinioside C (**63**) and then cultured with oxidized low-density lipoprotein (Ox-LDL). It was shown that reinioside C protected the HUVECs against the Ox-LDL-induced LDH leakage and exerted a protective effect on oxidative lesions induced by Ox-LDL, inhibited cholesteryl ester accumulation in macrophages, and decreased [Ca^{2+}] and SMC proliferation in vitro. These results suggested that reinioside C (**63**) may be a promising hypolipidemic candidate [34]. A study of Bai et al. (2006) showed that reinioside C (**63**) at 1, 3, and 10 μM could inhibit the increased expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) mRNA and LOX-1 protein induced by Ox-LDL in HUVECs [36]. Asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase (NOS) inhibitor, has been implicated in vascular inflammation through induction of reactive oxygen species (ROS) and proinflammatory genes in endothelial cells, but few studies were made on monocytes, important cells throughout all stages of atherosclerosis [37]. A recent study showed that reinioside C (**60**) dose-dependently inhibited tumor necrosis factor-α (TNF-α) production, an important cytokine involved in the development of atherosclerosis induced by ADMA in cultured human monocytic (THP-1) cells. This effect was suggested to be promoted through inhibiting ROS/NF-kB signaling pathway, more precisely through inhibiting the ROS rise at the cellular level [37]. More recently, reinioside C (**63**) was suggested to attenuate the upregulated expression of adhesion molecules and the adherence of monocytes to endothelial cells by inhibiting NADPH oxidase/ROS/NF-kB pathway [38]. Other saponins such as ginsenosides were well documented as inhibitors of monocyte-endothelial cell adhesion [39]. Therefore, it was postulated that the sugar linkage position and the sugar residue number may play a role in the anti-atherosclerosis activity of reinioside C. However, further investigations need to be done to address structure-activity relationship.

3.5 Activity on the Central Nervous System

3.5.1 *Polygala japonica*

P. japonica has been used in a folk medicine as an antidepressant agent in the South of China. Among the saponins isolated from its aerial parts, the medicagenic acid derivatives polygalasaponin H (**46)** and E (**47**) (100 mg/kg orally, once daily, for 4 days) significantly reduce the immobility status of mice by 51.3 % and 58.1 %, respectively, in the forced swimming test [25]. These results suggested that these derivatives may be antidepressant constituents of *P. japonica*. A bayogenin glycoside, polygalasaponin G (**55**, Fig. 104.6), was shown to have a potent neurotrophic activity on pheochromocytoma (PC)-12 cells and cultured cortical neurons. This compound could promote neurite outgrowth of neurons cultured on the myelin substrates and inhibit the activation of proteins belonging to the family of Ras homologous A (RhoA) [40]. Such results might represent a therapeutic approach to improve axon regeneration after CNS injuries. Another study reported the neuroprotective effects of saponins which were evaluated in vitro using the MTT method, on a nerve cell injury model by incubating neuron-like PC-12 cells, in presence of amyloid-β peptides 25–35, $A\beta_{25\text{-}35}$, the major constituent of Alzheimer disease plaques [18]. In this model, two bayogenin derivatives, polygalasaponin II (3-O-(*β*-D-glucopyranosyl)bayogenin 28-{O-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl} ester) [41] and F (**54**, Fig. 104.6) [25], showed moderate activity at 10 μM. Furthermore, polygalasaponin F (**54**) was shown to induce long-term potentiation in hippocampal dentate gyrus in anesthetized rats via N-methyl-D-aspartate (NMDA) receptor activation mediated by several signaling pathways [42]. This glutamate receptor is an important molecular device for controlling synaptic plasticity and memory function.

3.5.2 *Polygala tenuifolia*

P. tenuifolia has been widely used as a memory enhancer for people in Asian countries for many years [43] and is also included in many traditional prescriptions used for the treatment of amnesia and dementia. Recently, the ethanol extract of the dried roots of *P. tenuifolia* called BT-11 was shown to possess memory-enhancing effects in healthy [44] and elderly volunteers [45] with a randomized, double-blind, placebo-controlled study. The improvement of memory functions was evidenced in many animal models with learning and memory impairment induced chemically [46] or by stress [47]. This cognitive improvement by *P. tenuifolia* has been associated with polygalasaponins as its main constituents [46]. However, the toxicity of polygalasaponin fraction in animals seriously limit the application and development of an effective agent from *P. tenuifolia* for Alzheimer's disease (AD), a neurodegenerative disorder characterized by progressive memory loss and cognitive deterioration [48]. Therefore, the effect of the hydrolysate of polygalasaponins (HPS) with reduced toxicity was undertaken on cognitive improvement in amnesia C57BL/6J mice [49]. The effect was assessed by two behavioral tests (Morris water maze and step-through passive avoidance tests) on cognitive impairment induced by A$\beta_{25\text{-}35}$ injection into the hippocampus in comparison with the positive control galanthamine. In these assays, HPS (50 and 100 mg/kg) showed significant improvements [49]. Additionally, HPS increased the corresponding SOD activities (62.34 % and 22.09 %) and decreased MDA levels (28.21 % and 32.35 %) in both cortex and hippocampus as compared to model animals. These results showed that HPS may be a useful treatment against amnesia probably via its antioxidant properties. Another in vitro study showed that tenuigenin, mostly a mixture of onjisaponins extracted from *P. tenuifolia*, was shown to inhibit secretion of amyloid β-protein in transfected neuroblastoma (SH-SY5Y) cells [50]. This observation suggested that tenuigenin may be worthy of further in vivo studies as a possible anti-AD agent [50]. All these studies have been achieved with mixtures of saponins, but there are several pharmacological studies on pure constituents. Namely, polygalasaponin XXXII (**64**) [10, 13, 51] was shown to improve hippocampus-dependent learning and memory, possibly through improvement of synaptic transmission, activation of the mitogen-activated protein (MAP) kinase cascade, and enhancement of the level of brain-derived neurotrophic factor (BDNF) [52]. Therefore, this presenegenin derivative (**64**) might be considered as a potential cognition-enhancing therapeutic drug. The antagonistic effects of the saponins of *P. tenuifolia* were investigated on neurotoxicity induced by glutamate and serum deficiency in PC-12 cells by using the MTT method in vitro, with the nerve growth factor (NGF) as a positive control [13]. On the basis of this bioassay, the saponins of *P.* t*enuifolia* were considered to have neuroprotective effects at the concentration of 1×10^{-5} M. Six of them were the most active, onjisaponins Vg (**12**), Ng (**8**), J (**9**), L (**10**), B, and polygalasaponin XXXII (**64**) [9, 13]. The roots *P. tenuifolia* are used in folk medicine as a sedative-hypnotic drug. Therefore, the anxiolytic and sedative-hypnotic activities of polygalasaponins from *P. tenuifolia* (40, 80, 160 mg/kg, *p.o.*) were determined in mice using hole-board, elevated plus maze, open field, and sodium pentobarbital-induced hypnosis tests [53]. The results suggested that the polygalasaponins possessed evident anxiolytic and sedative-hypnotic activities with a relatively safe dose range, supporting the use of the roots in folk medicine.

4 Conclusion

Polygalaceae is a rich source of triterpene saponins, particularly in the genus *Polygala*. This chapter has summarized the newest achievements from a chemical and pharmacological point of view on Polygalaceae saponins, which were reported during 2005–2012. The family is reported to contain a diversity of complex triterpene glycosides, which have been classified into seven main classes according to the nature of the aglycone. Some 60 new saponins, triterpene saponins, having from three to seven sugar moieties were isolated and characterized from *Polygala*, *Carpolobia*, *Nylandtia*, *Securidaca*, and *Atroxima* genera, of which some 40 were derivatives of presenegenin (**1–41**). The other reported saponins have as aglycone structural analogs of presenegenin with a keto function at C-2 (**42,43**) or C-11 (**44**), medicagenic acid (**45-52**), its 2-oxo derivative (**53**), bayogenin (**54-56**), and 3*β*,23,27,29-tetrahydroxy-olean-12-en-28-oic acid (**57**). As we already observed [8], most saponins isolated from Polygalaceae shared the same sequence 3-O-(*β*-D-glucopyranosyl)presenegenin 28-{O-*β*-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-*β*-D-fucopyranosyl} ester, which represents a common structural feature in the family, and could be considered as a chemotaxonomic marker. Additionally, the above sequence is mostly completed by a glycosylation or acylation by an *E*-trimethoxycinnamoyl unit at the position 3 of fucose, whereas the position 4 of fucose is mainly acylated by either *E*/*Z*-4-methoxy-, or (*E*/*Z*)-3,4-dimethoxy-, or (*E*)-3,4,5-trimethoxycinnamoyl groups or by one acetyl group. The originality of some saponins is related to the presence of two apiofuranosyl units in the same oligosaccharidic chain which is very rare in the Polygalaceae family and was reported only in *Securidaca longepedunculata*. The results of the reported bioactivities have shown that some triterpene saponins mainly presenegenin glycosides from *Polygala* genus might be potent immunoadjuvant, antiangiogenic, hypolipidemic, anti-inflammatory, antidepressant, sedative, and neuroprotective agents. These observations indicate that the Polygalaceae saponins have multiple targets to show their pharmacological effects, deserving further additional studies to elucidate the mechanism of action and the structure-activity relationships.

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Plant Steroidal Saponins: A Focus on Open-Chain Glycosides

105

Victoria L. Challinor and James J. De Voss

Contents

1 Introduction 3226
2 Structural Diversity of Steroidal Saponins 3227
 2.1 Spirostanol and Furostanol Saponins 3228
 2.2 Open-Chain Steroidal Saponins 3232
 2.3 Other Classes of Steroidal Saponins 3236
3 Analytical Methods 3238
 3.1 Isolation and Purification 3238
 3.2 Multistage Mass Spectrometry: Preliminary Structural Information 3239
 3.3 NMR Spectroscopy: Determination of Planar Structure 3239
 3.4 Determination of Stereochemistry in Steroidal Saponins 3241
 3.5 Determination of Sugar Absolute Configuration 3242
4 Biosynthesis of Plant Steroidal Saponins 3242
5 Biological Activities of Plant Steroidal Saponins 3243
6 Conclusions 3244
References 3245

Abstract

Saponins are a large and structurally diverse class of phytochemicals that consist of a nonpolar steroidal or triterpenoid skeleton that is glycosylated by varying numbers of sugar residues at different positions. Steroidal saponins exhibit a large range of biological activities, including cytotoxic, anti-inflammatory, hemolytic, antifungal, and antibacterial properties. Saponins possessing a steroidal skeleton are usually divided into two main structural categories, namely, spirostanol and furostanol saponins. However, the open-chain steroidal glycosides represent

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a further structural class with numerous representatives, some of which possess potent cytotoxic activity. Different patterns of steroid oxygenation and glycosylation in furostanol, spirostanol, and open-chain steroidal saponins generate considerable structural diversity, which may account for their wide range of observed biological activities. The structures and stereochemistry of steroidal saponins are typically elucidated via a combination of multistage mass spectrometry, 1D and 2D NMR spectroscopy, and chemical degradation and synthesis.

Keywords

Cholestane glycosides • furostanol • open-chain steroidal glycosides • spirostanol • stereochemistry • steroidal saponins • structure elucidation

Abbreviations

AMP	Adenosine monophosphate
Ara	Arabinopyranose
CID	Collision-induced dissociation
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
ELSD	Evaporative light scattering detection
ESI	Electrospray ionization
F26G	Furostanol glycoside 26-*O*-β-glucosidase
FAB	Fast atom bombardment
Fuc	Fucopyranose
Gal	Galactopyranose
Glc	Glucopyranose
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum correlation
MALDI	Matrix-assisted laser desorption/ionization
MS^n	Multistage mass spectrometry
NOESY	Nuclear Overhauser effect spectroscopy
Rha	Rhamnopyranose
ROESY	Rotating frame nuclear Overhauser effect spectroscopy
SPE	Solid phase extraction
TFAA	Trifluoroacetic anhydride
TOCSY	Total correlation spectroscopy
UDP	Uridine 5′-diphosphate
Xyl	Xylopyranose

1 Introduction

Saponins represent a large and structurally diverse class of plant terpenoids and are common chemical constituents of, for example, medicinal herbs. The term *saponin* is derived from the Latin word *sapo*, meaning soap, in reference to the stable, foamy lather that these compounds form when shaken in water. This derivation is also evident in the names of saponin-containing plants that historically have been used as natural detergents, for example, members of the *Sapindus* genus (commonly known as soapberries or soapnuts, family Sapindaceae), *Quillaja saponaria* Molina (soap bark tree, family Quillajaceae), and *Saponaria officinalis* L. (soapwort, family Caryophyllaceae). Structurally, saponins consist of either a steroidal (C_{27}) or triterpenoid (C_{30}) skeleton, referred to as the aglycone or sapogenin, which is substituted by varying numbers of sugar residues at different positions. (The suffix *genin* is used in the trivial nomenclature to denote an aglycone structure, as in diosgenin (**2**) and yamogenin (**10**).) In general, steroidal saponins are found in the monocotyledonous angiosperms, while triterpenoid saponins are common in dicotyledonous angiosperms [1]. Steroidal saponins are particularly abundant in the yam (Dioscoreaceae), asparagus (Asparagaceae), solanum (Solanaceae), lily (Liliaceae), and onion (Amaryllidaceae) families [1, 2]. Both steroidal and triterpenoid saponins are biosynthesized via the isoprenoid pathway through the cyclization of a 2,3-oxidosqualene precursor, before structural elaborations via, for example, hydroxylation or glycosylation. The steroidal aglycone typically has monosaccharide units or oligosaccharyl chains attached at one (monodesmoside), two (bisdesmoside) or, rarely, three (trisdesmoside) different positions. The sugar residues most commonly found in saponins are the hexoses D-glucose and D-galactose, the deoxyhexoses L-rhamnose and D-fucose, and the pentoses L-arabinose and D-xylose. Saponins have been reported to possess a large range of biological activities, doubtless owing in part to the high degree of structural diversity in this class of phytochemicals. The range of reported bioactivities, including cytotoxic, anti-inflammatory, hemolytic, antifungal, and antibacterial properties [3], has also led to considerable interest in the synthesis of these compounds and their analogs.

2 Structural Diversity of Steroidal Saponins

The majority of steroidal saponins isolated from terrestrial plants fall into two structural subfamilies: the pentacyclic furostanol saponins, characterized by a hemiacteal moiety at C-22 and a glycosidic linkage at C-26; and the spirostanol saponins, which instead possess six rings including a bicyclic acetal at C-22 (Fig. 105.1). A further class of plant saponins, which we have termed open-chain steroidal glycosides, are based upon a cholesterol-derived aglycone that lacks the additional ring(s) formed from the C-17 side chain that are characteristic of spirostanol and furostanol saponins. Within each of these three classes, structural diversity is generated through differences in stereochemistry and different patterns of oxygenation and glycosylation of the steroid nucleus.

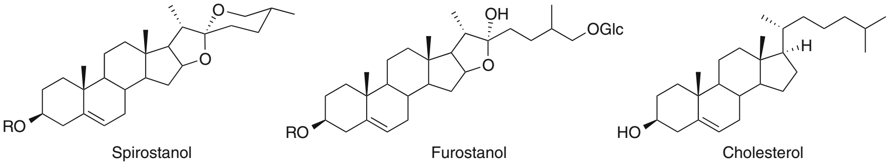
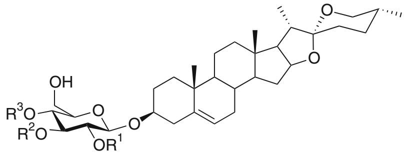


Fig. 105.1 Basic structures of spirostanol and furostanol saponins (*left* and *middle*, respectively) (R = sugar) and the structure of cholesterol (*right*)

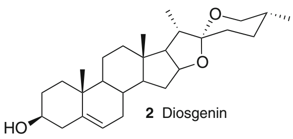
2.1 Spirostanol and Furostanol Saponins

Spirostanol saponins are characterized by the presence of a bicyclic spiroacetal moiety at position C-22 involving the steroid E and F rings as seen in dioscin (**1**), a characteristic metabolite of *Dioscorea* species [2]. Dioscin, an archetypal spirostanol saponin, consists of a diosgenin aglycone (**2**) with a branched trisaccharide moiety composed of an inner β-D-glucose residue substituted by two α-L-rhamnose units (chacotrioside) linked at position C-3. Spirostanols are usually monodesmosidic (glycosylated at one position), having attachment of sugar units at position C-3 only. Although C-5 is sp^2 hybridized in **1**, the steroid A/B ring junction is also commonly found with either *cis* or *trans* fusion, while the B/C and C/D ring junctions are usually *trans* fused. The C-21 methyl group is always α-orientated, while the 25*R* or 25*S* absolute configuration gives rise to either equatorial or axial orientation of the C-27 methyl group, respectively. The steroidal F ring can also be further hydroxylated, for example, at position C-27.

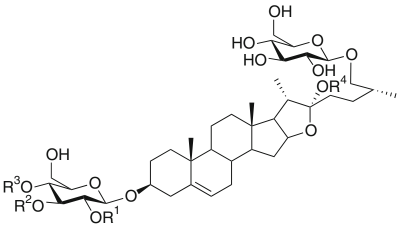
In contrast to spirostanol saponins, furostanols, such as protodioscin (**3**), another saponin commonly found in *Dioscorea* plants [2], instead possess a hemiacetal moiety at position C-22. Cyclization of the steroid to form an F ring is prevented by glycosylation of position C-26, typically by a single β-D-glucose unit as in **3**. The structure of protodioscin (**3**) is otherwise identical to that of dioscin (**1**), as it possesses the same branched chacotrioside moiety linked at position C-3. As in spirostanol saponins, C-25 can possess either *R* or *S* absolute configuration in furostanols, while the C-22 hydroxyl moiety is mainly α orientated. Pentacyclic "furostanol" saponins may be further divided into those that possess either a hemiacetal (trivially designated 22-hydroxy) or methyl acetal (22-methoxy) moiety at C-22 and those that possess $\Delta^{20(22)}$-unsaturation. In most cases, 22-methoxy saponins are considered to be artifacts formed during methanolic extraction.



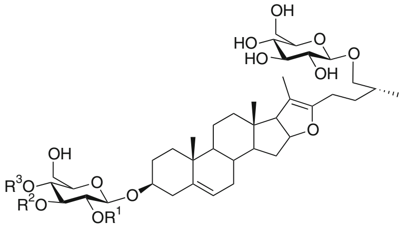
1 Dioscin $R^1 = R^3 = \alpha$-L-Rha $R^2 = H$
6 Gracillin $R^1 = \alpha$-L-Rha $R^2 = \beta$-D-Glc $R^3 = H$



2 Diosgenin

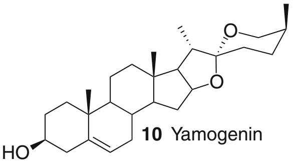


3 Protodioscin R^1 = R^3 = α-L-Rha R^2 = R^4 = H
4 Methylprotodioscin R^1 = R^3 = α-L-Rha R^2 = H R^4 = CH_3
7 Protogracillin R^1 = α-L-Rha R^2 = β-D-Glc R^3 = R^4 = H
8 Methylprotogracillin R^1 = α-L-Rha R^2 = β-D-Glc R^3 = H R^4 = CH_3

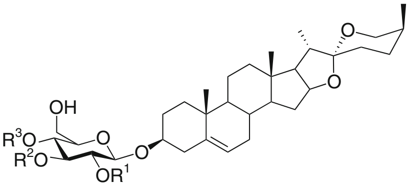


5 Pseudoprotodioscin R^1 = R^3 = α-L-Rha R^2 = H
9 Pseudoprotogracillin R^1 = α-L-Rha R^2 = β-D-Glc R^3 = H

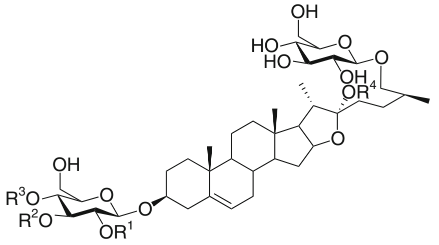
Many structurally analogous spirostanol/furostanol saponin “pairs” based on the same steroidal skeleton co-occur in the same plant species or genus, for example, dioscin (**1**) and protodioscin (**3**) are both found in *Dioscorea* [2]. The 22-methoxy and $\Delta^{20(22)}$-unsaturated analogs of protodioscin, methylprotodioscin (**4**) and pseudoprotodioscin (**5**) respectively, have also been reported from *Dioscorea* [2,4]. Another pair of structurally analogous saponins possessing the diosgenin aglycone are the spirostanol gracillin (**6**) and its corresponding furostanol protogracillin (**7**) [2]. Nicely illustrating the origins of structural diversity in saponins, these saponins differ from dioscin and protodioscin only in their glycosylation pattern; saponins **6** and **7** possess the gracillimatriose trisaccharide linked at position C-3 of the aglycone instead of the chacotrioside moiety. Again, the corresponding 22-methoxy (methylprotogracillin, (**8**)) and $\Delta^{20(22)}$-unsaturated (pseudoprotogracillin, (**9**)) saponins have been identified from *Dioscorea* [2]. A common point of difference in spirostanol and furostanol saponins is the stereochemistry of position C-25. Glycosides based on the 25*S* epimer of diosgenin, yamogenin (**10**), have also been isolated from the yam genus *Dioscorea*, as reflected in the trivial name of this aglycone [2]. The spirostanol saponin yamoscin (**11**) represents the 25*S* analog of dioscin (**1**) [5], while the corresponding furostanol protoneodioscin (**12**) bears the same relationship to protodioscin (**3**) [6]. In addition, methylprotoneodioscin (**13**) [7] and pseudoprotoneodioscin (**14**) [8] represent the 25*S* epimers of methylprotodioscin (**4**) and pseudoprotodioscin (**5**), respectively. The 25*S* glycosides that correspond to gracillin (**6**) and protogracillin (**7**) have also been characterized, as collettinside IV/zingiberenin B (**15**) [9] and protoneogracillin (**16**) [6], respectively.



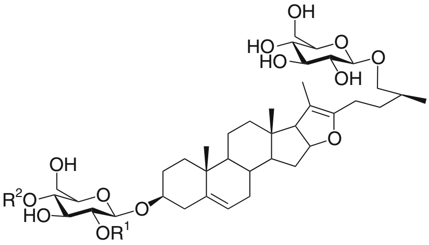
10 Yamogenin



11 Yamoscin R^1 = R^3 = α-L-Rha R^2 = H
15 Collettinside IV/Zingiberenin B R^1 = α-L-Rha R^2 = β-D-Glc R^3 = H



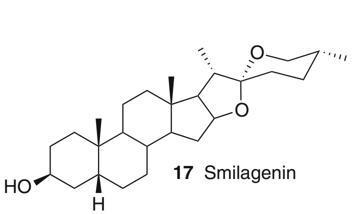
12 Protoneodioscin R^1 = R^3 = α-L-Rha R^2 = R^4 = H
13 Methylprotoneodioscin R^1 = R^3 = α-L-Rha R^2 = H R^4 = CH$_3$
16 Protoneogracillin R^1 = α-L-Rha R^2 = β-D-Glc R^3 = R^4 = H



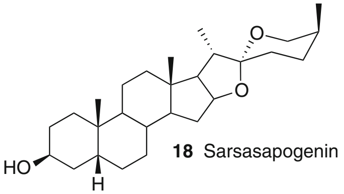
14 Pseudoprotoneodioscin R^1 = R^2 = α-L-Rha

As illustrated by consideration of **1-16**, a number of modifiers are commonly used in the trivial nomenclature of furostanol saponins. Where an analogous spirostanol glycsoide has been reported previously with a trivial name, the prefix *proto* can be added to give the name of the furostanol, for example protodioscin (**3**) derived from dioscin (**1**). The *methyl* prefix is used to indicate that the glycoside is a C-22 methyl acetal (22-methoxy) furostanol (as in methylprotodioscin, (**4**)), while *pseudo* indicates Δ$^{20(22)}$-unsaturation (pseudoprotodioscin, (**5**)). The *neo* modifier is used to indicate the 25*S* absolute configuration, for example in protoneodioscin (**12**) and protoneogracillin (**16**).

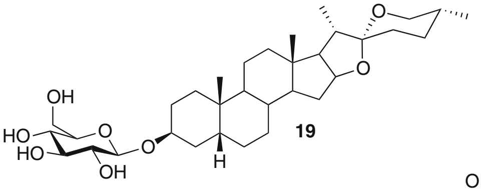
Though **1–16** all possess Δ$^{5(6)}$-unsaturation, spirostanol and furostanol saponins are also commonly found with either a *cis* (5β) or *trans* (5α) fused steroid A/B ring junction. For example, a number of glycosides have been reported with the 25*R* aglycone smilagenin (**17**) or its 25*S* analog sarsasapogenin (**18**), which differ from diosgenin (**2**) and yamogenin (**10**), respectively, only in possessing a *cis* (5β) A/B ring junction. Glycosides of smilagenin and sarsasapogenin are abundant in species of the *Smilax* (sarsaparilla) genus, again reflected in the trivial names of these aglycones and their glycosides. Smilagenin 3-*O*-β-D-glucopyranoside (**19**) isolated from *Smilax medica* Schltdl. & Cham (synonymous with *Smilax aristolochiifolia* Mill.) [10] is an example of a smilagenin-based spirostanol, while disporosides C and D (**20** and **21**) isolated from *Disporopsis pernyi* (Hua) Diels are examples of smilagenin furostanols [11]. Sarsasapogenin-based glycosides include the spirostanol parillin (**22**) and its corresponding 22-hydroxy and Δ$^{20(22)}$-unsaturated furostanols, sarsaparilloside (**23**), and Δ$^{20(22)}$-sarsaparilloside (**24**), respectively, first reported from *Smilax aristolochiifolia* Mill. [12–14]. The ^{1}H and ^{13}C NMR spectroscopic data for **22–24** were later reported along with the isolation of two additional sarsasapogenin-derived 22-hydroxy furostanols with different glycosylation patterns, sarsaparillosides B and C (**25** and **26**, respectively) [15]. Steroidal saponins having a *trans* (5α) A/B ring junction are also common in plants, for example, those based on the 25*R* tigogenin (**27**) or 25*S* neotigogenin (**28**) aglycones, which are otherwise analogous to diosgenin (**2**) and yamogenin (**10**), respectively.



17 Smilagenin



18 Sarsasapogenin



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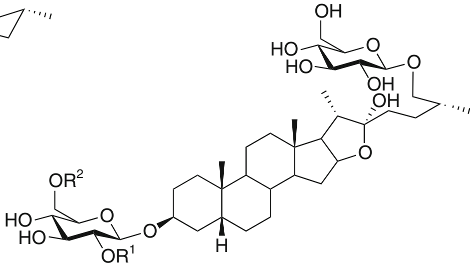
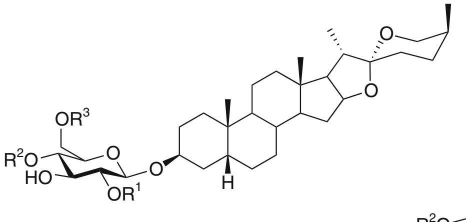
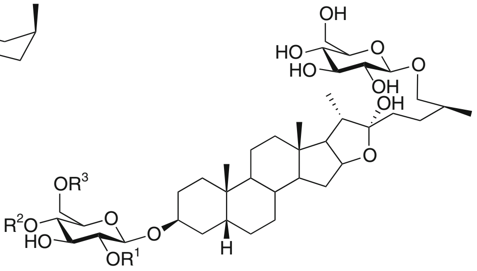


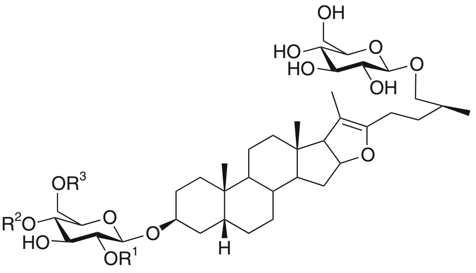
20 Disporoside C R^1 = β-D-Glc R^2 = H
21 Disporoside D R^1 = R^2 = β-D-Glc



22 Parillin R^1 = R^3 = β-D-Glc R^2 = α-L-Rha



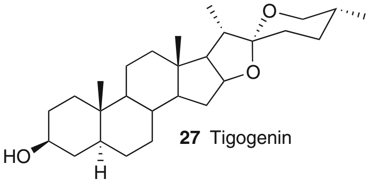
23 Sarsaparilloside R^1 = R^3 = β-D-Glc R^2 = α-L-Rha
25 Sarsaparilloside B R^1 = H R^2 = α-L-Rha R^3 = β-D-Glc
26 Sarsaparilloside C R^1 = R^2 = H R^3 = β-D-Glc



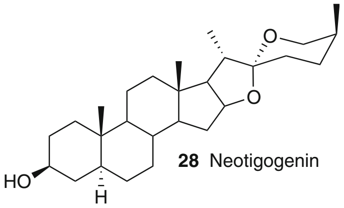
24 $\Delta^{20(22)}$-sarsaparilloside R^1 = R^3 = β-D-Glc R^2 = α-L-Rha

Another source of structural variation in steroidal saponins is further hydroxylation of the steroidal aglycone, for example, the 1β-hydroxylation present in the

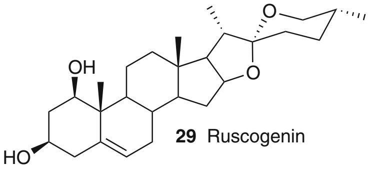
$\Delta^{5(6)}$-unsaturated aglycones ruscogenin (**29**) and neoruscogenin (**30**). There are many further examples of plant spirostanol and furostanol saponins based on aglycones having different oxygenation patterns, for example, the commonly occurring pennogenin (**31**), which possesses a 17α-hydroxyl group and hecogenin (**32**), which has a C-12 ketone moiety.



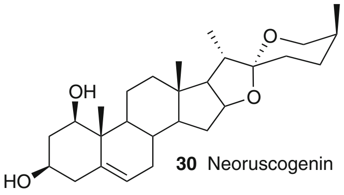
27 Tigogenin



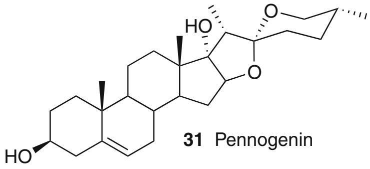
28 Neotigogenin



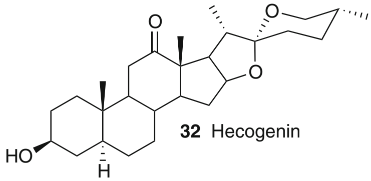
29 Ruscogenin



30 Neoruscogenin



31 Pennogenin



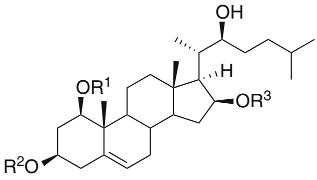
32 Hecogenin

The structural diversity of spirostanol and furostanol glycosides and their aglycones has been documented in a number of general reviews, for example, those of Vincken et al. [1] and Sahu et al. [16], as well as by Agrawal in his compilation of ^{1}H and ^{13}C NMR spectroscopic data for steroidal saponins and aglycones [17]. The saponins occurring in particular plant groups have also been cataloged, including those of *Dioscorea* [2, 18], *Tupistra* [19], *Ophiopogon* [20], *Tribulus terrestris* L. [21, 22], *Vernonia* [23], and the Agavaceae family [24], as well as the saponins found in plants of a particular geographical region, such as Brazil [25].

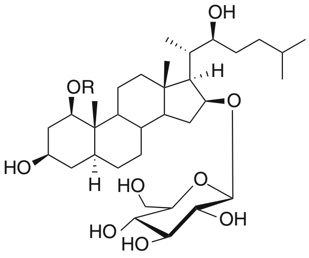
2.2 Open-Chain Steroidal Saponins

The open-chain steroidal saponins represent a further, less common structural class of steroidal glycosides. Like spirostanol and furostanol glycsoides, they possess a C_{27} aglycone, but they lack any additional ring(s) derived from the C-17 side chain. Saponins of this type, while abundant in marine organisms such as starfish, sponges, and cnidarians, appear to be less common in terrestrial plants. Open-chain steroidal glycosides are particularly prevalent in echinoderms, where they are the characteristic secondary metabolites of the classes Asteroidea (starfish) and Holothuroidea (sea cucumbers) [26]. While the structural diversity of open-chain steroidal saponins has been extensively cataloged in recent reviews of marine natural products [27–30], this class of glycoside has been largely overlooked in reviews of plant steroidal saponins, despite over 150 representatives having been reported to date. Plant open-chain steroidal glycosides are based upon a cholesterol-derived skeleton that possesses between three and five sites of oxygenation, typically at positions C-16 and C-22 as well as the C-3 oxygenation that is present as a consequence of their formation via the cyclization of 2,3-oxidosqualene. Plant open-chain saponins have been reported that possess one (monodesmosidic), two (bisdesmosidic), and three (trisdesmosidic) sites of sugar linkage with up to six attached sugar residues, most commonly β-D-glucose and α-L-rhamnose. Most open-chain steroidal saponins reported from terrestrial plants to date possess an aglycone with four sites of oxygenation, with a number of recurring oxygenation patterns emerging.

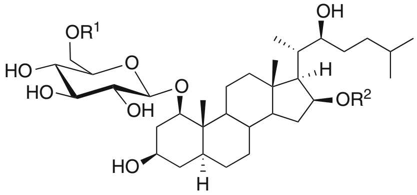
A significant proportion of the presently identified plant open-chain saponins are based on either the alliosterol aglycone, (22*S*)-1β,3β,16β,22-tetrahydroxy-cholest-5-ene (**33**), or a closely related skeleton having the same pattern of oxygenation. Alliosterol glycosides have been reported as natural products isolated from plants of the *Allium* (Amaryllidaceae), *Ruscus* (Asparagaceae), *Ornithogalum* (Asparagaceae), *Nolina* (Asparagaceae), *Reineckea* (Asparagaceae), *Tribulus* (Zygophyllaceae), and *Polianthes* (Asparagaceae) genera, with some saponins found in more than one genus. Alliosterol (**33**) was reported in 1991 as the aglycone of allosides A and B (**34** and **35**, respectively) from *Allium suworowii* Regel and *Allium stipitatum* Regel, from which the trivial names of this aglycone and its glycosides are derived [31]. The presence of a free hydroxyl group at position C-3 of the aglycone in **34** and **35**, while unusual in spirostanol and furostanol saponins, is common among glycosides of alliosterol. As in the spirostanol and furostanol saponins, differences in glycosylation pattern are a major source of structural diversity in open-chain steroidal glycosides. A total of 24 differently glycosylated alliosterol saponins have been reported to date, including monodesmosidic (**34**), bisdesmosidic (**35**), and trisdesmosidic (**36**) structures [31, 32]. Other common structural variations among open-chain steroidal glycosides are $\Delta^{5(6)}$-unsaturation or a *trans* A/B ring junction, and $\Delta^{24(25)}$-unsaturation. Interestingly, saponins based on aglycones that share the same oxygenation pattern as alliosterol uniformly possess the same 22*S* absolute configuration and β-orientation of the substituents at positions C-1, C-3, and C-16. A number of glycosides are based on aglycones that are structurally related to alliosterol but instead have *trans* (5α) A/B ring fusion (e.g., **37** and **38**) [33, 34], $\Delta^{24(25)}$-unsaturation (**39**) [35], or a C-22 ketone moiety (**40**) [36].



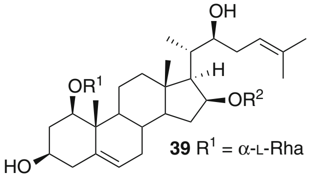
33 Alliosterol $R^1 = R^2 = R^3 = H$
34 Alloside A $R^1 = R^2 = H$ $R^3 = \beta$-D-Gal
35 Alloside B $R^1 = \beta$-D-Glc $R^2 = H$ $R^3 = \beta$-D-Gal
36 $R^1 = R^2 = \alpha$-L-Rha $R^3 = \beta$-D-Glc



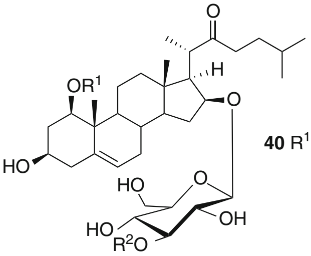
37 R = α-L-Rha



38 R^1 = 3,4,5-trimethoxybenzoyl $R^2 = \beta$-D-Glc

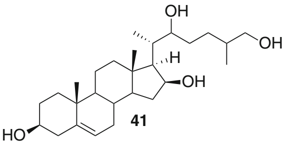


39 $R^1 = \alpha$-L-Rha $R^2 = \beta$-D-Glc



40 $R^1 = R^2 = \alpha$-L-Rha

Another recurring structural motif in open-chain steroidal glycosides is oxygenation of position C-26 as well the standard oxygenation of positions C-3, C-16, and C-22. This is seen in the 3β,16β,22,26-tetrahydroxy-cholest-5-ene skeleton (**41**) and its derivatives, which have been reported with varying stereochemistries at C-22 and C-25. The majority of glycosides derived from **41** or a structurally related aglycone are found in the *Solanum* (Solanaceae) and *Tribulus* (Zygophyllaceae) genera, though they have also been detected in *Trillium* (Asparagaceae) [37–39], *Chamaelirium* (Melanthiaceae) [40], *Dioscorea* (Dioscoreaceae) [41], and *Paris* species (Melanthiaceae) [42]. Again, structural diversity is generated among this family of steroidal saponins through differences in glycosylation pattern (e.g., in **42**–**44**) [38–40], *trans* A/B ring fusion as in pentandroside E (**45**) [43], and steroid oxidation states as in anguivioside XV (**46**) [44]. Abutilosides E and G (**47** and **48**) and their corresponding 5α glycosides abutilosides D and F (**49** and **50**, respectively) isolated from *Solanum abutiloides* (Griseb.) Bitter & Lillo are very unusual among the reported open-chain steroidal glycosides in possessing an α- rather than β-orientated C-16 hydroxyl group [45]. Glycosides such as **47–50** that possess both a C-22 ketone and a free C-16 hydroxyl group exist in an open-chain form because the α-orientation of the latter stereochemically prevents cyclization to form the five-membered E ring characteristic of furostanol and spirostanol saponins. In all of the reported saponins that instead possess a β-orientated C-16 hydroxyl group and a C-22 oxo moiety (e.g., **40**) cyclization is prevented by linkage of one or more sugar units at C-16 [36].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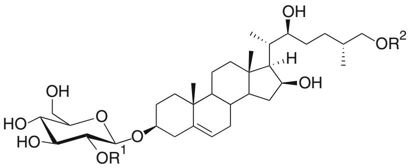
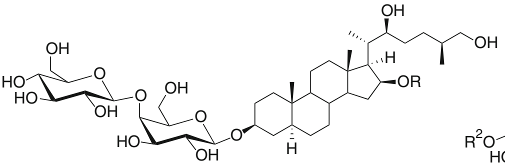
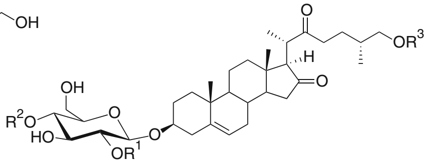
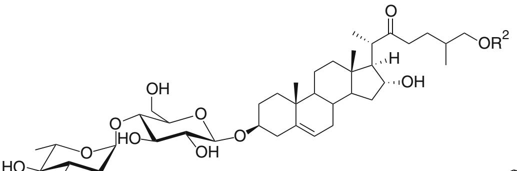
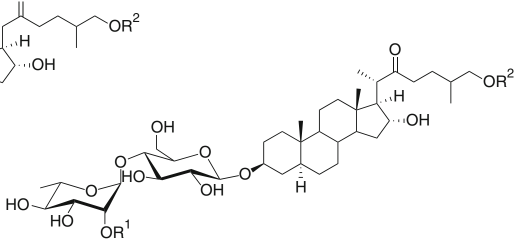

42 Bethoside B $R^1 = R^2$ = β-D-Glc
43 Bethoside C R^1 = β-D-Glc R^2 = H
44 11-deoxyheloside A R^1 = H R^2 = β-D-Glc

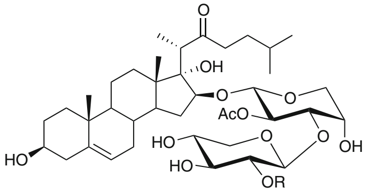

45 Pentandroside E R = β-D-Xyl


46 Anguivioside XV $R^1 = R^2$ = α-L-Rha R^3 = β-D-Glc

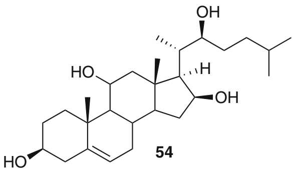

47 Abutiloside E R^1 = β-D-Xyl R^2 = β-D-Glc
48 Abutiloside G R^1 = H R^2 = β-D-Glc


49 Abutiloside D R^1 = β-D-Xyl R^2 = β-D-Glc
50 Abutiloside F R^1 = H R^2 = β-D-Glc

Another structural subfamily of open-chain steroidal glycosides is made up of OSW-1 or orsaponin (**51**) and related structures (e.g., **52** and **53**) derived from the 3β,16β,17α-trihydroxy-cholest-5-en-22-one aglycone [46]. So far, these have been found exclusively in species of *Ornithogalum* (Asparagaceae), specifically *Ornithogalum saundersiae* Baker [46–48], *Ornithogalum thyrsoides* Jacq. [49], and *Galtonia candicans* (Baker) Decne., which is synonymous with *Ornithogalum candicans* (Baker) J.C.Manning & Goldblatt [35]. This family of open-chain steroidal glycosides has generated particular interest because OSW-1 (**51**), as well as exhibiting potent inhibitory activity against cyclic AMP phosphodiesterase in the original literature report [46], has subsequently been shown to have potent cytotoxic activity against a number of malignant tumor cell lines [47, 48]. The potency and selectivity of **51** against a range of malignant tumor cell lines has led to considerable interest in the synthesis of this glycoside and its aglycone, as well as various derivatives [50–56].



51 OSW-1 R = *p*-methoxybenzoyl
52 R = H
53 R = 3,4-dimethoxybenzoyl



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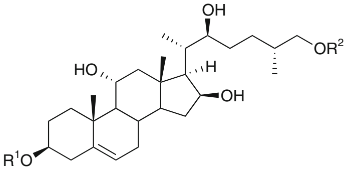
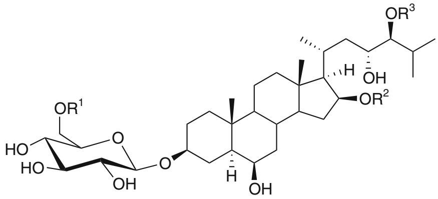
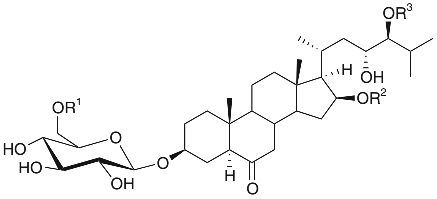


55 Heloside A R^1 = R^2 = β-D-Glc
56 Heloside B R^1 = β-D-Glc R^2 = H



57 Chamaeliroside A R^1 = β-D-Glc R^2 = R^3 = H
58 Chamaeliroside B R^1 = β-D-Glc R^2 = H R^3 = β-D-Fuc
59 Chamaeliroside C R^1 = β-D-Glc R^2 = H R^3 = 4-*O*-acetyl-β-D-Fuc
60 Chamaeliroside D R^1 = β-D-Glc R^2 = H R^3 = α-L-Ara
61 Chamaeliroside E R^1 = R^2 = β-D-Glc R^3 = H



62 6-dehydrochamaeliroside A R^1 = β-D-Glc R^2 = R^3 = H
63 6-dehydrochamaeliroside B R^1 = β-D-Glc R^2 = H R^3 = β-D-Fuc
64 6-dehydrochamaeliroside E R^1 = R^2 = β-D-Glc R^3 = H

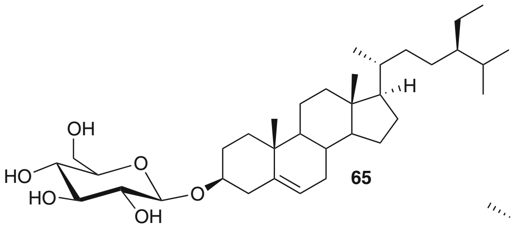
In addition to these three well-represented classes, many further structural types of open-chain steroidal glycoside have been detected in terrestrial plants. For example, glycosides based on the (22*S*)-3β,11,16β,22-tetrahydroxy-cholest-5-ene (**54**) aglycone, having characteristic oxygenation of position C-11, have been reported from *Ornithogalum* [57]. Closely related glycosides with hydroxylation of position C-26 are helosides A and B (**55** and **56**) isolated from *Chamaelirium luteum* (L.) A.Gray [58]. The majority of steroidal saponins isolated from *C. luteum*, chamaelirosides A–E (**57**–**61**) and 6-dehydrochamaelirosides A, B, and E (**62**–**64**), are based on comparatively highly oxygenated aglycones, with all possessing five sites of oxygenation (C-3, C-6, C-16, C-23, and C-24) of the steroidal aglycone [40].

2.3 Other Classes of Steroidal Saponins

In addition to the well-represented classes of spirostanol, furostanol, and open-chain steroidal glycosides, the broad steroidal saponin family can also be considered to encompass structures derived from less common aglycones, such as cycloartane, lanostanol, sitosterol, stigmasterol, steroidal alkaloids ("glycosteroidalkaloids"), homocholestanes, and withanolides. These all share an early biosynthetic precursor, 2,3-oxidosqualene, whose alternative cyclizations and rearrangements give rise to the divergent triterpene and steroid biosynthetic pathways.

Glycosides of cycloartenol are common in the *Astragalus* genus of the Leguminosae family, for example, those detected in *Astragalus membranaceus* (Fisch.) Bunge (synonymous with *A. propinquus* Schischkin) [59]. Saponins derived from lanosterol are more unusual, with only a few reported from *Muscari paradoxum* (Fisch. & C.A. Mey.) K.Koch, which is synonymous with *Bellevalia paradoxa* (Fisch. & C.A. Mey.) Boiss. [60]. Sitosterol glucoside (**65**) and various derivatives have been reported as glycosides isolated from *Punica granatum* L. [61], *Mentha longifolia* (L.) L. [62], and *Paris formosana* Hayata (synonymous with *P. polyphylla* var. *chinensis* (Franch.) H.Hara) [63, 64]. Stigmasterol glycosides have also been reported from *Trichosanthes dioica* (Roxb.) [65], *Asparagus adscendens* Roxb. [66], and *Ambroma augustus* L.f. [67], and *P. formosana* Hayata (synonymous with *P. polyphylla* var. *chinensis* (Franch.) H.Hara) [63].

The basic cholesterol skeleton can also be modified by the incorporation of a nitrogen heteroatom as in the steroidal alkaloids. Two examples are abutilosides A and B (**66** and **67**) isolated from *S. abutiloides* [68–70], which are closely related to the open-chain steroidal glycosides abutilosides D-G (**47–50**) [45] discussed above. Glycosteroidalkaloids such as these are particularly abundant in the *Solanum* (Solanaceae) [68–70], *Lycopersicon* (Solanaceae) [71–73], and *Fritillaria* (Liliaceae) [74–76].



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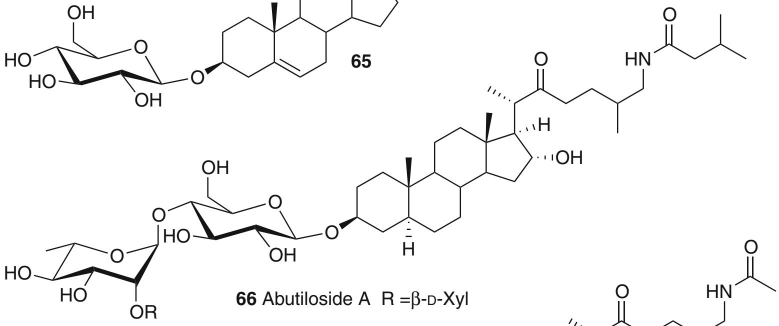
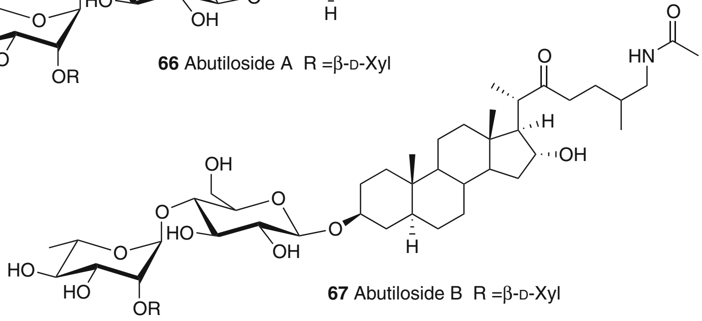


66 Abutiloside A R =β-D-Xyl



67 Abutiloside B R =β-D-Xyl

Additionally, several distinct classes of plant secondary metabolites also occur in glycosylated form and can be considered as saponins derived from a steroidal precursor, for example, glycosides of pregnane, androstane, homocholestane, the cardiac glycosides, and the withanolides. The triterpene biosynthetic pathway diverges from that of steroids at oxidosqualene and leads to the formation of glycosides based on the dammarane, tirucallane, lupane, hopane, oleanane, taraxasterane, ursanes, and cucurbitane aglycones, which have been cataloged by Vincken et al. [1].

3 Analytical Methods

Steroidal saponins are isolated from dried plant material including aerial parts, roots, and bark and are usually partitioned into a polar organic solvent before purification via reverse phase (RP) HPLC. The planar structure and stereochemistry of the isolated steroidal saponins is then typically determined using a combination of multistage mass spectrometry (MS^n), 1D and 2D NMR experiments, chemical degradation and derivatization, and enantioselective gas chromatography (GC).

3.1 Isolation and Purification

Steroidal saponins are typically extracted into aqueous methanol or acetonitrile or, less commonly, chloroform. The crude extract may then be partially purified via solid phase extraction (SPE) before further purification via RPHPLC. The general lack of a chromophore in steroidal saponins presents a challenge since UV detection cannot be used. Refractive index detection has been used in a number of recent reports of steroidal saponin isolation [77–81], but its application is limited since it cannot be used under a gradient elution regime. The utility of evaporative light scattering detection (ELSD) for steroidal saponins has been reviewed [82–84], making this now the preferred method of detection of steroidal saponins in gradient elution HPLC [15, 40, 58, 85–88]. Chromatographic separation of steroidal saponins can also be coupled with mass spectrometric analysis, for example, in HPLCESIMS^n. This can allow rapid dereplication since it provides information on the molecular weight of the saponin as well as the number and nature of attached sugar units. Given the wide range of biological activities of plant saponins and their potential application as pharmaceuticals or drug lead compounds, online or offline bioactivity-guided fractionation is also of interest. This method was employed in the isolation of the spirostanol orbiculatoside B from *Dioscorea deltoidea* var. *orbiculata* Prain & Burkhill (synonymous with *D. deltoidea* Wall. ex Griseb.), which showed antifungal and cytotoxic activities [89].

3.2 Multistage Mass Spectrometry: Preliminary Structural Information

Mass spectrometric data can provide important preliminary information about the structure of the steroidal aglycone and the number and type of attached sugar residues. Positive or negative electrospray ionization (ESI) is commonly used to detect steroidal saponins, since this “soft” ionization mode generates molecular ions that can be selected as precursor ions for further fragmentation in multistage mass spectrometry (MS^n). Other soft ionization modes such as fast atom bombardment (FAB) and matrix-assisted lazer desorption/ionization (MALDI) have also been used for the analysis of steroidal saponins [90, 91]. The MS^n experiment is now widely used as a powerful tool for structure elucidation of steroidal glycosides as well as identification of saponins present within a complex mixture [92–94]. The fragmentation of the intact saponin by stepwise dissociation of sugar residues from the aglycone, followed by examination of the fragment ion genealogy, indicates at once the number, nature, and linkage pattern (branched vs. linear) of attached monosaccharides. Analysis of the fragmentation cascade also enables predictions of aglycone structure by comparison with MS^n fragmentations of known compounds. The selective isolation of desired precursor ions also allows resolution of complex mixtures, even of compounds that co-elute in a coupled LC system.

3.3 NMR Spectroscopy: Determination of Planar Structure

The planar structure of steroidal saponins is routinely determined using 1D and 2D NMR experiments. Recent advances in instrumentation have meant that the acquisition of a complete set of 1D and 2D NMR spectra need not be prohibitively time or sample intensive. Further, structure elucidation may be performed on the intact glycoside, as opposed to the traditional, time-consuming procedure of degradation of the saponin into known structural elements.

A first step in the structure elucidation of a steroidal saponin is usually the acquisition of 1D ^{1}H and ^{13}C NMR spectra in either pyridine-d_5 or methanol-d_4 (solvents such as chloroform-d and dimethyl sulfoxide-d_6 are now used only rarely). Steroidal saponins are easily recognized by examination of their ^{1}H NMR spectrum, which usually displays characteristic signals for the steroidal methyl groups at positions C-18, C-19, C-21, and C-27, as well as a downfield region containing signals typical of the anomeric proton of a glycoside. The ^{13}C NMR spectrum of a steroidal saponin similarly displays the characteristic signals of the steroid methyl groups and sugar anomeric centers. In the spirostanol and furostanol saponins, the ^{13}C NMR spectrum additionally contains a hemiacetal/spiroacetal signal typically at around δ_C 110–114 ppm [17, 95]. In combination with MS data analysis, the ^{1}H and ^{13}C NMR chemical shifts of the isolated steroidal saponin can be compared with reference data, for example, those compiled by Agrawal et al. [17, 95], allowing rapid identification of known structures.

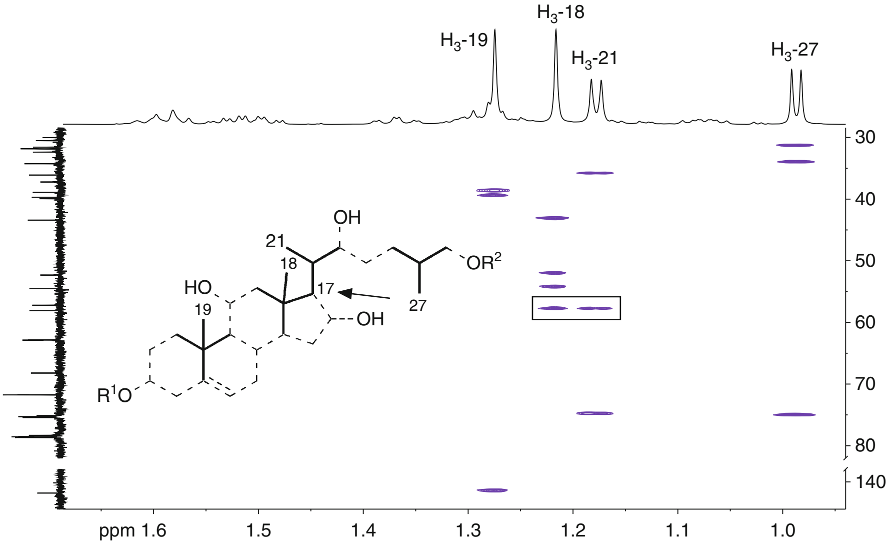


Fig. 105.2 Segment of the HMBC spectrum of heloside A (**55**) showing correlation of both H_3-18 and H_3-21 with C-17 ($R^1 = R^2 =$ β-D-glucose, pyridine-d_5, 500 MHz)

The structure determination of a new steroidal saponin usually requires the acquisition of a complete set of 1D and 2D NMR experiments including COSY, TOCSY, HSQC, HMBC, and ROESY spectra. A number of recent reviews provide information on the best choice of pulse sequences and post-acquisition processing strategies for the structure elucidation of natural products [96–98]. The examination of the long-range HMBC correlations of the characteristic steroid methyl group signals is a useful starting point for analysis. These correlations provide a means of rapid ^{1}H and ^{13}C NMR assignment of positions 18, 19, 21, and 27, since only those of H_3-18 and H_3-21 intersect (at position C-17) as illustrated in Fig. 105.2. The HMBC correlations of these methyl groups can then be used in combination with HSQC and COSY spectra to fully assign the steroidal aglycone. The gradient-selected HSQC experiment with spectral editing (i.e., CH and CH_3 up, CH_2 down) is particularly useful since it provides all short-range ^{13}C-^{1}H correlations and carbon multiplicity information, obviating the need for the separate acquisition of an edited DEPT spectrum.

The TOCSY experiment is invaluable for assignment of the saponin monosaccharide units as it allows the isolation of ^{1}H signals belonging to a single sugar from the original complex ^{1}H NMR spectrum. A 1D-selected TOCSY experiment is acquired by irradiation of a chosen, well-resolved signal followed by magnetization transfer throughout the mutually coupled spin system during the mixing period. Selective irradiation of a sugar anomeric doublet, for example, and acquisition of 1D TOCSY spectra with different mixing times, reveals the ^{1}H NMR signals of the entire sugar unit. Acquisition of a series of 1D TOCSY spectra with a range of mixing times allows the sequence of proton signals in a sugar to be assigned, which may be repeated for each attached sugar unit in a saponin and followed by assignment of ^{13}C NMR signals via HSQC. The 1D TOCSY experiment has two important advantages over 2D TOCSY; it provides improved resolution of ^{1}H signals, and the acquisition of a set of selected 1D experiments takes less time than acquisition of the corresponding 2D spectra. Correlations observed in an HMBC spectrum may then be used to map the sugar linkage pattern, greatly simplifying what was in the past a more onerous procedure involving permethylation followed by hydrolysis, with the non-methylated sites in the hydrolysis product revealing sites of sugar attachment.

3.4 Determination of Stereochemistry in Steroidal Saponins

Assignment of substituent and ring junction stereochemistry in a steroidal saponin is typically achieved through examination of through-space correlations in a 2D ROESY spectrum. The ROESY experiment is preferred over NOESY since the maximum observable nuclear Overhauser effect for compounds in the molecular weight range 750–2,000 Da (most steroidal saponins) is usually close to zero [96]. Again, the correlations of the steroidal angular methyl groups provide a helpful starting point for analysis, since they show through-space correlations with the nearby groups on the β (upper) face of the steroid. Along with correlations on the α (lower) face, these allow the unambiguous assignment of substituent and ring junction stereochemistry.

3.4.1 Determination of C-25 Absolute Configuration in Spirostanol and Furostanol Saponins

In spirostanols, the absolute configuration of C-25 can be determined via examination of the proton vicinal coupling constants for positions 24–26 as well as comparison of F ring ^{1}H and ^{13}C NMR chemical shifts with literature values [99, 100]. While stereochemistry is easily assigned within the rigid steroid ring system of spirostanols, it is more challenging in structures possessing a flexible side chain such as furostanols and open-chain glycsoides. A reliable but time-consuming method for the determination of C-25 stereochemistry in furostanol saponins is their conversion to the corresponding spirostanol, either through acid catalyzed hydrolysis or specific, enzymatic cleavage of the C-26 glucose residue to effect ring closure of the side chain [101]. The absolute configuration of C-25 in furostanols may also be more conveniently assigned by NMR spectroscopy. The geminal proton resonances of H_2-26 (δ_a and δ_b, $\Delta_{ab} = \delta_b - \delta_a$) are dependent on the configuration of C-25, being more resolved (greater Δ_{ab}) in 25*S* than 25*R* furostanols [102, 103]. Recently, the solvent dependency of this empirical rule has been highlighted; while 25*S* furostanols exhibit greater separation of the chemical shifts of H_2-26 in both pyridine-d_5 and methanol-d_4 (the two most commonly used solvents for acquisition of steroidal saponin NMR data), this behavior is less pronounced in methanol-d_4 [104]. The empirical rule proposed in the initial reports by Agrawal ($\Delta_{ab} \geq 0.57$ ppm for 25*S*; $\Delta_{ab} \leq 0.48$ ppm for 25*R*)

[102, 103] should be applied only when spectra are obtained in pyridine-d_5, while the revised rule ($\Delta_{ab} = 0.45–0.48$ ppm for 25*S*; $\Delta_{ab} = 0.33–0.35$ ppm for 25*R*) [104] should be used when spectra are acquired in methanol-d_4.

3.4.2 Side Chain Stereochemistry in Open-Chain Steroidal Saponins

The flexible nature of the steroidal side chain (C-20 to C-27) in open-chain glycosides hampers the assignment of stereochemistry via NMR spectroscopy. The absolute configurations of positions within the side chain of open-chain glycosides is often assigned via comparison of ^{1}H and ^{13}C NMR data with the literature, total synthesis, X-ray crystallography, or advanced Mosher ester analysis. For example, the (22*S*,25*R*) stereochemistry of helosides A and B (**55** and **56**) isolated from *C. luteum* was determined via X-ray crystallographic analysis of their aglycone helogenin [58]. For chamaelirosides A and B (**57** and **58**) isolated from the same plant, the stereochemistry of the vicinal C-23/C-24 diol was determined via total synthesis of the two *erythro* aglycone diastereomers with X-ray crystallographic analysis performed on an advanced synthetic intermediate (the aglycone acetonide) [88]. Comparison of the ^{1}H and ^{13}C NMR spectra of the two synthetic, diastereomeric aglycone acetonides with the acetonide derived from the natural aglycone of **57** and **58** revealed the (23*R*,24*S*) stereochemistry of the diol [88].

3.5 Determination of Sugar Absolute Configuration

The absolute configurations of attached monosaccharides in the saponin must be also be determined, as both D- or L- forms of some monosaccharides occur naturally. Methods reported for the determination of sugar absolute configuration in steroidal saponins typically involve acid hydrolysis of the saponin to yield free monosaccharide units, which are then subjected to either RPHPLC in line with refractive index/optical rotation detectors [34] or GC analysis of their trimethylsilyl thiazolidine derivatives from L-cysteine [44, 105]. An alternative procedure has been reported by König et al., in which methyl glycosides are generated via acid catalyzed methanolysis of the saponin before derivatization with TFAA and enantioselective GC analysis [106]. This method is preferred because the TFA-methyl glycosides give two or more peaks in enantioselective GC analysis (corresponding to formation of α- and β-anomers of the pyranoside/furanoside), allowing unambiguous identification of D- and L-enantiomers [106].

4 Biosynthesis of Plant Steroidal Saponins

The biosynthesis of plant sterols and saponins proceeds via the isoprene pathway with the cyclization and subsequent rearrangement of a C_{30} 2,3-oxidosqualene precursor, as discussed in reviews by Haralampidis et al. [107], Benveniste [108, 109], Kreis and Müller-Uri [110], and Osbourn et al. [111]. Following cyclization of 2,3-oxidosqualene (in the "chair-boat-chair" conformation), the resultant protosteryl carbocation is quenched in divergent pathways to yield either lanosterol (having $\Delta^{8(9)}$-unsaturation) or cycloartenol (having a cyclopropyl ring involving C-9, C-10, and C-19) [109, 110]. The C_{27} skeleton of plant sterols is then generated through the loss of three methyl groups mediated by sterol 4-demethylase and 14α-demethylase enzymes [109, 110]. The cycloartenol pathway of sterol biosynthesis appears to predominate in plants while the lanosterol pathway operates mainly in fungi and animals, although lanosterol glycosides have been reported from plants rarely (*vide supra*) [110]. Interestingly, the lanosterol synthases found in eudicots seem to have evolved independently of those found in fungi and animals, representing a catalytically unique class of lanosterol synthases [112]. The common plant metabolites sitosterol and stigmasterol are generated via C-24 alkylation of a steroidal precursor through the activity of sterol methyltransferases [113].

The basic C_{27} scaffold is elaborated through various enzymatic oxygenation and glycosylation reactions to generate the observed diversity of isolated steroidal saponins. The cholesterol aglycone is elaborated without further cyclizations in the case of open-chain steroidal glycosides, while one or two heterocyclic rings must be generated in furostanol and spirostanols, respectively. The 26-*O*-glycosylated furostanols are considered to be direct precursors to their spirostanol analogs, with specific cleavage of the 26-*O*-glucopyranose by a furostanol glycoside 26-*O*-β-glucosidase (F26G) leading to intramolecular spiroacetal formation [101]. It has been proposed that glycosylation of the steroidal nucleus can occur at various stages of saponin biosynthesis and it is known to be mediated by glycosyltransferases, such as the membrane-bound uridine 5′-diphosphate (UDP)-glucose: sterol glucosyltransferases [110]. There is some evidence that monosaccharide units are added successively to the aglycone substrate (as opposed to the attachment of a preformed polysaccharide) in triterpenoid saponin biosynthesis [114]; it remains to be seen whether this also applies in steroidal saponin biogenesis. Further structural diversity is generated by modifications such as specific acylation as seen in the OSW-1 family of glycosides (*vide supra*), perhaps carried out by a member of the BAHD family of acyltransferases [115]. (The BAHD family of acyltransferases is named for the first four members to be characterized: benzylalcohol *O*-acetyltransferase (BEAT); anthocyanin *O*-hydroxycinnamoyltransferase (AHCT); anthranilate *N*-hydroxycinnamoyl/benzoyltransferase (HCBT); and deacetylvindoline 4-*O*-acetyltransferase (DAT).)

5 Biological Activities of Plant Steroidal Saponins

Steroidal saponins isolated from plants have been reported to possess a wide range of biological activities. Early literature reports often describe steroidal saponins as having a bitter taste, perhaps reflecting an ecological function such as a protective effect against herbivory. A number of recent reviews have summarized the various bioactivities of steroidal and triterpenoid saponins, including cytotoxic/antitumor, hemolytic, hepatoprotective, antimicrobial, antibacterial, antifungal, and anti-inflammatory properties [3, 16, 116, 117]. Further, the therapeutic potential of saponins as anticancer agents has been brought into focus by reviews from Man et al. [118], Podolak et al. [119], Fuchs et al. [120], Bachran et al. [121], and Mimaki et al. [122].

As described in a review by Sautour et al. [2], a number of the above-discussed spirostanol and furostanol saponins isolated from *Dioscorea* species show cytotoxic activity against different human cancer cell lines. In particular, the cytotoxic activities of protodioscin (**3**) [123], methylprotodioscin (**4**) [124], gracillin (**6**) [125], methylprotogracillin (**8**) [126], protoneodioscin (**12**) [127], and methylprotoneodioscin (**13**) [128] were assessed against a panel of 60 human cancer cell lines as part of the National Cancer Institute anticancer drug screen. Compounds **3**, **8**, **12**, and **13** showed cytotoxic activity against most cell lines derived from leukemia and solid tumors [123, 126–128], while **4** was strongly cytotoxic against solid tumor cell lines and acted selectively against one colon cancer cell line and one breast cancer cell line [124]. Gracillin (**6**) was cytotoxic against most of the tested cancer cell lines, but displayed a lack of selectivity [125].

Among the open-chain steroidal glycosides, structures based on the 3β,16β,17α-trihydroxy-cholest-5-en-22-one aglycone, such as OSW-1 (**51**) and closely related saponins, exhibit potent bioactivities against a range of tumor cell lines. Compound **51** exhibits potent inhibitory activity against cyclic AMP phosphodiesterase [46] as well as cytotoxic activity against malignant tumor cell lines including leukemia HL-60, mouse mastrocarcinoma, human pulmonary adenocarcinoma, human pulmonary large cell carcinoma, and human pulmonary squamous cell carcinoma [47, 48]. A total of 20 glycosides have to date been reported to possess the 3β,16β,17α-trihydroxy-cholest-5-en-22-one aglycone. The evaluation of in vitro cytotoxic activity for this structurally related series of compounds allows their structure-activity relationships to be investigated; substitution of the C-16 diglycosyl unit by an aromatic ester group and the presence of a carbonyl group at C-22 appear to be required for cytotoxic activity [129].

6 Conclusions

Steroidal saponins, biosynthesized via the isoprene pathway, make up a large and structurally diverse class of plant natural product. Three main structural subfamilies of plant steroidal saponin exist: the spirostanol saponins, characterized by a bicyclic spiroacetal moiety at position C-22; the furostanol saponins, which instead possess a 22-hemiacetal, 22-methyl acetal, or $\Delta^{20(22)}$-unsaturation; and the open-chain steroidal glycosides, which lack the additional ring(s) present in spirostanol and furostanol saponins. The planar structure and stereochemistry of steroidal saponins is typically elucidated via a combination of multistage mass spectrometry (MS^n), 1D and 2D NMR spectroscopy, chemical degradation, and total synthesis. The observed structural diversity within this class of natural product is generated through differences in aglycone structure and glycosylation pattern, and is likely responsible for the wide range of reported biological activities for plant saponins.

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Tetraterpenes: Carotenoids 106

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Contents

1 Introduction 3252
2 Distribution and Occurrence 3254
3 Biosynthesis 3257
3.1 Carotenogenesis Genes and Gene Clusters 3257
3.2 IPP and Phytoene Synthesis 3257
3.3 Desaturation of Phytoene to Form Lycopene 3258
3.4 β-Carotene and α-Carotene Synthesis by Lycopene Cyclization 3258
3.5 Carotenogenesis in Land Plants 3264
3.6 Carotenogenesis in Algae 3264
3.7 Carotenogenesis in Cyanobacteria 3265
3.8 Carotenogenesis in Purple Bacteria 3268
3.9 Carotenogenesis in Green Sulfur Bacteria 3271
3.10 Carotenogenesis in Non-phototrophic Bacteria 3273
4 Conclusion 3274
References 3277

Abstract

Carotenoids are C_{40}-compounds consisting of eight isopentenylpyrophosphate units. More than 750 structurally defined carotenoids are found in nature. They are synthesized by oxygenic phototrophs (land plants, algae, and cyanobacteria), anoxygenic phototrophs (purple bacteria, green sulfur bacteria, green filamentous bacteria, and heliobacteria), some eubacteria, some archaea, and some fungi. Most of animals also contain carotenoids, which they obtain from foods. The type of carotenoid present depends on variation in carotenogenesis pathways, different characteristics of carotenogenesis enzymes, and the

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presence or absence of certain carotenogenesis genes. Some of the major carotenogenesis pathways have been elucidated, but some major and minor pathways remain unknown. These variations can be used for chemotaxonomical classifications of organisms. Here major carotenogenesis pathways, characteristics of enzymes and genes, and some biological functions of carotenoids are described.

Keywords
Algae • carotenogenesis • carotenoid • chemotaxonomy • cyanobacteria • green sulfur bacteria • phototroph • purple bacteria

Abbreviations

BChl	Bacteriochlorophyll
Chl	Chlorophyll
DOXP	1-Deoxy-D-xylulose-5-phosphate
IPP	Isopentenylpyrophosphate
MVA	Mevalonate

1 Introduction

Phototrophic organisms including land plants, algae, cyanobacteria, and photosynthetic bacteria grow by photosynthesis, and necessarily contain (bacterio)chlorophylls (BChls/Chls) as well as carotenoids, whose main functions are light-harvesting and photoprotection. Furthermore, most non-phototrophic organisms including non-photosynthetic eubacteria, archaea, fungi, and animals contain carotenoids mainly for protection from harmful reactive oxygen species (Table 106.1). More than 750 structurally defined carotenoids are reported from nature [1]. Except for animals, these organisms can produce many types of carotenoids, which are synthesized in diverse carotenogenesis pathways. These carotenoids and carotenogenesis pathways can be used as chemotaxonomic makers [2–8]. Attempts have been made to cultivate single-cell algae and bacteria for a long time, but the numbers of single-cell algae and bacteria cultivated were limited. With the recent development of culture techniques, some single-cell species can be cultured, and their characteristics including their pigments can be studied. Algal and bacterial phylogenetics have been developed with the development of taxonomic technology involving DNA base sequences of 16S or 18S rRNA and some genes.

In addition, characteristics of carotenogenesis enzymes and genes have been investigated. Some carotenogenesis genes have high similarity from bacteria to land plants, but some differ greatly. Some homologous genes can be searched from complete genome sequences of many organisms at present, but some new carotenogenesis enzymes and genes for unique structures have yet to be identified.

Table 106.1 Distribution of characteristic carotenoids and carotenoid derivatives

		Characteristic carotenoids				Carotenoid derivatives				
Organisms	Presence of carotenoids in species	Elimination of some carbons from C_{40}-carotenoid	C_{30}-carotenoid	C_{45}- and C_{50}-carotenoid	Aromatic-carotenoid	Carotenoid fatty acid ester	Carotenoid glycoside	Carotenoid glycoside fatty acid ester	Carotenoid sulfate	Carotenoid-protein complexes
Archaea	A part			+			+			
Eubacteria	A part		+	+	+		+	+	+	
Photosynthetic bacteria	All		+		+		+	+	+	+
Cyanobacteria	All						+			+
Algae	All	+				+	+			+
Land plants	All	+				+	+	+		+
Fungi	A part									
Invertebrate	Most	+			+	+		+	+	+
Vertebrate	Most	+				+				+

+ Means that there are species to have this carotenoid in the class

2 Distribution and Occurrence

Distributions of carotenoids can be used for chemotaxonomical classification (Tables 106.1, 106.2). Goodwin summarized the distribution of carotenoids in land plants, algae, and bacteria [2], and animals, especially invertebrates [3]. Rowan summarized the distribution in algae [4]. Recently, phytoplanktonic pigments have been summarized in a book by Roy et al. [9], and it compiles characteristics of algal carotenoids and Chls, including their structures, absorption spectra, and HPLC elution profiles [8].

Usually, "carotenoid" is used to indicate C_{40}-compounds consisting of eight isopentenylpyrophosphate (IPP) units. Some variations in carbon numbers of carotenoids are also observed (Table 106.1). C_{30}-carotenoids are synthesized by the combination of two farnesol instead of the usual geranylgeraniol unit. C_{45}- and C_{50}-carotenoids are synthesized by the addition of one and two IPP units to C_{40}-carotenoids, respectively. Unique carotenoid derivatives, such as carotenoid glycoside fatty acid esters and carotenoid sulfates, are also distributed in specific organisms (Table 106.1).

Land plants including moss, fern, gymnosperm, and angiosperm majorly contain the carotenoids, β-carotene **13**, violaxanthin **16**, 9′-*cis* neoxanthin **19**, and lutein **11** in chloroplasts for photosynthesis. Some unique carotenoids are found in specific organs, such as petals, fruits, and roots; these carotenoids are not involved in photosynthesis.

Algae are classified throughout many divisions (Table 106.2). Their sizes range from single cells of picoplankton to seaweeds. Some carotenoids are broadly distributed, and some are division specific. Allene (C=C=C), acetylene (C≡C), and acetyl (-O-CO-CH_3) groups are unique groups of carotenoids found in algae. Alloxanthin **66** is present only in Cryptophyta, fucoxanthin **75** is present mainly in Heterokontophyta, peridinin **72** and P457 **70** are present only in Dinophyta [10], and siphonaxanthin **63** is present in Chlorophyta [6, 7, 11].

Cyanobacteria are considered to originate from photosynthetic bacteria, and to have given rise to plant chloroplasts through evolution and symbiosis. The carotenoid structures and carotenogenesis pathways differ among photosynthetic bacteria, cyanobacteria, and chloroplasts, and accordingly, carotenoids seem to change discontinuously. It is interesting to speculate on how these different carotenogenesis pathways have evolved. Cyanobacteria produce unique ketocarotenoids and carotenoid glycosides (myxol glycoside **83** and oscillol diglycoside **87**) (Table 106.2) [12, 13].

Anoxygenic photosynthetic bacteria are classified into purple bacteria, green sulfur bacteria, green filamentous bacteria, and heliobacteria. Purple bacteria produce unique acyclic and tertiary methoxy carotenoids. Green sulfur and green filamentous bacteria mainly produce monocyclic carotenoids and its derivatives, and β-carotene **13**. Heliobacteria produce C_{30}-carotene 4,4′-dipaoneurosporene **81** and its derivatives [5, 14]. Carotenogenesis of purple and green sulfur bacteria are described later.

Table 106.2 Distribution of carotenoids in oxygenic phototrophs

Division	Carotenes		Major xanthophylls											Chlorophylls			
Class	β	α	Ze	Vi	Ne	Da	Dd	Fx	Va	Lu	Lo	Sx	Others	*a*	*b*	*c*	*d*
Cyanophyta	H	L	H										No, L; Ec, H; My, H	H	L		L
Glaucophyta	H		H											H			
Rhodophyta																	
Unicellular type	H		H											H			
Macrophytic type	L	L	H	L						H				H			
Cryptophyta		H	L										Al, L; Cr, L; Mo, L	H		H	
Heterokontophyta																	
Chrysophyceae	H		L			L	L	H	L					H		H	
Raphidophyceae	H		H	L		L	L	L						H		H	
Bacillariophyceae	H		L			L	L	H						H		H	
Phaeophyceae	H		H	H		L	L	H						H		H	
Xanthophyceae	H		L			H	H						Va-FA, L	H		H	
Eustigmatophyceae	H			H					L					H			
Haptophyta	H		L			L	H	H					Fx-FA, L	H		H	
Dinophyta	L		L			L	H						Pe, H	H		H	
Euglenophyta	H		L		L	L	H				L	L		H	H		
Chlorarachniophyta	H		L	L	L					L	L		Lo-FA, L	H	H		

(*continued*)

Table 106.2 (continued)

Division	Carotenes		Major xanthophylls											Chlorophylls			
Class	β	α	Ze	Vi	Ne	Da	Dd	Fx	Va	Lu	Lo	Sx	Others	*a*	*b*	*c*	*d*
Chlorophyta																	
Prasinophyceae	H	L	L	H	H					L	L	H	Pr, L; Lo-FA, L; Sx-FA, H	H	H		
Chlorophyceae	H	H	L	H	H					H	L	L	Sx-FA, L	H	H		
Ulvophyceae	H	L	L	H	H					L	L	L	Sx-FA, H	H	H		
Trebouxiophyceae	H		L	H	H					H				H	H		
Charophyceae	H		L	H	H					H				H	H		
Land Plants	H	L	L	H	H					H				H	H		

H Major carotenoid in most species of the division or class, *L* low content in most species or major carotenoid in limited species
α α-carotene **10**, *β* β-carotene **13**, *Al* alloxanthin **66**, *Cr* crocoxanthin **60**, *Da* diatoxanthin **65**, Dd diadinoxanthin **67**, *Ec* echinenone **51**, *-FA* fatty acid ester, *Fx* fucoxanthin **75**, *Lo* loroxanthin **62**, *Lu* lutein **11**, *Mo* monadoxanthin **59**, *My* myxol 2′-glycoside **83** and oscillol 2,2′-diglycoside **87**, *Ne* 9′-*cis* neoxanthin **19**, *No* nostoxanthin **77**, *Pe* peridinin **72**, *Pr* prasinoxanthin **61**, *Sx* siphonaxanthin **63**, *Sx-FA* siphonaxanthin ester **64**, *Va* vaucheriaxanthin **69**, *Vi* violaxanthin **16**, *Ze* zeaxanthin **14**

Archaea and eubacteria are divided into approximately 26 phyla, and at least 13 phyla contain carotenoids. Pigments in some species are identified as carotenoids, but those in many species have not been investigated. Consequently, the relation between the phylogenetic classification and presence of carotenoids is not known. These carotenoids may be photoprotective.

Usually carotenoids take all-*trans* forms. Some carotenoids take *cis* forms because of steric hindrance of some groups or for functions as indicated in the text.

3 Biosynthesis

3.1 Carotenogenesis Genes and Gene Clusters

In the 1980s, some carotenogenesis genes were cloned and functionally confirmed, and the characteristics of enzymes were investigated. Around 1990, carotenogenesis gene clusters were found in some eubacteria, such as *Rhodobacter capsulatus* [15] and *Pantoea ananatis* (previously *Erwinia uredovora*) [16]. Complete carotenogenesis pathways and the functions of each carotenogenesis gene involved in these pathways were investigated. All purple bacteria may have such a carotenogenesis gene cluster [14]. The aerobic photosynthetic bacterium *Bradyrhizobium* ORS278 has two sets of gene clusters for spirilloxanthin **35** and canthaxanthin **53** syntheses [17, 18]. Some eubacteria, such as *Paracoccus*, *Streptomyces*, and *Brevundimonas* contain carotenogenesis gene clusters. Furthermore, a part of the pathways was investigated in organisms lacking gene clusters, such as some eubacteria (including green sulfur bacteria, green filamentous bacteria, heliobacteria, and cyanobacteria), archaea, algae, land plants, and fungi.

3.2 IPP and Phytoene Synthesis

IPP, a C_5-compound, is the source of carotenoids, isoprenoids, terpenes, quinones, and phytol of BChls and Chls. There are two known independent pathways of IPP synthesis: the classical mevalonate (MVA) pathway and the alternative, non-mevalonate, 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway [19, 20]. In the MVA pathway, acetyl-coenzyme A is converted to IPP through mevalonate. The enzymes and genes involved in this pathway are well studied [21]. The DOXP pathway was found in the 1990s, and in this pathway, pyruvate and glyceraldehyde are converted to IPP.

The distribution of these two pathways among prokaryotes follows no obvious pattern of taxonomic classification. The MVA pathways are found in some eubacteria including green filamentous bacteria, archaea, fungi, plant cytoplasm, and animals, whereas the DOXP pathway is found in some eubacteria (including purple bacteria, green sulfur bacteria, heliobacteria, and cyanobacteria) and the plastids of algae and land plants [19]. Carotenoids are synthesized from the nuclear genes in plastids. Exceptionally among algae, Euglenophyceae has only the MVA pathway, and Chlorophyceae has only the DOXP pathway [19].

Most carotenoids consist of eight IPP units. Farnesyl pyrophosphate **1** (C_{15}) is synthesized from three IPPs, and then one IPP is added to farnesyl pyrophosphate by geranylgeranyl pyrophosphate synthase (CrtE, GGPS) to yield geranylgeranyl pyrophosphate **2** (C_{20}). In a head-to-head condensation of the two C_{20}-compounds, the first carotene phytoene **3** (C_{40}) is formed by phytoene synthase (CrtB, Pys, Psy) using ATP [22, 23]. This pathway has been confirmed by cloning genes from two species of *Rhodobacter* (purple bacteria) and two species of *Pantoea* (previously *Erwinia*) [16, 22, 23]. *crtE* and *crtB* have high sequence similarity from bacteria to land plants, respectively.

3.3 Desaturation of Phytoene to Form Lycopene

Four desaturation steps are involved in the conversion of phytoene to lycopene (Fig. 106.1). Oxygenic phototrophs such as cyanobacteria, algae, and land plants require three enzymes: phytoene desaturase (CrtP, Pds), ζ-carotene desaturase (CrtQ, Zds), and *cis*-carotene isomerase (CrtH, CrtISO). CrtP catalyzes the first two desaturation steps from phytoene to ζ-carotene **5** through phytofluene **4**, and CrtQ catalyzes two additional desaturation steps from ζ-carotene to lycopene through neurosporene **6**. During desaturation by CrtQ, neurosporene and lycopene are isomerized to poly-*cis* forms such as 7,9,7′,9′-poly-*cis* lycopene **8**, and then CrtH isomerizes the poly-*cis* forms to all-*trans* forms. Light causes photoisomerization of *cis* forms of neurosporene and lycopene to all-*trans* forms [24–27]. The functions of these enzymes have been mainly confirmed in cyanobacteria, green algae, and land plants (Table 106.3). CrtP of *Synechococcus elongatus* PCC 7942 is stimulated by NAD(P) and oxygen as a possible final electron acceptor [28].

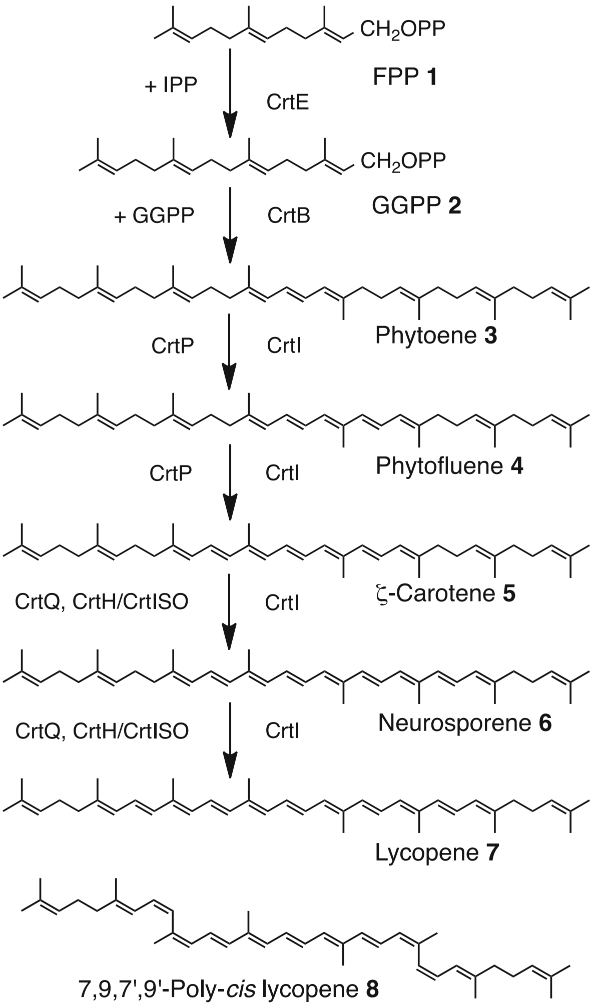
In contrast, eubacteria (including purple bacteria, green filamentous bacteria, and heliobacteria), archaea, and fungi use only one enzyme phytoene desaturase (CrtI) to convert phytoene **3** to lycopene **7**. Exceptionally, the primitive cyanobacterium *Gloeobacter violaceus* PCC 7421 uses this CrtI [29, 30]. *G. violaceus* is the first oxygenic phototroph that has been shown to use this enzyme (Table 106.3), whereas green sulfur bacteria use CrtP, CrtQ, and CrtH [14, 31].

Usually, almost all carotenoids in organisms are synthesized from lycopene **7**. Most carotenoids are dicyclic carotenoids, i.e., β-carotene **13**, α-carotene **10**, and their derivatives. Some eubacteria and some fungi produce the monocyclic carotenoids, γ-carotene **12**, and its derivatives. Acyclic carotenoids are mostly found in purple bacteria and some archaea [1–3].

3.4 β-Carotene and α-Carotene Synthesis by Lycopene Cyclization

All carotenoids in oxygenic phototrophs are dicyclic carotenoids, i.e., β-carotene, α-carotene, and their derivatives (Fig. 106.2, Table 106.2). Exceptionally, myxol

Fig. 106.1 Carotenogenesis pathway leading to conversion of phytoene to lycopene and functionally confirmed enzymes involved in this pathway. Oxygenic phototrophs require three enzymes (CrtP, CrtQ, and CrtH), whereas anoxygenic phototrophs, bacteria, and fungi use only CrtI. See the text and Table 106.3 for precise explanations


CH2OPP
FPP 1
+ IPP
CrtE
CH2OPP
GGPP 2
+ GGPP
CrtB
Phytoene 3
CrtP
CrtI
Phytofluene 4
CrtP
CrtI
ζ-Carotene 5
CrtQ, CrtH/CrtISO
CrtI
Neurosporene 6
CrtQ, CrtH/CrtISO
CrtI
Lycopene 7
7,9,7',9'-Poly-cis lycopene 8

glycosides **83** and oscillol diglycosides **88** in cyanobacteria are monocyclic and acyclic carotenoids, respectively.

Lycopene **7** is cyclized into either β-carotene **13** through γ-carotene **12** or α-carotene **10** through δ-carotene **9**. Three distinct families of lycopene cyclases have been identified in carotenogenetic organisms (Table 106.3) [13, 32–34]. One large family contains CrtY in eubacteria except most cyanobacteria, and CrtL (CrtL-b, Lcy-b) in some cyanobacteria and land plants. Lycopene ε-cyclases (CrtL-e, Lcy-e) from land plants and lycopene β-monocyclases (CrtYm, CrtLm) from eubacteria are also included in this family. The amino acid sequences of these lycopene cyclases exhibit a significant five conserved regions [32, 33, 35, 36] and

Table 106.3 Functionally confirmed carotenogenesis enzymes and genes from some organisms

Enzymes	Genes	Classification of organisms	Example of organisms
Geranylgeranyl-pyrophosphate synthase (EC 2.5.1.29)	*crtE*	Purple bacteria	*Rhodobacter sphaeroides* [82]
	crtE	Cyanobacteria	*Thermosynechococcus elongatus* [93]
	crtE	Eubacteria	*Pantoea ananatis* [16]
	crtE, ggps	Archaea	*Archaeoglobus fulgidus* [94]
	carG, ggps	Fungi	*Mucor circinelloides* [95]
	crtE, ggps	Land plants	*Arabidopsis thaliana* [96]
Phytoene synthase (EC 2.5.1.32)	*crtB*	Purple bacteria	*Rhodobacter sphaeroides* [82]
	crtB	Green sulfur	*Chlorobaculum tepidum* [31]
	crtB, psy	Cyanobacteria	*Synechocystis* sp. PCC 6803 [97]
	crtB	Eubacteria	*Pantoea ananatis* [16]
	crtB	Algae	*Chlamydomonas reinhardtii* [98]
	crtB, psy, pys	Land plants	*Arabidopsis thaliana* [99]
Phytoene synthase (bifunctional-fusion-type)	*crtYB, carRA*	Fungi	*Mucor circinelloides* [45]
Diapophytoene synthase	*crtM*	Eubacteria	*Staphylococcus aureus* [100]
Phytoene desaturase (bacterial type)	*crtI*	Purple bacteria	*Rhodobacter sphaeroides* [82]
	crtI	Cyanobacteria	*Gloeobacter violaceus* [29, 30]
	crtI	Eubacteria	*Pantoea ananatis* [16]
	carB	Fungi	*Mucor circinelloides* [101]
Diapophytoene desaturase	*crtN*	Eubacteria	*Staphylococcus aureus* [100]
Phytoene desaturase (plant type)	*crtP*	Green sulfur	*Chlorobaculum tepidum* [31]
	crtP, pds	Cyanobacteria	*Synechocystis* sp. PCC 6803 [102]
	pds	Algae	*Chlamydomonas reinhardtii* [103]
	pds	Land plants	*Arabidopsis thaliana* [104]
ζ-Carotene desaturase	*crtQ*	Green sulfur	*Chlorobaculum tepidum* [31]
	crtQ	Cyanobacteria	*Synechocystis* sp. PCC 6803 [105]
	zds	Land plants	*Arabidopsis thaliana* [104]
cis-Carotene isomerase	*crtH*	Green sulfur	*Chlorobaculum tepidum* [31]
	crtH	Cyanobacteria	*Synechocystis* sp. PCC 6803 [24, 25]
	crtISO	Land plants	*Arabidopsis thaliana* [26, 27]
Lycopene cyclase, Lycopene β-cyclase (Y- and L-type)	*crtY*	Purple bacteria	*Erythrobacter elongatus* [106]
	crtL	Cyanobacteria	*Synechococcus* sp. PCC 9742 [38]
	crtY	Eubacteria	*Pantoea ananatis* [16]
	lcy	Algae	*Haematococcus pluvialis* [107]
	crtL-b, lcy-b	Land plants	*Arabidopsis thaliana* [47]
Lycopene β-monocyclase	*crtYm, crtLm*	Eubacteria	*Paracoccus* P99-3 [78]
Lycopene cyclase (fusion-type)	*crtYcd*	Eubacteria	*Algoriphagus* KK10202C [44]
		Archaea	*Halobacterium salinarum* [42]

(*continued*)

Table 106.3 (continued)

Enzymes	Genes	Classification of organisms	Example of organisms
Lycopene cyclase (two peptide-type)	*crtYc* + *Yd*	Eubacteria	*Mycobacterium aurum* [41]
Lycopene cyclase (bifunctional-fusion-type)	*crtYB, carRA*	Fungi	*Mucor circinelloides* [45]
Lycopene cyuclase (*cruA*/*P*-type)	*cruA*	Green sulfur	*Chlorobaculum tepidum* [34]
	cruA	Cyanobacteria	*Synechococcus* sp. PCC 7002 [34]
	cruP		*Synechococcus* sp. PCC 7002 [34]
Lycopene ε-cyclase	*crtL-e*	Cyanobacteria	*Prochlorococcus marinus* [39]
	crtL-e, lcy-e	Land plants	*Arabidopsis thaliana* [47]
Desaturase/ methyltransferase (ϕ end group formation)	*crtU*	Green sulfur	*Chlorobaculum tepidum* [31]
	crtU	Eubacteria	*Streptomyces griseus* [89]
Desaturase/ methyltransferase (χ end group formation)	*cruE*	Cyanobacteria	*Synecococcus* sp. PCC 7002 [108]
β-Carotene hydroxylase (nonheme-type)	*crtR*	Cyanobacteria	*Synechocystis* sp. PCC 6803 [68, 69]
	crtZ	Eubacteria	*Pantoea ananatis* [16]
	crtR-b	Algae	*Haematocuccus luvialis* [54]
	crtR-b, bch	Land plants	*Arabidopsis thaliana* [109]
β-Carotene hydroxylase (P450-type)		Eubacteria	*Thermus thermophiles* [110]
		Land plants	*Arabidopsis thaliana* [52]
β-Carotene 2-hydroxylase	*crtG*	Cyanobacteria	*Thermosynechococcus elongatus* [67]
	crtG	Eubacteria	*Brevundimonas* sp. SD212 [70]
ε-Carotene hydroxylase (P450-type)		Land plants	*Arabidopsis thaliana* [52]
Zeaxanthin epoxidase	*zep, npq*	Algae	*Chlamydomonas reinhardtii* [55]
	zep, aba	Land plants	*Arabidopsis thaliana* [111]
Violaxanthin de-epoxidase	*vde*	Algae	*Mantonilla squamata* [56]
	vde	Land plants	*Spinicia oleracea* [112]
Neoxanthin synthase	*nsy, nxs*	Land plants	*Lycopersicon esculentum* [49, 50]
Capsanthin-capsorubin synthase	*ccs*	Land plants	*Capsicum annuum* [113]
β-Carotene ketolase, β-Carotene oxygenase (*crtI*-type)	*crtO*	Cyanobacteria	*Synechocystis* sp. PCC 6803 [71]
	crtO	Eubacteria	*Deinococcus radiodurans* [92]
β-Carotene ketolase, β-Carotene oxygenase (*crtW*-type)	*crtW*	Purple bacteria	*Bradyrhizobium* ORS278 [17]
	crtW	Cyanobacteria	*Anabaena* sp. PCC 7120 [73]
	crtW, bkt	Algae	*Haematococcus pluvialis* [61]
Astaxanthin synthase	*crtS, asy*	Fungi	*Xanthophyllomyces denrorhous* [91]
Spheroidene monooxygenase	*crtA*	Purple bacteria	*Rhodobacter sphaeroides* [82]

(*continued*)

Table 106.3 (continued)

Enzymes	Genes	Classification of organisms	Example of organisms
Acyclic carotenoid C-1,2 hydratase	*crtC*	Purple bacteria	*Rhodobacter sphaeroides* [82]
	crtC	Green sulfur	*Chlorobaculum tepidum* [31]
	crtC	Eubacteria	*Myxococcus xanthus* [114]
Carotenoid C-1,2-hydratase	*cruF*	Cyanobacteria	*Synechococcus* sp. PCC 7002 [115]
	cruF	Eubacteria	*Deinococcus radiodurans* [116]
Acyclic carotenoid C-3,4-desaturase	*crtD*	Purple bacteria	*Rhodobacter sphaeroides* [82]
	crtD	Eubacteria	*Deinococcus radiodurans* [78]
Acyclic 1-hydroxycaroteniod methyltransferase	*crtF*	Purple bacteria	*Rhodobacter sphaeroides* [82]
Neoxanthin cleavage enzyme	*nce*	Land plants	*Zea mays* [117, 118]
β-Carotene-15,15′-monooxygenase (bacterio-opsin)	*diox*	Cyanobacteria	*Synechocystis* sp. PCC 6803 [119]
	brp	Archaea	*Halobacterium salinarum* [120]
	carX	Fungi	*Fusarium fujikuroi* [121]
(retinal-synthesis)	*ninaB, bcd*	Animal	*Drosophila melanogaster* [122, 123]
Zeaxanthin glucosylase	*crtX*	Eubacteria	*Pantoea ananatis* [16]
Glucosyltransferase	*cruC*	Green sulfur	*Chlorobaculum tepidum* [79]
Glucosyl esterase, acyltransferase	*cruD*	Green sulfur	*Chlorobaculum tepidum* [79]

have an NAD(P)/FAD-binding motif [37]. Maresca et al. [34] divided this family into two, CrtY and CrtL families.

Two cyanobacteria also contain these enzymes (Table 106.3). *Synechococcus elongatus* PCC 7942 contains a functional CrtL [38]. *Prochlorococcus marinus* MED4 contains two lycopene cyclases, CrtL-b and CrtL-e. CrtL-b exhibits lycopene β-cyclase activity, whereas CrtL-e is a bifunctional enzyme having both lycopene ε-cyclase and lycopene β-cyclase activities [39]. The combination of these two cyclases allows the production of β-carotene, α-carotene, and ε-carotene. Both enzymes might have originated from the duplication of a single gene. The characteristics of this CrtL-e are somewhat different from those observed in land plants [40].

The second family of lycopene cyclases, heterodimer (*crtYc* and *crtYd*) or monomer (*crtYc-Yd*), has been found in some eubacteria, archaea, and fungi [32, 33, 41–44], but not in phototrophs. CrtYc-CrtYd of some fungi is fused with CrtB and is named CrtYB (Table 106.3) [45].

A new third family of functional lycopene cyclase CruA has been found in the green sulfur bacterium *Chlorobaculum* (previously *Chlorobium*) *tepidum*, and the main product of CruA is γ-carotene **12** in *Escherichia coli* with a lycopene **7** background [34]. Homologous genes *cruA* and *cruP* have been found in the genome of *Synechococcus* sp. PCC 7002, and the main product of CruA and CruP is

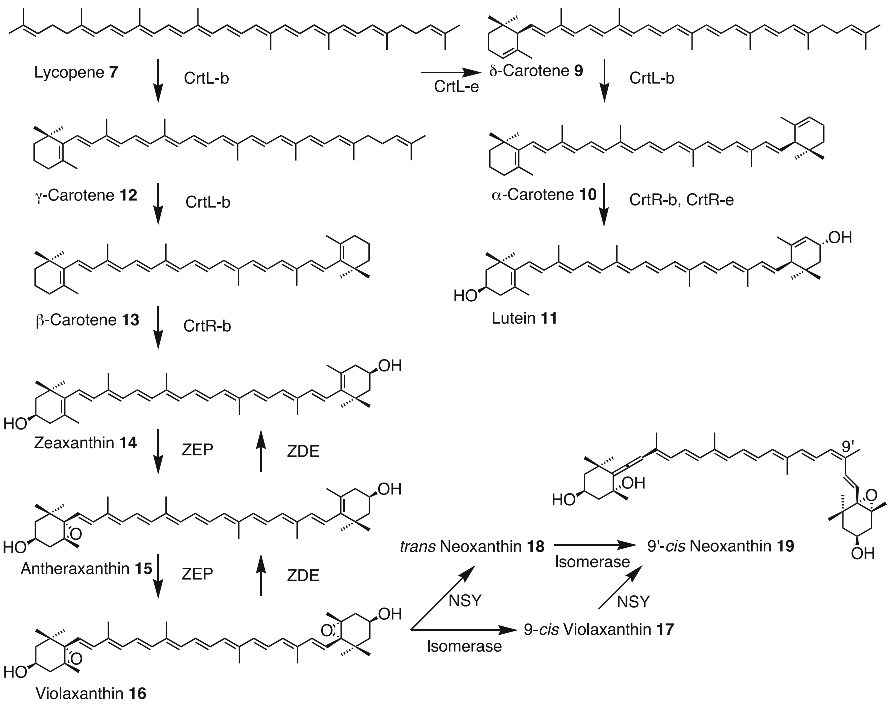

Lycopene 7
CrtL-b
CrtL-e
δ-Carotene 9
CrtL-b
γ-Carotene 12
CrtL-b
α-Carotene 10
CrtR-b, CrtR-e
β-Carotene 13
CrtR-b
Lutein 11
HO
OH
Zeaxanthin 14
ZEP
ZDE
Antheraxanthin 15
ZEP
ZDE
O
trans Neoxanthin 18
Isomerase
9'-cis Neoxanthin 19
9'
NSY
NSY
9-cis Violaxanthin 17
Isomerase
Violaxanthin 16

Fig. 106.2 Carotenogenesis pathways in land plants and functionally confirmed enzymes involved in these pathways. These are commonly found in land plants

β-carotene **13** and γ-carotene, respectively, in *E. coli* with a lycopene background (Table 106.3) [34]. In addition, the homologous genes of *cruA* and *cruP* are widely distributed in cyanobacteria such as *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120; however, these *cruA*- and *cruP*-like genes from both species did not show lycopene dicyclase or monocyclase activities [46]. *Synechococcus elongatus* PCC 6301 and PCC 7942 possess *crtL*-, *cruA*- and *cruP*-like genes; consequently, distributions of functional lycopene cyclases in cyanobacteria are unknown.

Because *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 lack *crtL*-like genes and possess nonfunctional *cruA*-like genes, there is the possibility of a fourth new family of lycopene cyclases in these cyanobacteria. Further studies of functional lycopene cyclases (e.g., CrtL- and CruA-like) in cyanobacteria are needed.

Distribution of α-carotene **10** and its derivatives is limited in some algae classes and land plants (Table 106.2). CrtL-e and *crtL-e* have not yet been identified in algae. However, in some species of land plants, the characteristics of CrtL-e have been investigated [40, 47], and *crtL-e* has been shown to have sequence homology with *crtL-b*. Lycopene **7** is first converted to δ-carotene **9** by CrtL-e and then to α-carotene by CrtL-b. γ-Carotene **12** produced by CrtL-b is not a suitable substrate for CrtL-e (Fig. 106.2).

3.5 Carotenogenesis in Land Plants

Most of the carotenogenesis pathways and the functionally confirmed enzymes in land plants have been studied (Fig. 106.2, Table 106.3). Hydroxyl groups are introduced into β-carotene **13** by β-carotene hydroxylase (CrtR, CrtR-b, BCH) to produce zeaxanthin **14**. Epoxy groups are introduced into zeaxanthin by zeaxanthin epoxidase (Zep, NPQ) to produce violaxanthin **16** through antheraxanthin **15**. Under high light conditions, violaxanthin is converted to zeaxanthin by violaxanthin de-epoxidase (Vde) for dispersion of excess energy from excited Chls. These reactions catalyzed by Zep and Vde constitute the xanthophyll cycle [48]. One end group of violaxanthin is converted to an allene group of neoxanthin **18** by neoxanthin synthase (Nsy) [49, 50]. Because all neoxanthin in chloroplasts has the 9′-*cis* form of 9′-*cis* neoxanthin **19**, an unknown 9′-isomerase converting all-*trans* neoxanthin to 9′-*cis* neoxanthin and/or all-*trans* violaxanthin to 9-*cis* violaxanthin **17** should be present [51].

In *Arabidopsis thaliana*, β-carotene **13** is hydroxylated mainly by the non-heme di-iron enzymes, BCH1 and BCH2 (CrtR-b), to produce zeaxanthin **14**, whereas α-carotene **10** is mainly hydroxylated by the cytochrome P450 enzymes CYP97A3 for the β-end group and CYP97C1 for the β- and ε-end groups to produce lutein **11** [52, 53].

3.6 Carotenogenesis in Algae

Little is known about the carotenogenesis pathways among algae, but some are proposed based on the chemical structures of carotenoids (Fig. 106.3). Functionally confirmed enzymes are mainly reported in Chlorophyceae including *Chlorella*, *Chlamydomonas*, *Dunaliella*, and *Haematococcus* for CrtB, CrtP, CrtL-b, CrtR-b [54], Zep [55], Vde [56], and CrtW (Table 106.3) [6].

In the cell-free preparation of the Dinophyta *Amphidinium carterae*, ^{14}C-labellled zeaxanthin **14** was incorporated into neoxanthin **18**, and then into acetylenic diadinoxanthin **67** and C_{37} peridinin **72** (Fig. 106.3). In addition, the three carbon atoms (C-13′,14′,20′) of peridinin were eliminated from neoxanthin (C-13,14,20) [57, 58]. In organic chemistry, the C-7,8 double bond of zeaxanthin can be oxidized to the triple bond (acetylene group) of diatoxanthin **65** [59]. Enzymes for these reactions remain unknown.

Allenic carotenoids are very limited in algae (Table 106.2). From their chemical structures, all-*trans*-neoxanthin **18** might be converted to fucoxanthin **75**, dinoxanthin **74**, peridinin **72**, vaucheriaxanthin **69**, and diadinoxanthin **67**, but the pathways and enzymes remain unknown (Fig. 106.3) [6].

Both xanthophyll and diadinoxanthin cycles are active in algae (Fig. 106.3) [60]. Diatoxanthin **65** is converted to diadinoxanthin **67** by diatoxanthin epoxidase, and under high light conditions, diadinoxanthin is converted to diatoxanthin by diadinoxanthin de-epoxidase. The process is similar to the xanthophyll cycle in land plants, but the enzymes and genes involved have not yet been isolated.

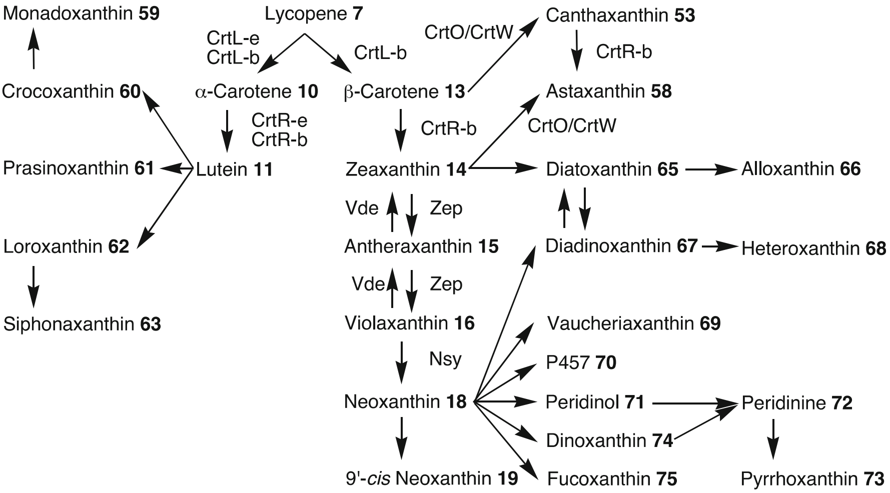

Monadoxanthin 59
Lycopene 7
CrtL-e
CrtL-b
CrtL-b
CrtO/CrtW
Canthaxanthin 53
CrtR-b
Crocoxanthin 60
α-Carotene 10
β-Carotene 13
Astaxanthin 58
CrtR-e
CrtR-b
CrtR-b
CrtO/CrtW
Prasinoxanthin 61
Lutein 11
Zeaxanthin 14
Diatoxanthin 65
Alloxanthin 66
Vde
Zep
Loroxanthin 62
Antheraxanthin 15
Diadinoxanthin 67
Heteroxanthin 68
Vde
Zep
Siphonaxanthin 63
Violaxanthin 16
Vaucheriaxanthin 69
Nsy
P457 70
Neoxanthin 18
Peridinol 71
Peridinine 72
Dinoxanthin 74
9'-cis Neoxanthin 19
Fucoxanthin 75
Pyrrhoxanthin 73

Fig. 106.3 Carotenogenesis pathways in algae and functionally confirmed enzymes involved in these pathways. The pathways and enzymes vary with algal divisions and classes (Table 106.2) [6]

Under a stressful environment such as high light, UV irradiation, and nutrition stress, some Chlorophyceae such as *Haematococcus*, *Chlorella*, and *Scenedesmus*, accumulate the ketocarotenoids canthaxanthin **53** and astaxanthin **58**, which are synthesized by the combination of CrtR-b and β-carotene ketolase (CrtW, BKT) (Fig. 106.3, Table 106.3) [61–65]. Note that although β-carotene ketolase of *Haematococcus* and *Chlorella* were named CrtO at first [62, 63], they are CrtW-type not CrtO-type from amino acid sequences (Table 106.3).

Lutein **11** and its derivatives are found only in Rhodophyta (macrophytic type), Cryptophyta, Euglenophyta, Chlorarachniophyta, and Chlorophyta (Table 106.2), but nothing is known about α-carotene **10** hydroxylation. The chemical structures of siphonaxanthin **63** [66], loroxanthin **62**, prasinoxanthin **61**, and monadoxanthin **59** suggest that they are derived from lutein, but the pathways and enzymes remain unknown (Fig. 106.3) [6].

3.7 Carotenogenesis in Cyanobacteria

Cyanobacteria contain unique ketocarotenoids, such as echinenone **51** and 4-ketomyxol glycoside **84**, and carotenoid glycosides. Usually, the glycoside moieties are methylpentoses, fucose, rhamnose, and chinovose [13].

3.7.1 Zeaxanthin 14, Nostoxanthin 77, and Echinenone 51 Synthesis

Some cyanobacteria produce zeaxanthin, and some produce both zeaxanthin and nostoxanthin (Fig. 106.4, Table 106.3). First, the C-3 and C-3′ hydroxyl groups of zeaxanthin are introduced into β-carotene **13** by CrtR, and then the C-2 and C-2′

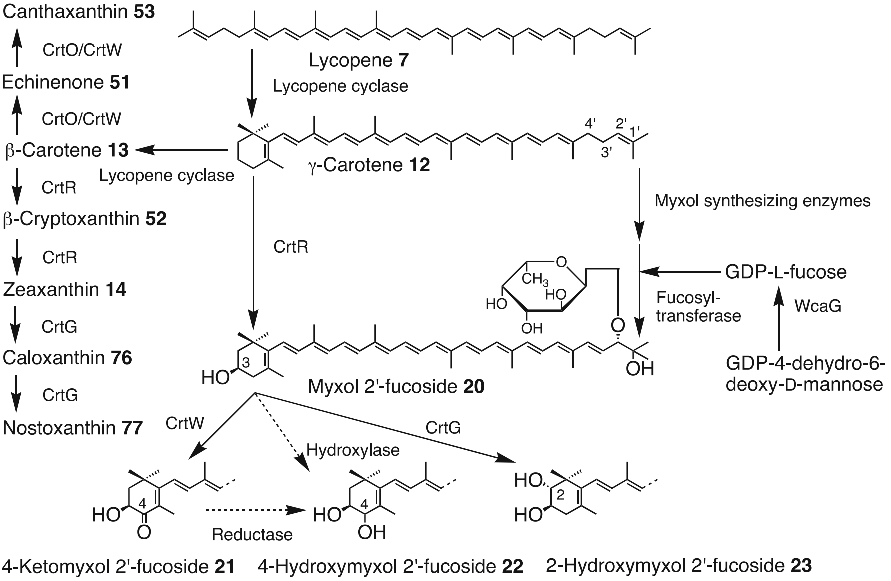

Canthaxanthin **53**
CrtO/CrtW
Echinenone **51**
CrtO/CrtW
β-Carotene **13**
Lycopene cyclase
CrtR
β-Cryptoxanthin **52**
CrtR
Zeaxanthin **14**
CrtG
Caloxanthin **76**
CrtG
Nostoxanthin **77**
Lycopene **7**
Lycopene cyclase
4'
2'
1'
3'
γ-Carotene **12**
Myxol synthesizing enzymes
CrtR
GDP-L-fucose
CH3
O
HO
HO
OH
O
Fucosyl-
transferase
WcaG
3
HO
OH
Myxol 2'-fucoside **20**
GDP-4-dehydro-6-
deoxy-D-mannose
CrtW
Hydroxylase
CrtG
4
HO
O
Reductase
HO
4
OH
HO
2
HO
4-Ketomyxol 2'-fucoside **21**
4-Hydroxymyxol 2'-fucoside **22**
2-Hydroxymyxol 2'-fucoside **23**

Fig. 106.4 Carotenogenesis pathways in cyanobacteria and functionally confirmed enzymes involved in these pathways. The pathways and enzymes vary with cyanobacterial species [6]

hydroxyl groups of nostoxanthin are introduced into zeaxanthin by 2,2′-β-hydroxylase (CrtG) [67].

CrtR from *Synechocystis* sp. PCC 6803 catalyzes the conversions of β-carotene **13** to zeaxanthin **14**, echinenone **51** to 3′-hydroxyechinenone **54**, and deoxymyxol 2′-dimethyl-fucoside **86** to myxol 2′-dimethyl-fucoside **83** (Fig. 106.4) [68, 69]. The CrtR activity for deoxymyxol 2′-fucoside from *Anabaena* sp. PCC 7120 and *Anabaena variabilis* ATCC 29413 is high, while that for β-carotene is very low, judging from trace or none of zeaxanthin present. Consequently, deoxymyxol **85** is a good substrate for CrtR in cyanobacteria because deoxymyxol is absent, while β-carotene is or is not a substrate, depending on the characteristics of CrtR in these species. On the other hand, β-carotene is a good substrate for CrtR in *Synechococcus* species, *Acaryochloris marina*, and *Prochlorococcus marinus* because of the presence of large amounts of zeaxanthin. Because CrtR in land plants and CrtZ in bacteria are known to catalyze the conversion of β-carotene to zeaxanthin, further studies of characteristics, including substrate specificities, functions, and amino acid sequence comparisons, are still needed for these enzymes [13].

Some cyanobacteria such as *Thermosynechococcus elongatus* and three *Synechococcus* species contain both caloxanthin **76** and nostoxanthin **77**, which have additional hydroxyl group(s) at C-2 and C-2′ of the β end group (Fig. 106.4, Table 106.3). CrtG has been found to catalyze C-2 hydroxylation of zeaxanthin **14** in three species of eubacteria *Brevundimonas* [44, 70], and recently also from *T. elongatus* [67]. In *T. elongatus*, CrtG also catalyzes the conversion of myxol

2′-fucoside **20** to 2-hydroxymyxol 2′-fucoside **23** [67]. Previously, hydroxymyxol 2′-glycosides were found in some cyanobacteria and named aphanizophyll, which were believed to be 4-hydroxymyxol 2′-glycosides **22**. A more definitive determination of 2-hydroxymyxol 2′-glycoside or 4-hydroxymyxol 2′-glycoside should be performed.

Echinenone **51** is a major carotenoid in some cyanobacteria, while canthaxanthin **53** is a minor one (Fig. 106.4). Echinenone might play a role in photosynthesis. CrtO and/or CrtW catalyze the introduction of the keto group as described below.

At present, with regard to enzymes involved in the above mentioned reaction, two distinct β-carotene ketolases, CrtO and CrtW, have been found, and only seven β-carotene ketolases have been functionally confirmed in four species of cyanobacteria (Fig. 106.4, Table 106.3). In *Synechocystis* sp. PCC 6803, CrtO catalyzes the conversion of β-carotene **13** to echinenone **51** [71], and 4-ketomyxol 2′-glycoside **84** is absent [72]. *Anabaena* sp. PCC 7120 has two functional enzymes: CrtO catalyzes the conversion of β-carotene to echinenone and CrtW catalyzes the conversion of myxol 2′-fucoside **20** to 4-ketomyxol 2′-fucoside **21** [73]. *Nostoc punctiforme* PCC 73102 has two CrtW-type enzymes, CrtW38 and CrtW148 [74], as well as echinenone and 4-ketomyxol 2′-fucoside [75]. Based on their substrate specificities, CrtW38 might catalyze the conversion of β-carotene to echinenone, and CrtW148 might catalyze the conversion of myxol 2′-fucoside to 4-ketomyxol 2′-fucoside [75]. In *Gloeobacter violaceus* PCC 7421, both CrtO and CrtW function to catalyze the conversion of β-carotene to echinenone. Oscillol 2,2′-di(α-L-fucoside) **88** is present but myxol 2′-glycoside **83** is absent [29, 30]. It is not known whether both or either gene function in the cells. In *Thermosynechococcus elongatus*, genes coding for ketocarotenoids, *crtO*- and *crtW*-like genes, are absent. In total, in cyanobacteria, the conversion of myxol 2′-glycoside to 4-ketomyxol 2′-glycoside is catalyzed by CrtW in two species, while that of β-carotene to echinenone is catalyzed by CrtO in three species and by CrtW in two species. The composition of the products, echinenone and canthaxanthin **53**, might depend on the characteristics of the enzyme. Consequently, two distinct β-carotene ketolases, CrtO and CrtW, are used in two pathways, β-carotene and myxol 2′-glycoside, depending on the species. It would be interesting to determine just how cyanobacteria obtain both or either β-carotene ketolases, and how they utilize them properly [13].

3.7.2 Myxol Glycoside Synthesis

Lycopene **7** is cyclized to γ-carotene **12** by one of lycopene cyclase (Fig. 106.4). The left half (β end group) of the γ-carotene is hydroxylated by CrtR. Its function has been confirmed by deletion mutants of *Synechocystis* sp. PCC 6803 [69] and *Anabaena* sp. PCC 7120 [46], which produce deoxymyxol 2′-glycosides **86** not myxol 2′-glycosides **83**. Because deoxymyxol **85** is almost absent in cyanobacteria, CrtR activity for deoxymyxol should be very high. Further, a keto group is introduced by the CrtW of *Anabaena* sp. PCC 7120 [73] and *Nostoc punctiforme* PCC 73102 [74] to form 4-ketomyxol 2′-glycoside **84**, and a 2-hydroxyl group is introduced by CrtG of *Thermosynechococcus elongatus* to form 2-hydroxymyxol

2′-fucoside **23** [67]. It is not known that whether 4-hydroxymyxol **22** is produced directly from myxol by hydroxylation or from 4-ketomyxol **21** by reduction, because the stereochemistry of 4-hydroxyl group from *Anabaena variabilis* ATCC 29413 is a mixture of (4*R*)- and (4*S*)-chiralities (Fig. 106.4) [76].

The right half (ψ end group) of myxol **82** has a very unique glycosidic linkage (Fig. 106.4). Although certain enzymes should be involved in myxol synthesis, little is known about this process. A deletion mutant of GDP-fucose synthase (WcaG) of *Anabaena* sp. PCC 7120 produces myxol 2′-rhamnoside, but not the usual myxol 2′-fucoside, and contains relatively little free myxol [46]. GDP-rhamnose could be the substrate for GDP-fucose transferase, which has yet to be identified, instead of the usual GDP-fucose. In contrast, the deletion mutant of GDP-fucose synthase of *Synechocystis* sp. PCC 6803 produces only free myxol, instead of the usual myxol 2′-dimethyl-fucoside **20** [46, 73]. This might be due to the absence of the substrate of GDP-fucose transferase. A *crtD* (3,4-dehydrogenase of *Rhodobacter*) homolog from a marine bacterium strain P99-3 (MBIC 03313; previously *Flavobacterium* sp.) [77], which produces free myxol, and *Deinococcus radiodurans* [78] are known to have a CrtD function, whereas this homolog from *Synechocystis* sp. PCC 6803 does not have CrtD functions [34, 79]. Furthermore, a *crtD* homolog in *Anabaena* sp. PCC 7120 (*all5123*) has no function in carotenogenesis [46]. In case of oscillol 2,2′-diglycoside **88**, enzymes involved in myxol synthesis catalyze both end groups of lycopene **7**, and this might be due to the characteristics of myxol-synthesizing enzymes and/or lycopene cyclase (Fig. 106.4, Table 106.3). Thus, further studies of myxol-synthesizing enzymes and associated genes are needed.

In *Anabaena variabilis* ATCC 29413, myxol glycosides are absent, while the free forms of myxol **82** and 4-hydroxymyxol are present [76]. Another strain of *A. variabilis* IAM M-3 produces myxol 2′-fucoside **20** and 4-ketomyxol 2′-fucoside **22** [75]. Thus, *A. variabilis* ATCC 29413 is the first cyanobacterium found to have free myxol and not myxol glycosides, and it seems to lack the gene for or activity of glycosyl transferase. It also has the GDP-fucose synthase homologous gene. Thus, this strain is considered to be of potential use in investigating the characteristics of myxol glycosides in cyanobacteria.

3.8 Carotenogenesis in Purple Bacteria

3.8.1 The Normal Spirilloxanthin Pathway

The biosynthesis of spirilloxanthin **35**, a final product of the normal spirilloxanthin pathway, is found in around half species of purple bacteria [5, 14]. Spirilloxanthin is a symmetrical compound containing the methoxy groups at C-1 and C-1′, and additional double bonds in the C-3,4 and C-3′,4′ positions. It has 13 conjugated double bonds (Fig. 106.5).

A sequence of the reactions leading from lycopene **7** to spirilloxanthin **35** has been confirmed (Fig. 106.5, Table 106.3). This sequence includes successive reactions of (1) hydration at C-1,2 by hydratase (CrtC); (2) desaturation at C-3,4

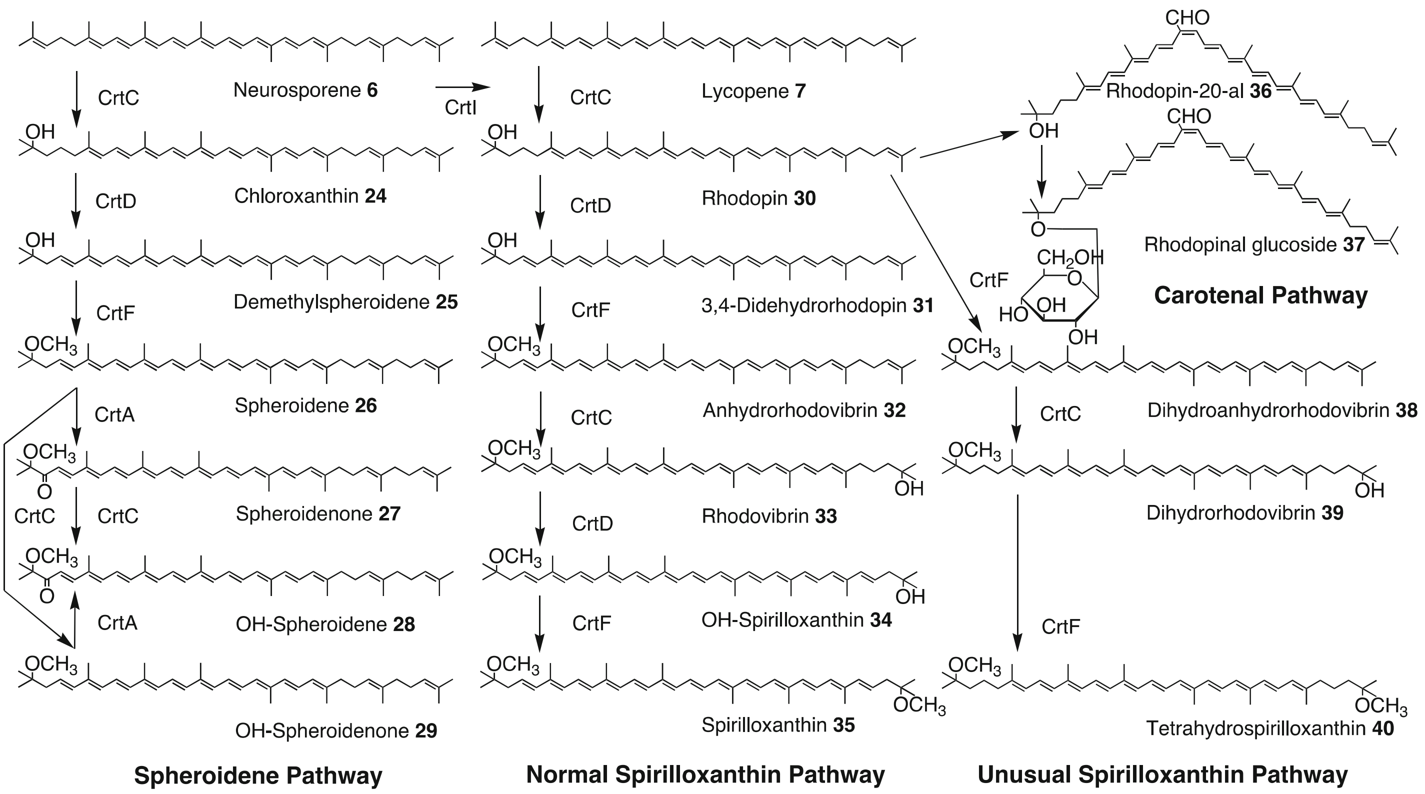

Neurosporene 6
CrtI
Lycopene 7
CrtC
Chloroxanthin 24
CrtD
Demethylspheroidene 25
CrtF
Spheroidene 26
CrtA
Spheroidenone 27
CrtC
OH-Spheroidene 28
CrtA
OH-Spheroidenone 29
Rhodopin 30
3,4-Didehydrorhodopin 31
Anhydrorhodovibrin 32
Rhodovibrin 33
OH-Spirilloxanthin 34
Spirilloxanthin 35
Rhodopin-20-al 36
Rhodopinal glucoside 37
Dihydroanhydrorhodovibrin 38
Dihydrorhodovibrin 39
Tetrahydrospirilloxanthin 40
OH
OCH3
O
CHO
CH2OH
HO
Spheroidene Pathway
Normal Spirilloxanthin Pathway
Unusual Spirilloxanthin Pathway
Carotenal Pathway

Fig. 106.5 Carotenogenesis pathways in purple bacteria and functionally confirmed enzymes involved in these pathways. The pathways and enzymes vary with bacterial genera and/or species [14]

by desaturase (CrtD); and (3) methylation of the tertiary hydroxyl group at C-1 by methyltransferase (CrtF). These reactions occur initially on one half of the molecule to yield anhydrorhodovibrin **32**, and then on the other half. The major product is spirilloxanthin, and usually small amounts of all or a few of five intermediates (Fig. 106.5) are also found. These genes are found in the carotenogenesis gene clusters from *Rhodopseudomonas palustris* and *Rhodospirillum rubrum*. At present, the manner in which the successive reactions are controlled, with reactions first on one half of the carotenoid and then the other, has not been elucidated.

3.8.2 The Unusual Spirilloxanthin Pathway

When one enzyme of the normal spirilloxanthin pathway is lacking or is present with reduced activity, the carotenoid composition of the bacterium is expected to change (Fig. 106.5) [5, 14]. Indeed, some species have been reported to have such unusual compositions. Lycopene **7** is accumulated in *Rhabdochromatium marinum* and *Thiobaca trueperi*, and this may be due to low CrtC activity. Rhodopin **30** is the major carotenoid in some species: *Rhodopseudomonas palustris*, *Allochromatium vinosum*, *Thermochromatium tepidum*, and *Ectothiorhodospira marismortui* [5]. This may be due to low CrtD activity, and rhodopin may not be a suitable substrate for CrtF because of the single bond at C-3,4. On the other hand, 3,4,3′,4′-tetrahydrospirilloxanthin **40** is the major component in *Rhodospira trueperi*, *Thiocapsa pfennigii*, and *Thioflavicoccus mobilis* [5] probably because of lack of CrtD, but in these cases, rhodopin can be methylated by CrtF in spite of the single bond at C-3,4. The same carotenoid is also found in a *crtD* mutant of *Rhodospirillum rubrum* [80]. *Rhoseospira* accumulates 3,4-didehydrorhodopin **31** may be because of low CrtF activity [81].

3.8.3 The Spheroidene Pathway

Three genera *Rhodobacter*, *Rhodobaca*, and *Rhodovulum* produce spheroidene **26** and its derivatives [5, 14]. Spheroidene is an asymmetrical compound containing the same end group as spirilloxanthin **35** on one side and the 7,8-dihydro-ψ end group on the other side (Fig. 106.5, Table 106.3).

All seven carotenogenesis genes in *Rhodobacter capsulatus* [15] and *Rba. sphaeroides* [82] have been found to form a gene cluster. The characteristics of their products have been investigated. CrtI produces neurosporene **6** instead of lycopene **7** from phytoene **3**. Then, the sequence includes successive reactions of hydration at C-1,2 by CrtC to yield chloroxanthin **24**, desaturation at C-3,4 by CrtD to yield demethylspheroidene **25**, and methylation at the C-1 hydroxyl group by CrtF to yield spheroidene **26**. Further, CrtC can more or less hydrate at the 7′,8′-dihydro-ψ end group to yield OH-spheroidene **29** depending on species. Under semi-aerobic conditions, spheroidene monooxygenase (CrtA) introduces the keto group at C-2 to yield spheroidenone **27**. The 1′-hydroxy-7′,8′-dihydro-ψ end group cannot be modified further because of the single bond at C-7′,8′. The keto group of spheroidenone from *Roseobacter denitrificans* is the single bond *cis*-conformation around the conjugated double bond. Water is a substrate for CrtC in *Rba. sphaeroides*. The hydrogen acceptor of CrtD in *Rba. sphaeroides* is molecular oxygen. The methyl residue in the methoxy groups arises from *S*-adenosylmethionine in *Rba. sphaeroides* and *Rba. capsulatus*. The oxygen of the keto group at C-2 is derived directly from the atmosphere in *Rba. sphaeroides* [5].

Because *Rhodobaca bogoriensis* accumulates demethylspheroidene **25** and demethylspheroidenone **91**, CrtF activity in this bacterium may be low (Fig. 106.5).

Rubrivivax gelatinosus produces carotenoids using both the spheroidene and the spirilloxanthin pathways (Fig. 106.5). When *Rvi. gelatinosus crtI* is expressed in *Escherichia coli* with a phytoene **3** background, the products include approximately 90 % neurosporene **6** and 10 % lycopene **7** [83]. Accordingly, *Rvi. gelatinosus* produces spheroidene **26** and spirilloxanthin **35** from neurosporene and lycopene, respectively. Although the reaction of ketolation at C-2 of the ψ end group (CrtA) is usually involved in the spheroidene pathway and not in the normal spirilloxanthin pathway (Fig. 106.5), spirilloxanthin is also oxidized to 2,2′-diketospirilloxanthin **92** by CrtA in this bacterium.

Thus, the spheroidene pathway is a variant of the unusual spirilloxanthin pathway as a result of the different properties of CrtI and the additional enzyme CrtA [5, 14].

3.8.4 The Carotenal Pathway

Four types of cross-conjugated carotenals (Fig. 106.5) are found in 8 genera that include 12 species [5, 14]. Most species contain rhodopinal **36** as a major component, which has an aldehyde group at C-20 with the 13-*cis* form.

From the structure of these carotenals, Francis and Liaaen-Jensen [84] have postulated a branched pathway for each of the derivatives of cross-conjugated carotenals. To date, no other study supporting this postulate has been reported, and the partially modified carotenogenesis pathway is shown in Fig. 106.5. Rhodopin **30** and lycopene **7** are hydroxylated at C-20 to yield rhodopinol **78** and lycopenol **79**, and then the hydroxyl groups are oxidized to the aldehyde groups to yield rhodopinal **36** and lycopenal **80**, respectively. Because all of these carotenals have C-3,4 single bond, CrtD may be inactive. Furthermore, CrtF activity is absent or low. Additional enzymes for hydroxylation at C-20 and for oxidation to the aldehyde group may be involved in this pathway, whereas nothing is known about how the aldehyde group is introduced under anaerobic conditions. The position is necessarily at C-20 rather than C-20′ or C-19 and the 13-*cis* form, because it is more stable than the all-*trans* form as a result of the hydroxyl or aldehyde groups at C-20.

3.9 Carotenogenesis in Green Sulfur Bacteria

Green sulfur bacteria produce some types of carotenoids, β-carotene **13**, chlorobactene **46**, and their derivatives including their glucoside, and/or isorenieratene **50** (Fig. 106.6) [5, 14].

Carotenoids of *Chlorobaculum* (previously *Chlorobium*) *tepidum* have been precisely investigated [85], and some enzymes involved in the synthesis of these carotenoids have been functionally confirmed (Fig. 106.6, Table 106.3) [31, 34, 79].

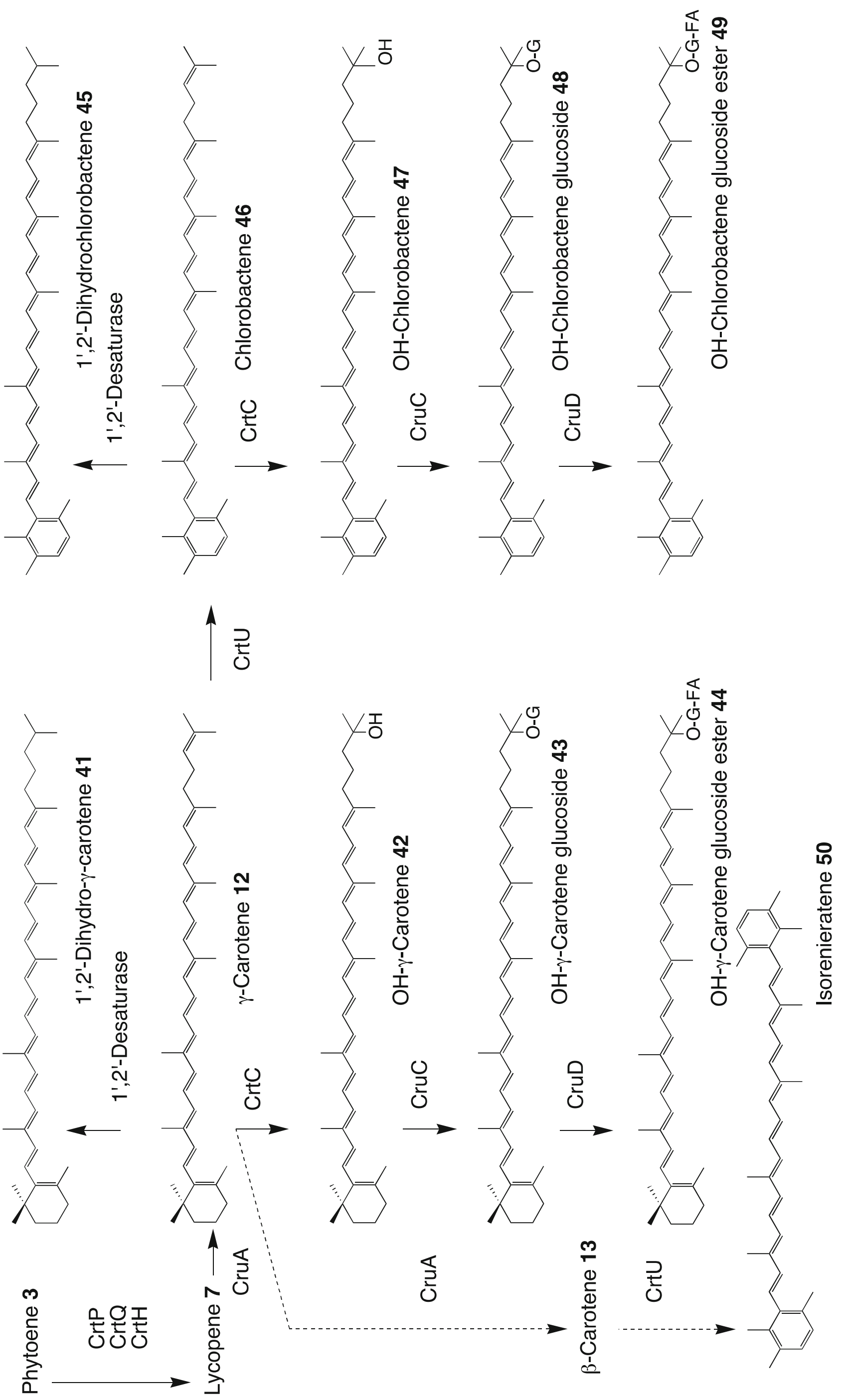

Phytoene 3
CrtP
CrtQ
CrtH
Lycopene 7
CruA
1',2'-Desaturase
1',2'-Dihydro-γ-carotene 41
γ-Carotene 12
CrtC
OH-γ-Carotene 42
OH
CruC
OH-γ-Carotene glucoside 43
O-G
CruD
OH-γ-Carotene glucoside ester 44
O-G-FA
CrtU
1',2'-Desaturase
1',2'-Dihydrochlorobactene 45
Chlorobactene 46
CrtC
OH-Chlorobactene 47
OH
CruC
OH-Chlorobactene glucoside 48
O-G
CruD
OH-Chlorobactene glucoside ester 49
O-G-FA
CruA
β-Carotene 13
CrtU
Isorenieratene 50

Fig. 106.6 Carotenogenesis pathways in the green sulfur bacterium *Chlorobaculum tepidum* and functionally confirmed enzymes involved in these pathways [5, 85]

Lycopene **7** is converted to γ-carotene **12** by a new lycopene cyclase (CruA). The β end group is changed to the ϕ end group by bifunctional desaturase/ methyltransferase (CrtU) to form chlorobactene **46**. γ-Carotene and chlorobactene are hydrated by CrtC. The hydroxyl group is glucosylated by glucosyltransferase (CruC), and then glucoside is esterified by lauroyltransferase (CruD). The enzyme that catalyzes 1′,2′-dihydrocarotene synthesis remains unknown. Both end groups of isorenieratene **50** are ϕ end groups.

3.10 Carotenogenesis in Non-phototrophic Bacteria

Pantoea ananatis (previously *Erwinia uredovora*) produces zeaxanthin diglucoside **89**. The complete carotenogenesis pathway has been determined, and the characteristics of enzymes and genes investigated, since it has a carotenogenesis gene cluster including *crtE, crtB, crtI, crtY, crtZ*, and *crtX* [16] (Table 106.3).

All or some of the genes in the gene cluster have been functionally confirmed, such as zeaxanthin diglucoside **89** in *Pantoea stewartii* (previously *Erwinia herbicola*) [86], two clusters for spirilloxanthin **35** and canthaxanthin **53** in *Bradyrhizobium* ORS278 [17, 18], astaxanthin **58** in *Paracoccus* sp. N81106 (previously *Agrobacterium aurantiacum*) [87], zeaxanthin **14** in *Paracoccus zeaxanthinifaciens* (previously *Flavobacterium* sp. R1534) [88], isorenieratene **50** in *Streptomyces griseus* [89] and *Brevibacterium linens* [33], and 2-hydroxyastaxanthn **90** in *Brevundimonas* sp. SD212 [70] (Table 106.3).

Some eubacteria and archaea do not possess carotenogenesis gene clusters. The genes for early steps can be found from homologous searches. Some genes and enzymes are found by molecular biological techniques (Table 106.3), but some genes for the final steps of complex carotenoids remain unknown.

Astaxanthin **58** synthesis has mainly been investigated using *Paracoccus* [87]. Lycopene **7** is cyclized to β-carotene **13**, and then by the combination of CrtW and CrtZ, keto and hydroxyl groups are introduced into β-carotene, respectively; usually, the intermediates are also found (Figs. 106.7, 106.8, Table 106.3). These enzymes are found in some bacteria and some algae, and their characteristics including activity and substrate specificity are somewhat different from each other. For high production of astaxanthin, pathway engineering including a combination of these enzymes from different organisms has been investigated [90]. Astaxanthin from these organisms has (3*S*,3′*S*)-chirality, while that from a red yeast, *Xanthophylomyces* shows the opposite (3*R*,3′*R*)-chirality. It is produced by one bifunctional enzyme astaxanthin synthase (ASY, CrtS), which has both β-carotene hydroxylase and β-carotene ketolase activities (Table 106.3) [91].

Two distinct β-carotene ketolases CrtO and CrtW are widely distributed in eubacteria, including cyanobacteria, and green algae [13, 63, 64, 70, 92]. Although the reactions catalyzed by both CrtO and CrtW involve the same β-carotene ketolation, the characteristics of these enzymes are different. CrtO is almost twice the size of CrtW, and does not share significant amino acid sequence homology with CrtW. CrtO acts only on compounds with the β end group (β-carotene **13** and

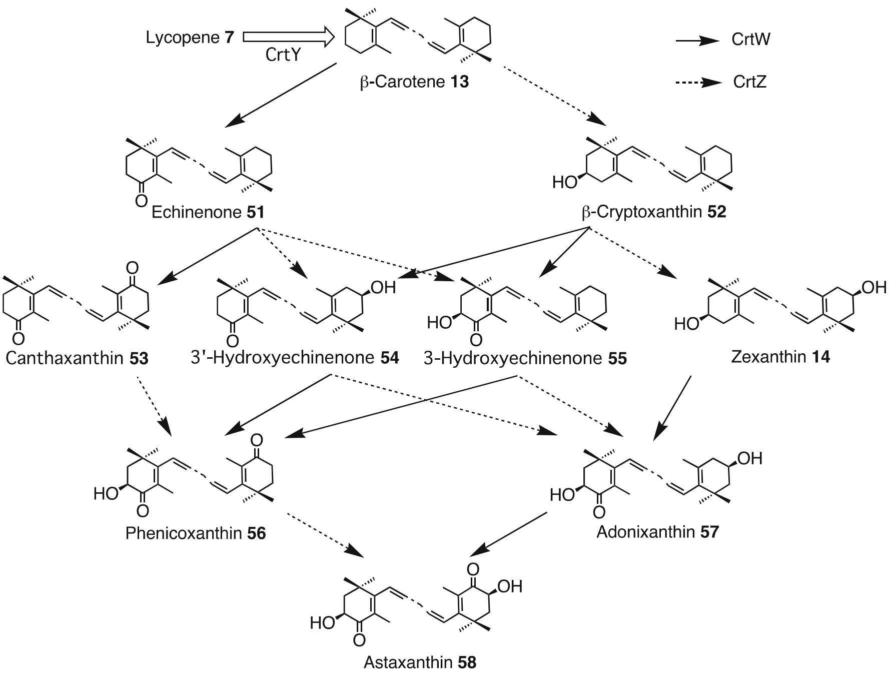

Lycopene 7
CrtY
β-Carotene 13
CrtW
CrtZ
Echinenone 51
β-Cryptoxanthin 52
Canthaxanthin 53
3'-Hydroxyechinenone 54
3-Hydroxyechinenone 55
Zexanthin 14
Phenicoxanthin 56
Adonixanthin 57
Astaxanthin 58
O
HO
OH

Fig. 106.7 Carotenogenesis pathways of astaxanthin and functionally confirmed enzymes involved in these pathways

γ-carotene **12**) and mainly exhibits monoketolase activity. CrtW acts on compounds with the β end group (β-carotene) and 3-hydroxy-β end groups (zeaxanthin **14** and myxol **82**), and mainly exhibits diketolase activity. CrtO has six conserved regions including the FAD-binding motif [92], while CrtW has iron-binding motifs [74]. Two β-carotene ketolases might have evolved convergently from different ancestors to acquire the same functions, although further studies are needed to confirm this assumption [73].

4 Conclusion

Almost all organisms including plants, algae, bacteria, and animals contain various carotenoids (Tables 106.1, 106.2). Variation in carotenoids depends on the variation in carotenogenesis pathways, characteristics including substrate specificities of carotenogenesis enzymes, and presence or absence of certain carotenogenesis genes. These variations can be used for chemotaxonomical classification of organisms. Some of major carotenogenesis pathways have been elucidated, but some major and minor pathways remain unknown. Almost all animals may need carotenoids embedded in foods and may be able to modify them. Because some carotenoids are phylogenetically specific to certain groups, some carotenogenesis

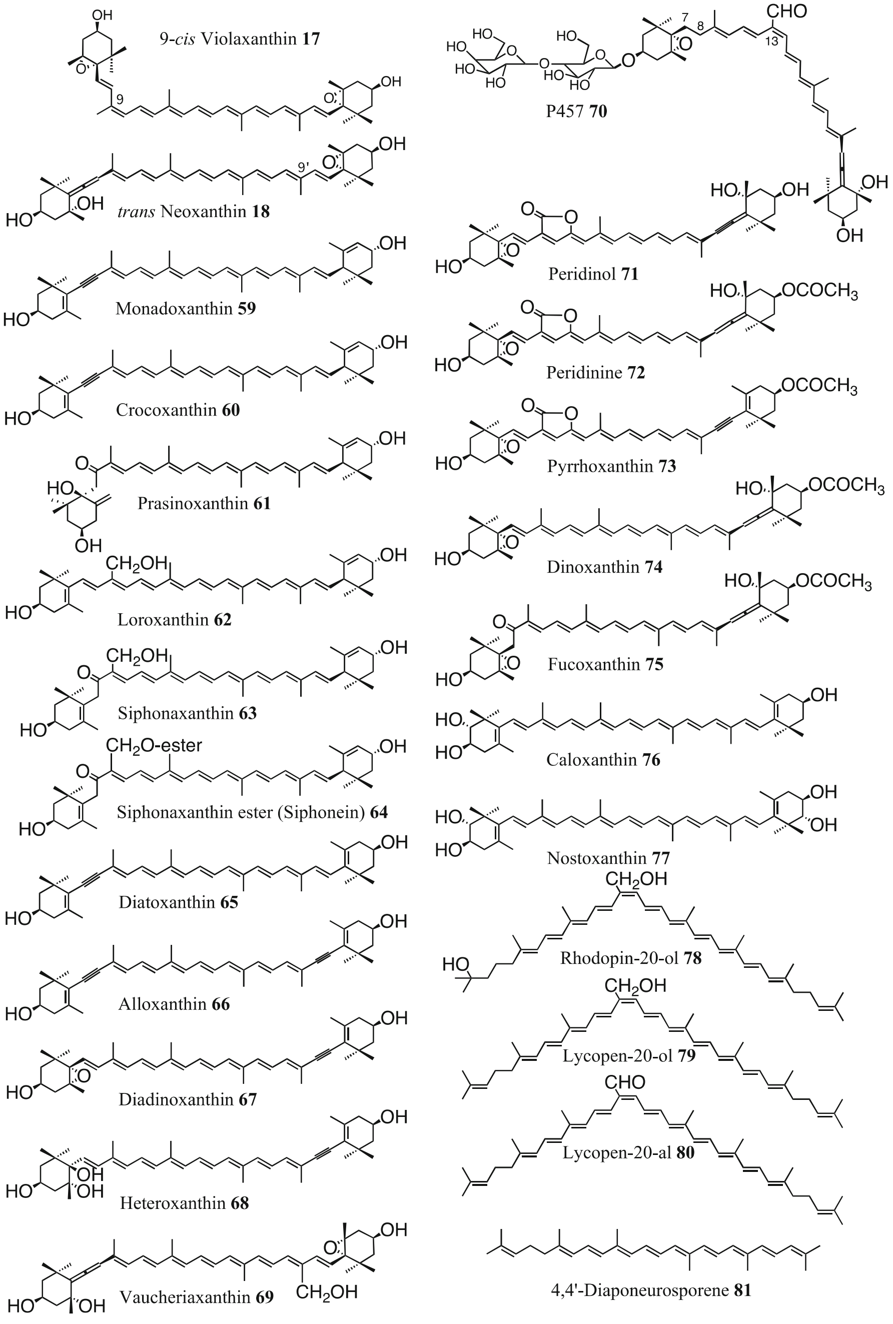
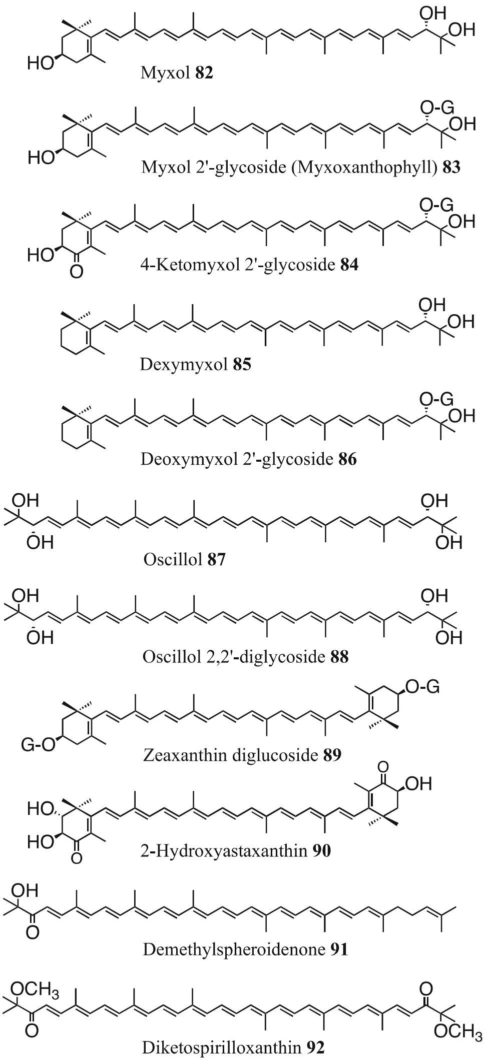

9-*cis* Violaxanthin **17**
9
trans Neoxanthin **18**
9'
Monadoxanthin **59**
Crocoxanthin **60**
Prasinoxanthin **61**
Loroxanthin **62**
CH_2OH
Siphonaxanthin **63**
CH_2O-ester
Siphonaxanthin ester (Siphonein) **64**
Diatoxanthin **65**
Alloxanthin **66**
Diadinoxanthin **67**
Heteroxanthin **68**
Vaucheriaxanthin **69**
CH_2OH
CHO
7
8
13
P457 **70**
Peridinol **71**
Peridinine **72**
$OCOCH_3$
Pyrrhoxanthin **73**
Dinoxanthin **74**
Fucoxanthin **75**
Caloxanthin **76**
Nostoxanthin **77**
Rhodopin-20-ol **78**
Lycopen-20-ol **79**
Lycopen-20-al **80**
4,4'-Diaponeurosporene **81**

Fig. 106.8 (continued)



Myxol **82**

Myxol 2'-glycoside (Myxoxanthophyll) **83**

4-Ketomyxol 2'-glycoside **84**

Dexymyxol **85**

Deoxymyxol 2'-glycoside **86**

Oscillol **87**

Oscillol 2,2'-diglycoside **88**

Zeaxanthin diglucoside **89**

2-Hydroxyastaxanthin **90**

Demethylspheroidenone **91**

Diketospirilloxanthin **92**

Fig. 106.8 Structure of carotenoids not found in Figs. 106.1 to 106.7

genes might be lost and/or added. At present, some carotenogenesis genes have not been identified, the functional confirmed genes are limited, and only a few species have been examined (Table 106.3), although genome sequences have been determined in many organisms. Thus, further studies of carotenoids, characteristics of carotenogenesis enzymes and genes, and the carotenogenesis pathways are needed.

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Part XII

Terpenes: Methods of Analysis

Essential Oils: Analytical Methods to Control the Quality of Perfumes

107

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Contents

1 Introduction 3289
2 Regulatory Aspects for Fragrance Chemicals in Cosmetics and Household Products 3292
3 Analytical Aspects of Fragrance-Related Compounds 3293
3.1 Determination of the Potentially Allergenic Fragrance-Related Substances 3295
3.2 Determination of Musks 3299
3.3 Determination of Phthalates 3302
4 Conclusions 3305
References 3305

Abstract

Cosmetic products, including perfumes, are regulated by a single law concerning important aspects, such as composition or labeling, in order to protect public health. A revision on the regulatory aspects for fragrance chemicals in cosmetics and household products is exposed here.

Quality control of both, commercial perfumes and raw materials, is important for perfume manufacturers to assure that the finished perfume is the one that was formulated. On the other hand, analytical methods are necessary to assure, for safety purposes, that there are no undesired or banned compounds present in the finished product. Methods for perfume analysis are compiled and revised with special emphasis on the potentially allergenic fragrance-related substances and some other groups of substances such as musks and phthalates, being some of them restricted or forbidden.

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Abbreviations

AHTN	6-acetyl-1,1,2,4,4,7-hexamethyltetralin
AITI	5-acetyl-3-isopropyl-1,1,2,6-tetramethylindane
ALEX	Automated liner exchange
ANN	Artificial neuronal networks
BBP	Benzyl butyl phthalate
CE	Capillary electrophoresis
DBP	Dibutyl phthalate
DEHP	Bis(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DIBP	Diisobutyl phthalate
DIPP	Diisopentyl phthalate
DMEP	Bis(2-methoxyethyl) phthalate
DMP	Dimethyl phthalate
DNOP	Di-n-octyl-phthalate
DNPP	Di-n-pentyl-phthalate
DPP	Dipropyl phthalate
ECD	Electron capture detector
EESI	Electrospray ionization mass spectrometry
EIC	Extracted ion chromatogram
FDA	Food and Drug Administration
FID	Flame ionization detector
FS	Full scan
GC	Gas chromatography
HHCB	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran
HS	Head space
IFRA	International Fragrance Association
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MA	Musk ambrette
MD	Multidimensional
MEEKC	Microemulsion electrokinetic chromatography
MK	Musk ketone
MM	Musk moskene
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion
MT	Musk tibetene
MX	Musk xylene

NPIPP	n-pentyl-isopentylphthalate
PAS	Potentially allergenic substance
PCA	Principal component analysis
PLE	Pressurized liquid extraction
PTV	Programmed temperature vaporizing
RIFM	Research Institute for Fragrance Materials
SBSE	Stir bar sorptive extraction
SIM	Selected ion monitoring
SPD	Solid-phase dispersion
SPE	Solid-phase extraction
TIC	Total ion chromatogram
TLC	Thin-layer chromatography
TOF	Time-of-flight
UV	Ultraviolet

1 Introduction

"Scent is one of the most powerful of senses." This phrase can be read in the website of one of the more remarkable associations related to perfumes, the International Fragrance Association (IFRA), and we are all aware that this is true. Scent can alter mood, stress, or pain. We are not always aware of them, but odors are around us all the time and affect our daily life. People, food, plants, and everything have a particular odor, and we even add aromatic substances to many other things. Cosmetic products, such as shampoos, deodorants, soaps, and fine fragrances, or household products such as laundry detergents, cleaners, and bleaches contain perfumes (Fig. 107.1) which are responsible for their characteristic odors. Moreover, we sometimes scent the air with air fresheners or fragrance candles.

It is worth mentioning that the use of perfumes is not new, as they have been used for thousands of years since the Egyptian civilization, when they were used in religious rites. After the roman civilization, they became an oriental art, and it was not until the thirteenth century that all this knowledge arrived to Europe due to Arabic influences. Louis XV, in the eighteenth century, used it so much that his court was called *"le cour parfumee."* Nowadays, France is still the heart of the European perfume industry. Since then, the perfume market has become very important, with sales of billions of dollars.

Perfumes are composed of hundreds of aromatic chemicals, namely, fragrance chemicals, or just simply fragrances. Among these compounds, it is very common to find compounds with the characteristic five-carbon isoprene unit, giving them the names of terpenes, such as terpene aldehydes, monoterpene alcohols, sesquiterpene alcohols, terpene ketones, terpene esters, monoterpene hydrocarbons, or sesquiterpene hydrocarbons. In addition, other compounds, such as aldehydes, alcohols, ketones, esters, phenols, or lactones, are also very common.

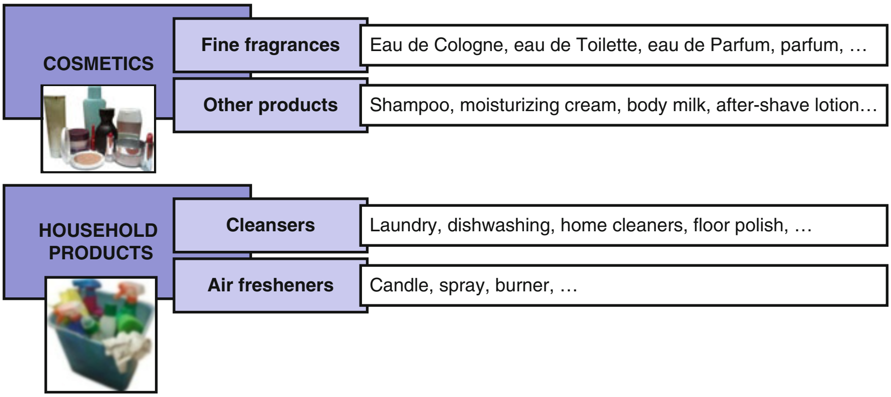

COSMETICS
Fine fragrances
Eau de Cologne, eau de Toilette, eau de Parfum, parfum, …
Other products
Shampoo, moisturizing cream, body milk, after-shave lotion…
HOUSEHOLD PRODUCTS
Cleansers
Laundry, dishwashing, home cleaners, floor polish, …
Air fresheners
Candle, spray, burner, …

Fig. 107.1 Containing perfume products

Perfumes can be classified according to their nature, in *natural* or *synthetic*. *Natural perfumes* are obtained from plants (e.g., lavender, geranium) or from some of its parts, like flowers (e.g., jasmine, rose, gardenia), fruits (e.g., lemon, orange, vanilla), roots (e.g., vetiver, cistus, angelica), leaves (e.g., violet, patchouli, peppermint), wood (e.g., vetiver, sandalwood, cedarwood), bark (e.g., cinnamon, nutmeg), resin (e.g., benjui, tolu, galbanum), and seeds (e.g., angelica, celery, anis). Also, they may be obtained from animal glands and organs, for example, musk, which is obtained from the testicles of the musk deer; civet, which is a secretion from glands of the civet cat; ambergris, which is obtained from a secretion from the intestine of the sperm whale; and finally, castoreum, which is obtained from glands near the reproductive organs of the beaver. These natural perfumes, or essential oils, are extracted by several methods, which depend on the raw material and the chemical fragrances to be extracted [1].

Thus, *steam distillation* consists in passing water vapor through the raw material to perform the extraction. Once the steam condenses, the oil is easily separated from the water as it floats on the top. In *hydrodistillation*, the raw material is boiled in water, and the volatile essential oil is also obtained from the water vapor by condensation. In the case of *solvent extraction*, raw materials are submerged in organic solvents, such as hexane, ethanol, or petroleum ether. After extraction, the solvent is eliminated by distillation resulting in the extract known as “concrete.” The concrete can be further purified by a second extraction with ethanol. In this case, the resulting extract is known as “absolute.” When the extraction solvent is animal fat, the process is known as *enfleurage*. This is a very time-consuming and expensive way of extraction. *Maceration* is similar to enfleurage, but in this case, the raw material is immersed in warmed oil. In *rectification*, the raw materials are directly heated without a solvent. This method is used when a toasted scent is wanted. Another way of extraction is named *expression* and consists in manually or mechanically pressing the plants. This is very suitable for thermolabile compounds.

Finally, a more modern way of extraction is *supercritical fluid extraction*. In this process, carbon dioxide in supercritical conditions is used to extract the hydrophobic aromatic compounds from the raw material. It does not alter the nature of the aromatic compounds as it takes place at a low temperature and CO_2 vaporizes after depressurization. However, it is an expensive process and needs special instrumentation [2].

In the case of *synthetic perfumes*, they are obtained by mixing synthetic fragrance chemicals, which are synthesized in the laboratory, usually searching for a similar odor of a natural fragrance and sometimes searching for something new and original. In practice, this type of fragrances has many advantages, specially its lower cost compared to natural perfumes. The quantity and quality of natural source supplies are in many ways unpredictable, due to their dependence on crop quality or weather. Thus, synthetic perfumes do not have this inconvenience, but unfortunately, they have some others. For example, a natural essential oil is made up by hundreds or thousands of different compounds, which makes difficult to reproduce the desired perfume exactly by just mixing different synthetic fragrances. Moreover, all the compounds contribute with their characteristic odors, and then, not only the main components but also those minor components, including those at trace levels, can affect considerably the odor of the perfume. In addition, in those cases where two isomeric forms of a compound smell different, chiral synthesis is needed.

On the other hand, perfumes itself, and also the pure fragrance chemicals that compose the perfumes, are classified according to the note they provide, that is, according to the fragrance type. So, one can find different types, like floral, which reminds one of scents similar to jasmine, rose, heliotrope, etc.; citrus, which are aromas reminiscent of lemon, orange, lime, grapefruit, etc.; fruity, based on non-citrus fruity odors like peach, apple, banana, etc.; green, which creates the sensation of smelling recently cut grass and leaves; woody, which reminds one of dry wood and trees; oriental refers to those sweet strong fragrances reminiscent of vanilla, ambergris, etc.; spice, giving off a redolence coming from clove, cinnamon, thyme, pepper, etc.; animal refers to scents provided by musk, civet, and castoreum; and leather, which tries to reproduce the characteristic smell of leather, tobacco, smoke, etc.

It should be also emphasized that the perfume content depends of the product. Fine fragrances, which are hydroethanolic solutions of perfumes, are usually classified according to their perfume content in eau de cologne (1–5 %), eau de toilet (5–8 %), eau de parfum (8–15 %), and parfum (>15 %). Other cosmetic-products present lower perfume content. Moisturizing creams and body milks (0.1–0.5 %), aftershave (0.5–2 %), toothpastes (0.5–1 %), and hair care products (0.01–0.1 %) are just some examples. Regarding cleansing household products, nearly all, as, for example, laundry or dishwashing products and bathroom or furniture cleaners, contain perfume, in variable amounts, to make them smell clean and mask unpleasant odors from other components of the product.

Thus, perfumers rarely use pure fragrance chemicals, but they usually mix different raw materials, natural or synthetic, to create a perfume that fulfills the market expectations, that is, type of product to which it is to be added, product image, target consumer, originality, fashion, etc. For fine fragrances, high content of pungent and elegant perfumes are used, whereas more sweet and refreshing ones are found in eau de toilets and eau de colognes. For skin and hair care cosmetic products, sweet, tenuous, and refreshing perfumes are combined, whereas high refreshing notes are employed in toothpastes or mouthwashes. In the case of household products, detergents and softeners contain refreshing notes, while different home cleaners and air fresheners can be found with refreshing or on the contrary with pungent and elegant or sweet notes.

2 Regulatory Aspects for Fragrance Chemicals in Cosmetics and Household Products

The establishment of the single market in the European Union meant the free movement of goods in the member states. Cosmetic products were not an exception, but the great exposure of the population to these products enhanced the need of a single law concerning important aspects, such as cosmetic composition or labeling, in order to protect public health. As a result of many discussions between experts, Council Directive 76/768/EEC was adopted on the 27th of July of 1976. Since then, many amendments and adaptations have been made. Last recast of all these changes as one single text is Regulation 1223/2009 [3]. Among other aspects, this regulation controls and regulates the substances banned in cosmetics, as well as the maximum allowable amount of certain restricted substances according to their intended use. Moreover, there is also the so-called European Inventory of Cosmetic Ingredients [4] which lists the substances usually employed in cosmetic products. This inventory is divided into two sections where Sect. 2 deals with those ingredients used in perfume and aromatic raw materials.

Regarding the USA, cosmetics are regulated by the US Food and Drug Administration (FDA), but regulation is much more permissive [5].

A cosmetic product is defined by the European regulation as “any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odors” and by the FDA as “an article intended to be applied to the human body for cleansing, beautifying, promoting attractiveness, or altering the appearance without affecting the body’s structure or functions.” Therefore, fragrances and perfumes are closely related to cosmetic products as, besides being a cosmetic product itself, they are many times an ingredient added to make the product smell nice or mask any undesirable odor of the product.

Both regulations specify that all ingredients of cosmetic products should be indicated on the label. In the case of perfumes, they are either elaborated by mixing different natural raw materials which could contain hundreds or thousands of different compounds or based on complexes synthetic formulations. This is why perfume components do not necessarily have to be specified, but they can be grouped and labeled under the word “perfume” or “aroma” in the EU or “fragrance” in the USA.

Nevertheless, in 2003, the 2003/15/EC Directive [6] listed 26 fragrances classified as potentially allergenic substances (PASs). Since then, any cosmetic product containing any of these 26 substances have to declare its presence in the label when present at a higher concentration than 10 μg mL^{-1} in those products to remain on the skin or 100 μg mL^{-1} in those intended to be rinsed off. This information should improve the diagnosis of contact allergies among consumers and should enable them to avoid the use of cosmetic products which they do not tolerate.

On the other hand, the European regulation also prohibits and restricts the amount used of more than 50 fragrance substances, like some extracts or synthetic musks, in cosmetic products. Other substances related to fragrances, such as phthalate esters, commonly known as phthalates, which have been used as solvents and vehicles for fragrance ingredients, are also restricted as a consequence of their undesirable side effects.

Regarding the US FDA’s list of prohibited ingredients, it bans only a few compounds, but none of them are used as fragrance ingredients. Nevertheless, the USA and other countries, such as Canada, will probably adopt the recommendations given in the EU regulation in the future [7].

The EU also regulates household products, and detergents are regulated by Regulation 648/2004 [8] where similar labeling to cosmetic products is required in the case of perfume content. A perfume or an essential oil shall be considered to be a single ingredient and will be labeled as “parfum” listing none of the substances that it contains. Additionally, as in the case of cosmetic products, there is an exception for those allergenic fragrance substances that appear on Annex III of the cosmetic regulation. If their concentrations exceed 0.01 % by weight, the allergenic fragrances must be labeled separately.

Despite the regulations in law in each country, the fragrance industry is in some way self-regulated by some independent entities. The Research Institute for Fragrance Materials (RIFM) evaluates and distributes scientific data on the safety assessment of fragrance substances found in cosmetics and other products. On the other hand, the aforementioned IFRA establishes usage guidelines for fragrance ingredients based on RIFM evaluation results. Recommendations to avoid many ingredients are given, although this organization has no legal authority, and therefore, the final decision is mostly conditioned on legislation [7].

3 Analytical Aspects of Fragrance-Related Compounds

Quality control of commercial perfumes and raw materials is very important for perfume manufacturers. This is the way to assure that the amount of perfume is the one that was initially formulated or that there are no undesired or banned compounds. In addition, characterizing an essential oil is also very interesting.

Different tests, depending on the instrumentation available, are performed in the industry. There are many easy and cheap quality control techniques that provide overall information about their quality, as measurements of physical (density, color, optical rotation, etc.) and chemical properties (acidity or carbonyl indexes). Spectrometric techniques, such as ultraviolet/visible or infrared spectrometry can also provide overall qualitative, or even quantitative, useful information for the quality control of a perfume, by comparing the obtained spectra with those previously recorded and kept in the databases. However, all these techniques do not provide information about each component separately, but overall information. For these purposes, more selective techniques are needed. As most of the perfume ingredients are volatile or semi-volatile compounds, gas chromatography (GC) is, by far, the technique of choice [9]. The identification of the individual compounds is then made by using the Kovats indexes and comparing them with those reported in the databases for the same chromatographic columns, although it can sometimes be a difficult task taking into account the high number of compounds that can be present in the perfume composition. In this sense, the mass spectrometry (MS) detector can be a useful tool since the identity of a compound can be elucidated from the MS spectra obtained.

The so-called electronic noses are also very useful. This device mimics human olfactory system and consists of a sample system in head space, a sensor array as detection system, and a computer for data treatment. It is not a separative mechanism; it just gives signal patterns for a particular odor. In a way, it measures smells. It is worth mentioning that an individual sensor is unspecific and could give exactly the same signal with two different odors. Thus, an array of sensors has to be used to reduce the probability of this happening [10]. In order to use an electronic nose, a database of reference has to be firstly built. To do so, a set of known samples would have to be analyzed and the data statistically treated, for example, with pattern recognition methods, such as principal component analysis (PCA), artificial neural network (ANN), or clusters. New samples could then be recognized by comparing them with the database. This technology is commonly used in the food and beverage industry [11]. It has also been used for medical or environmental applications and, of course, in the cosmetic and fragrance industry. In the last years, some works that use electronic nose in fragrance analysis have been published. For example, Carrasco et al. [12] discriminated between different families of Yves Saint Laurent perfumes by an electronic nose. Branca et al. [13] developed an electronic nose device combined with PCA and ANN to detect the presence of a perfume note called mangone in fragrances. Recently, Cano et al. [14] developed a PCA method to detect between original and counterfeit perfumes. Ye et al. [15] also used an electronic nose combined with PCA to discriminate between natural musk and adulterated. Although this is not an electronic nose, Chingin et al. [16] used extractive electrospray ionization mass spectrometry (EESI-MS) for the same purpose in a very simple and sample-preparation-free method which could be applied for quality control.

3.1 Determination of the Potentially Allergenic Fragrance-Related Substances

As mentioned before, 26 substances were declared as potentially allergenic substances (PASs) by the European regulation [6]. Of these 26 substances, listed in Table 107.1, 24 are chemically defined volatile compounds, whereas the other two are natural moss extracts, thus not defined chemicals, but a natural mixture of many of them.

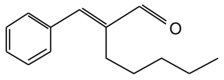
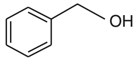
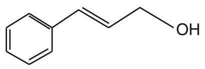
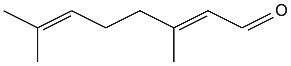
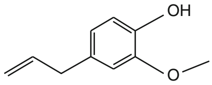
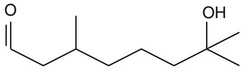
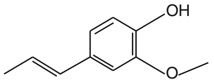
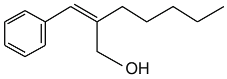
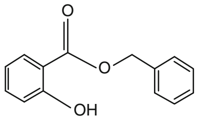
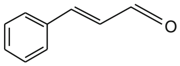
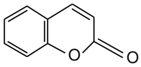
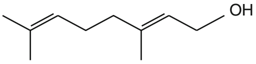
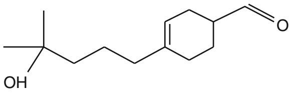
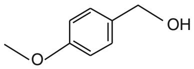
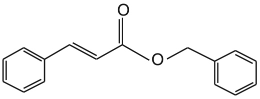
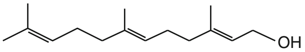
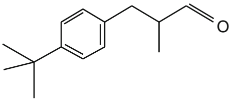
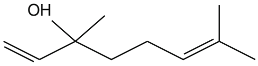
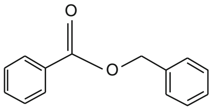
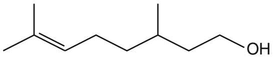
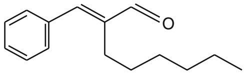
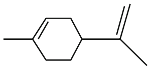
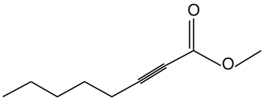
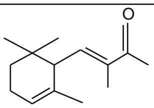
Since this declaration took place in 2003, the determination of these skin-sensitizing ingredients has become a challenge. Literature deals separately with the determination of the 24 chemically defined PASs and the two natural moss extracts.

The group of the 24 chemically defined allergens belongs to very different classes of compounds. These diverse groups include alcohols (such as farnesol), carbonyl compounds (such as citral), esters (such as benzyl benzoate), lactones (such as coumarin), cyclic hydrocarbons (such as limonene), or phenols (such as eugenol). Their volatile characteristics make gas chromatography (GC) the better technique of analysis. Although universal detectors like flame ionization detector (FID) have been used, a more selective detector, like a mass spectrometer (MS), is also a necessity, as one of the biggest difficulties to face in their determination is the coelution between the target compounds and other of the many components of the perfume. The literature deals with these using different solutions, operating either in selected ion monitoring (SIM) or extracted ion chromatogram (EIC) mode, but it is difficult to completely eliminate false positives and negatives, due to coelution. Other solutions are the use of comprehensive GC or clean up steps before the determination to extract the target compounds [17].

Being aware of the problematic skin-sensitizing chemicals in perfumes, Rastogi proposed for the first time, even before the approval of the regulation, a solid-phase extraction (SPE) followed by GC method for the determination of 11 of these allergens [18]. GC-MS was used for previous identification, while GC-FID was chosen for quantification. Some years later, the author published another work [19] using the same GC-MS method, with some modifications, to survey the content of the 24 PASs in cleaning products and other consumer products such as cosmetics or toys. In this method, the sample treatment depended on the type of sample, but in summary, samples were shaken with methanol, and the lixiviated fraction was purified with a silica gel column before its injection into the GC port. Detection was performed in full scan (FS) and quantification in EIC mode. In the same way, Ellendt et al. [20] did also use GC-MS, FS mode for identification and SIM mode for quantification, for the determination of the 24 PASs in deodorants. However, coelution problems were encountered in complex fragrance mixtures.

Later, Chaintreau et al. [21] made a significant progress presenting a method to determine PASs in perfume concentrates, with direct injection of the samples. This work delves in the coelution problem between the target compounds and other components of the perfume. Four different columns were used to try to solve this problem. SIM mode of three different ions per compound (i.e., one ion

Table 107.1 List of the 26 potentially allergenic substances according to the European regulation

 Amyl cinnamal [CAS 122-40-7]	 Benzyl alcohol [CAS 100-51-6]	 Cinnamyl alcohol [CAS 104-54-1]
 Citral [CAS 5392-40-5]	 Eugenol [CAS 97-53-0]	 Hydroxycitronellal [CAS 107-75-5]
 Isoeugenol [CAS 97-54-1]	 Amylcinnamyl alcohol [CAS 101-85-9]	 Benzyl salicylate [CAS 118-58-1]
 Cinnamal [CAS 104-55-2]	 Coumarin [CAS 91-64-5]	 Geraniol [CAS 106-24-1]
 Hydroxyisohexyl 3-cyclohexene carboxaldehyde [CAS 31906-04-4]	 Anise alcohol [CAS 105-13-5]	 Benzyl cinnamate [CAS 103-41-3]
 Farnesol [4602-84-0]	 Butylphenyl methylpropional [80-54-6]	 Linalool [78-70-6]
 Benzyl benzoote [CAS 120-51-4]	 Citronellol [CAS 106-22-9]	 Hexyl cinnamal [CAS 101-86-0]
 Limonene [CAS 5989-27-5]	 Methyl 2-octynoate [CAS 111-12-6]	 Alpha-isomethyl ionona [CAS 127-51-5]
Oakmoss extract (*Evernia prunastri* extract) [CAS 90028-68-5]	Treemoss extract (Evernia furfuracea extract) [CAS 90028-67-4]	

for quantification and the other two as qualifiers) was also used and their relative abundances taken into account for correct identification, thus minimizing false positives and false negatives, by comparing the relative abundances with respect to those of the reference compound. Even though all this helped, false positives, due to coelution, still occurred. Moreover, false negatives can occur as a consequence of retention time shifts, that is, that a target compound shifts out of the acquisition SIM window. The work also claims ethanol is not a good candidate as solvent of the standard solutions since protic solvents react with some of the allergens. Thus, isooctane and o-fluorotoluene were chosen. This is an important fact which not all later works take into account. Lastly, in this paper, Chaintreau and coworkers highlighted the need for cleanup techniques as the nonvolatile parts of the cosmetic products remain in the injector disturbing future injections. It should be emphasized that this work was accepted as the official IFRA method for the quantification of potential fragrance allergens in fragrance compounds [22], with two recommendations: (1) in case of suspected coelutions, one of the qualifier ions should be used as quantization ion; (2) in case of retention time shifts, a qualitative FS run should be done before each sample in order to adjust the time windows. These recommendations were applied in a paper published some years later [23], which concluded that coelution problems could still occur and recommended to inject each sample in two different polarity columns as well as to monitor three ions per compounds to assure about the positive identification/quantification. A ring test was conducted using this strategy, and the results were published years later [24], in which the investigation of an automated data treatment procedure to aid the analyst during the interpretation of the analytical results was also proposed.

A similar strategy was followed by Leijs et al. [25] for the analysis of these 24 compounds in fragrance raw materials and perfume oils. These authors used a chromatograph with two injectors and connected each one to a different polarity column. Both columns were connected to a MS interface using a dual-hole ferrule, thus carrying out a sequential dual-column analysis for each sample. However, they preferred operating in FS mode and quantifying in EIC mode, to avoid additional acquisition in case of coelutions and to prevent false negatives as a result of retention time shift. Obviously, this mode of acquisition significantly increases the limit of quantification (LOQ).

Another work, from Mondello et al. [26], determined the PASs using fast GC-MS, by employing shorter and narrower columns besides high-speed MS detector. The MS operated in FS mode, and quantification was performed in EIC mode. The main novelty of the work basically consisted in a very short run time (less than 5 min). However, the same above-mentioned problems regarding coelutions are expected to occur.

Taking into account the Chaintreau and coworkers' recommendation dealing with cleanup steps to separate the target compound from the matrix and thus avoid dirtiness of the system, several works can be found in the literature describing cleanup steps before the determination of the 24 restricted allergens. For example, as mentioned before, Rastogi [19] used a silica gel column for purifying the extracts. Niederer et al. [27] proposed a cleanup step using size-exclusion chromatography prior to GC-MS for the determination of the 24 PAS in different cosmetic products, such as creams, body lotions, and oils. David et al. [28, 29] classified samples into four different groups based on matrix characteristics. Simple samples, with no presence of nonvolatile material, like fragrances, were diluted and injected.

This is not possible with dirtier matrixes, where a programmed temperature vaporizing (PTV) inlet with an automated liner exchange (ALEX) was proposed. Thus, fractioning of the target compounds from this nonvolatile matrix took place in the liner. When handling aqueous samples with the target compounds at very low concentrations, a stir bar sorptive extraction (SBSE) step was added before the PTV approach. Regarding cleanup steps, Chen et al. [30] used solid-phase microextraction (SPME) followed by GC-FID for the quantitative analysis of geraniol, a restricted allergen, and other flavor and perfume compounds in shampoo. Lamas et al. [31] proposed the use of solid-phase dispersion-pressurized liquid extraction (SPD-PLE) followed by GC-MS in EIC mode for the determination of the 24 regulated fragrance allergens and two other fragrance compounds in cosmetic products (creams and lotions). A year later, the same authors presented a method based on matrix solid-phase dispersion (MSPD) as concentration and cleanup step followed by GC-MS in SIM mode for PASs determination in cosmetic products [32] and a GC-MS method with no pretreatment of samples other than dilution for their determination among other fragrance-related components in perfumes [33].

Development in the field of comprehensive GC (GCxGC) also seems a good way to handle the coelution problem. In this sense, Chaintreau and coworkers [34] presented a GCxGC-FID method for the determination of the 24 regulated substances plus two other compounds. The method could not completely eliminate component overlap, leading to similar results than using 1D-GC coupled to MS. Later, the same authors [35] presented the first work dealing with the quantification of the PASs based on comprehensive GC coupled to a quadrupole MS (GCxGC-MS). As obviously expected, selectivity is improved compared to GC-MS and GCxGC-FID. Comprehensive GC can overcome coelution problems but is very time-consuming during the interpretation of the analytical results. The same authors published another paper [36] presenting a software prototype to reprocess the data which shortens this step. Leco [37] presented a method for allergens in perfumes using GCxGC-(TOF)MS (time-of-flight mass spectrometer). Identification was better achieved than using a simple quadrupole, but the equipment is too expensive to be used in routine analysis. Dunn et al. [38] compared two approaches for this analysis, GCxGC and multidimensional gas chromatography (MDGC), in which selected portions of the primary separation, rather than the whole sample, are transferred to a secondary column. MDGC seems to be a better option as data processing for the output is almost identical to a single column analysis, while the data of a GCxGC experiment is still relatively labor intense. Finally, Cordero et al. [39] published a work where determination by GCxGC-qMS and GCxGC-FID is accomplished.

Only two papers dealing with the determination of these compounds with a different technique than gas chromatography have been found. Villa et al. [40] propose the first work where liquid chromatography and UV detection (LC-UV) are used for the determination of the 24 suspected allergens in different cosmetic products. A C18 column and a gradient acetonitrile/water were used. Samples are basically weighted, diluted, sonicated, and filtered before injection. This can be a useful method for routine analysis in cosmetic industries where more expensive equipment is not available. However, coelution problems are present, not only between compounds but also for matrix components. In this sense, Furlanetto et al. [41] published a method for 18 of the 24 allergens by microemulsion electrokinetic chromatography (MEEKC) and UV detection. The method was applied to two rinse-off cosmetics, a shampoo, and a bath gel.

On the other hand, the number of publications regarding the determination of the two natural moss extracts restricted as potentially allergenic – treemoss and oakmoss – is rather scarce. The industrial processing varies considerably, but basically, harvested lichens, from oak trees in the case of oakmoss and pine and cedar trees in the case of treemoss, are extracted, usually with hexane or more polar solvents. Then, they are further diluted in ethanol and usually submitted to physical treatments intended to remove their original color. Taking into account their natural conditions and that industrial processing is not standardized, it is not surprising to find variability in the chemical composition of these extracts. Joulain et al. published two comprehensive reviews on the composition of these extracts. One hundred seventy constituents are identified in oakmoss extracts and 90 in the case of treemoss [42, 43].

The nature of these extracts makes them unsuitable to GC although Bernard et al. [44] searched for allergenic or sensitizing molecules in oakmoss using GC-MS after chemical fractionation of the extract by gel permeation chromatography (GPC). Some other works can be found in the literature dealing with the determination of some of the oakmoss components that show allergenic properties, like atranorin and chloroatranorin, a lichen depside, or atranol and chloroatranol, formed by transesterification and decarboxylation of atranorin and chloroatranorin during industrial processing. Hiserodt et al. [45] used LC-MS/MS to identify atranorin and some related potential allergens in oakmoss absolute. No quantification was performed in this work. Bossi et al. [46] used this method to quantify atranol and chloroatranol in perfumes. Rastogi et al. [47] also quantified the presence of these two allergenic compounds in different commercial perfumes based on this method. It is worth mentioning that these individually allergenic components of oakmoss are not listed by the European regulation, which only restricts total concentration of oakmoss and treemoss extracts.

3.2 Determination of Musks

Musk compounds have been widely used as fragrance chemicals in many consumer products such as cosmetics, detergents, food additives, or household products [48, 49]. They are valuable compounds not only for their unique odor but also for their fixative properties.

Natural musk, itself, was already used in ancient times and was obtained as a secretion produced by a gland of the musk deer. Traditionally, musk deer are killed to remove the musk gland, although musk can be extracted through the external orifice of the musk gland without the need of killing the animal. These facts and its difficult availability make this natural product very expensive [50].

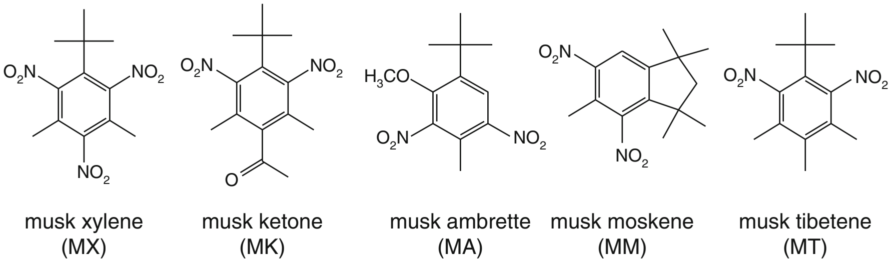


Fig. 107.2 Chemical structure of nitro musk compounds

On the other hand, the term musk also refers to other compounds, with totally different chemical structure but possessing musk-like odor properties. These are commonly named synthetic musks and appeared as a response to economical and ethical motives. Artificial musks have been generally divided in three subgroups: nitro musks, polycyclic musks, and macrocyclic musks.

The *nitro musks*, which are characterized by a nitro-aromatic moiety, were accidentally discovered by Baur in 1888 [51]. This group is basically formed by five compounds, that is, musk ambrette (MA), musk xylene (MX), musk moskene (MM), musk tibetene (MT), and musk ketone (MK). Figure 107.2 shows the chemical structure of these compounds.

Despite their pleasant aroma, nitro musks are believed to be persistent pollutants due to their strong tendency to bioaccumulate [52]. Many papers have been written about their health risks, showing they are related with different types of dermatitis, carcinogenic effects, and endocrine disruption [49, 53–58]. In fact, the use in cosmetic products of MA, MT, and MM is banned in the European Union, while the use of MX and MT is restricted. Nevertheless, its use is permitted in North America.

Regarding the *polycyclic musks*, these are composed of several cycles and do not have nitro substituents. Figure 107.3 shows the structure of some of the most commonly used polycyclic musks, such as 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran (HHCB) commonly known as galaxolide®, 6-acetyl-1,1,2,4,4,7-hexamethyltetralin (AHTN) commonly known as tonalide®, 5-acetyl-3-isopropyl-1,1,2,6-tetramethylindane (AITI) commonly known as traseolide®, and 1,1,2,3,3-pentamethyl-2,5,6,7-tetrahydroinden-4-one commonly known as cashmeran.

These musks were developed in 1950 and have slowly replaced the nitro musk compounds in Europe, since they are believed to have less harmful and toxic effects. However, in the later years, many studies show their bioaccumulation in the aquatic system and living beings [52, 59–61]. Nonpolycyclic musk is banned in the European Union, and only some of them are restricted. The rest can be freely used in cosmetic products. Thus, polycyclic musks are more used in Europe, while nitro musks are the common ones in North America [62].

Finally, *macrocyclic musks*, which are much larger compounds than the other two groups, have been developed in the recent years. Even though they have synthetic nature, they are synthesized trying to imitate the natural musk

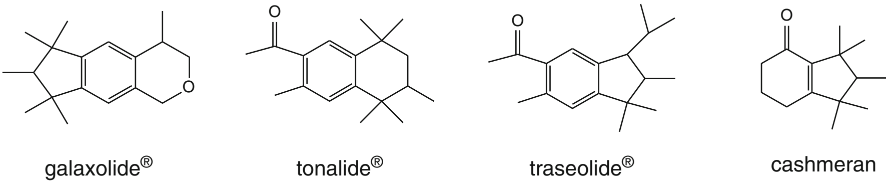


Fig. 107.3 Chemical structure of polycyclic musk compounds

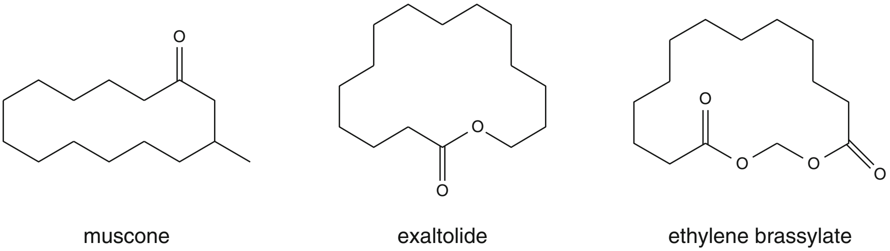


Fig. 107.4 Chemical structure of macrocyclic musk compounds

compounds. As these compounds are not restricted by law and seem not to have biodegradability problems, their use has increased considerably and is replacing the polycyclic musk compounds [50]. Some of the most important macrocyclic musks are shown in Fig. 107.4.

Many articles about the determination of artificial musks in different matrixes can be found in the literature. The most abundant are environmental samples such as air, water, and sediments [52, 59], but other matrixes such as biological fluids (blood [60, 63] or human milk [51]) or aquatic species [64, 65] are also available. However, the literature dealing with the determination of musks in cosmetic samples is rather scarce. The preferred technique is GC, with MS or electron capture detector (ECD) in the case of nitro musks.

Sommer [66] presented a method for quantification of nitro musks in cosmetics and detergents using GC with both, ECD and MS. Wineski et al. [67] determined MA in fragrance products with an internal standard addition technique by GC-ECD. The same author published another paper [68] some years later with a similar method to determine MA, MX, and MK. Struppe et al. [69] proposed a headspace solid-phase microextraction (HS-SPME) and GC with atomic-emission detection method for the determination of MX, MK, and MA in cosmetic samples. Four different commercially available fiber coatings were tested, and a 100-μm polydimethylsiloxane gave the best results. To the best of our knowledge, Eymann et al. [70] published the first paper where the determination of polycyclic musks in cosmetics was achieved, together with nitro musks. GC was used, with ECD in the case of nitro musks and MS for polycyclic musks. Some years later, Sommer and Juhl [71] proposed a GC with FID and MS detection to determine macrocyclic musks in alcohol-containing cosmetics.

More recently, several papers have been published on the determination of musks in cosmetic products. Roosens et al. [72] determined AHTN, HHCB, MX, and MK in 82 personal care products, 19 of them perfumes, with GC-MS and combined these results with the average usage to estimate exposure profiles through dermal application concluding that exposure to the polycyclic musks is much higher than to the nitro musk compounds. Ma et al. [73] proposed a SPE-isotope dilution-GC-MS/MS for the determination of MX in cosmetics. Martinez-Girón [74] achieved the enantiomeric separation of four chiral polycyclic musks using capillary electrophoresis (CE). The method was applied to determine these enantiomers in perfumes. Finally, as already mentioned before, Sanchez-Prado et al. [33] also developed a method that allows the simultaneous determination of many compounds, including nitro musks and polycyclic musks, in perfumes.

3.3 Determination of Phthalates

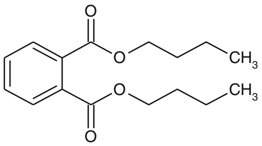
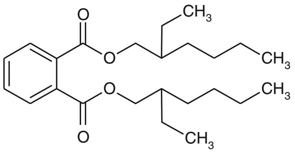
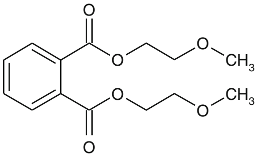
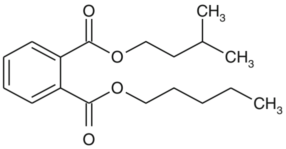
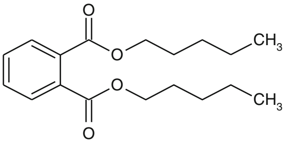
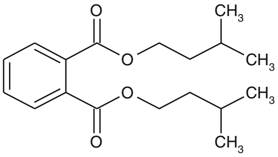
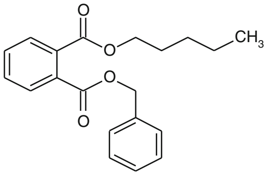
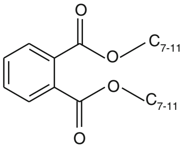
The term phthalate is used to designate the dialkyl or alkyl aryl esters of phthalic acid. These compounds are used in the cosmetic industry mainly as denaturants of the alcohols used in their manufacture, as fixatives or solvents for some fragrances (e.g., synthetic musks) and as film formers. Thus, it is usual to find them in nail polish, hairsprays, and perfumes [75]. Moreover, these substances are mainly used in the manufacture of plastics to increase their flexibility. Therefore, they can be found in different manufactured products, not only as part of its formulation but as a result of migration during manufacture or storage [1].

Phthalates have been proven to be harmful to living organisms [76–78]. These lipophilic compounds are rapidly metabolized by humans and biotransformed in their respective monoesters and other oxidative products, which are excreted in urine and feces [79]. Studies show that some phthalates and their metabolites are potentially toxic in animals due to endocrine-disruptive effects, which can cause adverse health effects, particularly for fertility and reproduction [80–82]. Moreover, some of them cause cancer in rats and mice [83]. Therefore, the presence of some phthalates is forbidden by the European legislation in some vulnerable products, such as toys [84] or personal care products [3]. Specifically, current European regulation of cosmetic products forbids the presence in cosmetic products of the phthalates shown in Table 107.2.

Other phthalates like diethyl phthalate (DEP), dimethyl phthalate (DMP), or di-n-octyl-phthalate (DNOP) are allowed in cosmetic products in Europe without any restriction, even though they are defined as pollutants by the US Environmental Protection Agency [85] and are also related to adverse health effects [86, 87].

Concerning the phthalate determination, the number of publications dealing with these compounds shows their great interest and their widespread presence in many consumer products and the environment. Specifically, in the case of the determination of phthalates in cosmetic products, several analytical methods for the identification and determination at the level of μg mL^{-1} and ng mL^{-1} have been published. The most commonly used techniques are liquid chromatography (LC)

Table 107.2 List of the forbidden phthalates in cosmetics by the European regulation

 dibutyl phthalate (DBP) [CAS 84-74-2]	 bis(2-ethylhexyl) phthalate (DEHP) [CAS 117-81-7]
 bis(2-methoyethyl) phthalate (DMEP) [CAS 117-82-8]	 1,2-benzenedicarboxylic acid, dipentylester, branched and linear (NPIPP) [CAS 84777-06-0]
 di-n-pentyl phthalate (DNPP) [CAS 131-18-0]	 diisopentylphthalate (DIPP) [CAS 605-50-5]
 benzyl butyl phthalate (BBP) [CAS 85-68-7]	 1,2-benzenedicarboxylic acid, di-$C_{7\text{-}11}$, branched and linear alkyl esters (DHNUP) [CAS 68515-42-4]

combined with UV-visible and gas chromatography (GC) with flame ionization detector (FID) or coupled to mass spectrometry (MS). Most of the publications deal with the determination of dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate (DEHP), benzyl butyl phthalate (BBP), and di-n-pentyl-phthalate (DNPP) within other non-prohibited phthalates. Less common are studies that include bis (2-methoxyethyl) phthalate (DMEP), the most polar prohibited phthalate. Finally, no analytical methods for the determination of all three isomers, DNPP, diisopentyl phthalate (DIPP), and n-pentyl-isopentylphthalate (NPIPP), or the mix of $C_{7–11}$ branched and linear alkyl esters have been found.

The determination of phthalates in cosmetic samples started many years ago, as shown in a work dated in 1973 where different phthalates were determined by GC-FID [88]. Some years later, Markovic et al. [89] published a work where the phthalate DMP and N,N-diethyl-m-toluamide, both used as insect repellent compounds, were determined in cosmetic samples by thin-layer chromatography (TLC) and UV scanning densitometry. Koo and Lee [90] estimated the median daily human exposure to phthalates by determining the presence of the phthalates DEHP, DEP, DBP, and BBP by HPLC in a hundred different cosmetic samples, like perfumes, deodorants, or nail polishes. Samples were weighted, dissolved in methanol, vortexed, and centrifuged before injection. A run time of 50 min in isocratic elution was needed. As expected, DEP, the non-banned phthalate, was found at high concentrations. Nevertheless, the other phthalates were also present in rather high concentrations in many samples.

Chen et al. [91] developed the first method for the determination of six phthalate esters, DMP, DEP, DBP, BBP, DEHP, and DNOP, in cosmetics by GC-FID. MS was also used but just to qualitatively confirm the presence of the target compounds. Sample pretreatment, similar to Koo and Lee's work, consisted in weighting the samples, dissolving them in methanol, sonicating, and centrifuging. Once water from the upper clean layer was eliminated with anhydrous sodium sulfate it was directly injected. Compared to the previous works, this method showed better sensitivity, with lower limits of detection (LOD). De Orsi et al. [92] continued with LC coupled to UV detection and presented a method where DMP, DEP, dipropyl phthalate (DPP), diisobutyl phthalate (DIBP), BBP, DBP, and DEHP were determined in nail cosmetics. This time, a gradient was used, and run time was reduced to 30 min. Shen et al. [93] studied the determination of seven phthalates and four parabens in different kinds of cosmetic samples, using both LC-UV and GC-MS in SIM mode. A cleanup step using C18 SPE was carried out, which improved the LOD. However, the method is time-consuming and uses high volumes of methanol, so it could not be considered a green method suitable for routine analysis. The IFRA has a phthalate quantification procedure, available in its website [94]. The determination is performed in a GC-MS in SIM mode, with both a quantifier and a qualifier ion per compound.

Regarding the sample pretreatment step, traditional extraction techniques that involve the use of high amounts of toxic solvents should be avoided. In this sense, Chingin et al. [95] presented a novel procedure based on extractive electrospray ionization mass spectrometry (EESI-MS) for the detection of DEP in perfumes with no need of sample pretreatment. Su et al. [96] determined DMP, DBP, dicyclohexyl phthalate (DCHP), and DNOP in perfumes and lacquer removers based on polymer monolith microextraction (PMME) and LC-UV. The conditions for extraction were optimized leading to a sensitive method.

Recently, Koniecki et al. [97] determined the phthalate levels in 252 cosmetic products from the Canadian market by GC-MS to estimate the dermal exposure. Sanchez-Prado et al. [33] also developed a method that allows the simultaneous determination of many compounds, including phthalates, in perfumes. The method consists in diluting the sample in ethyl acetate and injecting it directly into a GC-MS system.

All studies with real perfume samples agree that the presence of prohibited phthalates like DBP, BBP, and DEHP, even in several hundred parts per million, is very common. In this sense, the US Consumers Association [98] conducted a study with eight different brands of perfumes where basically all tested perfumes contained at least DEP and the prohibited phthalate DEHP. A Greenpeace study [75] of different commercial perfumes by GC-MS also found prohibited phthalates in the mg L^{-1} range. Cosmetic industry should try to avoid this issue using free phthalate plastics and solvents.

4 Conclusions

This work resumes the most important regulatory aspects for fragrance chemicals. Different legislations concerning cosmetic products or household products can be found all over the world. This should encourage analytical chemists to develop new methods that enable the determination of all the substances mentioned in these regulations. This would surely help manufacturers and authorities to perform quality control and to assure that no restricted substances are present in the product. Different methods for perfume analysis have been revised here, especially those on restricted or forbidden perfume-related substances, such as potentially allergenic fragrance-related substances, musks, and phthalates. All these works are a very good start, but much more work can be done in perfume analysis.

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Methods for Analysis of Triterpenoid Saponins 108

Jinlan Zhang and Feng Qu

Contents

1 Introduction 3312
2 Sample Preparation 3313
3 Analytical Method 3314
3.1 UV–Vis Spectrophotometry 3314
3.2 Thin-Layer Chromatography 3314
3.3 HPLC Coupled with UV and Evaporative Light Scattering Detector 3314
3.4 HPLC Coupled with Mass Spectrometry 3315
3.5 Mass Spectrometry 3316
3.6 Determination of the Absolute Configuration of Monosaccharides in Saccharide Chain and the Substituted Groups of Triterpenoid Sapogenins 3319
4 Conclusion 3320
References 3320

Abstract

Analytical techniques are very important for promoting the development of the research field of triterpenoid saponin. Although the traditional thin-layer chromatography and spectrophotometry lack sensitivity, resolution, and specificity, they are still widely used due to their high-throughput capacity, simplicity, and rapidity. The development of high-performance liquid chromatography and mass spectrometry, especially high-performance liquid chromatography coupled with hybrid mass spectrometry, has made it easy to comprehensively probe the triterpenoid saponins in matrix, as well as rapidly identify and simultaneously determine minor and multiple triterpenoid saponins from one sample. The type,

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sequence, and absolute configuration of monosaccharide in saccharide chain and structural type of some triterpenoid sapogenins can be determined. Here, we review analytical methods available for qualitative and quantitative analysis of triterpenoid saponins.

Keywords

Analytical method • high-performance liquid chromatography • high-performance liquid chromatography and mass spectrometry • thin-layer chromatography • triterpenoid saponins • ultraviolet visible spectrophotometry

Abbreviations

CID	Collision-induced dissociation
ELSD	Evaporative light scattering detection
ESI	Electrospray ionization
FTICRMS	Fourier transform ion cyclotron resonance mass spectrometry
HPLC	High-performance liquid chromatography
HPLC-MS	High-performance liquid chromatography tandem mass spectrometry
IS	Internal standard
IT	Ion trap
MALDI	Matrix-assisted Laser Desorption Ionization
MS/MS	Two-stage mass analysis
MS^n	Multi-stage mass analysis
Q	Quadrupole
QQQ	Triple quadrupole
TOF	Time of flight

1 Introduction

Triterpenoid saponin exists widely in plants of Araliaceae, Campanulaceae, Polygalaceae, Leguminosae, Umbelliferae, etc. Triterpenoid saponin is consisted of triterpenoid sapogenins and saccharide chain. Tetracyclic and pentacyclic triterpenoid saponins are very common and showed extensive activity on immune system, cardiovascular system, even with antitumor effect. They have long been under study to elucidate their activity and mechanism, new structural compounds, and develop rapid and accurate analytical methods. The isolation of triterpenoid saponins is tedious due to their high polarity, and so is the structure identification due to the presence of more than three monosaccharides. New analytical tools and approaches have been developed for qualitative and quantitative analysis of triterpenoid saponins in food, medicinal plant and health care products. This chapter describes spectrophotometry, mass spectrometry, and chromatography coupled with different detector for triterpenoid saponin analysis in matrix.

2 Sample Preparation

The interference of the coexisting components is easily observed due to the weak ultraviolet absorbance and difficult volatility of triterpenoid saponins. The sample preparation is very important for analysis of triterpenoid saponins in matrix for high resolution, accuracy, and selectivity, regardless of the analytical techniques subsequently used. In general, the solid sample is dried and pulverized before extraction. Alcoholic solvents such as methanol, ethanol, and *n*-butanol are commonly used to extract triterpenoid saponins. For triterpenoid saponins with rich hydroxyl groups and strong hydrophobicity, water [1], *n*-butanol [2], and aqueous alcohol [3–19], such as 50 %, 70 %, and 80 %, showed good extract efficiency. However, for some triterpenoid acids and esters, chloroform [10–12, 20–24] and dichloromethane [25, 26] have good extract efficiency. Heat extraction [1, 3, 4, 6, 7, 9, 10, 12, 14, 19, 20, 26–28] and ultrasonic extraction [6, 8, 12, 13, 15–18, 22–25, 29] are typical extraction methods. Heat extraction involves extraction in a Soxhlet's extractor [3, 4, 26, 27] and under reflux [1, 6, 7, 9, 10, 12, 14, 19, 20, 28] at 50–80 °C.

Since coexisted lipophilic compounds decrease the resolution and efficiency for analysis of triterpenoid saponins, solid sample or extraction solution is usually defatted with lipophilic solvents such as petroleum ether [3, 9, 10, 20], ethyl acetate [11], and chloroform [2].

In order to further concentrate and separate target triterpenoid saponins and remove the bulk of the major co-extract compounds from extraction solution, the liquid–liquid extraction and chromatographic separation are attempted. For the most part, the extract of aqueous alcohol is filtered and then evaporated to dryness. The residue is dissolved in water, then extracted with *n*-butanol [9–11] or subjected to chromatographic column [1, 19, 27–29] such as macroporous resin column or to thin-layer chromatographic plate [25]. The *n*-butanol extraction solution and target eluents from chromatographic column or thin-layer chromatographic plate are collected and evaporated to dryness. The residue is dissolved in appropriate solvent for analysis.

For biological samples protein precipitation with acetonitrile [30] or methanol [5], liquid-liquid extraction [5, 31], and solid-phase extraction [32] are used to extract triterpenoid saponins from rat or human plasma. The extraction solution or collected eluents are evaporated and the residues are reconditioned by suitable solvent for analysis.

The optimum extraction conditions are obtained by experiments as follows: extraction solvent, the ratio of material to solvent (W/V), extraction temperature, extraction time, and elution or development solvents for chromatographic column or thin-layer chromatography. The sample preparation procedure, either one-step extraction or multiple-step extraction, needs to be carefully tried according to the analytical procedure subsequently used.

Accelerated solvent extraction is a new extraction technique and is performed at high temperature and high pressure. It demonstrates high efficiency, short extraction time, and low solvent usage. Diatomaceous earth was extracted on an accelerated solvent extraction extractor [33] and different solvents (dichloromethane, *n*-hexane, methanol, and ethanol/water (80:20)) are tested. The temperature

(5–130 °C) and number of extraction cycles are optimized. The extracts of each extraction cycle are evaporated separately. Prior to HPLC analysis, the dry residues resulting from different cycles are redissolved in ethyl acetate.

3 Analytical Method

3.1 UV–Vis Spectrophotometry

With unsaturated bond or 3-hydroxy and 3-carbonyl group, triterpenoid saponins can easily react with strong acids, such as glacial acetic acid, sulfuric acid, and perchloride acid, and form carbonium ion with visible color under anhydrous condition [34]. Carbonium ion can form stable visible color with vanillin by the nucleophilic addition reaction, so 5 % of vanillin-glacial acetic acid and perchloric acid are usually used as color-developing agents for the determination of triterpenoid saponin by ultraviolet visible (UV–vis) spectrophotometry [9, 11, 27]. The detection wavelength is set at 500–600 nm. In order to create the anhydrous reaction condition, the extract solution was evaporated to dryness and the residues were redissolved in anhydrous solvents. UV–Vis spectrophotometry can only be used to determine the total amount of triterpenoid saponins due to the lack of resolution and specificity. The total amount of triterpenoid saponins is calculated with the standard curves of reference compound. In order to improve the specificity and reduce the interference, the multiple-steps sample preparation is commonly performed to concentrate and separate target triterpenoid saponins.

3.2 Thin-Layer Chromatography

Thin-layer chromatography (TLC) is simple and fast, so it is still used as identification test for authentication of the medicinal plants containing triterpenoid saponins, such as Ginseng [2]. Silica gel thin-layer plate is usually employed and the lipophilic solvent is used as developing solvent. Ten percent of sulfuric acid in ethanol is color-developing reagent. With the development of thin-layer chromatogram scanner, TLC is used to determine triterpenoid saponins [20]. In order to avoid the noise and interference, two detection wavelengths are set, one for reference and the other for detection.

3.3 HPLC Coupled with UV and Evaporative Light Scattering Detector

High-performance liquid chromatography (HPLC) coupled with different types of detectors is often used to quantitatively determine or profile triterpenoid saponins. Most of triterpenoid saponins lack chromophores. Their ultraviolet absorbance depends on the degree of unsaturation. For triterpenoid saponins with independent double bonds, their ultraviolet absorbance is close to the 200–250 nm range with weak intensity [8, 14–16, 18, 21, 22, 25, 29, 31, 32, 35]. For ultraviolet detector, it is difficult to choose suitable mobile phase. For triterpenoid saponins with α,β-unsaturated carbonyl or conjugated diene bond, their ultraviolet absorbance is close to the 240–285 nm range [12, 24, 36]. Reversed phase C18 column seems more suitable to separate triterpenoid saponins than normal phase column [33]. Mobile phase for C18 column is consisted of water containing acids, such as phosphoric acid [14, 18, 22, 24, 31, 35, 36], phosphate [15, 16], acetic acid [21, 24], formic acid [17], and perchloric acid [24], and methanol or acetonitrile. Sometimes tetrahydrofuran [21] is added in mobile phase to improve chromatographic resolution. The gradient elution [8, 15–18, 22, 24, 25, 29, 31, 36–38] is often used for simultaneous determination of multiple triterpenoid saponin compounds in matrix [8, 15, 16, 18, 22, 25, 29, 31, 36, 38] or fingerprint analysis [17, 22, 24, 37] of triterpenoid saponins in medicinal plants. The limitation of detection and quantitation is in the range of 0.01–50 μg/mL.

Evaporative light scattering detection (ELSD) is an alternative technique for triterpenoid saponins with weak UV absorbance. In ELSD detector, the eluent from HPLC column is evaporated and the nonvolatile solute components as droplets are illuminated by laser. The scattered light of droplets is measured. Reversed-phase C18 column is commonly used and mobile phase consisted of water-containing volatile acids such as acetic acid, formic acid, trifluoroacetic acid, and methanol or acetonitrile containing above volatile acid is employed [3, 4, 6, 12, 13]. To improve chromatographic resolution, mobile phase is modified with tetrahydrofuran [3], isopropyl alcohol [3], and methyl tert-butyl ether [13]. The gradient elution is often used and the temperature of drift tube is set at 59–105 °C. The limitation of detection and quantitation seems better than that of ultraviolet detector. Evaporative light scattering detector can detect any compound that does not evaporate, so it lack of specificity as ultraviolet detector.

To get reliable quantization of triterpenoid saponins in extract solution, calibration curves for each target analyte must be generated, due to different signal responses. For determination of triterpenoid saponins in complex matrix with good accuracy and precision [25, 31], selection of internal standard (IS) is key. The ideal IS would be chemically similar to the target analytes. Calibration curve is constructed using the peak area ratios of analyte to IS versus the concentration of analyte.

3.4 HPLC Coupled with Mass Spectrometry

The high separation power of HPLC, together with excellent selectivity and sensitivity of mass spectrometry (HPLC-MS), overcame the above problem in the analysis of triterpenoid saponins. HPLC-MS [1, 5, 7, 10, 17, 19, 28–30, 37, 39–42] plays an important role in the analysis of triterpenoid saponins.

At present many mass analyzers are available, such as single quadrupole (Q), triple quadrupole (QQQ), ion trap (IT), time of flight (TOF), and fourier transform ion cyclotron resonance mass spectrometry (FTICRMS). In addition, new types of hybrid mass spectrometers, such as Q-TOF, IT-TOF, and LTQ-FTICRMS, provide the perfect connection between high-flow chromatography and mass spectrometry with high accuracy and resolution. The use of ion trap or quadrupole permits easy control and selection of the amounts of target parent or product ions sent to the mass spectrometry with high accuracy and resolution (TOF or FTICRMS). Mass data of target parent or product ions with good accuracy and resolution can be obtained to determine the elemental composition and to improve the resolution for the poor chromatographic separation. HPLC-TOF and HPLC-QTOF have been successfully applied for the identification of triterpenoid saponins in the complex constituents of crude herb extract [1, 5, 29, 42].

The multiple-stage MS, such as QQQ [41] and ion trap [1, 5, 10, 17, 19], can provide two-stage MS (MS/MS) analysis and multiple-stage MS (MS^n) analysis. The MS^n analysis is carried out automatically, by the selected data-dependent scan mode, that first-stage mass analyzer collects full scan mass data, then picks the parent or product ion of interest for fragmentation in collision cell by collision-induced dissociation (CID), and fragment ions are analyzed in second-stage mass analyzer. The cycle is repeated throughout the duration of the acquisition and provides a great deal of MS^n data, which is very useful for structure elucidation or confirmation of triterpenoid saponins [1, 5, 10, 19, 41]. Different aglycons have the characteristic fragmentation, and the aglycon structures of some triterpenoid saponins can be differentiated on the basis of diagnostic fragments [1, 5, 10, 19, 28, 29, 41, 42]. If the characteristic fragment ions of saccharide chain are obtained, glycosylation sites at aglycon, the sugar types, and the sequence of the sugar units can be determined [1, 5, 10, 19, 41, 42]. In order to get enough diagnostic fragment information, the mobile phase of HPLC is optimized and formic acid, acetic acid, and ammonium acetate are used to improve the mass signal response. The reference standards of the known triterpenoid saponins was primarily used to optimize mass parameters. The diagnostic fragment ions and fragmentation rules of known triterpenoid saponins are summarized. Based on the information of diagnostic fragment ions and fragmentation rules, the triterpenoid saponins in matrix, even the new triterpenoid saponins, might be identified. It is found that the positive ion mode is more sensitive than negative electrospray (ESI) mode for most of the triterpenoid saponins with TOFMS. The negative ESI mode of ITMS easily provided extensive fragmentation information by CID. Therefore, the positive ion mode of TOFMS [1, 5] and the negative mode of ITMS were selected to obtain the characteristic fragment ions and fragmentation rule which might differentiate isomers and determine the monosaccharide and the linkage of the saccharide chain.

3.5 Mass Spectrometry

Different kinds of tandem mass spectrometry techniques have been applied to different types of purified triterpenoid saponins from natural medicinal plants [23, 28, 29, 33, 39, 43–47]. ESI [23, 28, 29, 33, 39, 43, 44, 46, 47] and Matrix-assisted

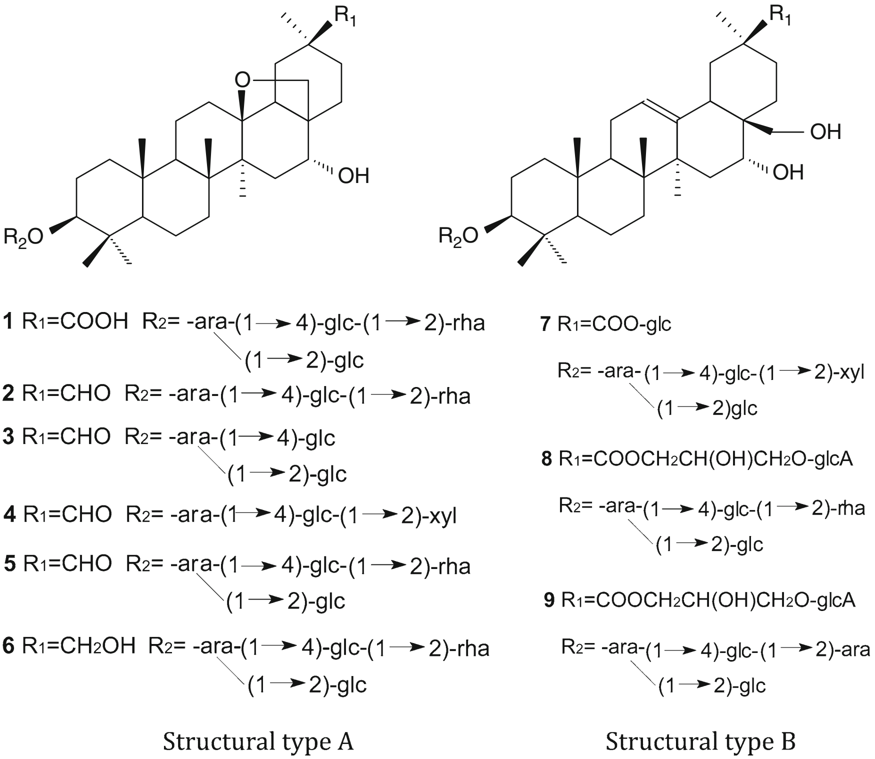
R1
O
OH
R2O
R1
OH
OH
R2O
1 R1=COOH R2= -ara-(1→4)-glc-(1→2)-rha
(1→2)-glc
2 R1=CHO R2= -ara-(1→4)-glc-(1→2)-rha
3 R1=CHO R2= -ara-(1→4)-glc
(1→2)-glc
4 R1=CHO R2= -ara-(1→4)-glc-(1→2)-xyl
5 R1=CHO R2= -ara-(1→4)-glc-(1→2)-rha
(1→2)-glc
6 R1=CH2OH R2= -ara-(1→4)-glc-(1→2)-rha
(1→2)-glc
7 R1=COO-glc
R2= -ara-(1→4)-glc-(1→2)-xyl
(1→2)glc
8 R1=COOCH2CH(OH)CH2O-glcA
R2= -ara-(1→4)-glc-(1→2)-rha
(1→2)-glc
9 R1=COOCH2CH(OH)CH2O-glcA
R2= -ara-(1→4)-glc-(1→2)-ara
(1→2)-glc
Structural type A
Structural type B

Fig. 108.1 Structures of eight triterpene saponins [1–9]

Laser Desorption Ionization (MALDI) [45, 46] combined with hybrid types of mass spectrometers, such as ion trap [44], Q-LTQ [23], QTOF [28, 43], IT-TOF [46], TOF/TOF [46], and FTICRMS [47], are used to elucidate the fragmentation mechanism and rule of triterpenoid saponins and to discover diagnostic fragment ions for differentiating the aglycon and determine the substitute site, sequence, and linkage of saccharide chain. It is observed that the type of ionization method, either ESI or MALDI, does not influence the fragmentation of identical precursor ions in low-energy CID [46]. The energy of CID must be optimized so as to obtain useful structural information. The sugar linkage information resulting from cross-ring cleavage is easily available under low-energy CID. On the other hand the high-energy CID tends to yield diagnostic fragment information of the aglycon due to internal cleavage. We have developed the negative ESI-LTQ/FTICRMS method to analyze of triterpene saponins from *Ardisia crenata* Sims [47]. Eight isolated triterpene saponins belonging to two structural types A and B (Fig. 108.1) are analyzed using parent mass list-triggered data-dependent multiple-stage accurate mass analysis at a resolving power of 100,000. The accurate m/z values of precursor and product ions are obtained and used to calculate the chemical formulae (Tables 108.1–108.4). Characteristic mass fragmentation rules are summarized.

Table 108.1 Mass data from ten saponin compounds

Compounds	[M-H]-	Proposed formula	RDB	Calculated mass (m/z)	Error (ppm)
1	1089.5509	$C_{53}H_{85}O_{23}^{-}$	11.5	1089.5476	3.015
2	911.5063	$C_{47}H_{75}O_{17}^{-}$	10.5	911.5017	7.047
3	927.4954	$C_{47}H_{75}O_{18}^{-}$	10.5	927.4948	0.656
4	897.4831	$C_{46}H_{73}O_{17}^{-}$	10.5	897.4842	−1.256
5	1073.5579	$C_{53}H_{85}O_{22}^{-}$	11.5	1073.5527	4.843
6	1075.5703	$C_{53}H_{87}O_{22}^{-}$	10.5	1075.5684	1.813
7	1237.5906	$C_{58}H_{93}O_{28}^{-}$	12.5	1237.5848	4.696
8	1339.6194	$C_{62}H_{99}O_{31}^{-}$	13.5	1339.6165	2.178
9	1325.6111	$C_{53}H_{85}O_{22}^{-}$	13.5	1325.6008	7.746
10	1355.6156	$C_{53}H_{85}O_{22}^{-}$	13.5	1355.6114	3.101

Table 108.2 MS^2 data from ten saponin compounds

Compounds	Precursor ion	Product ion	Relative intensity (%)	Proposed formula	RDB	Calculate mass (m/z)	Error (ppm)
1	1089.57	943.4913	100	$C_{47}H_{75}O_{19}^{-}$	10.5	943.4897	1.689
		781.4401	22	$C_{41}H_{65}O_{14}^{-}$	9.5	781.4369	3.349
2	911.51	765.4444	100	$C_{41}H_{65}O_{13}^{-}$	9.5	765.4420	3.177
		603.3912	18	$C_{35}H_{55}O_{8}^{-}$	8.5	603.3891	3.406
3	927.49	765.4434	100	$C_{41}H_{65}O_{13}^{-}$	9.5	765.4420	1.870
		603.3909	17	$C_{35}H_{55}O_{8}^{-}$	8.5	603.3891	2.908
4	897.48	765.4442	100	$C_{41}H_{65}O_{13}^{-}$	9.5	765.4420	2.915
		603.3912	9	$C_{35}H_{55}O_{8}^{-}$	8.5	603.3891	2.908
5	1073.55	927.4983	100	$C_{47}H_{75}O_{18}^{-}$	10.5	927.4948	3.783
		765.4448	30	$C_{41}H_{65}O_{13}^{-}$	9.5	765.4420	3.699
6	1075.57	929.5143	100	$C_{47}H_{77}O_{18}^{-}$	9.5	929.5104	4.151
		767.4604	41	$C_{41}H_{67}O_{13}^{-}$	8.5	767.4576	3.885
7	1237.59	1075.5359	100	$C_{53}H_{87}O_{22}^{-}$	11.5	1075.5684	3.659
8	1339.62	1321.6022	100	$C_{62}H_{97}O_{30}^{-}$	14.5	1321.6059	−2.283
		943.4892	20	$C_{47}H_{75}O_{19}^{-}$	10.5	943.4897	−0.537
		781.4376	24	$C_{41}H_{65}O_{14}^{-}$	14.5	781.4369	0.717
9	1325.61	1307.5986	31	$C_{61}H_{95}O_{30}^{-}$	14.5	1307.5903	6.372
		943.4942	70	$C_{47}H_{75}O_{19}^{-}$	10.5	943.4897	4.763
		781.4395	100	$C_{41}H_{65}O_{14}^{-}$	14.5	781.4369	0.948
10	1355.62	1337.6071	23	$C_{62}H_{97}O_{31}^{-}$	13.5	1337.6008	3.101
		959.4894	83	$C_{47}H_{75}O_{20}^{-}$	10.5	959.4846	4.981
		797.4339	100	$C_{41}H_{65}O_{15}^{-}$	9.5	797.4318	2.637

The triterpene saponins of structural type A undergo a sequential loss of sugar from the substituted saccharide chain at the C-3 position in the FTICR–MS^n experiment. The triterpene saponins of structural type B readily lose the substituted group at the C-30 position, with the subsequent loss of the terminal monosaccharide group from

Table 108.3 MS^3 data from ten saponin compounds

Compounds	Precursor ion	Product ion	Relative intensity (%)	Proposed formula	RDB	Calculate mass (*m*/*z*)	Error (ppm)
1	943.49	781.4398	100	$C_{41}H_{65}O_{14}^{-}$	9.5	781.4369	3.733
		619.3858	34	$C_{35}H_{55}O_{9}^{-}$	8.5	619.3841	2.810
2	765.44	603.3912	100	$C_{35}H_{55}O_{8}^{-}$	8.5	603.3891	3.406
3	765.44	603.3909	100	$C_{35}H_{55}O_{8}^{-}$	8.5	603.3891	2.908
4	765.44	603.3909	100	$C_{35}H_{55}O_{8}^{-}$	8.5	603.3891	2.908
5	927.50	765.4448	100	$C_{41}H_{65}O_{13}^{-}$	9.5	765.4420	3.699
6	929.51	767.4604	100	$C_{41}H_{67}O_{13}^{-}$	8.5	767.4576	3.885
7	1075.54	943.4921	100	$C_{47}H_{75}O_{19}^{-}$	10.5	943.4897	2.537
8	1321.60	1131.5618	32	$C_{55}H_{87}O_{24}^{-}$	12.5	1131.5582	5.409
		943.4935	65	$C_{47}H_{75}O_{19}^{-}$	10.5	943.4897	4.021
		781.4376	100	$C_{41}H_{65}O_{14}^{-}$	9.5	781.4369	2.965
		619.3854	46	$C_{35}H_{55}O_{9}^{-}$	8.5	619.3841	3.133
9	781.44	619.3861	100	$C_{35}H_{55}O_{9}^{-}$	8.5	619.3841	3.294
10	797.43	635.3817	100	$C_{35}H_{55}O_{10}^{-}$	8.5	635.3790	4.290

Table 108.4 MS^4 data from six saponin compounds

Compounds	Precursor ion	product ion	Relative intensity (%)	Proposed formula	RDB	Calculate mass (m/z)	Error (ppm)
1	781.44	619.3862	100	$C_{35}H_{55}O_{9}^{-}$	8.5	619.3841	3.456
5	765.44	603.3910	100	$C_{35}H_{55}O_{8}^{-}$	8.5	603.3891	3.074
6	767.46	605.4069	100	$C_{35}H_{57}O_{8}^{-}$	8.5	605.4048	3.477
7	943.50	781.4401	100	$C_{41}H_{65}O_{14}^{-}$	9.5	781.4369	4.117
		619.3861	9	$C_{35}H_{55}O_{9}^{-}$	8.5	619.3841	3.294
8	781.44	619.3860	100	$C_{35}H_{55}O_{9}^{-}$	8.5	619.3841	3.778
9	619.39	571.3646	100	$C_{34}H_{51}O_{7}^{-}$	9.5	571.3629	2.927

the substituted saccharide chain at the C-3 position, which is similar to the tendency observed in the triterpene saponins of structural type A. On the basis of characteristic fragmentation rule and feature of fragment ions, two unknown traces of triterpene saponins were identified in the mixture 9 and 10.

3.6 Determination of the Absolute Configuration of Monosaccharides in Saccharide Chain and the Substituted Groups of Triterpenoid Sapogenins

The absolute configuration of monosaccharides in saccharide chain and the substituted groups of triterpenoid sapogenins is difficult to determine. Chemical analyses combined with chiral chromatographic method have been used to ascertain the absolute configuration. The absolute configuration of seven new pentacyclic triterpenoid saponins, named dianversicosides from the aerial parts of *Dianthus versicolor* is determined [48]. Isolated dianversicosides are hydrolyzed in 1 mol/L HCl at 80 °C. The reaction solutions were extracted with ethyl acetate. The water-soluble parts containing monosaccharides were neutralized and then concentrated to dryness. The dried residue was made to react with L-cysteine methyl ester hydrochloride and then trimethylchlorosilane. The reaction solutions were extracted with *n*-hexane. The supernatant was subjected to GC-MS analysis. The absolute configurations of the monosaccharide units were confirmed by comparison of the retention times with standard monosaccharide samples. A solution of $LiEt_3BH$ in dry tetrahydrofuran was added to solutions of isolated compounds in dry tetrahydrofuran, under an ice bath with an inflow of Argon for 30 min. H_2O was added to reaction mixture and 0.1 mol/L HCl was used to adjust the pH to 3. The reaction product lactones were extracted with ethyl acetate and analyzed by chiral HPLC. The retention times of isolated compounds were compared with those of (3S)- and (3R)-mevalonolactones standards, then the absolute configuration of the 3-hydroxyl-3-methylglutaryl group of some dianversicosides was elucidated as (3S)-3-hydroxy-3-methylglutaryate.

4 Conclusion

This chapter describes the different types of analytical methods in use for the analysis of triterpenoid saponins. HPLC coupled with mass spectrometry shows more merit than other analytical methods on selectivity, sensitivity, and resolution. The diversified HPLC-MS technique provides rich mass information to determine the type and sequence of monosaccharide in saccharide chain and structural type of some triterpenoid sapogenins. This technique is also of interest to develop the reliable and sensitive analytical method for minor triterpenoid saponins in complex matrix. However, due to the lack of standards, there are not many analytical methods for triterpenoid saponins in biological samples. Development of mass spectrometry–based method for triterpenoid saponins in biological samples is necessary to understand the effect and mechanism of triterpenoid saponins.

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Methods of Isolation and Analysis of Forskolin from *Coleus forskohlii* 109

Mohamed Saleem A.

Contents

1 Introduction 3326
1.1 Physicochemical Properties of Forskolin 3328
2 Isolation by Column Chromatography 3328
3 Isolation by Vacuum Liquid Chromatography 3329
4 Isolation by Charcoal Column Chromatography 3329
5 Isolation by Immunoaffinity Column Chromatography 3330
6 Isolation by Hydrotropic Extraction 3330
7 Isolation by Microwave-Assisted Extraction 3331
8 Isolation by Using Selective Adsorbent Designed by Molecular Simulation 3332
9 Extraction by Three-Phase Partitioning 3333
10 TLC Analysis of Forskolin 3334
11 HPTLC Analysis of Forskolin 3336
12 HPLC Analysis of Forskolin 3336
13 GC Analysis of Forskolin 3337
14 Analysis of Forskolin by ELISA 3338
15 Hyphenated Techniques 3339
16 Conclusion 3339
References 3340

Abstract

Forskolin is a well-known activator of adenylyl cyclase, obtained from the roots of *Coleus forskohlii*. Several methods were reported for the isolation of pure forskolin from the roots of *C. forskohlii*. The various methods of isolation, the purity, and yield of isolated forskolin are described. The methods included column chromatography, vacuum liquid chromatography, charcoal

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column chromatography, immunoaffinity column chromatography, hydrotropic extraction, and adsorption on a selective adsorptive ligand designed by molecular simulation. Forskolin with a maximum purity of 98 % w/w was obtained through adsorption on a selective adsorptive ligand designed by molecular simulation. Similarly, several analytical techniques like, TLC, HPTLC, HPLC, GLC, and ELISA are described for the quantitative determination of forskolin in standardized crude extracts and pharmaceutical formulations. Among the analytical methods, HPLC and HPTLC appear to be more popular and widely used in the industry and academia. Analysis of forskolin by ELISA appears to be highly sensitive than other techniques.

Keywords

Analytical techniques • Charcoal column chromatography • Chromatography • *Coleus forskohlii* • Forskolin • ELISA • GLC • HPLC • HPTLC • Hydrotropic extraction • Immunoaffinity chromatography • Microwave-assisted extraction • Molecular simulation • TLC

Abbreviations

BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CNBr	Activated Sepharose 4B – Cyanogen bromide-activated-Sepharose 4B
ELISA	Enzyme-linked immunosorbent assay
GC	Gas chromatography
GLC	Gas liquid chromatography
HAS	Human serum albumin
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
IgG	Immunoglobulin G
LC-MS	Liquid chromatography mass spectrometry
MAb	Monoclonal antibody
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
PBS	Phosphate buffered saline
TLC	Thin layer chromatography
UV	Ultraviolet

1 Introduction

Coleus forskohlii Briq. (Lamiaceae) is a perennial herb, occurs naturally in Indian subcontinent and is also distributed in Egypt, Arabia, Ethiopia, tropical East Africa, and Brazil [1, 2]. In traditional medicine, *C. forskohlii* is commonly used in different countries for various disorders. In Egypt and Africa, the leaf is used as an expectorant, emmenagogue, and diuretic [1]. In Brazil, it is used as a stomach

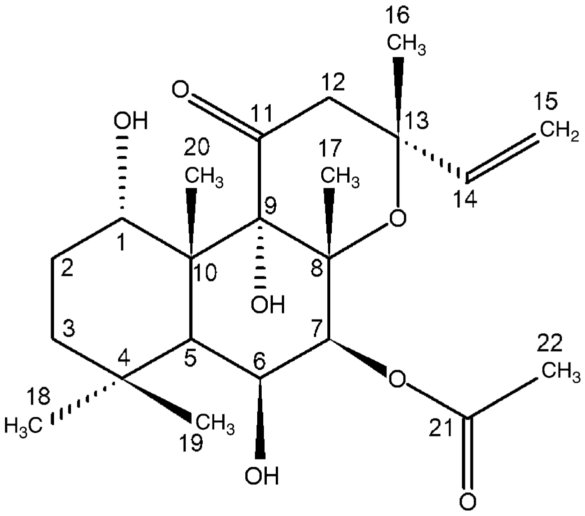

Fig. 109.1 Structure of forskolin

aid, for treating intestinal disorders and to interrupt pregnancy [1, 3]. In India, it is used as a condiment and the root tubers are prepared as pickle and eaten. In traditional Ayurvedic systems of medicine, *C. forskohlii* has been used for treating heart diseases, abdominal colic, respiratory disorder, insomnia, convulsions, asthma, bronchitis, intestinal disorders, burning sensation, constipation, epilepsy, and angina [4]. The root tubers are employed in the treatment of worms and to reduce burning sensation in festering boils. The root paste is applied to treat eczema and other skin infections after mixing with mustard oil. The plant is also used for veterinary disorders [5]. The root tubers of *C. forskohlii* are included in various Ayurvedic formulations and listed in Ayurvedic Pharmacopoeia of India as *Gandira* [6].

Forskolin is a bioactive compound obtained mainly from the tuberous roots of *C. forskohlii*. Recently, stem of *C. forskohlii* was also found to contain appreciable quantities of forskolin [7]. *C. forskohlii* roots have long been used in Ayurvedic medicine for treating heart and lung disease, intestinal spasms, insomnia, and convulsions [4]. Forskolin (Fig. 109.1), a labdane diterpene, has exhibited positive effects in asthma, glaucoma, hypertension, cancer, heart disease, diabetes, and obesity [4]. Forskolin was isolated during 1970s by Indian researchers and initially referred to as coleonol and later changed to forskolin [8–10]. Its structure and stereochemistry were established by several studies [8, 10–13]. Forskolin occurs exclusively in *C. forskohlii* and could not be detected in other *Coleus* species [14]. Forskolin's major pharmacological mechanism of action is linked to its action on adenylyl cyclase enzyme. Forskolin activates various isoforms of adenylyl cyclase, which results in the increase in intracellular cAMP. Forskolin is used in many biochemical and pharmacological experiments as an activator of adenylyl cyclase [15].

The roots contain approximately 0.15–1.5 % w/w of forskolin. The content of forskolin in stems was found to be around 0.02 % w/w [7]. Apart from the

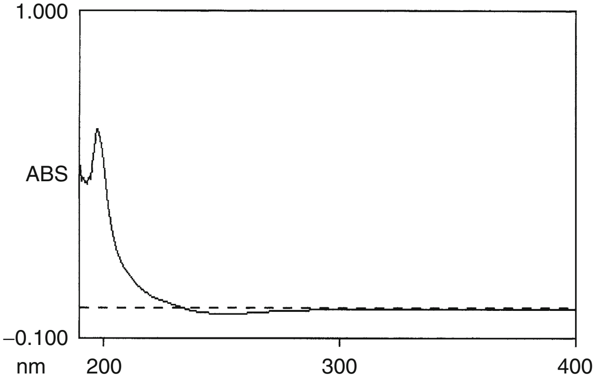

Fig. 109.2 UV spectrum of pure forskolin isolated from *C. forskohlii* roots [17]

Ayurvedic formulations which contain *C. forskohlii* as one of the ingredients, standardized extracts of *C. forskohlii* have been marketed in the recent days as nutraceutical supplements mainly for weight loss. Several analytical methods were developed for the quantitation of forskolin in crude extracts as well as in formulations. There is one clinical study in obese men indicating the efficacy of forskolin in inducing weight loss, promoting lean body mass, bone mass, and increasing serum free testosterone levels [16].

1.1 Physicochemical Properties of Forskolin

Forskolin is relatively a nonpolar compound, thus sparingly soluble in water. However, it is also insoluble in petroleum ether and xylene. Thus, it is soluble in solvents like toluene, chloroform, ethanol, and methanol. This property is exploited in the fractionation and isolation of forskolin. Also, forskolin is not very much UV sensitive. Its UV absorption maximum is around 200 nm (Fig. 109.2). Hence, during isolation process, monitoring of the fractions is done usually by TLC. However, TLC is cumbersome, as it involves spraying with either anisaldehyde-sulfuric acid reagent or vanillin-sulfuric acid reagent and heating [7, 17].

2 Isolation by Column Chromatography

Column chromatography was used extensively for the isolation of forskolin during 1970s–2000s. Forskolin was isolated by using column chromatography on silica gel from methanolic extract of root powder by Bhat et al. and other researchers [8, 9, 18, 19]. The crude extract was chromatographed several times before pure forskolin was obtained. The mobile phase system consisted of eluting with nonpolar solvents first to remove impurities, followed by gradually increasing the polarity of solvents [7].

3 Isolation by Vacuum Liquid Chromatography

Vacuum liquid chromatography was employed to separate the forskolin-rich fractions from the crude extract [Unpublished reports]. This vacuum liquid chromatographic method was a modification of the published method for the flash chromatography [20]. The crude extract was obtained by extracting the root powder with toluene or chloroform. Here, the polar solvents like ethanol and methanol were avoided to reduce the extractable impurities. The crude extract, after the solvent removed, was subjected to vacuum liquid chromatography in a sintered glass Buchner funnel, which was connected to a Buchner flask. Silica gel (230–400 mesh) was used as an adsorbent. The vacuum (100–200 mmHg) was applied to suck the eluate. The mobile phase was selected based on the TLC profile of crude extract. The TLC method of crude extract was optimized to have forskolin's R_f value around 0.30. For TLC, aluminum-backed silica gel GF_{254} was used as the stationary phase and anisaldehyde-sulfuric acid reagent was used as the spray reagent. The mobile phase which gave the forskolin's R_f value around 0.30 was a combination of toluene and ethyl acetate (80:20 % v/v). Instead of toluene, *n*-hexane also can be used. Elution was carried out first with 100 % v/v of low-polar solvent (toluene or *n*-hexane) to elute the impurities out. Then, exhaustive elution was carried out with the selected mobile phase. After monitoring by TLC, similar fractions were combined and the solvent was evaporated. The residue obtained thus was brownish yellow in color. This crude forskolin was repeatedly crystallized in a mixture of *n*-hexane and ethyl acetate (75:25 % v/v) to obtain white or off-white crystals of pure forskolin [Unpublished reports].

4 Isolation by Charcoal Column Chromatography

Activated charcoal, a nonpolar adsorbent, has been extensively used for the separation and purification of organic compounds for its easy regeneration, low cost, high adsorption capacity. It is mainly used in the fractionation of monosaccharide-oligosaccharide mixtures and in the isolation of sugars and their polar derivatives using aqueous solvents. The adsorbent surface consists mainly of carbon, which enables adsorption to take place on the principle of dispersion forces [21]. Adsorption on charcoal is largely governed by the molecular size of the sample. Charcoal more strongly adsorbs higher molecular weight dissolved organic molecules than lower molecular weight molecules and nonpolar molecules than polar molecules [22]. Recently, several applications of charcoal column chromatography to separate low-polar compounds with nonaqueous solvents have been reported [17, 23, 24].

Charcoal column chromatography was used to purify forskolin [17]. Activated charcoal used in this study was untreated, granular carbon prepared from chemically activated wood with a particle size of less than 75 μm (80–90 %) (100–400 mesh). One hundred grams of powdered root material was extracted with toluene. The toluene extract was concentrated to 15 ml under reduced pressure at 40 °C. To this extract, 150 ml of *n*-hexane was added slowly under continuous stirring to obtain crude forskolin (brown solid precipitate) in powder form (1.6 g). This crude forskolin was placed on a column (7.5 cm × 1.7 cm i.d.), packed with activated charcoal (6 g), placed on a Buchner flask of suitable size connected to a vacuum source (100–200 mmHg), eluted with methanol and acetonitrile:methanol (50:50, v/v). Forskolin was detected by TLC as a major spot in the eluates obtained with acetonitrile:methanol (50:50, v/v) and as a minor spot in the eluates obtained with methanol. Hence, the yellowish brown colored eluates obtained with acetonitrile:methanol (50:50, v/v) were combined and concentrated under vacuum at 40 °C to obtain a residue (500 mg). This residue was stirred with *n*-hexane to remove *n*-hexane soluble impurities, and *n*-hexane was removed by filtration. After drying the residue, it was dissolved in 5 ml of chloroform. To this solution, 50 ml of *n*-hexane was added with continuous stirring to remove most of the coloring matter and crude forskolin was obtained as a pale yellow precipitate. This precipitate was crystallized in ethyl acetate:light petroleum (b.p. 40–60 °C) mixture (25:75, v/v) to obtain the forskolin. This crystallization step was repeated once to get forskolin as off-white color precipitate. This precipitate was again crystallized with diethyl ether:*n*-hexane (1:15, v/v) to obtain pure forskolin (yield – 0.0971 % w/w with respect to the weight of dried root powder; purity – 96.9 % w/w) [17].

5 Isolation by Immunoaffinity Column Chromatography

Yanagihara et al. developed an immunoaffinity column chromatography method to isolate forskolin from the roots of *C. forskohlii*, by using the anti-forskolin monoclonal antibody [25]. Anti-forskolin monoclonal antibody was prepared by injecting the forskolin-bovine serum albumin conjugate into BALB/c mice followed by cell culture techniques. Purified IgG in phosphate buffered saline (PBS) solution was added to the slurry of CNBr-activated Sepharose 4B in coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl), and used as the affinity gel. The dried powder of tuberous root was extracted with diethyl ether. After evaporation of solvent, the residue was dissolved in PBS containing 6 % methanol and subjected to the immunoaffinity column separation. Elution was carried out with PBS containing 45 % methanol. The forskolin content was estimated by ELISA. The recovery of forskolin was found to be 95.6 % w/w. However, forskolin purified in this way by the immunoaffinity column was still contaminated by a small amount of 7-deacetyl forskolin because this compound has cross-reactivity against anti-forskolin monoclonal antibody. Therefore, the mixture was treated with pyridine and acetic anhydride at 4 °C for 2 h to give pure forskolin [25].

6 Isolation by Hydrotropic Extraction

Hydrotropes are highly water-soluble small molecular weight organic salts (e.g., sodium salicylate and sodium cumene sulfonate). They possess ability to dissolve other organic compounds in aqueous solutions in a concentration-dependent manner. Hydrotropes exhibit their solubilization capacity for organic compounds above a characteristic minimum hydrotrope concentration (MHC). Hydrotropic solubilization technique is one of the methods used to enhance the aqueous solubility of insoluble or slightly soluble bioactive compounds. Hydrotropes dissolve well the organic compounds above their MHC in aqueous solution. Hence, if the hydrotrope solution containing organic compound is diluted below its MHC, then the solubility of that organic compound in aqueous solution decreases and the organic compound may separate out [26–29]. Gaikar et al. employed this technique to isolate piperine [30], boswellic acids [31], diosgenin [32], and andrographolide [33]. This technique was successfully employed to isolate forskolin by Mishra et al. [34].

Pulverized roots of *C. forskohlii* were suspended in aqueous hydrotrope solution of sodium cumene sulfonate and agitated vigorously. After the extraction, the solution was subsequently filtered under vacuum. A clear brown color solution was obtained as filtrate, while the insoluble sticky solid portion was collected as residue. The filtrate was diluted with water to the respective minimum hydrotrope concentration (MHC) of the hydrotrope. Solid brown color crystals of forskolin that precipitated out from the hydrotrope solutions were isolated by centrifugation or filtration. The purity of isolated forskolin was 85 % w/w with maximum extraction of 70 % w/w [34].

7 Isolation by Microwave-Assisted Extraction

Microwave is used to irradiate the plant material during the extraction process. Microwave irradiation is reported to cause rupture of cells, thereby facilitating the action of solvents on the cells [35]. The usage of microwave irradiation was employed for the isolation of curcumin from turmeric [36–39], piperine from *Piper nigrum* [40], solanesol from tobacco leaves [41], and artemisin from *Artemisia annua* [42].

Microwave irradiation was employed to extract forskolin from the roots of *C. forskohlii* by Devendra et al. [35]. The raw material was soaked in water before subjecting it to microwave irradiation and it was irradiated with microwave, after it was evenly and thinly spread over a Petri dish, in a microwave oven. After the irradiation, the material was extracted by suspending it in 150 ml of methanol with vigorous agitation. The methanolic extract was concentrated by evaporating methanol up to 80 % of its original volume. Crude forskolin was crystallized from the concentrated solutions by simultaneous addition of petroleum ether and water in a 1:1 volumetric ratio. Forskolin crystallized from the above process was a free-flowing and brown colored powder with 30–35 % w/w purity. The crude forskolin was dissolved in acetonitrile and further decolorized by adsorption on an alumina column. When forskolin extract in acetonitrile was passed over an alumina bed, all brown colored organic impurities of the crude extract were strongly retained by the column. The light yellow eluate from the column contained 78 % (w/w) forskolin, 15 % (w/w) of 7-deacetylforskolin, and 6 % (w/w) of

1,9-dideoxyforskolin. No separation of forskolin and its analogues was, however, achieved with the alumina column, but the product contained no other impurities. This fraction was further purified to isolate forskolin by using a chloromethylated polystyrene polymer loaded with diethanolamine [35].

8 Isolation by Using Selective Adsorbent Designed by Molecular Simulation

Devendra et al. reported the usage of selective adsorbent designed by the molecular simulation technique to isolate forskolin and its analogues based on the interaction between the adsorbent and the target molecules [35].

Diethanolamine was loaded on chloromethylated polystyrene matrix by chemical synthesis. Diethanolamine was selected as a ligand for interacting with the target molecules based on the hypothesis that the hydroxyl groups of diethanolamine would form hydrogen bonding with the hydroxyl groups of forskolin and its analogues in a differential manner because of relative spatial positions of different hydroxyl groups on the structures of interacting molecules. This hypothesis was tested in silico by using a computer modeling software, Material Studio 3.2 (MS, Acclerys, USA). Each solute and two units of the amine-loaded polystyrene were allowed to interact in different solvent environments. In the case of interaction studies in the solvated state in acetonitrile, none of the functional groups of forskolin showed any interaction with the amine-loaded polymer, whereas in the case of 7-deacetylforskolin, all the four hydroxyl groups interact with the hydroxyl groups of the amine-loaded polymer. In the case of 1,9-dideoxyforskolin, the acetyl group at seventh position interacts with the hydroxyl groups of the amine-loaded polymer. These theoretical calculations indicated the possibility of separating forskolin from its analogues by selective adsorption of the latter on a diethanolamine-loaded polystyrene matrix from acetonitrile solutions.

Devendra et al. performed the experimental work to confirm the predictions of in silico molecular simulation. The methanolic extract of the root was concentrated by evaporating methanol up to 80 % of its original volume. Crude forskolin was crystallized from the concentrated solutions by simultaneous addition of petroleum ether and water in a 1:1 volumetric ratio. Forskolin crystallized from the above process was a free-flowing and brown colored powder with 30–35 % w/w purity. The crude forskolin was dissolved in acetonitrile and further decolorized by adsorption on an alumina column. The eluate obtained after decolorization of the crude forskolin extract through an alumina column mainly contained forskolin and its analogues and, therefore, was used as a feed solution for adsorption on the amine-loaded polymer, loaded in a column to investigate their adsorption behavior. The uptake by the polymer from the acetonitrile solution was found to be maximum for 1,9-dideoxyforskolin (91 %), intermediate for 7-deacetylforskolin (37 %), and minimum for forskolin (12.2 %) until five bed volumes. These differential uptakes validated the hypothesis from molecular simulation that analogues shall be adsorbed more preferentially than forskolin on the polymer. Almost 90 % w/w of forskolin was recovered from this technique and the purity of forskolin was 94 % w/w.

In another study, Devendra et al. [43] used different selective polymers designed by molecular simulation to have interaction with forskolin to isolate forskolin. *N*-propinoyl aspartic acid (NPAA), phenyl glycine-*o*-carboxylic acid (PGOCA), and phenyl glycine-*p*-sulfonic acid (PGPSA) were used as the ligands for selective adsorption of forskolin. Extraction and decolorization processes of crude extract were carried out as per the previous report [35]. The ligands were synthesized and loaded on chloromethylated polystyrene matrix cross-linked with divinylbenzene in dimethyl sulfoxide (DMSO) solutions. The ligand-loaded polymer was packed in a glass column. The decolorized extract solution was pumped through the packed adsorption bed by a peristaltic pump. Samples were collected at regular time intervals at the exit of the column. The PGPSA-loaded polymer gave almost 98 % w/w pure forskolin during desorption, with the yield of 16 % w/w. About 95 % w/w pure forskolin was achieved with 46.54 % w/w recovery with PGOCA-loaded polymer.

9 Extraction by Three-Phase Partitioning

Three-phase partitioning (TPP) is a bioseparation technique useful for the fractionation and concentration of proteins from plant materials and microorganisms. It involves the partitioning of hydrophilic constituents, proteins, and hydrophobic constituents in three phases comprising of water, ammonium sulfate, and organic solvent [44, 45]. TPP was utilized for extraction of forskolin from *C. forskohlii* roots. Aqueous slurry was prepared by mixing 5 g of *C. forskohlii* root powder in 50-ml distilled water by mild stirring with a magnetic stirrer. Calculated quantities of ammonium sulfate was added to the aqueous slurry prepared, followed by addition of known amount of *t*-butanol. The extraction was carried out for 1 h by gentle stirring with magnetic stirrer. The mixture was allowed to stand for 1 h for the formation of three phases. The three phases so formed were separated by centrifugation. The upper organic layer was collected and the solvent (*t*-butanol) was evaporated to dryness. Forskolin content was estimated in the extract thus obtained by HPLC. A maximum of 30.83 % recovery of forskolin was obtained under the optimized conditions. Ultrasonication and enzyme pretreatment with commercial enzyme preparations of Stargen® 002 (contains *Aspergillus kawachi* alpha-amylase expressed in *Trichoderma reesei* and a glucoamylase from *T. reesei* that work synergistically to hydrolyze granular starch substrate to glucose) and Accellerase® 1500 (mixture of cellulase and glucosidase that works synergistically to hydrolyze cellulosic substrate to glucose) followed by TPP gave 79.95 % and 83.85 % recovery (when compared to conventional soxhlet extraction which is taken as 100 %), when used individually within 4 h [46]. This study only described extraction procedure for the extraction of crude extract which contains forskolin and not the isolation of pure forskolin from *C. forskohlii* roots [46]. The summary of all isolation procedures is given in Table 109.1.

Table 109.1 Overview of isolation of forskolin by several methods

Isolation method	Principle of separation/isolation	Yield from plant roots	Purity	Reference
Column chromatography	Adsorption	0.103 % w/w [7]	b	[7–9, 18, 19]
Vacuum liquid chromatography	Adsorption	a	b	[Unpublished reports]
Charcoal column chromatography	Adsorption	0.0971 % w/w	96.9 % w/w	[17]
Immunoaffinity column chromatography	Antigen-antibody interaction	a	b	[25]
Hydrotropic extraction	Solubility	a	85 % w/w	[34]
Microwave-assisted extraction	Solubility/partition/ adsorption	a	b	[35]
Selective adsorbent designed by molecular simulation	Adsorption	a	94 % w/w [35], 98 % w/w [43]	[35, 43]

a – yield not stated in the literature; b – purity not stated in the literature

10 TLC Analysis of Forskolin

Generally, TLC method is used to monitor the presence of forskolin during the isolation process. However, it can also be used to determine the quantity of forskolin present in the crude extract as well as in formulations. Inamdar et al. described a TLC method for the quantification of forskolin in pharmaceutical preparations [47]. In this method, forskolin was detected by using vanillin in acetic acid and perchloric acid as the detection agent. Forskolin gave violet color spot and the intensity of violet color was quantitatively estimated.

A validated TLC method was reported by Ahmad et al. [48]. This method was developed on TLC aluminum plates precoated with silica gel $60F_{254}$ using solvent system benzene:methanol (9:1, v/v), which gave compact spot of forskolin (R_f value 0.25 ± 0.02). Densitometric analysis of forskolin was carried out in the absorbance mode at 545 nm after spraying with anisaldehyde-sulfuric acid visualization reagent. After spraying with anisaldehyde-sulfuric acid regent, the TLC plate was dried and heated to 110–120 °C. Forskolin was detected as a dark violet or purple color spot. This method was applied for determination of forskolin in *C. forskohlii* root and in capsule dosage forms, which showed 0.18 % and 0.57 % w/w of forskolin [48]. In a stability test, forskolin was subjected to acid and alkali hydrolysis, oxidation, photodegradation, and heat degradation. It was observed that the drug was susceptible to acid, base hydrolysis, oxidation, photo-oxidation, and heat degradation. This TLC method effectively resolved the forskolin from components of *C. forskohlii* root, from excipients of capsule as well as the degradation products of forskolin [48].

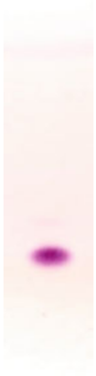

Fig. 109.3 Thin layer chromatogram of pure forskolin

Another TLC method was reported by our group, which was mainly used as a qualitative identification method [7, 17]. TLC was performed with precoated plates of silica gel $60F_{254}$ (Merck, Darmstadt, Germany) in toluene:ethyl acetate (80:20, v/v) as mobile phase. Anisaldehyde-sulfuric acid was used as the spraying reagent (1 ml concentrated H_2SO_4 was added to 0.5-ml anisaldehyde in 50-ml acetic acid). After developing the plate, the plate was dried and heated to 110–120 °C for 5–10 min in a hot air oven with careful and periodical monitoring to avoid excessive heating and subsequent charring of the plate. After the plates were taken out from the hot air oven, the plates were allowed to cool to room temperature and scanned immediately in a digital photoscanner and the scanned image was stored in a computer. Furthermore, the plate was preserved by wrapping with cellophane tape. It was observed that if the plates were left exposed to air, the spots disappeared after few days. R_f value of 0.27 was obtained for forskolin as a single, compact dark violet/purple spot (Fig. 109.3). Similar TLC method was reported by Selima et al. [49] but with a different R_f value of forskolin (0.48).

There is a monograph available in Indian Pharmacopoeia Commission (IPC) website (http://www.ipc.gov.in) for the characterization of *Coleus* dry extract [50]. A TLC method is described in that monograph. The procedure consisted of coating the plate with silica gel GF_{254} and developing in a mobile phase consisting of a mixture of 40 volumes of ethyl acetate and 60 volumes of hexane. Test solution was prepared by dissolving about 500 mg of the extract under examination with 10-ml methanol and filtered. Reference solution was prepared by making a 0.1 % w/v solution of forskolin RS in methanol; 10 μl of each solution was applied to the plate as bands of 10 mm by 2 mm. The mobile phase was allowed to rise up to 8 cm. The plate was air dried and sprayed with anisaldehyde-sulfuric acid reagent solution. The plate was heated at 110 °C for 10 min and examined under 365 nm and under day light. The chromatogram obtained with the test solution showed a band corresponding to the band obtained by using reference solution, indicating the presence of forskolin.

11 HPTLC Analysis of Forskolin

HPTLC analysis of crude extracts for the quantitation of marker compounds has gained wider acceptance by the industrial scientists in the recent days for the relative ease of the technique and its capability to analyze multiple samples at the same time in a single plate [51]. An automated multiple development HPTLC method for the separation of several forskolin derivatives was developed and validated. The HPTLC development used a 25-step gradient with a polarity range of methylene chloride–methanol to hexane and detection by chlorosulfonic acid reagent [52].

Pushpa et al. described a HPTLC method in which benzene extract of *C. forskohlii* roots was applied to HPTLC plate. Benzene: ethyl acetate (80:20 v/v) was used as the mobile phase and anisaldehyde-sulfuric acid was used as the spray reagent. After developing and spraying, the plate was dried at 100–105 °C. Forskolin gave violet colored spot with R_f value of 0.45 [53].

Vijay et al. described a HPTLC method for estimation of forskolin in ophthalmic preparations. A stock solution of forskolin (marker compound) equivalent to 1 mg ml^{-1} was prepared and different quantities of this solution, namely, 1, 2, 3, 4, 5 μl, were spotted onto precoated silica gel GF plates (10 × 10 cm size) using automatic sample syringe (Linomat IV Application mode) of the CAMAG-HPTLC equipment in order to develop calibration between 1 and 4 μg drug concentrations. The plate was placed in the twin-trough development chamber, which was presaturated with the mobile phase consisting either of a mixture of toluene:ethyl acetate (7:3) or chloroform:methanol (8:2) separately. The plate was developed for about 20 min and dried with a current of hot air. The plate was then scanned at 292 nm in the densitometer, and the area under the curve (AUC), of each concentration, was determined. Calibration curve was developed by plotting a graph between the concentration and the area under the curve [54]. Several other HPTLC methods were also reported in the literature [55–58]. An overview of HPTLC parameters for the analysis of forskolin is given in Table 109.2.

12 HPLC Analysis of Forskolin

HPLC analysis of forskolin has gained wider acceptance among academic and industrial researchers due to its high accuracy and repeatability. It is used for the quantitative estimation of forskolin in *C. forskohlii* root crude extracts and in formulations. Inamdar et al. described a HPLC method for the determination of forskolin in crude extracts and in pharmaceutical formulations [47].

A rapid method was developed for the evaluation of forskolin in the root and stem of dried *C. forskohlii* and in 17 market products by reversed-phase high performance liquid chromatography (RP-HPLC) with a photodiode array detector at 210 nm. The temperature was held constant at 30 °C, and the retention time of forskolin was approximately 6.8 min. The samples were extracted with acetonitrile by sonication.

Table 109.2 Overview of HPTLC conditions for the analysis of forskolin

Mobile phase	Stationary phase	Detection/scanning wavelength	R_f value of forskolin	Reference
Benzene: Ethyl acetate (80:20 v/v)	HPTLC plate	anisaldehyde-sulfuric acid/ 560 nm	0.45	[53]
Toluene:ethyl acetate (7:3) or chloroform: methanol (8:2)	Precoated silica gel GF plates	292 nm	a	[54]
Benzene:ethyl Acetate (85:15)	Silica gel $60F_{254}$	Vanillin-sulfuric acid/550 nm	a	[59]
Toluene:methanol (18:1.5, v/v)	Silica gel $60F_{254}$	Anisaldehyde sulfuric acid/ 545 nm	0.27 ± 0.02	[60]
Benzene:ethyl acetate (75:25, v/v)	Precoated plates	200 nm	0.49 ± 0.01	[58]

a – R_f value not stated in the literature

The method was found to be precise and accurate. The response was linear through zero from 6.3 to 630 μg ml^{-1} with a correlation coefficient (R^2) of 0.9998. Identity of the marker compound was confirmed by a LC-MS experiment [61].

Another rapid reverse-phase HPLC method with evaporative light scattering detection (ELSD) was reported for the determination of forskolin in weight-loss multi-herbals products. The analysis was performed by water-acetonitrile gradient elution at a flow rate of 1.0 ml min^{-1}. The evaporator tube temperature of ELSD was set at 35 °C, and with the nebulizing gas flow rate (pressure) of 3.0 bar. Good linear relationships were obtained with correlation coefficients exceeding 0.9995. The average recovery of forskolin ranged from 99.4 % to 100.4 % with RSDs below 3 %. The percent relative standard deviations (% RSD) of intra- and inter-day precision varied by less than 2.1 %. LOD and LOQ were 0.95 and 3.21 μg ml^{-1}, respectively. The method was found to be accurate, precise, and repeatable [62]. Several other similar HPLC methods were also reported in literature especially for the analysis of forskolin in tissue culture studies and in formulations [63–72].

A HPLC method was used by our group to quantify the purity of isolated forskolin. Actually, this method was adapted from a previously reported method [61]. In this method, C18 (250 × 4 mm i.d., 5 μ) column was used with a mobile phase consisting of water (A):acetonitrile (B) (50:50, v/v) at a flow rate of 1.0 ml min^{-1}. The gradient elution was 50A/50B to 43A/57B in 10 min. The UV detection was carried out at 210 nm. The retention time of forskolin was found to be 10.3 min [17]. An overview of various HPLC methods is given in Table 109.3.

13 GC Analysis of Forskolin

Usually gas chromatographic (GC) analysis requires an analyte to be volatile. Forskolin is not naturally volatile. Hence, it needs to be converted into its volatile

Table 109.3 HPLC conditions for the analysis of forskolin

Stationary phase	Mobile Phase	Flow rate	Detection	Reference
C18	water-acetonitrile gradient elution	1.0 ml min^{-1}	ELSD evaporator tube temperature at 35 °C, nebulizing gas flow rate (pressure) of 3.0 bar	[62]
C18 (150 × 4.6 mm i.d., 5 μ)	Water (A): Acetonitrile (B) (50:50, v/v); The gradient elution was 50A/50B to 43A/57B in 10 min.	1.0 ml min^{-1}	210 nm	[61]
C18 (250 × 4.6 mm i.d., 5 μ)	Acetonitrile:water (50:50, v/v)	1.6 ml min^{-1}	220 nm	[59]
C18 (250 × 4.6 mm i.d., 5 μ)	Acetonitrile:methanol (80:20, v/v)	1.0 ml min^{-1}	254 nm	[58]
C18 (250 × 4.6 mm i.d., 5 μ)	Acetonitrile:water (45:55, v/v)	1.0 ml min^{-1}	220 nm	[67]
C18 (250 × 4 mm i.., 5 μ)	Water (A): Acetonitrile (B) (50:50, v/v); The gradient elution was 50A/50B to 43A/57B in 10 min.	1.0 ml min^{-1}	210 nm	[17]
C18 (300 × 3.9 mm i.d.)	Acetonitrile:water (65:35, v/v) at pH 2.5 by adding orthophosphoric acid	1.0 ml min^{-1}	202 nm	[73]

derivatives. Since, this is a limiting factor especially for the analysis of crude extracts, only one gas chromatographic method was reported in literature. A GLC method was reported by Inamdar et al. for the quantitative estimation of forskolin in crude extracts and in pharmaceutical formulations [74].

14 Analysis of Forskolin by ELISA

Immunoassay systems using monoclonal antibody (MAb) against natural products have become an important tool for studies on receptor binding analysis, enzyme assay, and quantitative and/or qualitative analytical techniques in animals or plants [75]. Immunogenic forskolin-bovine serum albumin (BSA) conjugate was synthesized via 7-deacetyl-7-hemisuccinyl forskolin. BALB/c male mice were injected intraperitoneally with forskolin-BSA conjugate emulsified with Freund's complete adjuvant. Splenocytes were isolated from the hyperimmunized BALB/c mice and fused with the P3-X63-Ag8-U1 myeloma cells. Six hybridomas producing monoclonal antibodies (MAbs) reactive to forskolin were obtained.

One of the original clones secreted anti-forskolin antibody which was purified by protein A affinity column chromatography and its purity was confirmed by MALDI mass spectrometry. The MAb was bound to polystyrene microtitration plates precoated with a forskolin-human serum albumin (HAS). The full measuring range of the assay extended from 6 ng to 200 ng ml^{-1} of forskolin [68, 76]. The ELISA system established in this study was more sensitive compared to TLC [47], GLC [74] or HPLC [47, 77].

15 Hyphenated Techniques

Hyphenated techniques like GC-MS and LC-MS techniques are mainly used for identification of the compound and confirmation of its structure and stereochemistry. A LC-MS technique was employed for the identification of forskolin in crude extracts and in marketed formulations. A C18 column (150 × 4.6 mm, 5 μm) was used at ambient temperature. The mobile phase consisted of water (A) and acetonitrile (B). At a flow rate of 0.5 ml min^{-1}, the gradient elution was programmed to change from 50A/50B to 43A/57B in 10 min and to remain constant at 43A/57B for next 10 min. The detection wavelength was 210 nm, and the injection volume was 5 μl. Best results were obtained in positive electrospray ionization (ESI) mode, with ionization voltage set to 25 V, source voltage to 3.0 kV, and probe temperature to 350 °C [61].

16 Conclusion

Several diversified techniques are summarized for the isolation of forskolin from the tuberous roots of *C. forskohlii*. However, the purity of the isolated forskolin remains a matter of concern for the biologists, since forskolin with high purity is required in biological tests. Higher the purity of a bioactive compound, lesser would be the effect of the impurities present and the observed pharmacological effect can be safely attributed to the major compound. Only few techniques like charcoal column chromatography, selective adsorption using phenyl glycine-*p*-sulfonic acid–loaded polymer, and selective adsorption using phenyl glycine-*o*-carboxylic acid–loaded polymer could achieve purity greater than 95 % w/w. Even though hydrotropic extraction process seems to be simple to perform, the purity of forskolin was only 85 % w/w, thus disabling its potential application. Further research is required to identify processes which will yield high purity forskolin in less amount of time in an environment-friendly manner. One of those approaches may focus on using molecular simulation technique to identify a suitable hydrotrope or adsorbent for separating forskolin from its structurally similar analogues.

Standardized crude extract of *C. forskohlii* and forskolin are marketed as weight-loss products. Several analytical techniques were reported in the literature for the quantification of forskolin from crude extracts as well as in formulations.

Among them, many were HPLC methods. In addition, HPTLC technique was also reported in many papers. These indicate the popularity of HPLC and HPTLC techniques in the analysis of forskolin. The analysis of forskolin by ELISA technique appears to be more sensitive than other methods and may be further explored for regular utilization.

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Quassinoids: Chemistry and Novel Detection Techniques

110

Dipjyoti Chakraborty and Amita Pal

Contents

1 Introduction 3346
2 Structure: Quassinoids 3347
3 Structure–Activity Relationship 3348
4 Isolation, Detection, and Characterization 3352
4.1 Isolation Methods 3352
4.2 Separation/Purification Techniques 3355
4.3 Structure Determination 3359
5 Conclusion 3361
References 3361

Abstract

Natural products play a dominant role in pharmaceutical industry, providing resources for the discovery of new drug molecules. Quassinoids are degraded triterpenes reported from the members of the Simarouboidaea subfamily of Simaroubaceae. The importance of quassinoids as antiplasmodial, anticancer and anti-HIV compounds has revived interest in them as potential drug candidates. Its efficacy as combinatorial drug coupled with the structure–activity analysis has reclaimed novel structural leads for new drug development. Quassinoids are categorized into five distinct groups according to their basic

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skeleton, viz., C-18, C-19, C-20, C-22, and C-25. This present entry reviews the structure, structure–activity relationship, and methods of isolation, detection, and characterization of quassinoids. Recent LC–MS/MS-based techniques could effectively provide more lead molecules which would act in synergism with other compounds.

Keywords

Mass spectrometry • quantitative structure–activity relationship • Quassinoid

Abbreviations

ESI	Electrospray ionization
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
QESAR	Quantitative electronic structure–activity relationship
QSAR	Quantitative structure–activity relationship
SAR	Structure–activity relationship

1 Introduction

Natural products play a dominant role in pharmaceutical industry, and systematic investigation of natural resources for the discovery of new drug molecules is the primary objective for bioprospection programs [1]. The profound knowledge of herbal remedies in traditional culture has been developed through trial and error over many centuries based on the verbal knowledge transferred from one generation to the next. Majority of bioactive constituents isolated from plants fall into the category of secondary metabolites and can be divided into three chemically distinct groups – terpenoids, phenolics, and nitrogen-containing compounds.

Quassinoids are degraded triterpenes, reported from the members of the Simarouboidaea subfamily of Simaroubaceae. Legend states that a man named “Quassi” treated fever using the bitter extracts derived from the bark of these trees and hence named “quassin” [2]. Quassinoids have received attention due to their antiplasmodial, anticancer, and anti-HIV properties. Its efficacy as combinatorial drug coupled with the structure–activity analysis has reclaimed novel structural leads for new drug development.

Chromatographic techniques such as paper chromatography (PC), thin layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC) are utilized to isolate and quantify secondary metabolites.

These techniques when coupled with detectors, the most popular being the UV–Vis detector for HPLC, may detect a separated compound, by matching the retention time to that of a known standard compound and pattern of UV–Vis scan. In addition to univariate identification, e.g., retention time in chromatography, and wavelength/frequency in spectrometry, the use of mass spectrometry is an excellent tool to define the chemical identification.

This present entry reviews the structure, structure–activity relationship, and methods of isolation, detection, and characterization of quassinoids. Recent LCMS/MS-based techniques could effectively provide more lead molecules which would act in synergism with other compounds.

2 Structure: Quassinoids

Quassinoids are categorized into five distinct groups according to their basic skeleton, viz., C-18, C-19, C-20, C-22, and C-25 (Fig. 110.1). The C-19 and C-20 quassinoids have received particular attention in view of their bioactive properties in vitro and in vivo. The majority of isolated quassinoids have a C-20 skeleton and are δ-lactones, while the few C-19 skeletal-type quassinoids that have been isolated are γ-lactones with the lactonic linkage either at C-12 or at C-7 [3].

In the 1940s, it was reported that compounds extracted from the barks of plants belonging to the Simaroubaceae are effective against malaria [4], and subsequently, a strong antimalarial activity of many derivatives has been demonstrated. C-20 compounds are associated with antiplasmodial activity and are of two types, tetracyclic (without oxygenation at C-20) and pentacyclic (with additional oxygenation at C-20 allowing the formation of an additional ring). An α-, β-unsaturated ketone in ring one and an oxymethyl bridge are associated with providing the antimicrobial activity [5]. Quassin (**1**) and neoquassin (**2**) are the first of the quassinoids to be isolated and characterized, and all the other compounds isolated later from the members of the Simaroubaceae family are chemically related to quassin (Table 110.1).

Quassinoids have attracted much attention due to the wide spectrum of biological activities that they display, viz., antimalarial, antifeedant, anti-inflammatory, antiulcer, antipyretic, antifertility, antileukemic, cytotoxic activities, and induction of murine stem cell differentiation which have been widely reported [54–59]. We have demonstrated that quassin is a potent immunomodulatory tool that efficiently controls the establishment of leishmanial parasite within the host macrophages [60]. Our contention was that quassin could be used as a potential immunomodulator to generate the required immunity not only for the treatment of leishmaniasis but also for the treatment of other chronic infectious diseases. Subsequently, we had shown that both quassin and neoquassin can be used along with artesunate (a derivative of artemisinin) as combinatorial therapy against malaria [61].

Several novel quassinoids are being reported every year with bioactive properties, keeping the possibility of obtaining a lead molecule within the realm of possibility. Recently, six new bioactive quassinoids have been reported [62];

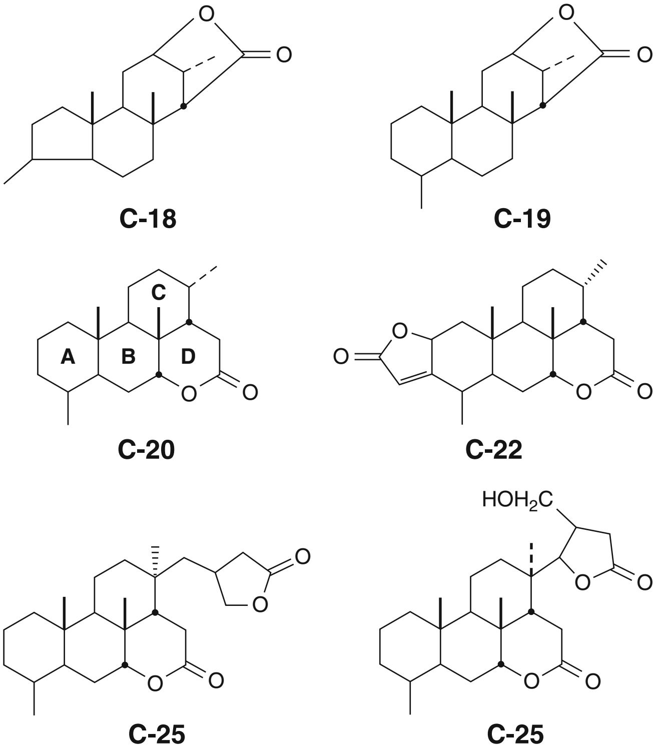


Fig. 110.1 Structural representation of C-18, C-19, C-20, C-22, and C-25 quassinoids

among these, five have unusual structure with a 2,3-seco A-ring. A new quassinoid picrasin K (**3**) was isolated from *Quassia amara* leaves with low activity on *Plasmodium falciparum* and on human cancerous cell line [41]. A compound simalikalactone E (**4**) was reported to be less cytotoxic than simalikalactone D (**5**) [63]. Two new quassinoids, javanicolide E and javanicolide F, have been isolated from the seeds of *Brucea javanica* with potential antitobacco mosaic virus activity [64]. Delaumonones A and B isolated from *Laumoniera bruceadelpha* show antimalarial activity [65].

3 Structure–Activity Relationship

In spite of rapid advances in isolation and characterization of natural compounds that have been the mainstay of traditional medicine for centuries, only a few of the thousands of potent bioactive compounds make it to the stage of clinical trials. This has led to a decline in mining of potent bioactive compounds in the latter half of the twentieth century [66]. Although natural products have provided the best antimalarial compounds known till date, the activity of the isolated compound at several

Table 110.1 Types of quassinoids and their chemical constituent and plant source

Type	Chemical constituent	Reference	Source Plant
C-18	Samaderin A (**6**)	[6]	*Samadera indica*
C-19	Samaderins C (**7**), B (**8**)		
	Indaquassin C		
	(+)-Polyandrol	[7]	*Castela polyandra*
	15-O-acetyl-5(S)-polyandrol	[8]	
	1-epi-holacanthone		
	15-O-acetylglaucarubol		
	15-O-acetyl-Δ4,5-glaucarubol		
	1-epi-5-iso-glaucarubolone		
	1-epi-glaucarubolone		
	$\Delta^{4,5}$-glaucarubol		
	5(R)-polyandrol	[7]	
	Holacanthone	[9]	
	Glaucarubolone	[10]	
	Glaucarubol	[5]	
	11-*O*-*trans*-*p*-coumaroylamarolide	[11]	*Castela texana*
	Castelatin	[12]	*Castela tortuosa*
	Castelalene-11-*O*-β-D-glucopyranoside		
	Chaparramarin		
	Castelosides A, B		
	Eurylactone A, B		*Eurycoma longifolia*
C-20	6-Dehydroxylongilactone	[15]	*Eurycoma longifolia*
	7-α-Hydroxyeurycomalactone		
	15β-Hydroxyklaineanone		
	6-α-Hydroxyeurycomalactone	[16–18]	
	longilactone		
	4,15- β-Dihydroxyklaineanone		
	6-Dehydro longilactone	[19]	
	Euryconolactones A, E–F	[20–21]	
	Euryconolactones B–D		
	11-Deydroklaineanone	[22]	
	12-*epi*-11-Deydroklaineanone		
	14,15β-Dihydroxyklaineanone	[17]	
	15β-O-Acetyl-14-hydroxyklaineanone	[23]	
	Pasakbumins A, B, C, D	[24]	
	13alpha(21)-Epoxyeurycomanone	[25]	
	Eurycomanone		
	13alpha,21-Dihydroeurycomanone		
	eurycomanol		
	Iandonosides A, B	[26]	*Eurycoma harmandiana* Pierre
	Iandonone		
	2-*O*-Glucosylsamaderine C	[27]	*Quassia indica*

(*continued*)

Table 110.1 (continued)

Type	Chemical constituent	Reference	Source Plant
	Cedronolactones B–D	[28]	*Simaba cedron*
	Cedronolactone E	[29]	
	Ailanquassin A, B	[30]	*Ailanthus malabarica*
	Ailantinol A, B	[31]	
	Perforaquassins A, B, C	[32]	*Harrisonia perforata*
	Picrasinoside H	[33]	*Picrasma ailanthoides*
	Picrasinol D	[34]	
	Picrasinol C	[35]	
	20(*R*)- and 20(*S*)-Simarolides	[36]	*Picrasma crenata*
	16-β-*O*-Methylneoquassin		
	16-β-*O*-Ethylneoquassin		
	Javanicin Z, dihydrojavanicin Z, hemiacetaljavanicin	[37]	*Picrasma javanica*
	Javanicinosides I, J, K, L	[38]	
	Picraqualides A–E	[39]	*Picrasma quassioides*
	Nigakilactone B, C, E, F		
	Kusulactonne		
	Javanicin U		
	12-Norquassin		
	Quassin		
	2,3-Didehydropicrasin B		
	Picrasin B		
	Simalikalactone C		
	Samaderines X, Y, Z	[27]	*Quassia indica*
	Indaquassin X		
	11-α-*O*-(β-D-glucopyranosyl)-16-α-*O*-Methylneoquassin	[40]	*Quassia amara*
	1-α-*O*-Methylquassin		
	12-α-Hydroxy-13,18-dehydroparain		
	Picrasin K	[41]	
	6-α-Hydroxychaparrinone	[22]	*Quassia multiflora*
	14-Hydroxychapparinone	[39]	*Hannoa chlorantha*
	chaparrinone	[42]	
	14-Hydroxychaparrinone		
	15-Desacetylundulatone		
	6-α-Tigloyloxyglaucarubol		
	glaucarubolol	[43]	*Simaba cedron*
	Vilmorinone A	[44]	*Ailanthus vilmoriniana*
	Vilmorinone B–F	[45]	
C-22	Nilocitin	[43]	*Simaba cedron*
	Dihydronilocitin		
	Piscidinol		

(*continued*)

Table 110.1 (continued)

Type	Chemical constituent	Reference	Source Plant
	Bourjutinolone A		
	Glaucarubolone		
C-23	11-Acetylamarolide	[46]	*Simarouba versicolor*
C-25	Niloticin	[17]	*Castela polyandra*
	Peninsularinone	[47]	
	Gutolactone	[48]	*Simaba guianensis*
	Cedronolactone A	[45]	*Simaba cedron*
	Bruceanols D	[49]	*Brucea antidysenterica*
	Bruceanols G	[50]	
	Bruceoside C	[51]	*Brucea javanica*
	Bruceosides D, E, F	[52]	
	20(*R*)- and 20(*S*)-Simarolides	[53]	*Eurycoma longifolia* Jack

Table 110.2 Analysis of structure activity relationship in quassinoids

Activity	SAR analysis	Reference
Antileukemic	Inhibition of protein synthesis by binding to ribosome	[67]
Inactivation of rabbit reticulocyte ribosomes	Double bonds in the A-ring and lactone ring	[68]
Inhibitors of induced inflammation and arthritis in rodents	3-Hydroxy-delta 3-2-oxo moiety and C-15 ester-bearing delta-lactone ring in brusatol, 1-hydroxy-delta 3-2-oxo moiety in brucein-D, C-11 and C-12 free hydroxyl groups	[69]
Inhibitory effect on Epstein–Barr virus activation	Methyleneoxy bridge and side chain enhance activity, sugar moiety-reduced activity	[70]
Herbicide	Oxymethyl bridge	[71]
Antimalarial activity	Ring A functionality, oxygenation at C-2	[56]
Eukaryotic protein synthesis	Nature of C-15 side chain, nature of A-ring modifications, presence or absence of sugar moiety, the presence of an epoxymethano bridge	[51]
Antitrypanosomal activity	Presence of a diosphenol moiety in ring A, the nature of the C-15 side chain	[72]
Antimalarial activity	Unsaturated lactone on ring A, oxymethyl bridge between C-8 and C-11	[63]

instances do not account on their own for the activity of the crude extract [66]. Structure–activity relationship (SAR) analysis enables the determination of chemical groups responsible for evoking a particular biological effect and as such is immensely important in understanding its potency. Recently, the method has been refined as quantitative structure–activity relationship (QSAR), which provides a mathematical relationship between the chemical structure and biological activity.

Several SAR studies have been conducted on the wide range of activities attributed to quassinoids (Table 110.2) and is discussed in detail in recent review articles [55, 56]. A strong correlation between the potent antileukemic activity and the ability to inhibit protein synthesis by bruceantin (**9**) has also been reported [67]. For the potent use of quassinoids as herbicides, the oxymethyl bridge is reported to be required by computer modeling [71]. SAR analysis reveal that quassinoids affect eukaryotic protein synthesis depending on the nature of C-15 side chain, nature of A-ring modifications, presence or absence of sugar moiety, and the presence of an epoxymethano bridge [51]. Enhancement of biological activity is reported to be due to the free hydroxyl at C-1 or C-3 because of the intramolecular hydrogen bonding between the hydroxyl and oxygen of the enone which further activated it toward a nucleophilic attack [71, 73]. Several workers have addressed the SAR in C-, D-, and E-rings (Fig. 110.2) of C-20 quassinoids whereby oxygenation pattern and integrity of the D-ring and E-ring are essential for antitumor activity [74–79]. It has also been suggested that the C-ring is flattened due to the E-ring which holds the quassinoid in a stable, biologically active confirmation.

In addition, several extensions of the SAR studies by multilinear regression, artificial neuron network, regression through origin, etc. have enabled better understanding of the mechanism of binding bioactive compounds to potential targets [80]. The utilization of techniques such as QESAR (quantitative electronic structure–activity relationship) has facilitated the designing of novel derivatives [81, 82]. The mode of action of quassinoids on the GABA receptor for potential nematocidal activity has been evaluated by comparative molecular field analysis, a method of three-dimensional quantitative structure–activity relationship analysis [83]. Several potent antitumor and anti-HIV drug candidates based on natural compounds have been developed employing bioactivity and mechanism of action-directed isolation and characterization coupled with rational drug design-based modification and analogue synthesis [84, 85]. Chemical modification of quassin into γ-lactone is reported to enhance its antiplasmodial activity with a derivative 15α-hydroxy,16-O-m-chlorobenzoyl analogue showing 506-fold enhanced activity [3]. Quassinoids are potent molecules against cancer and is known to downregulate c-Myc, a transcription factor that regulates induction of differentiation and controls apoptotic pathways [86, 87]. In view of above findings, quassinoid derivatives have a fresh lease of life as bioactive natural products. Although the effectiveness of several quassinoids is unquestionable, some are too toxic to be used without any modification and synthesis of suitable analogues. About 65% of the novel small molecule drugs introduced from 1981 to 2006 are either natural products or their derivatives.

4 Isolation, Detection, and Characterization

4.1 Isolation Methods

Crude plant extracts contain several hundreds of secondary metabolites of varying chemical nature and spectroscopic parameters. Several of these compounds are also

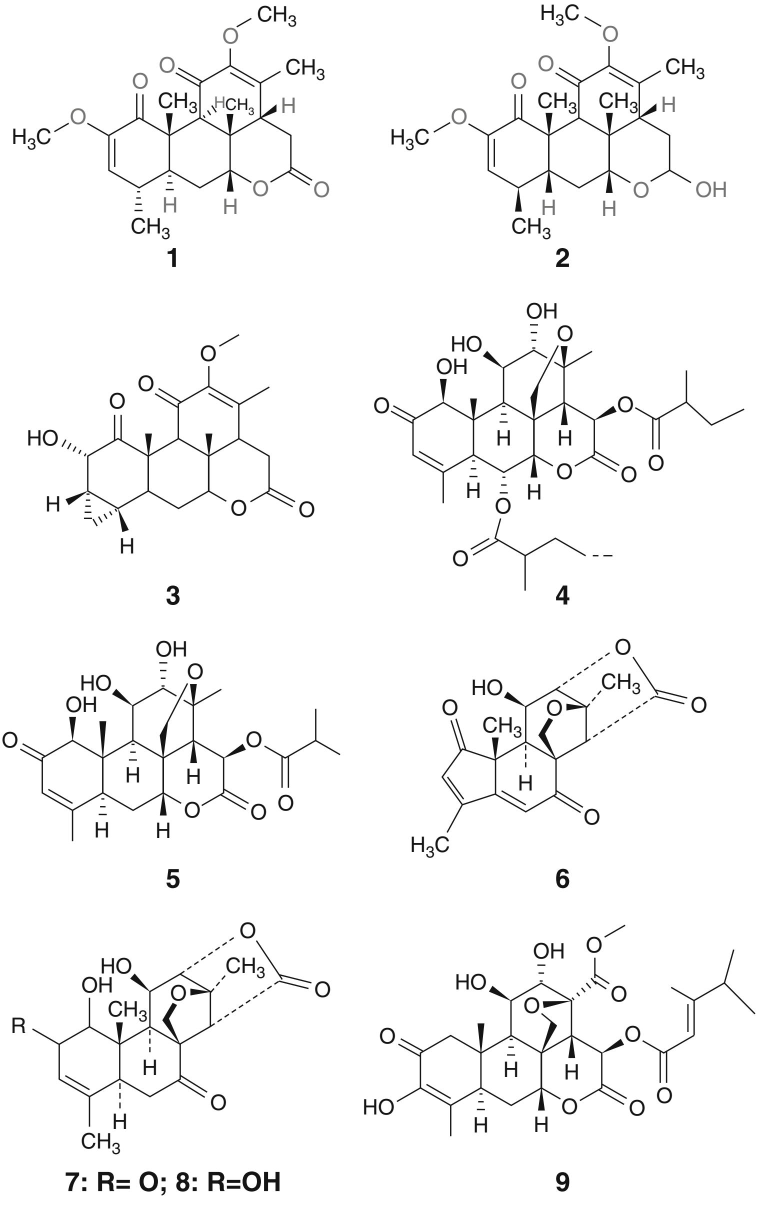
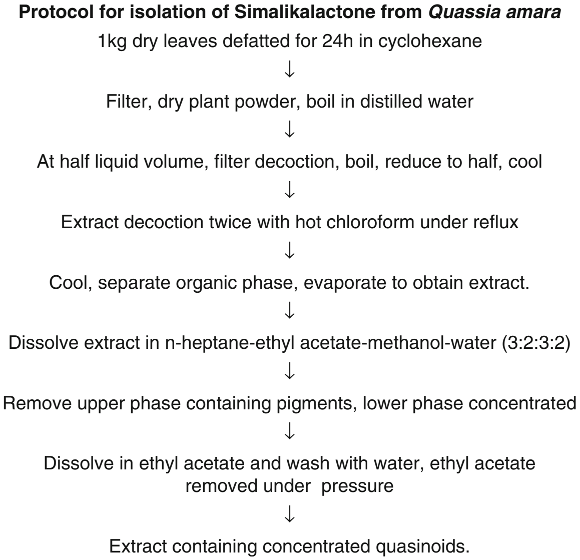


Fig. 110.2 Quassinoids. *1*. Quassin, *2*. neoquassin, *3*. picrasin K, *4*. simalikalactone E, *5*. simalikalactone D, *6*. samaderin A, *7*. samaderin C, *8*. samaderin B, *9*. bruceantin

present as conjugates within the cell and are difficult to separate from the tissue matrix. Extraction, chromatographic purification, or isolation is thus important for the detection, identification, and quantification of such compounds. Several chromatographic methods such as paper chromatography (PC), thin layer


Protocol for isolation of Simalikalactone from *Quassia amara*

1kg dry leaves defatted for 24h in cyclohexane

↓

Filter, dry plant powder, boil in distilled water

↓

At half liquid volume, filter decoction, boil, reduce to half, cool

↓

Extract decoction twice with hot chloroform under reflux

↓

Cool, separate organic phase, evaporate to obtain extract.

↓

Dissolve extract in n-heptane-ethyl acetate-methanol-water (3:2:3:2)

↓

Remove upper phase containing pigments, lower phase concentrated

↓

Dissolve in ethyl acetate and wash with water, ethyl acetate removed under pressure

↓

Extract containing concentrated quasinoids.

Scheme 110.1 Protocol for isolation of simalikalactone from *Quassia amara*

chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC) have been standardized for the purpose of isolation and quantification of secondary metabolites.

Optimization of extraction methods for bioactive compounds from plants often encounters several challenges in effectively isolating it from the complex matrix. Extraction rate expressed as the amount of a compound isolated from a given amount of raw material provides an indication of the suitability of the method in the recovery of said phytochemical. Quassinoids are generally extracted in alcoholic solvents or in a combination of methanol–water and methanol–chloroform at different ratio. Extraction of plant material with hexane and several times with boiling water followed by subsequent extraction in chloroform is used in some extraction protocols. Using several solvents sequentially for removing the interfering compounds have also been followed for some of the quassinoids. In *P. amarus* [88], extraction of the plant material is performed in 50% methanol for 72 h followed by rinsing with extraction solvent (1:10) for another 72 h prior to filtration. The supernatant is concentrated and extracted three times with hexane and the residual water extract utilized for analysis of quassinoids. During the extraction step with hexane, chlorophyll is removed by liquid-phase extraction. The crude extract is generally freeze dried. However, several modifications are reported for various quassinoids, and purification of the aqueous decoction [63] is now routinely done (Scheme 110.1) with the advent of highly sophisticated detection techniques.

4.2 Separation/Purification Techniques

Chromatographic techniques are routinely used for separation and also when coupled to spectrographic detectors for identification of components in a complex mixture of extracted compounds. Components of the crude/partially purified extract are carried through stationary phase by the mobile phase and separations based on the differences in migration rates between the sample components.

4.2.1 Thin Layer Chromatography (TLC)

TLC is a simple, inexpensive technique that can be effectively used for the analysis of natural products including quassinoids. In addition to being used as an analytical tool, TLC is also used to partially purify and isolate quassinoids as in column chromatography. The effectiveness of the separation depends on the extract, choice of mobile phase, and absorption layer such as silica gel, alumina, cellulose powder, polyamides, and ion-exchangers supported on glass or metal plate. R_f values or the retention factor is commonly used to describe chromatographic behavior and is defined as distance moved of a particular spot divided by the distance moved by the solvent front. Comparative R_f values can be utilized to analyze extracts both qualitatively and quantitatively. Quassinoids give red color on TLC plate when sprayed with concentrated sulfuric acid and brown when heated. Various stationary and mobile phases have been utilized in the separation of quassinoids, viz.:

- Whatman linear-K preparative plate with methylene chloride–methanol–acetic acid (65:10:1) as the mobile phase [23]
- Silica gel with toluene–ethyl acetate as solvent phase (4:1) and detection by a radiochromatoscanner [89]
- Silica gel eluted with toluene–acetone-acetic acid (70:30:0.05) or n-butanol–acetic acid–water (65:15:25) and spots identified under short-wave UV light and sprayed further with iodoplatinate and anisaldehyde–sulfuric acid reagent [90]
- Silica gel with diethyl ether–methanol (9:1), detection under UV light and after spraying subsequently with 5% phosphomolybdic acid and 60% sulfuric acid in methanol, followed by heating at 120 °C for 10 min

4.2.2 High-Performance Liquid Chromatography

With the advent of HPLC techniques, the separation and detection of natural products with better resolution is possible. HPLC techniques for quassinoids were developed in the early 1980s [91, 92] and thereafter have been extensively used (Table 110.3). Several protocols are available for the HPLC separation of quassinoids employing different columns and mobile phases for a specific set of compounds. HPLC technique requires the comparative profile of the standard for identification which is generally based on UV–Vis detection but with the advent of mass spectrometers coupled to the LC system that has given a definite edge to this popular technique over the others. Both isocratic and complex gradient programs have been used for the separation of quassinoids.

Table 110.3 HPLC separation and identification of quassinoids

Column	Mobile phase	Detection	Plant	Compound detected	Reference
uBondapack C18 (10 cm × 8 mm)	Methanol–water–n-heptane sulfonic acid (45:55:5), gradient of 45–65% methanol in 20 min	UV	*Picrasma quassioides*	Quassin	[91]
Merck LiChrospher 100 RP-18 endcapped (250 × 4 mm), LiChrosorb CN; 5μm; 250 × 4 mm	40 % chloroform, 60% hexan–acetonitrile	UV, 254 nm	Standard compounds	Quassin	[92]
Microsorb^{TM} phenyl 5-μm column (250 × 4.6 mm) Resolve^{TM} C-18 column (300 × 3.9 mm, 5 μm)	Gradient flow: A – methanol, B – water–methanol–acetic acid (90:9:1) containing 0.1% ammonium acetate, C – acetonitrile	Photodiode detector	Standard compounds	Glaucarubolone; chaparrinone; holacanthone; glaucarubinone; picrasin B; paraine; neoquassin; quassin	[93]
Waters RP Nova-Pak C18, (15 cm × 4.6 mm)	Acetonitrile–water (65/35), postcolumn addition of 0.1% of 1 m ammonium acetate	UV–Vis detector, 254 nm, MS	–	Eco-triterpene lactone type quassinoids	[94]
Supelcosil LC-18 analytical column (250 mm × 4.6 mm,5 μm)	Gradient: acetonitrile, 0.5% aq. trifluoroacetic acid	UV–Vis detector, 254 nm	*Picrolemma sprucei*	Neosergeolide, isobrucein B	[95]
RP-18 Phenomenex (25 cm × 4.6 mm, 5 μm)	Not mentioned	ES-MS (+ve mode)	*Quassia amara*	Quassin and neo quassin	[90]
Atlantis^{TM} dC18 (100 mm × 4.6 mm, 5 μm)	Methanol–water (40:60)	255 nm, LC/MS	*Quassia excels*, *Picrasma quassioides*	Quassin, two isomers of neoquassin	[96]
LiChrospher 100 column RP-C18 (Merck, 250 mm × 4 mm, 5 μm) RP C18 precolumn (Merck, 30 mm × 4 mm, 5 μm)	Gradient elution system, methanol–water	UV	*Picrasma crenata*	Quassin, α-neoquassin, β-neoquassin	[97]

Phenomenex Luna RP C18 column	Gradient run of 40–100% methanol	Diode array detector, MALDI MS		Quassin, two isomers of neoquassin	[61]
Zorbax Eclipse XDB-C8 column	Isocratic elution, water methanol, 0.1 % formic acid	ESI-MS/MS		Quassin, neoquassin, picrasinoside B	[98]
RP C18 Acclaim PA column (1 mm × 5 mm, 3 μm)	Gradient system, 2 % acetonitrile in water with 0.1 % formic acid, 2 % water in acetonitrile with 0.1 % acetonitrile	LC-QTOF MS	*Eurycoma longifolia*	Eurycolactone A; eurycolactone B; eurycolactone C; eurycolactone D; eurycolactone E; eurycomalide A; eurycomalide B; eurycomalactone; 6α-hydroxyeurycomalactone; 7 α-hydroxyeurycomalactone; eurycomanone; 13α-(21)-epoxyeurycomanone; 12,15-diacetyl-13α(21)-epoxy-eurycomanone; 12-acetyl-13α (21)-dihydroeurycomanone; 15-acetyl-13α(21)-epoxyeurycomanone; 3,4ε-dihydroeurycomanone; 13,21-dihydroeurycomanone; eurycomanol; 13β,18-dihydroeurycomanol; 13β, 21-dihydroxyeurycomanol; eurycomanol-2-o-β-D-glycopyranoside; 11-dehydroklaineanone; 15_-hydroxyklaineanone; 14,15β-dihydroxyklaineanone; 5β,14β,15β-trihydroxyklaineanone; 15_-O-	[99]
Waters Xbridge C18 column (2.1 mm × 150 mm, 3.5 μm).	Binary solvent gradient, water with 0.1 % formic acid, solvent B (methanol with 0.1 % formic acid)	UFLC-triple TOF MS			
A C18-reserved phase Acquity column (4.6 mm × 150 mm, 1.7 μm)	Binary solvent system, water with 0.1 % formic acid, acetonitrile	UPLC-QTRAP MS			

(*continued*)

Table 110.3 (continued)

Column	Mobile phase	Detection	Plant	Compound detected	Reference
				acetyl-14-hydroxyklaineanone; 6_-acetoxy-14,15_-dihydroxyklaineanone; 6α-acetoxy-15β-hydroxyklaineanone; laurycolactone A; laurycolactone B; longilactone; dehydroxylongilactone; 2,3-dehydro-4α-hydroxylongilactone; ailanthone; (α/β-epoxide) ailanthone; chaparrinone (α-methyl); 3,4ε-dihydrochaparrinone; picrasinoside B; klaineanolide B; iandonoside B; 16-α-o-methylneoquassin; samaderin B, glaucarubolone	
Inertsil ODS-3 column of 2.1 mm × 50 mm and 3μm, guard column ODS-3, 7 μm particle size, 1.5 mm × 10 mm cartridge guard column	Acetonitrile–water, methanol–water	LC-ESI-MS	*Eurycoma longifolia*	Eurycomanone, 13α(21)-epoxyeurycomanone, eurycomanol, eurycomanol-2-*O*-*β*-D-glucopyranoside, and 13,21-dihydroeurycomanone	[100]

Although RP HPLC provides the most efficient separation of biological samples, a survey of available literature indicates that most commonly used solvent systems involving acetonitrile/methanol–water give unsatisfactory resolution of quassinoids necessitating addition of n-heptane and sulfonic acid in gradient flow [101]. Such solvents also hinder further processing of the eluted compounds for mass spectrometry confirmation. HPLC method for analysis and purification of quassinoids from *Q. amara* dates back to the early 1980s [102]. Solid-phase extraction method on a phenyl sorbent was used for the extraction of quassin from spirits and quantitative determination carried out using HPLC on a RP-18 column with acetonitrile as a mobile phase and detection using UV and fluorescence [92]. A linear gradient of methanol–water–ammonium acetate–acetonitrile in a Microsorb phenyl column was used to separate quassinoids [11] with neoquassin eluting at 27 min and 28 min and quassin at 30 min with a total run time of 55 min. Separation of neoquassin and quassin is reported in a Waters RP Nova-Pak C18 column using acetonitrile–water (65/35) with neoquassin eluting at 6.0 and 6.4 min and quassin at a very short interval of 6.8 min [94]. Although the solvent system is compatible to LC–MS, collection of compound fractions was very difficult. Also, the resolution of the peaks could not be improved using methanol. Another protocol was developed to separate quassin from *P. crenata* extract with linearity for quassin in the range 13.13–100 μg/mL [97]. The aqueous extract showed linear response within the range 0.978–6.520 mg/mL. Recently, an isocratic protocol has been reported with a simple gradient run of 40–100% methanol–water in a Phenomenex Luna RP C18 column with well-separated peaks of quassin eluted at 14 min and isomers of neoquassin eluted at 21 and 23 min [61]. The separated compounds can be directly applied for mass analysis. Methanol is less toxic as compared to acetonitrile and is the preferred solvent in RP HPLC [103].

4.3 Structure Determination

Classical methods of structure determination including UV and IR spectroscopy, optical rotatory dispersion and circular dichroism, and later on NMR spectroscopy were instrumental in the structure elucidation of natural products including quassinoids. Early in the application of NMR spectroscopy, chemical shift and multiplicity of signals on chemical changes provide useful methods for assigning signals and interrelating protons. As many terpenoid compounds differ only in the number of the hydroxyl group or esters, the changes in spectra associated with changes in functionality provide means for establishing structures [104].

Novel mass spectrometric techniques are increasingly being used for the identification of quassinoids. These techniques have the advantage as they can be coupled to chromatographic techniques and allow comparative analysis with a database or library of previously reported structures. In 1958, the first application of mass spectrometry to the analysis of biomolecules was reported [105]. Mass spectrometry (MS) is an excellent analytical tool to measure the mass-to-charge (m/z) ratio of charged particles [106]. Many ionization methods are available, viz., atmospheric-pressure chemical ionization (APCI), electron impact ionization (EI), chemical ionization (CI), thermospray ionization (TSP), field desorption ionization (FDI), fast atom bombardment (FAB), electrospray ionization (ESI), and matrix-assisted laser desorption ionization (MALDI). The ionization procedure is involved in the formation of molecular ions; both positively and negatively charged ions are generated by depending on the proton affinity of the sample.

With the development of sensitive LC/MS methods, it has been possible to differentiate and make comparative studies as demonstrated in a study with Jamaica quassia (*Quassia excels*) and quassia extract (*P. quassioides*) whereby the main constituents isolated were quassin and two isomers of neoquassin; however, there was a difference in the minor components. This finding is of significance to evaluate the quality and suitability of the extracts when used as food additives. An ultraperformance liquid chromatography–time-of-flight mass spectrometry (UPLC–TOFMS) protocol for multiresidue determination of the sum of quassin and neoquassin in strawberry has been developed [107].

A combination of column chromatography, ODS HPLC, IR, ^{1}H and ^{13}C NMR, ESI-MS (neg.), and HR-ESI-TOF-MS (pos.) was utilized in isolation two novel quassinoids, delaumonones A (HR-ESI-MS [*m/z* 531.187 $(M + Na)^{+}$]) and B (HR-ESI-MS [*m/z* 523.2180, $(M + Na)^{+}$]) from *Laumoniera bruceadelpha* [108]. LC ESI-MS/MS has been utilized in the determination of quassin and neoquassin in fruits and vegetables [98] of *Q. amara*. Limit of determination (LOD) and limit of quantification (LOQ) for both quassinoids were 0.5 and 1 μg/kg, respectively, while for picrasinoside B, they were 5 and 10 μg/kg. This study demonstrated that multiple reaction transitions of precursor ions can be used for analytical purposes, and it is possible to detect and quantify quassinoids at trace levels.

Three LC–MS/MS hybrid systems (QTof, TripleTof, and QTrap) have been used to profile small metabolites (*m/z* 100–1,000) and to detect quassinoids along with alkaloids, triterpene, and biphenylneolignans from the aqueous extracts of *Eurycoma longifolia* collected from two different geographical terrains – Pahang and Perak [99]. The use of LC–MS/MS hybrid system enabled the simultaneous detection of 38 quassinoids (particularly eurycomanone and its derivatives) that were present in higher concentration compared to other group of metabolites. It was also possible to tag particular quassinoid to a particular geographical location, e.g., 3,4ε-dihydroeurycomanone was detected only in the Pahang extract, whereas canthin-6-one-3N-oxide was detected only in the Perak extract. Another study on the same plant compares electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) mass spectrometry using automated flow injection analysis (FIA) to the determination of eurycomanone, 13α (21)-epoxyeurycomanone, eurycomanol, eurycomanol-2-*O*-*β*-D-glucopyranoside, and 13,21-dihydroeurycomanone [100]. The detection limits (LODs) determined in isolation mode (selected ion monitoring, SIM) ranged between 0.03 and 0.1 μg mL^{-1}, while the intraday and interday precisions were less than 5.72% and 4.82%, respectively. Mass spectrometric techniques provide a robust and high-throughput method for the detection of quassinoids in minute compounds.

5 Conclusion

The importance of quassinoids as anticancer and anti-HIV compounds has revived with the isolation and characterization of new bioactive compounds. Its efficacy as combinatorial drug coupled with the structure activity analysis has provided novel structural leads for drug development. The present methods of detection including LC–MS/MS techniques could effectively provide more lead molecules which might act in synergism with other compounds. Rigorous evaluation of traditional medicines involving controlled clinical trials in parallel with agronomical development for enhanced in vivo production of active compounds could alleviate availability of phytochemicals at an acceptable cost that would ameliorate disease control and would also serve as a source of income generation.

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Methods for Extraction and Analysis of Carotenoids

111

Siti Machmudah and Motonobu Goto

Contents

1 Introduction 3368
2 Solvent Extraction and Isolation of Carotenoids 3371
2.1 Carotenoids Extraction 3371
2.2 Carotenoid Isolation 3375
3 Prepurification of Carotenoids by Crystallization 3376
4 Analytical Method of Carotenoids 3377
4.1 Analysis of Carotenoids by UV-Visible Spectrophotometry 3380
4.2 Chromatographic Methods for Separation and Analysis of Carotenoids 3384
4.3 Mass Spectrometry of Carotenoids 3397
5 Conclusions 3407
References 3407

Abstract

Carotenoids have received much attention because of their various functions. They are natural pigments synthesized by plants and some microorganisms. Carotenoids exhibit yellow, orange, and red colors but when they are bound to proteins, they acquire green, purple, or blue colors. They are found in a large number of natural products, such as fruits, vegetables, and sea products.

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This chapter presents method of extraction/isolation and analysis of carotenoids in natural products. General procedures of solvent extraction/isolation and analysis of carotenoids are described. Preparation and determination of carotenoids are also explained.

Keywords

Analysis • carotenoids • extraction • isolation

Abbreviations

APCI	Atmospheric pressure chemical ionization
BHT	Butylated hydroxytoluene
CI	Chemical ionization
CID	Collision-induced dissociation
DAD	Diode array
DHP	Dihydroxy pigment
EI	Electron impact
ESI	Electrospray ionization
FAB	Fast atom bombardment
GC/MS	Gas chromatography MS
HPLC	High-performance liquid chromatography
IPA	Indole-3-propionic acid
LC	Liquid chromatography
LC/MS	Liquid chromatography MS
MALDI	Matrix-assisted laser desorption/ionization
MALDI/TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight MS
MHP	Monohydroxy pigment
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
OCC	Open-column chromatography
SFE	Supercritical fluid extraction
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin-layer chromatography

1 Introduction

Carotenoids have been of great interest, and their importance in food coloration has been reviewed by some researchers [1–5]. Carotenoids are notable for their wide distribution, structural diversity, and various functions. More than 600 carotenoids, not including cis and trans isomers, have been isolated and characterized from natural sources, including the enormous array of carotenoids in algae, bacteria, yeast, and fungi [6].

Hydrocarbon carotenoids (e.g., β-carotene, lycopene) are known as carotenes, and oxygenated derivatives are called xanthophylls. Common oxygen substituents are the hydroxy (as in β-cryptoxanthin), keto (as in canthaxanthin), epoxy (as in violaxanthin), and aldehyde (as in β-citraurin) groups. Carotenoids can be acyclic (e.g., lycopene), monocyclic (e.g., γ-carotene), or dicyclic (e.g., α- and β-carotene). In nature, carotenoids exist primarily in the more stable all-trans (or all E) form, but small amounts of cis (or Z) isomers do occur.

Because plants are able to synthesize carotenoids de novo, the carotenoid composition of plant is enriched by low levels of biosynthetic precursors and derivatives of the main components. Carotenoids are not as widely distributed in animals and are present at much lower levels. Animals are incapable of carotenoid biosynthesis and hence depend on dietary carotenoids, which are selectively or unselectively absorbed, converted to vitamin A, and deposited as such or slightly altered to form carotenoids typical of animal species.

Figure 111.1 shows the principal carotenoids found in natural products, together with zeaxanthin, which is not as ubiquitous. β-Carotene, α-carotene, β-cryptoxanthin, lutein, and lycopene are also the carotenoids most commonly found in human plasma. These carotenoids, together with zeaxanthin, have been shown to have health-promoting effects.

β-Carotene, α-carotene, and β-cryptoxanthin are provitamins A. Structurally, vitamin A (retinol) is essentially one-half of the β-carotene molecule. Consequently, β-carotene is the most potent provitamin A; it is also the most widespread [7, 8]. The minimum requirement for a carotenoid to have vitamin A activity is an unsubstituted β-ring with an 11-carbon polyene chain. Thus, α-carotene and β-cryptoxanthin exhibit about 50% of the vitamin A activity of β-carotene.

Carotenoids, whether provitamins A or not, have been credited with other beneficial effects on human health: enhancement of the immune response and reduction of the risk of degenerative diseases such as cancer, cardiovascular diseases, cataract, and macular degeneration [9–19]. The action of carotenoids against diseases has been attributed to an antioxidant property, specifically, their ability to quench singlet oxygen and interact with free radicals [20]. However, other mechanisms have been reported: modulation of carcinogen metabolism, inhibition of cell proliferation, enhancement of cell differentiation, stimulation of cell to cell communication, and filtering of blue light [15, 21].

The ability of carotenoids to quench singlet oxygen has been linked to the conjugated double bond system, the maximum efficiency being shown by carotenoids with nine or more conjugated double bonds [22]. The acyclic carotenoid lycopene was found to be more efficient than the dicyclic β-carotene [23], despite both compounds possessing 11 conjugated double bonds. The effects of lycopene on human health have drawn considerable interest in recent years [24–33], the current evidence being stronger for lycopene protection against lung, stomach, and prostate cancer.

Recently, food processors and technologists have shown a great interest in the extraction, identification, and purification of natural colorants, including carotenoids. This unit describes a practical way of extracting, isolating, and purifying

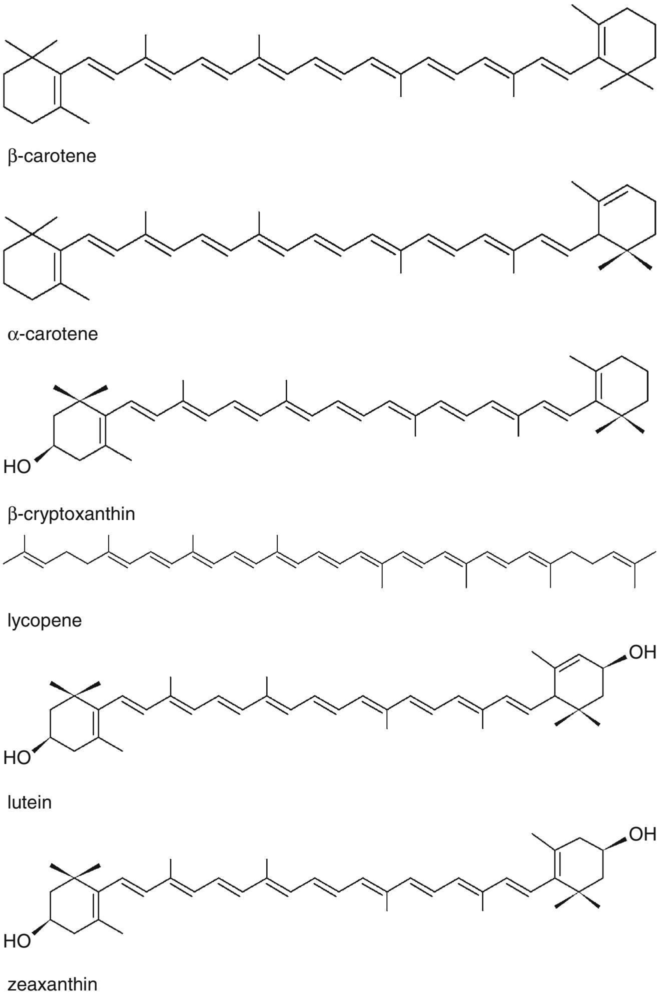

Fig. 111.1 Stuctures of the principal carotenoids in natural products and zeaxanthin

carotenoids from plant materials. The method is based mainly on the natural form in which the carotenoids are found (esterified or free) and to some extent on their polarity and/or solubility in the solvents used. Common and readily available solvents and laboratory equipment are suggested.

The extraction and isolation of three groups of carotenoids of different polarities are described in Sect. 2. A method for prepurifying carotenoids using crystallization is described in Sect. 3. Carotenoids may be purified further by chromatographic techniques and characterized (Sect. 4). The preparation of the sample before extraction is described in Sect. 2.1.2. This process consists mainly of removing water from the sample, followed by sample grinding or homogenizing.

In this chapter, extraction is carried out using solvents. Other methods for extracting carotenoids are available. Supercritical fluid extraction (SFE) has been used for extracting and isolating carotenoids from natural product samples since this technique shows several advantages, for example, more speed in extraction, the evaporation step is not required, and carbon dioxide, the fluid most used in SFE, is nontoxic, its cost is low, it is non-flammable, and it is environmentally acceptable [34]. In addition, carbon dioxide has a low critical temperature (31°C), making it ideal for extraction of thermally labile compounds. Nevertheless, carbon dioxide presents a low polarity and makes the extraction of polar compounds very difficult. This limitation can be solved by adding an organic modifier such as methanol or ethanol in order to increase its solvation power [35]. The use of supercritical carbon dioxide is described by Spanos et al. [36], Mendes et al. [37], de Franc et al. [38], Cadoni et al. [39], Canela et al. [40], Rozzi et al. [41], Machmudah et al. [42–44], Macias-Sanchez et al. [45], Prado et al. [46], and Ruen-ngam et al. [47]. The SFE conditions are summarized in Table 111.1.

Enzymes may also be used to help digest plant material and then facilitate carotenoid extraction by physical or chemical means [53–55]. Ultrasound may also be used to aid the extraction process; it is used in laboratory and industrial work for extracting carotenoids and other compounds [56, 57]. Vegetable oil may be used as an extractant and carotenoid carrier [44, 58, 59]. However, using oils for extraction produces low carotenoid concentrates. By introducing organic solvents, pigments are more easily solubilized, and extracts of high purity may be obtained.

2 Solvent Extraction and Isolation of Carotenoids

This section begins with the extraction of a dehydrated sample. It continues with a saponification procedure to initiate the isolation of the carotenoid mixture. During saponification, the esters are hydrolyzed and the free pigments released. Then, to continue the isolation, open-column chromatography (OCC) is suggested as a simple and fast means of separating the three main groups of carotenoids based on their different polarities.

2.1 Carotenoids Extraction

Numerous organic solvents such as acetone [60–64], tetrahydrofuran (THF) [65, 66], n-hexane [67, 68], pentane [51], ethanol [69, 70], methanol [71, 72], and chloroform [41], as well as solvent mixtures such as dichloromethane:methanol (6:1, v/v) [73], acetone:petroleum ether (50:50, v/v) [74], THF:methanol (1:1, v/v) [75, 76], n-hexane:toluene (5:4, v/v) [77], *n*-hexane:acetone (6:4, v/v) [78], 2-propanol:dichloromethane (2:1, v/v) [79], *n*-hexane:ethyl acetate (85:15, v/v) [80] and *n*-hexane:acetone:ethanol (50:25:25, v/v/v) [81] have been widely used. Thus, Taungbodhitham et al. [82] evaluated six different solvent combinations: acetone:hexane (4:6, v/v), ethanol:hexane (4:3, v/v), chloroform:methanol (2:1, v/v), dichloromethane:methanol (2:1, v/v), hexane:isopropanol (3:2, v/v),

Table 111.1 Example of supercritical fluid extraction conditions employed in carotenoid analysis

Sample	Carotenoid	Supercritical fluid	SFE conditions (temperature, pressure, flow rate)	References
Tomato	All-trans-lycopene	CO_2 without modifier	T = 40°C Pressure: 281 bar Flow rate: 4 ml/min	[48]
Tomato seeds and skins	Lycopene, β-carotene, α-carotene, α-tocopherol, γ-tocopherol, δ-tocopherol	CO_2 without modifier	T = 86°C Pressure: 345 bar Flow rate: 2.5 ml/min	[41]
Algae *Dunaliella bardawil*	Geometrical isomers of β-carotene	CO_2 without modifier	T = 40°C Pressure: 448 bar Flow rate: 0.5 − 1 ml/min	[49]
Tomato paste waste	Lycopene, β-carotene	CO_2 and 5% ethanol as modifier	T = 55°C (lycopene), 65°C (β-carotene) Pressure: 300 bar Flow rate: 4 kg/h	[50]
Vegetables (carrots, collard, turnip greens, kale, mustard greens, broccoli flowerets, zucchini, yellow squash)	α- and β-carotene	CO_2 and ethanol as modifier	T = 40°C Pressure: 342 bar Flow rate: 1.5 ml/min	[51]
Spirulina pacifica algae	β-Carotene, β-cryptoxanthin, zeaxanthin	CO_2 and 15% ethanol as modifier	T = 80°C (zeaxanthin), 76°C (β-cryptoxanthin), 60°C (β-carotene) Pressure: 342 bar Flow rate: 1.5 ml/min	[35]
Carrots (*Daucus carota* L. var. Caro Pride)	α- and β-carotene	CO_2 and 5% chloroform as modifier	T = 40°C Pressure: 606 bar Flow rate: 1 ml/min	[52]
Haematococcus pluvialis algae	Astaxanthin	CO_2 and ethanol as modifier	T = 40–80°C Pressure: 200–550 bar Flow rate: 2–4 ml/min	[42]
Rose hip fruit	Lycopene, β-carotene, lutein	CO_2 without modifier	T = 40–80°C Pressure: 150–450 bar Flow rate: 2–4 ml/min	[43]
Tomato peel waste	Lycopene, β-carotene	CO_2 and tomato seed oil as modifier	T = 70−90°C Pressure: 200−400 bar Flow rate: 2−4 ml/min	[44]

and acetone:petroleum ether (50:50, v/v) to extract lycopene and α- and β-carotene from canned tomato juice, and the best recoveries were obtained with the ethanol: hexane mixture. Deli et al. [83] used methanol followed by diethyl ether to extract carotenoids from fruits of pepper (Capsicum annuum var. lycopersiciforme rubrum). Gandul-Rojas et al. [67] extracted chlorophylls and free and monoesterified xanthophylls with dimethylformamide and carotenoids with hexane from olive fruits.

A good extraction procedure should release all the carotenoids from the natural products matrix and bring them into solution, without altering them. Because carotenoids are found in a variety of natural products, the extraction procedure should be adapted to suit the plant being analyzed. The solvent chosen should efficiently extract all carotenoids present in the sample. Because the solvents used in extraction will ultimately be removed or at least reduced by evaporation, solvents with low boiling points should be chosen to avoid prolonged heating.

The solvent used for extraction must be chosen according to the polarity of the pigments presumably present. If this characteristic is unknown, an acetone/hexane (1:1, v/v) mixture is suitable [84]. When it is known that the carotenoids in the sample are nonpolar or are in the ester form, hexane is a good choice for extraction. Ethanol will extract polar carotenoids, and a nonpolar solvent like hexane will promote crystallization. Simple procedure for extraction of carotenoids is describe here.

2.1.1 Extraction Procedure

1. Place dried/dehydrated plant material (see Sect. 2.1.2) and filter paper in a 500-ml extraction vessel.
2. Add 3 vol (v/w) solvent (1:1 (v/v) hexane/acetone or hexane alone) and mix for 15 min using an explosion-proof shaft mixer to suspend paste in solvent.

   Solvent is 1:1 (v/v) hexane/acetone or hexane alone.

   The type of solvent should be chosen based on the carotenoids of interest. No heating is necessary.
3. Vacuum filter mixture using Whatman no. 42 filter paper. Save filtrate.
4. Separate filtrant cleanly from filter paper, if possible, and extract a second time using 2 vol solvent. Continue extractions using smaller volumes of solvent until no appreciable color is observed in filtrate. Save and pool all filtrates.

   Note:

   If filtrant cannot be separated from paper; both should be placed in the extraction vessel.

   At this point, concentrated filtrate from the sample preparation (see Sect. "Removal of Water from Carotenoid-Containing Samples Using Alcohol," step 8) may be pooled with the filtrates.
5. Concentrate extracts to ~40 ml in a rotary evaporator attached to a vacuum pump at ≤55°C.
6. Add 3 ml saponifying solution and stir for 45 min at 56°C.
   - This is the temperature recommended for hot saponification of pigments by the AOAC [85].
   - Saponifying solution: 40% (w/v) KOH in methanol (cool to room temperature before bringing up to volume)

7. Transfer saponified extract to a 125-ml separatory funnel and add 1 vol salting-out solution.
    - Salting-out solution: 10% (w/v) Na_2SO_4
8. Remove bottom layer and wash upper layer three times with 10 ml water.
9. Add 3 g anhydrous Na_2SO_4 and filter using Whatman no. 42 filter paper.

2.1.2 Sample Preparation

For extraction, water content is considered an important factor. It has been found that working with low-moisture samples simplifies the extraction process. Industrial extraction normally is done with dry material, which reduces complications arising from the solvents used for processing and their recovery.

The plant material as prepared in sections "Removal of Water from Carotenoid-Containing Samples Using a Vacuum Oven and "Removal of Water from Carotenoid-Containing Samples Using Alcohol," is ready for extraction. It may contain some water (≤10%), which will not affect extraction. Freeze-drying may be another way of eliminating water with little pigment damage and may be an acceptable alternative to drying in a vacuum oven as long as the moisture content is taken to an appropriate level for efficient extraction. Freeze-drying is not adequate if sugar or other water-soluble compounds must be eliminated by physical separation (e.g., by filtration or centrifugation).

Removal of Water from Carotenoid-Containing Samples Using a Vacuum Oven

Plant tissues contain variable amounts of water, which is easily eliminated in order to facilitate extraction with organic solvents. Using a low-temperature vacuum oven is a good way of removing water from materials that are easily pulverized. This is true for many vegetables [86]. The procedure for removing water from materials is described below.

Procedure:

1. Weigh plant tissue of interest and record weight.
    Note:
    The initial wet weight is important as, in many cases, extraction and purification of carotenoids are done to quantify pigment concentrations, which must be related to the fresh material.
    The amount of tissue used is dependent on the expected pigment concentration. Usually 100–500 g is appropriate.
2. Place sample inside a vacuum oven at 60°C. Maintain set temperature.
3. Turn on and control the vacuum between 12 and 20 in.Hg (305–508 mmHg).
4. Open oven door occasionally during drying and stir material to obtain uniform dehydration.
5. Dry to ~8% moisture content.
    Note:
    Overdrying can damage the carotenoids present in the sample.

A moisture balance calibrated to indicate an 8% moisture content can be used to monitor weight loss and may be useful until experience is gained. The calibration can be performed as a gravimetric determination on a sample analyzed in parallel. This measurement can also be used to estimate an 8% moisture content relative to the initial sample weight.

6. Grind material to an appropriate size (≤30 mesh) with a laboratory mill.
7. Weigh powder for extraction.

   The weight will also be indicative of residual moisture if the initial amount is known.

Removal of Water from Carotenoid-Containing Samples Using Alcohol

This procedure is a practical way of removing water from plant tissues when water is needed for homogenization or when the dry material cannot be ground to a powder due to the presence of lipids, waxes, or sugars [86].

Procedure:

1. Weigh plant tissue of interest and record weight.
2. Mix plant tissue to a paste with an explosion-proof shaft mixer. If the material does not lend itself to mixing, add sufficient water to homogenize sample.
3. Add 2 vol of 95% ethanol to homogenate, and mix thoroughly for 5 min.
4. Vacuum filter homogenized sample using Whatman no. 41 filter paper. Save filtrate.
5. If filtrate is cloudy, repeat steps 3 and 4 using 1 vol of 95% ethanol. If filtrate is clear, continue with step 6.

   Note:

   A cloudy filtrate is an indication that additional alcohol is needed.
6. Remove and save filtrant along with the filter paper. Store ≤24 h at −20°C.
7. Pool filtrates in a separatory funnel. Add 1 vol hexane.

   Note:

   Some of the more polar pigments are usually carried into the filtrate and must be recovered by extraction.
8. Remove bottom layer and concentrate upper layer containing the lipophilic carotenoids in a rotary evaporator attached to a vacuum pump at ≤55°C. Store ≤24 h at −20°C.

2.2 Carotenoid Isolation

Carotenoid isolation is carried out in open-column chromatography (OCC). OCC should be carried out under a fume hood to protect the analyst from inhaling solvent vapor. Breathing hexane, for example, should be avoided due to neurotoxicity of some of its oxidative metabolites [87]. The isolation procedure is as follows.

Procedure

1. Plug the bottom of a 600 × 40-mm chromatography column with glass wool. Mount the column on a vacuum filtration device using a 1-l filtration flask as a receiving vessel.

2. Add adsorbent to obtain a 20-cm layer while applying vacuum.
   Note:
   Adsorbent: Mix equivalent weights of silica gel 60 GF_{254} (Merck) and diatomaceous earth (Hyflo Super-Cel; Celite, World Minerals) in a mechanical blender or in a large plastic bag for 2 h. Store $\leq$3 months in a sealed container at room temperature [85].
3. Level the surface of the adsorbent and place a firm 2-cm layer of anhydrous Na_2SO_4 on top.
4. Pour carotene eluant into column until eluant wets all of the adsorbent.
   Note:
   Carotene eluant: 4% (v/v) acetone in hexane.
5. Replace receiving vessel with a clean flask and pour filtrate (2.1.1 step 9) into column.
6. Allow all the sample to penetrate into the adsorbent and then add carotene eluant until the first carotenoid band that separates is completely collected in the flask.
   - The carotenes and esters present in the sample are contained in this fraction. Other carotenoids remain on the column.
   - The chromatography process is monitored visually. The exact volume of eluant added to the column will vary depending on sample concentration and composition.
7. Elute monohydroxy pigments with monohydroxy pigment (MHP) eluant and dihydroxy pigments and more polar pigments with dihydroxy pigment (DHP) eluant using a clean receiving flask for each. If necessary, store pigments $\leq$24 h at 0–5°C and protect from light.
   Eluates should be processed as soon as possible.
   Note:
   - MHP eluant: 1:9 (v/v) acetone/hexane
   - DHP eluant: 1:8 (v/v) acetone/hexane

3 Prepurification of Carotenoids by Crystallization

Once the carotenoids have been isolated as described in Sect. 2.2, they can generally be crystallized as an initial step to purification. Actually, what is most likely to happen is a co-crystallization. When working with a nonpolar fraction, α- and β-carotene may co-crystallize. In the same way, a polar fraction may yield lutein – zeaxanthin crystals. A pure carotenoid product may be obtained by crystallization of a fraction derived from a preparatory chromatographic procedure, which can be done using TLC, HPLC (Sect. 4), or in some cases column chromatography [84]. The crystallization procedure is shown here.

Procedure:

1. Place isolated carotenoid eluate (Sect. 2.2, steps 6 and 7) in a 50- or 125-ml pear-shaped flask and concentrate eluate to near saturation in a rotary evaporator attached to a vacuum pump under a stream of nitrogen from a nitrogen gas tank.

Note:

- Only one carotenoid eluate (carotenes, MHPs, or DHPs) should be prepurified at a time.
- The flask size will depend on the volume of eluate to be concentrated.

2. Add precipitating solvent drop by drop until the solution starts turning cloudy, indicating that precipitation has begun.
   Note:
   - A solvent mixture should be selected for crystallization that will easily solubilize the carotenoid of interest and also, when added in small quantities, will force the carotenoid out of solution and start crystallization. The extractants from Sect. 2.1.1 (hexane/acetone or hexane alone) are appropriate.
   - Precipitating solvent: 1:1 (v/v) hexane/acetone or hexane alone, room temperature and cold (0–5^{o}C)
3. Refrigerate (0–5°C) solution overnight.
   Crystallization can be accelerated by placing solution at −20 to 0^{o}C for 3 or 4 h. Usually better crystals are produced by a slow crystallization.
4. Vacuum filter crystals using Whatman no. 42 filter paper. Wash crystals with cold (0–5°C) precipitating solvent.
5. *Optional*: Repeat crystallization sequence to obtain crystals of higher purity, beginning with a saturated solution of crystals (step 4) in acetone and continuing with step 2.
6. Dry crystals in a vacuum oven at 40°C, flushing drying chamber occasionally with nitrogen.
7. Pack crystals under nitrogen and store for ≤6 months at −20°C if further purification work is anticipated.

4 Analytical Method of Carotenoids

This section includes identification and measurement of carotenoids by UV/visible spectrophotometry and column chromatography. The UV/visible absorption and the chromatographic behavior spectrum provide the first clues for the identification of carotenoids. Both the wavelengths of maximum absorption (λ_{max}) and the shape of the spectrum (spectral fine structures) are characteristics of the conjugated unsaturated part of the carotenoid molecule containing delocalized π-electrons called the “chromophore.” The λ_{max} values of the carotenoids commonly found in natural products in various solvents are listed in Table 111.2.

Most carotenoids absorb maximally at three wavelengths, resulting in a three-peak spectrum. As the number of conjugated double bonds increases, the λ_{max} shifts to longer wavelengths. Thus, the most unsaturated acyclic carotenoid, lycopene, with 11 conjugated double bonds, is red and absorbs at the longest wavelengths (λ_{max} at 444, 470, 502 nm in petroleum ether) (Fig. 111.2). The dicyclic molecule β-carotene, despite possessing the same number of conjugated double bonds as lycopene, is yellow-orange and exhibits absorption peaks at 450 and 477 nm and a mere shoulder at 425 nm (Fig. 111.2). Monocyclic γ-carotene is red-orange and exhibits a spectrum

Table 111.2 UV-Visible absorption data for common natural product carotenoids

Carotenoid	Solvent	λ_{max} (nm)			%III/II
α-Carotene	Acetone	424	448	476	55
	Chloroform	433	457	484	–
	Ethanol	423	444	473	–
	Hexane	422	445	473	55
	Petroleum ether	422	445	473	55
β-Carotene	Acetone	(429)	452	478	15
	Chloroform	(435)	461	485	–
	Ethanol	(425)	450	478	–
	Hexane	(425)	450	477	25
	Petroleum ether	(425)	450	477	25
α-Cryptoxanthin/Zeinoxanthin	Chloroform	435	459	487	–
	Ethanol	423	446	473	60
	Petroleum ether	421	445	475	60
β-Cryptoxanthin	Chloroform	(435)	459	485	–
	Ethanol	(428)	450	478	27
	Hexane	(428)	450	478	25
	Petroleum ether	(425)	449	476	25
Lutein	Chloroform	435	458	485	–
	Ethanol	422	445	474	60
	Petroleum ether	421	445	474	60
Lycopene	Acetone	448	474	505	–
	Chloroform	458	484	518	–
	Ethanol	446	472	503	65
	Hexane	444	470	502	65
	Petroleum ether	444	470	502	65
Zeaxanthin	Acetone	(430)	452	479	–
	Chloroform	(433)	462	493	–
	Ethanol	(428)	450	478	26
	Hexane	(425)	450	478	25
	Petroleum ether	(424)	449	476	25

Refs. [84, 88–90]

Parentheses indicate a shoulder. %III/II is the ratio of the height of the longest-wavelength absorption peak, designated III, to that of the middle absorption peak, designated II, taking the minimum between the two peaks as the baseline, multiplied by 100

intermediate to those of lycopene and β-carotene, in both λ_{max} and shape, reflecting a structure that is intermediate with respect to the other two carotenoids. α-Carotene is light yellow, and its absorption spectrum is slightly better defined with λ_{maxs} at slightly shorter wavelengths (422, 445, 473 nm) compared with the spectrum of β-carotene.

The absorption spectra of carotenoids are markedly solvent dependent (Table 111.2). This characteristic of carotenoids must be remembered when analyzing spectra recorded by the photodiode-array detector in HPLC because such spectra are taken in mixed solvents in isocratic elution and in varying mixed solvents in gradient elution.

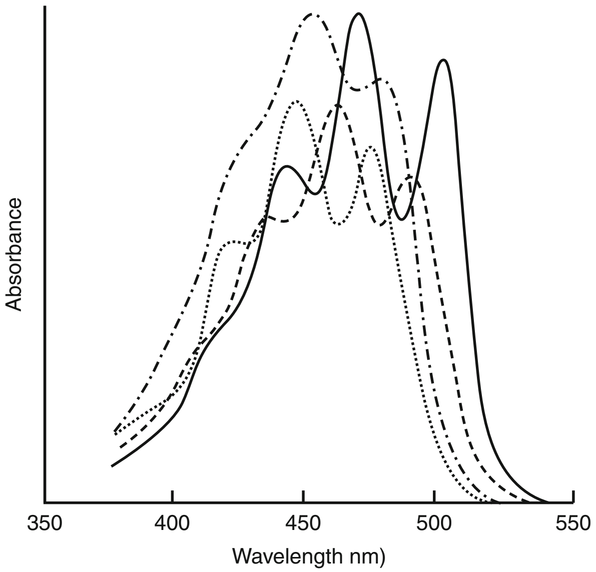

Fig. 111.2 Visible absorption spectra of lycopene (———), γ-carotene (- - - -), β-carotene (—·—·), and α-carotene (·······) in petroleum ether [88]

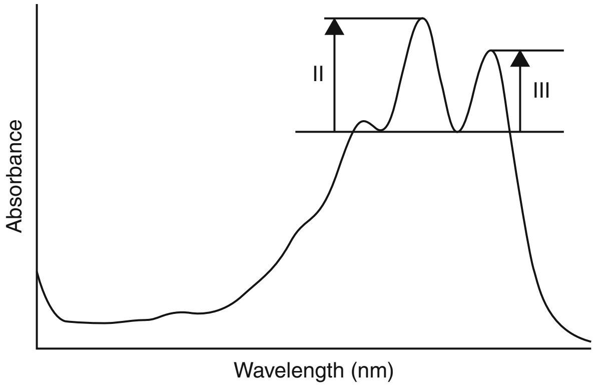

Fig. 111.3 Calculation of % III/II (III/II × 100) as an indicator of spectral fine structure [84, 88]

The λ_{max} values of carotenoids in hexane or petroleum ether are practically the same as in diethyl ether, methanol, ethanol, and acetonitrile and higher by 2–6 nm in acetone, 10–20 nm in chloroform, 10–20 nm in dichloromethane, and 18–24 nm in toluene [89].

To obtain the absorption spectral fine structure, the %III/II (Fig. 111.3) can be presented, along with the λ_{max} values. The %III/II is the ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as the baseline, multiplied by 100 [89].

The chromatographic behavior of carotenoids bears a definite relationship with their structures; however, carotenoids cannot be identified based on chromatographic data alone. Nevertheless, these data serve as useful complementary information.

4.1 Analysis of Carotenoids by UV-Visible Spectrophotometry

The majority of carotenoids exhibit absorption in the visible region of the spectrum between 400 nm and 500 nm. Carotenoids in solution obey the Beer-Lambert law, where absorbance (A) equals concentration multiplied by absorption coefficient ($A^{1\%}$), where the absorption coefficient ($A^{1\%}$) is defined as the absorbance of a 1% (10 g/l) solution of carotenoid, in a defined solvent, in a 1-cm path-length cuvette, at a specific wavelength (λ). This information can be used to quantify the concentration of a pure (standard) carotenoid (see Sect. 4.1.1) or to "estimate" the total carotenoid concentration in a mixture or extract of carotenoids in a sample (see Sect. 4.1.2). The absorption coefficient may also be expressed in terms of molarity.

The absorption coefficient values of selected carotenoids in certain solvent are presented in Table 111.3. The values may contain significant levels and error or uncertainty [89], and some discrepancies can be noted in Table 111.3. Given that different authors choose different coefficients for some carotenoids (in the same solvents), this alone can account for a good part of the variations in analytical results.

As seen in Table 111.3, a 1% solution (10 g/l) of β-carotene in hexane has an absorbance of 2,592 AU and a molecular weight of 537 g/mol (i.e., 1 mM = 0.537 g/l); therefore, the absorbance expected for a 1 mM solution is (0.537 g/l)/(l0 g/l) × (2,592 AU) = 139 AU/1 mM β-carotene. Using this ratio, if a β-carotene solution has an absorbance of 5, then the concentration is given as (5 AU)/(139 AU/mM) = 0.036 mM. It is important to note that due to the inherent difficulties in procedures for accurate determination of absorption coefficients, there may be a significant level of uncertainty in some published values. Small variations (e.g., 2–3 nm) may also occur in published data of absorption maxima. Whenever possible, the spectrum of a compound under investigation should be compared directly with an authentic pure standard. The spectra of the unknown and the standard should be identical for both the λ_{max} and the fine structure (%III/II).

4.1.1 Quantification and Calibration of Individual Carotenoid Standards

In this section, commercially purchased carotenoid standards are dissolved in a suitable solvent and the absorbance measured at its maximum wavelength (λ_{max}). Using published absorption coefficients (Table 111.2) and taking into consideration the dilution factor, the concentration of the standard carotenoid is calculated. The spectrum is also scanned in order to evaluate the fine structure.

Table 111.3 Absorption coefficients ($A^{1\%}$) of selected carotenoids

Carotenoids	MW	Solvent	λ_{max} (nm)	$A^{1\%}$	$\varepsilon^{1\ mM}$
α-Carotene	537	Hexane	445	2,710	145
		Petroleum ether	444	2,800	–
β-Carotene	537	Hexane	450	2,592	139
		Ethanol	450	2,620	–
		Chloroform	465	2,396	–
		Petroleum ether	450	2,592	–
α-Cryptoxanthin/zeinoxanthin	553	Hexane	445	2,636	–
β-Cryptoxanthin	553	Hexane	450	2,460	136
		Petroleum ether	449	2,386	–
Lutein	569	Ethanol	445	2,550	145
		Diethyl ether	445	2,480	–
		Diethyl ether	445	2,600	–
Lycopene	537	Hexane	470	3,450	185
		Petroleum ether	470	3,450	–
Zeaxanthin	569	Hexane	450	2,480	141
		Ethanol	450	2,480	–
		Ethanol	450	2,540	–
		Acetone	452	2,340	–
		Petroleum ether	449	2,348	–
Natural carotenoids as food colors					
Bixin (Bixa orellana)	395	Petroleum ether	456	4,200	166
Capsanthin (paprika)	585	Benzene	483	2,072	121
Capsorubin (paprika)	601	Benzene	489	2,200	132
Synthetic food colors					
β-Apo-8′-carotenal	417	Petroleum ether	457	2,640	110
Cantaxanthin	564	Petroleum ether	466	2,200	124

Refs. [84, 88, 89]

The carotenoid solution should ideally be assayed by HPLC as described in Sect. 4.2 to establish chromatographic purity and thus correct the calculated concentration. Procedure for quantification of individual carotenoids sample/standard is described below.

Procedure:

1. Dissolve carotenoid (e.g., ~1–5 mg) in a suitable solvent (see Table 111.2). Make to an accurate volume (e.g., 10–50 ml) in a volumetric flask.
   Note:
   A larger volume may be used if desired.
   It is essential that the carotenoid be completely dissolved. With crystalline samples, dissolution can be aided by initial addition of a small amount of a more effective solvent (e.g., dichloromethane) prior to making to volume for

spectrophotometric measurement (for most commonly assayed carotenoids, no more than 10% of the total volume should be required; however, lycopene may require up to 100%). Where a carotenoid is supplied in a sealed ampule, dissolution can be conveniently achieved by adding successive small aliquots of the more effective solvent to the ampule, and transferring to a volumetric flask. It is not easy to assess complete dissolution visually; therefore, it is advisable to filter the solution through a suitable solvent-compatible 0.45-μm filter.

Stock solutions that have been stored (e.g., in a refrigerator), should be allowed to warm up to room temperature, refiltered, and a "new" concentration calculated prior to preparing a new working solution, which should be checked for chromatographic purity.

2. Dilute the solution (e.g., 1:50) in desired solvent if necessary to give ~0.3–0.7 AU as measured on a spectrophotometer at λ_{max}.

   Note:

   It is recommended that at least two independent dilutions be made to ensure confidence in the measurement.

   If a larger final volume is used (see step 1) reduce the dilution accordingly.
3. Warm up the spectrophotometer per manufacturer's instructions.
4. Zero the spectrophotometer with solvent in a cuvette.

   Note:

   - It is not necessary to completely fill the cuvettes, ~$^2/_3$ full is normally sufficient. This helps to avoid accidental spillage.
   - Cuvettes must be kept scrupulously clean; avoid handling the surfaces of the cells.
5. Place a cuvette containing the carotenoid solution into the sample cell holder of the spectrophotometer.

   Note:

   Before placing the cells in the cell holder, the optical faces can, if necessary, be polished carefully (avoiding any spillage) with a fine tissue.
6. Measure the absorbance at λ_{max}. Take reading immediately.

   Note:

   Spectrophotometric readings should be carried out immediately after the solution has been placed in the cuvette to avoid evaporation of the solvent or degradation.
7. Scan to allow measurement of the fine structure (see Table 111.1).
8. Calculate the concentration of carotenoid as shown in the example below for all-*trans* β-carotene:

$$\frac{A \times V_1}{A^{1\%}} \times C^{1\%} = \frac{0.5\text{AU} \times 50}{2,592\text{AU}} \times 10\frac{\text{mg}}{\text{ml}} = 96.5\mu\text{g/ml} \qquad (111.1)$$

Where A is the absorbance reading of the diluted sample (0.5 AU), V_1 is the dilution factor (50×), $A^{1\%}$ is the absorbance of a 1% solution (i.e., the absorption coefficient; 2,592 AU), and $C^{1\%}$ is the concentration of a 1% solution (10 mg/ml). Using this formula, the concentration of the original solution in this example is = 96.5 μg/ml.

9. Subsequent to measurement of the carotenoid concentration, the solution should be assayed by HPLC to establish the chromatographic purity (see Sect. 4.2).

   For example, assuming the same values as (Eq. 111.1), if the total chromatographic area is 10,000, and the area of all-trans β-carotene peak is 9,500, then the chromatographic purity is 9,500/10,000 × 100 or 95%, and the actual concentration of β-carotene is 96.5 μg/ml × 95/100 or 91.7 μg/ml.

4.1.2 Measurement of Total Carotenoid Concentration in Food Colorants, Pharmaceuticals, and Natural Extracts

The same principle as described above can be used for the "estimation" of the carotenoid content of extracts of food colorants, pharmaceuticals, foods, biological samples, or chromatographic fractions. This procedure employs calculations used for individual carotenoids of high purity and thus will estimate the "total carotenoids" present in a food, biological, or natural extract, where a mixture of carotenoids would be expected. Greater accuracy can be obtained as extracts are purified to contain single components. A spectrum scan is not employed in this procedure as the fine structure of a mix of carotenoids can only be identified after HPLC separation and identification.

Procedure:

1. Prepare the sample as detailed in Sect. 2.1.2. Dilute the sample appropriately using a suitable solvent.

   Samples containing esterified carotenoids, chlorophyll, or high levels of fat may require saponification (Sect. 2.1).
2. Warm up spectrophotometer per manufacturer's instructions.
3. Zero the spectrophotometer with solvent in a cuvette.
4. Place a cuvette containing a suitably diluted (i.e., ~0.3–0.7 AU) extract of pharmaceutical, food, biological, or natural material into the sample cell holder of the spectrophotometer.
5. Measure absorbance at selected λ_{max} (Tables 111.2 and 111.3). Read immediately.
6. Estimate the total carotenoid concentration of the sample (see Sect. 4.1.1 procedures, step 9, and Eq. 111.1).

   With the exception of individual food colorants and single carotenoid pharmaceutical products, it is unlikely that the extract will be composed of only one predominant carotenoid; therefore, a specific λ_{max} or absorption coefficient (Table 111.3) cannot be used. In this case it is convenient to use a λ_{max} of 450 nm and a typical $A^{1\%}$ value of 2,500. Alternative values and other considerations are discussed below.

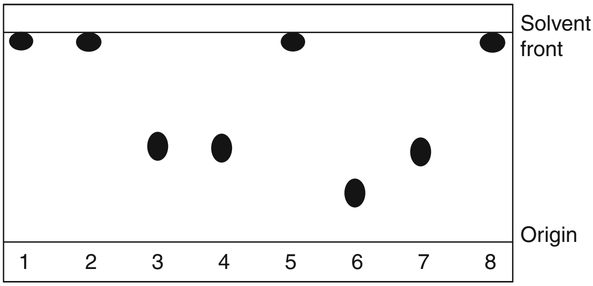

Fig. 111.4 TLC silica gel chromatogram of carotenoids and reaction products, developed with 5% methanol in toluene. *1* β-carotene, *2* lycopene, *3* β-cryptoxanthin, *4* β-cryptoxanthin methylated with acidic methanol – negative response, *5* β-cryptoxanthin acetylated with acetic anhydride, *6* lutein, *7* lutein methylated with acidic methanol, and *8* lutein acetylated with acetic anhydride [88]

4.2 Chromatographic Methods for Separation and Analysis of Carotenoids

Natural product samples typically contain both the nonpolar carotenes and the more polar xanthophylls. Whatever the method used, the chromatographic process should be able to cope with this polarity range.

Chromatography in descending, gravity-flow (often with slight pressure provided by a water aspirator) columns, known as open-column chromatography (OCC), is the classical method for separating carotenoids for quantitative analysis. It is also useful in isolating (See Sect. 2.2) and purifying carotenoids to be utilized as standards for HPLC. Separation of the carotenoid pigments is followed visually. Low pressure may also be applied at the top of the column (e.g., with N_2 gas); this technique is called flash column chromatography.

Thin-layer chromatography (TLC), although efficient in monitoring the progress of chemical tests for identification purposes, is not adequate for quantitative analysis because of the danger of degradation and isomerization on a highly exposed plate [91–93]. Carotenoids are particularly prone to oxidation by air when adsorbed on TLC plates. Additionally, it is not easy to quantitatively apply the sample on the plate and quantitatively recover the separated carotenoids from the plate for measurement. For qualitative purposes, TLC can separate the presence of oxygen substituents in carotenoids. This is demonstrated in a silica thin layer developed with 3% methanol in benzene or 5% methanol in toluene, in which all carotenes elute with the solvent front, whereas the xanthophylls are distributed along the plate according to the number and type of substituents present (Fig. 111.4). The hydroxyl group greatly influences absorption behavior. For instance, in the TLC chromatogram in Fig. 111.4, the hydroxycarotenoid appears near the origin, whereas the monohydroxycarotenoid appears near the middle of the chromatogram. The effect of more than one oxygen substituent is not always additive; a second substituent in the same end group tends to have less influence than the first.

In OCC, the column has to be packed for each analysis. A definite advantage of HPLC over OCC is that reproducible separations can be performed by using a reusable column under controlled conditions without undue exposure to air or light.

Reversed-phase HPLC on C18 columns has been the preferred mode for separating carotenoids for quantitative analysis. The popularity of the C18 column derives from its weak hydrophobic interactions with the analytes (which should make it less destructive than the polar forces in normal-phase OCC), compatibility with most carotenoid solvents and with the polarity range of carotenoids, and wide commercial availability. Many different C18 reversed-phase materials are available from different manufacturers, varying in the degree of carbon loading, end capping, and the nature of the bonded phase (i.e., monomeric or polymeric).

The majority of carotenoid separation has been carried out with 5 μm C18 spherical particles packed in a 250 × 4.6-mm column. However, shorter and narrower (narrow bore) columns, smaller particles, and C30 stationary phases are increasingly used.

Monomeric phases are simpler to use and more reproducible. Polymeric C18 phases have been found to have excellent selectivity for structurally similar carotenoids such as the geometric isomers of β-carotene [94, 95] and lutein and zeaxanthin [96]. However, the total carbon load is lower in the wide-pore polymeric phases, resulting in weak retention of carotenoids [97]. Additionally, compared with monomeric columns, the peaks tend to be broader, and columns from different production lots are more variable.

Guard columns, which should be changed frequently, are used for pigment samples to prevent particulate material and impurities from entering the analytical column, thus prolonging the column's life. Guard columns can, however, cause band broadening and could potentially retain carotenoids.

The most important properties to be considered in selecting the mobile phase are polarity, viscosity, volatility, and toxicity. In addition, it must be inert with respect to the carotenoids. Many solvent systems have been suggested as mobile phases for carotenoids, but the primary solvents are acetonitrile and methanol, with most systems being slight modifications of some basic combinations [97]. Acetonitrile has been widely used because of its lower viscosity and slightly better selectivity for xanthophylls when a monomeric C18 column is used [98]. Epler et al. [96] reported, however, that methanol-based solvents gave higher recoveries of carotenoids than acetonitrile-based solvents in almost all of 65 columns tested. Methanol is also more available, less expensive, and less toxic than acetonitrile. Addition of triethylamine to acetonitrile-based solvents was found to enhance carotenoid recovery [99].

Small amounts of other solvents are added to obtain the desired retention, increase solubility, and improve resolution. Chlorinated solvents (e.g., chloroform, dichloromethane) are frequently used for this purpose on account of their good solvent properties and effects on selectivity, although these solvents can be contaminated with traces of HCl. Other solvents used as modifiers are THF, ethyl acetate, hexane, acetone, and water. In some cases, methanol has been added to an acetonitrile-based mobile phase. Craft [100] investigated nine solvent modifiers and found THF to be the most beneficial modifier of methanol. Analysts tend to use mixtures of three or more solvents; however, Craft [100] cautioned against this practice because it can complicate the method, enhance demixing, and result in different evaporation rates, causing variation in the retention times during the course of the day.

Gradient elution should only be employed when the analysis cannot be done isocratically. Isocratic separation is rapid, can be performed with simple equipment (a single high-pressure pump and premixed solvent), and results in a stable baseline and more reproducible retention times. It is usually sufficient for the determination of provitamin A carotenoids or the principal carotenoids of natural samples.

Gradient elution has the advantages of greater resolving power, improved sensitivity, and elution of strongly retained compounds. It is more likely to resolve the whole range of carotenoids found in a given natural product. However, it has several disadvantages: (1) increased complexity, (2) requirement for more sophisticated and expensive equipment, (3) need for column reequilibration between runs, (4) greater differential detector response (i.e., different detector signals for the same concentration of different compounds), and (5) often poor reproducibility. The column must be brought back to the starting solvent and equilibrated for 10–30 min in this solvent before a new run is commenced. Good solvent miscibility is required to prevent baseline disturbance due to outgassing and refractive index effects [100].

Because *cis* isomers have different biological potencies than their *trans* counterpart, it is necessary to separate and quantify *cis* isomers when they are present in appreciable amounts. This level of detail, however, makes the analysis even more complicated. The polymeric C30 column was developed specifically for this purpose [101, 102]. This column, with an isocratic solvent system consisting of methanol:methyl-tert-butyl ether (89:11), was used for the quantification of *cis-trans* isomers of provitamin A carotenoids in fresh and processed fruits and vegetables [103].

The injection solvent must be compatible with the HPLC mobile phase. If the carotenoids are much more soluble in the injection solvent than in the mobile phase, the carotenoids will precipitate on injection, leading to peak tailing, or they will remain in the injection solvent while passing though the column, resulting in broad bands and doubled peaks [100]. These problems are particularly pronounced if the carotenoid solution is nearly saturated. The carotenoids will not dissolve completely if the solvent is too weak. Samples can be injected in the mobile phase to avoid this incompatibility problem. However, because of the solubility range of carotenoids in food samples, another solvent may be preferred for solubilization and injection.

Temperature regulation is recommended to maintain within-day and day-to-day reproducibility. Variations in column temperature result in substantial fluctuation of the carotenoid retention times. Temperature may also influence selectivity.

This section describes several liquid chromatographic methods for separating and analyzing carotenoids. The first procedure incorporates a reversed-phase separation using a wide-pore, polymerically synthesized C18 column with visible detection at 450 nm (see Sect. 4.2.1). The first alternate procedure is also isocratic C18 reversed phase but permits simultaneous analysis of retinol, tocopherols, and carotenoids using both a programmable UV–Vis detector and fluorescence detector, or a single diode-array detector (see Sect. 4.2.2). The second alternate procedure is oriented toward more detailed carotenoid analysis of geometric isomers (see Sect. 4.2.3); it incorporates a unique C30 "carotenoid" column with gradient separation and visible detection at 450 nm. The final basic protocol described in this unit is a normal-phase separation, permitting more complete quantitation of xanthophylls and their isomers (see Sect. 4.2.4). The detail preparation of standards for generating a calibration curve was described in Sect. "Standards Preparation and Calibration," while the guidelines for sample preparation based on knowledge of the sample matrix were performed in Sect. "Sample Preparation."

4.2.1 Isocratic Carotenoid Analysis Using Wide-Spore, Polymeric C18

This method requires the least sophisticated equipment and relies heavily on the unique characteristics of the column to separate the carotenoids [95, 96]. It incorporates the use of a polymeric C18 column, which has been shown to offer unique selectivity for structurally similar compounds such as geometric isomers. The addition of a second detector or use of a diode-array detector permits the simultaneous analysis of tocopherols, but not retinol. If the method is modified to incorporate a solvent gradient, retinol can be measured also [104].

HPLC system:

- Column: Vydac 201TP or 218TP C18 column, 5 µm, 250-mm × 4.6-mm (Vydac/Separations Group), Bakerbond WP C18 (J.T. Baker), or HiPore RP 318 (Bio-Rad Laboratories) and guard column containing similar packing material
- Pump: isocratic
- Detector: fixed, variable, programmable, or diode-array (DAD) UV–Vis detector
- Injector: manual

Procedure:

1. Prepare mobile phase by mixing 900 ml HPLC grade methanol, 100-ml HPLC grade acetonitrile, and 1 ml of HPLC grade triethylamine (TEA).
   Note: Triethylamine serves as a modifier to prevent both nonspecific adsorption and oxidation.
2. Degas the mobile phase via vacuum filtration, ultrasonic agitation, or inline vacuum degasser.
3. Set the HPLC pump flow rate at 1.0 ml/min.
4. Set UV–Vis detector at 450 nm (436 nm if using filter photometer).
5. Inject individual standards and the standard mixtures, including any optional internal standard, as described to generate a standard curve (see Sect. "Standards Preparation and Calibration").
6. Inject 10–50 µl of sample (see Sect. "Sample Preparation") and any optional internal standard dissolved in a solvent miscible with the mobile phase (e.g., ethanol, methanol, acetonitrile).

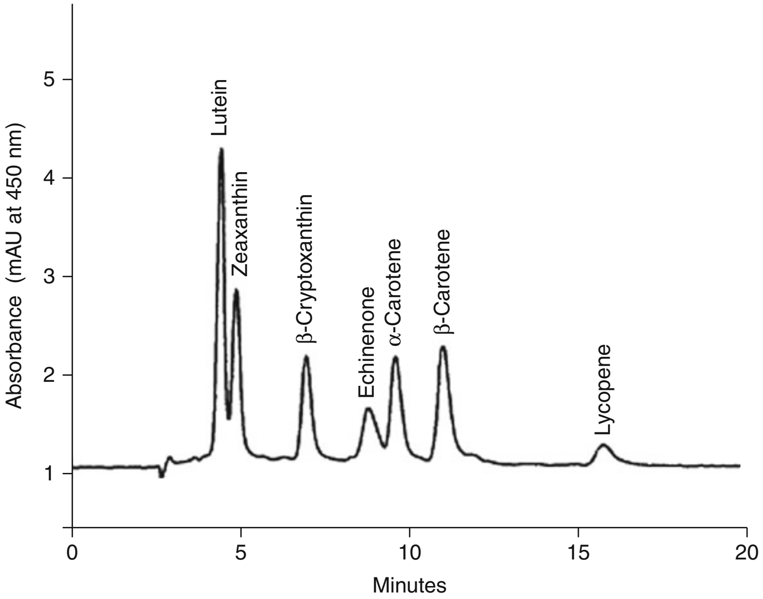

Fig. 111.5 Isocratic HPLC analysis of carotenoid standards. Condition: 5-μm × 250-mm × 4.6-mm Vydac 201TP column, 90:10 methanol/acetonitrile mobile phase, 1.0 ml/min flow rate, visible detection at 450 nm, and column temperature 25°C [84]

Note:

The complete separation from lutein to lycopene requires ~20 min. Figure 111.5 illustrates the separation of carotenoid standards using this system. The elution order using this method (i.e., lutein, zeaxanthin, β-cryptoxanthin, echinenone, α-carotene, β-carotene, lycopene) differs from many other methods.

Carotenoid retention and separation are influenced by column temperature; at temperatures above 20–25°C, lutein and zeaxanthin may not be well separated. Tocopherols can be measured simultaneously by using a diode array detector, a second UV detector set at 280–300 nm, or a fluorescence detector set at 296 nm excitation and 336 nm emission.

7. Calculate the final concentrations of carotenoids in samples by multiplying the peak areas of analytes by the calibration response factors. Apply sample weight and dilution factors to arrive at the concentration of carotenoids in the original sample (i.e., initial concentration).

   Note: The response factor is the ratio of the analyte concentration to the peak area (or height) produced under the defined set of conditions during calibration (e.g., wavelength, solvent composition, column). Typically, data systems will generate response factors for each analyte from calibration data. For example, to calculate the initial concentration, where the sample weight (W_1) is 0.5 g, the initial dilution (D_1) is 25 ml, and the dilution factor for injection (D_2) is 10×.

The final concentration of the injected sample (F_c) equals the response factor (R_f) multiplied by the peak area (P_a or height). The initial concentration (I_c) equals the final concentration multiplied by the initial dilution multiplied by the dilution factor for injection divided by the sample weight ($I_c = [F_c \times D_1 \times D_2]/W_1 = (5.0\ \mu g/ml \times 25\ ml \times I0)/0.5\ g = 2.5\ mg/g$, where $F_c = P_a \times R_f = 25{,}000$ units $\times\ 0.002 = 5.0\ \mu g/ml$).

Standards Preparation and Calibration

Analytical methods are only as good as the initial calibration; therefore, it is essential that the calibration for the accompanying HPLC methods be performed carefully. Carotenoids are labile compounds that are seldom obtained in pure form and degrade readily upon exposure to oxygen and light. Precautions should be taken to minimize standard and sample exposure to UV light and air. Given the above considerations, it is never recommended that carotenoid calibrants be prepared gravimetrically without verification using a spectrophotometer. This can lead to serious errors in quantitation [105]. Many impurities contribute mass but not color to the standards. In addition, carotenoids tend to dissolve slowly in many solvents. Thus, accuracy can be significantly improved by applying absorptivities ($A^{1\%}$) and Beer's Law to filtered solutions of carotenoids to obtain concentration. The value assigned can be further refined by correcting for peak purity. This is accomplished by injecting individual standard solutions into the HPLC column while monitoring the wavelength maximum of each standard. Once the standard solution concentrations have been established, the individual carotenoid standard solutions can be mixed to form calibration solutions. The general procedure is provided below.

Procedure:

1. Dissolve ~1–2 mg lutein, zeaxanthin, β-cryptoxanthin, and other xanthophylls directly in 100 ml reagent alcohol containing 30 ppm butylated hydroxytoluene (BHT). Dissolve ~1–2 mg lycopene, α-carotene, and β-carotene in 10 ml HPLC grade THF stabilized with 250 ppm BHT and then dilute to 100 ml with reagent alcohol.
   Note:
   For normal-phase separations, solutions should be prepared using hexane.
   Crystalline carotenoid standards are available from Sigma, Indofine Chemical, Atomergic Chemetals, Fluka Chemical, Kemin Industries, Wako, and others.
2. Vacuum filter stock solutions through a 0.45-μm membrane to remove any undissolved material.
3. Measure the absorbance of the solutions at the wavelength maximum, as described in Table 111.4, against an appropriate blank (i.e., reagent alcohol) on a spectrophotometer. Dilute appropriately with reagent alcohol to measure between 0.5 AU and 1.0 AU.
4. Inject each standard individually into the appropriate HPLC column (see Sects. 4.2.1 to 4.2.4), monitoring its wavelength maximum to determine the necessary purity correction.

Table 111.4 Commonly used absorptivity values for carotenoids and their corresponding wavelength maxima

Analyte	Wavelength (nm)	Absorptivity ($A^{1\%}$)[a]
α-Carotene	444	2,800
β-Carotene	452	2,592
δ-Carotene	456	3,290
α-Cryptoxanthin	445	2,636
β-Cryptoxanthin	452	2,386
Lutein	445	2,550
Lycopene	472	3,450
Neoxanthin	439	2,243
Phytoene	285	1,250
Phytofluene	347	1,577
Violaxanthin	443	2,250
Zeaxanthin	452	2,350

[a] Ref. [84]. For some carotenoids, $A^{1\%}$ is provided in petroleum ether or hexane and is not significantly different from those in ethanol

For example, if the area of the analyte peak constitutes 90% of the total peak areas, then the concentration calculated using spectrophotometric absorbance is adjusted to 90%.

5. (Optional) Inject the internal standard(s) to determine if there are any degradation products or impurities that may co-elute and absorb at the wavelength of the analytes and to determine the purity correction of the internal standard.
   Note: An internal standard is a compound that is not present in the sample, but is chemically and physically similar to the analytes of interest. A fixed quantity is incorporated into the calibration solutions. The same concentration of internal standard is added to the samples during extraction to compensate for analyte recovery and injection variability. As seen in Fig. 111.5, Echinenone, which is not typically found in natural products, is used as the internal standard. Unfortunately, compounds which may be used as internal standards for carotenoid analysis are not readily available commercially.
6. Mix standards in the range expected for each analyte in the sample matrix (see Table 111.5), optionally adding a fixed amount of internal standard. Dilute to a known volume (usually 5 ml) with ethanol to provide the desired final concentration.
   Note: A minimum of three concentrations should be prepared although five concentrations weighted toward the lower end are preferred. Total carotenoids in any single solution should not exceed 20 μg/ml. For normal-phase separations use hexane rather than ethanol for dilution.
7. Inject mixture (volume as recommended in the procedures for the particular column and conditions; see Sects. 4.2.1 to 4.2.4) and generate calibration curves.
   Note:
   Most HPLC instruments include computer data systems which automatically plot peak response versus concentration to generate response factors; however;

Table 111.5 Calibration range for carotenoids

Analyte	Range (μg/ml)[a]
α-Carotene	0.05–5.0
β-Carotene	0.05–10.0
β-Cryptoxanthin	0.05–5.0
Lutein	0.05–10.0
Lycopene	0.05–10.0
Zeaxanthin	0.05–5.0

[a] Ref. [84]

if using an older system, the standard curve can be plotted manually. The calibration curves should be linear with a correlation coefficient of >0.98 and intersect very near the origin.

Sample Preparation

Natural materials are variable and complex matrices. Knowledge of the sample matrix is critical for accurate carotenoid quantification. The type and chemical form of carotenoids and the composition of the natural products matrix are critical to the amount of sample preparation that is necessary prior to sample analysis. Many factors regarding the natural products matrix must be considered for efficient carotenoid extraction. For instance, the relative content of lipid to carotenoid in the natural products matrix influences the method of sample preparation. If both the lipid and carotenoid content are high, it may be possible to dilute the sample in an organic solvent that is miscible with the HPLC mobile phase for direct injection; however, when the lipid content of the sample is high and the carotenoid content is low, saponification is useful to separate the lipid (primarily triglycerides) from the carotenoids. Another factor is the form of the carotenoid that is present in the sample. Carotenes (hydrocarbon carotenoids) do not form ester linkages and can be directly extracted by homogenizing in the presence of lipophilic solvents (e.g., hexane, ethyl acetate, and toluene); however, the xanthophylls frequently form esters which will readily extract into lipophilic solvents. The xanthophylls are more easily quantified in the free form, which requires hydrolysis. Saponification (alkaline hydrolysis) is necessary to remove chlorophylls that are present in many foods because they can interfere with the detection of carotenoids. In other words, there is not one given sample preparation that will apply to all natural products; therefore, to provide general guidelines, sample preparation has been broken into the following three categories: (1) oil-based natural product samples containing only hydrocarbon carotenoids or nonesterified xanthophylls that are visibly yellow to red in color (e.g., palm oil), (2) direct extraction for natural product samples that do not contain xanthophyll esters or chlorophylls and have a low lipid and high carotenoid content (e.g., carrots), and (3) saponification for natural product samples containing xanthophyll esters, chlorophylls, or high lipid and low carotenoid content (e.g., spinach).

Procedure:

For oil-based natural products samples containing only hydrocarbon carotenoids or nonesterified xanthophylls that are visibly yellow to red in color (e.g., palm oil)

1a. Weigh 0.5–5.0 g of sample.

2a. Dissolve in 25 ml of HPLC grade THF stabilized with 250 ppm BHT and then dilute to volume in a 50-ml volumetric flask. Dilute the sample further with reagent alcohol until the carotenoid concentration is ~5–10 mg/l.

Note: The carotenoid concentration is approximated by diluting and measuring the absorbance at 450 nm on a spectrophotometer where 1 AU = ~4 mg/l

3a. (Optional) Filter through 0.45-μm filter prior to injection.

Note: If the sample is free of particulate, it may be directly injected into the HPLC column for analysis.

For direct extraction of natural products samples that do not contain xanthophyll esters or chlorophylls, and have a low lipid and high carotenoid content (e.g., carrots)

1b. Homogenize ~5 g of ground sample with 10% (w/w) magnesium carbonate and 25 ml of 50:50 HPLC grade methanol/THF.

Note: Lyophilized and dry samples should be reconstituted with water prior to extraction. They may require saponification if the samples contain chlorophylls or xanthophyll esters. Beadlet materials should be suspended in hot water, saponified, or enzymatically hydrolyzed before extraction. Follow the manufacturer's recommendations before analyzing by HPLC.

2b. Centrifuge in a swinging bucket rotor for 10 min at 6,000 × g, 4°C and remove the supernatant.

3b. Repeat steps lb and 2b until the extracting solvent is colorless.

4b. Combine the extracts and vacuum filter through Whatman paper no. 42 to remove particles.

5b. Dilute to a known volume with reagent alcohol so that the THF represents <10% of the total solution (e.g., if total volume is 90 ml dilute to 500 ml).

6b. (Optional) Filter through 0.45-μm filter prior to injection.

For saponification of natural products samples containing xanthophyll esters, chlorophylls, or high lipid and low carotenoid content (e.g., spinach)

1c. Proceed with a direct sample extraction as instructed in steps 1b–5b.

2c. Transfer a 5-ml aliquot to a 30–50-ml capped tube for saponification.

3c. Add 1 ml of 10% (w/v) pyrogallol in reagent alcohol.

4c. Add 2 ml of 40% (w/v) KOH in methanol.

Note: The final concentration of reagents in the mixed solution should be ~5–10% KOH and >1% pyrogallol.

5c. Flush the tubes with nitrogen gas and cap.

6c. Saponify the samples at 60°C for 1 h in a shaking water bath or 30 min in an ultrasonic bath.

7c. After saponification, dilute the samples with 8 ml of saturated NaCl solution.

8c. Extract by vigorous mixing with 10 ml 75:25 hexane/THF.

Note: Free xanthophylls, both endogenous and present in the saponified samples, are more polar and extract less eficiently into lipophilic solvents.

Frequently, the addition of a polar organic solvent (tetrahydrofuran, methylene chloride, diethyl ether) is required to thoroughly extract them from the sample matrix and aqueous phase.

9c. Remove the upper phase.

10c. Repeat the extraction step until the upper phase is colorless.
Note: For some samples the phases will not separate on standing and may require centrifugation to break emulsions.

11c. Wash the combined extract with water to remove traces of KOH and pyrogallol that may have been co-extracted.

12c. Place 3-in. solid sodium sulfate in the bottom of a 1.5 × 12-in. glass column plugged with sintered glass frit or plugged with glass wool. After removing the water, pass the extract through the sodium sulfate to remove any traces of water remaining.

13c. Remove the solvent from the extract using a solvent evaporation apparatus.

14c. Dissolve residue in a known volume of reagent alcohol to yield 5–20 mg/l (an absorbance of 1.5–5.0 AU at 450 nm).
Note: Samples extracted into strong organic solvents (hexane, ether, petroleum ether, ethyl acetate, etc.) must be transferred into a solvent miscible with the mobile phase. A small volume (e.g., 1 ml) of the organic extract should be evaporated under N_2 gas and dissolved in reagent alcohol. Further dilutions with alcohol may be necessary to obtain 5–10 mg/l concentration before HPLC injection.

15c. (Optional) Filter through 0.45-μm filter prior to injection.
Note: If this sample is free of particulate, it may be directly injected into the HPLC column for analysis.

4.2.2 Isocratic Carotenoid Analysis Capable of Simultaneous Separation of Retinol and Tocopherol Using Spherisorb ODS2

This method is the simplest approach for simultaneous carotenoid, retinol, and tocopherol analysis. Both the column and mobile phase have been chosen to provide efficiency and selectivity for the analysis of these components without the use of a gradient. The method uses the Spherisorb ODS2 column, in which the C18 chain is monomerically bound to the silica particles (i.e., the C18 chain binds at one site to the silica particles).

HPLC system (see Sect. 4.2.1):

- Column: 3-μm × 150-mm × 4.6-mm Spherisorb ODS2 (ES Industries, Phenomenex, or Waters) and guard column containing similar packing material

Procedure:

1. Prepare mobile-phase solution:
   - Dissolve 0.385 g ammonium acetate in 50 ml methanol.
   - Mix 830 ml acetonitrile, 130 ml *p*-dioxane, and 1 ml triethylamine (TEA).
     Note: TEA serves as a modifier to prevent both nonspecsific adsorption and oxidation.

- While stirring, slowly add 40 ml methanol/ammonium acetate solution prepared in step 1.
- Vacuum filter through 0.45-μm PTFE or polypropylene membrane.

2. Set the HPLC pump flow rate at 1.5 ml/min.
3. Set UV–Vis detector at 450 nm (436 nm if using filter photometer).
4. Inject 10–30 μl individual standards and the standard mixtures, including any optional internal standard, as described to generate a standard curve (see Sect. "Standards Preparation and Calibration").

   Column temperature should be maintained at a fixed temperature between 22°C and 29°C in either a room with well regulated temperature, or a column oven for above ambient temperatures.

   If the injection solvent is of comparable solvent strength to that of the mobile phase, up to 30 μl may be used as the sample injection volume; however, if the injection solvent is more lipophilic than the mobile phase, the injection volume is limited to 10 μl.
5. Inject 10–30 μl standard or sample dissolved in a solvent miscible with the mobile phase (e.g., methanol, acetonitrile).

   The complete separation from retinol to β-carotene requires ~15 min. Figure 111.6 illustrates the separation of vitamins and carotenoids in the mixed natural products extract using this liquid chromatography (LC) system. The elution order using this method is: lutein, zeaxanthin, β−cryptoxanthin, lycopene, α−carotene, and β−carotene.
6. Detection:
   (a) For carotenoids: Monitor 450 nm.
   (b) For retinol and carotenoids: Monitor 325 nm for 3.2 min, then program the detector to change the wavelength to 450 nm.
   (c) For tocopherols (optional): Place fluorescence detector after UV–Vis detector and monitor excitation at 296 nm and emission at 336 nm.
   (d) For diode array detectors (optional): monitor 296 nm for tocopherols, 325 nm for retinol, and 450 nm for carotenoids.
   7. Calculate the final concentrations as described above (see 4.2.1).

4.2.3 Gradient HPLC Analysis Using C30 Carotenoid Column

This method requires the most sophisticated equipment and yields the most detailed results by utilizing the C30 column that was created specifically for carotenoid separation. The C30 column is polymerically bonded, yielding selectivity similar to the polymeric C18. The column has an intermediate pore diameter (200 Å) and a 30 carbon alkyl chain, which results in higher carbon content and therefore stronger retention of the carotenoids. Due to the strong retention of carotenoids on this column, a gradient must be employed unless the sample is previously fractionated or only contains a specific group of carotenoids. It is possible to separate a wide polarity range of carotenoids and their isomers.

HPLC system:

- Pump: ternary gradient
- Column: 3-μm × 250-mm × 4.6-mm C30 with 3-μm guard column (water)

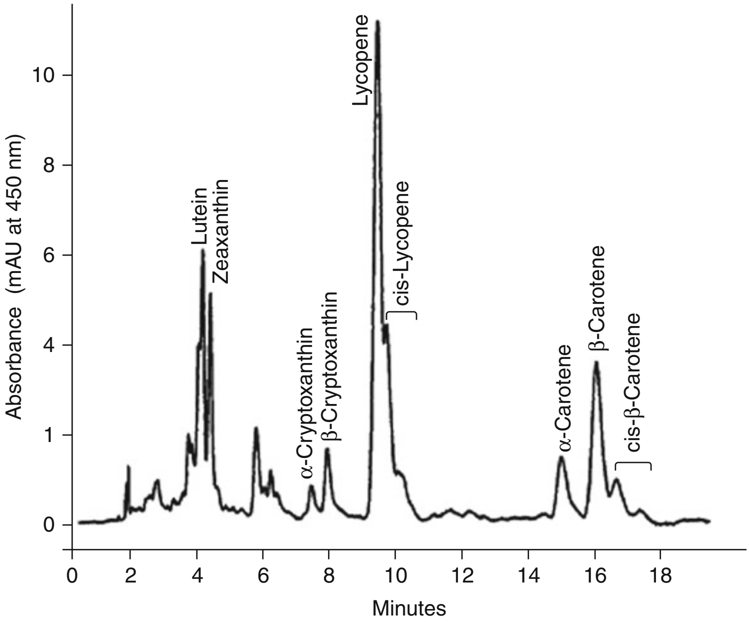

Fig. 111.6 Isocratic HPLC analysis of the natural material carotenoids using procedure 4.2.2. Conditions: Spherisorb 3-μm × 150-mm × 4.6-mm ODS2 column, 83:13:4:0.1 acetonitrile/dioxane/150 mM ammonium acetate in methanol/TEA mobile phase, 1.5 ml/min flow rate, visible detection at 450 nm, and column temperature 29°C [84]

- Column oven: 35°C
- Mobile phase:
  - Solvent A: 0.05% TEA and 50 mM ammonium acetate in methanol
  - Solvent B: 0.05% TEA in isopropyl alcohol
  - Solvent C: 0.05% TEA in HPLC grade THF stabilized with 250 ppm BHT

Procedure:

1. Set the HPLC pump flow rate at 1.0 ml/min.
2. Set UV–Vis detector at 450 nm (436 nm if using filter photometer).
3. Inject 10–50 μl individual standards and the standard mixtures, including any optional internal standard as described to generate a standard curve (see Sect. "Standards Preparation and Calibration").
4. Inject 10–50 μl sample (see Sect. "Sample Preparation") dissolved in a solvent miscible with the mobile phase (e.g., ethanol, 90% ethanol/10% isopropanol). Note: The complete separation from lutein to lycopene requires ~40 min with an additional 15 min to equilibrate the column back to the initial mobile phase. Figure 111.7 illustrates the separation of carotenoid in the mixed natural products extract using this LC system. The elution order using this method difers from many other methods: lutein, zeaxanthin, β-cryptoxanthin,

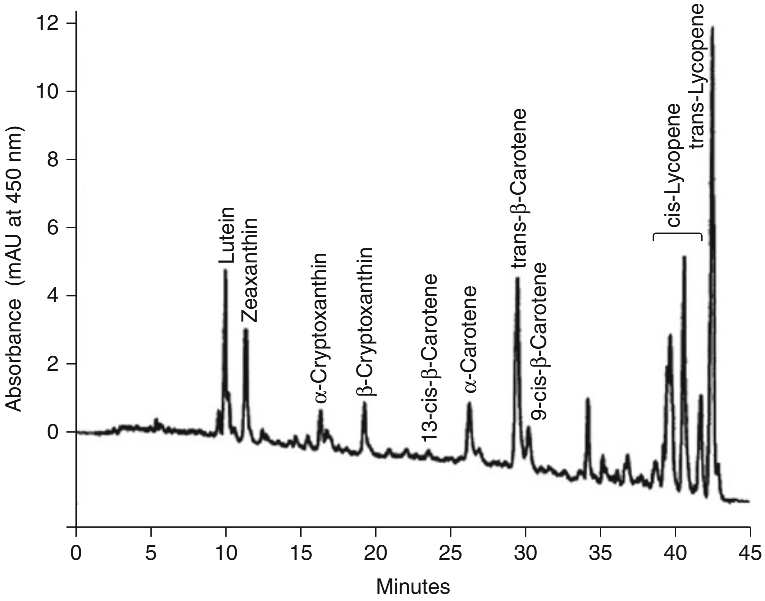

Fig. 111.7 Gradient HPLC analysis of the natural products reference material carotenoids using procedure 4.2.3. Conditions: 3-μm × 250-mm × 4.6-mm waters C30 column, 1.0 ml/min flow rate, visible detection at 450 nm, column temperature 35°C, solvent A = 50 mM ammonium acetate in methanol, B = isopropyl alcohol, and C = THF (all solvent contain 0.1% TEA). Flow program: 90% A/10% B linear gradient, 54% A/35% B/11% C over 24 min, linear gradient to 30% A/35% B/35% C over 11 min, hold for 8 min, and then return to initial conditions over 10 min [84]

15-*cis*-β-carotene, 13-*cis*-β-carotene, αcarotene, *trans* β−carotene, 9-*cis*-β-carotene, *cis*-lycopene, and *trans*-lycopene.

5. Prepare gradient:

   Starting with 90% solvent A/10% solvent B, establish a linear gradient over 24 min to 54% solvent A/35% solvent B/11% solvent C, followed by a second linear gradient over 11 min to 30% solvent A/35% solvent B/35% solvent C. Hold 5 min, return to initial conditions over 10 min. Hold 5 min before next injection.

   Note: Triethylamine in the solvent serves as a modifier to prevent both non-specific adsorption and oxidation.

6. Detection:
   - For carotenoids: Monitor 450 nm.
   - For tocopherols (optional): Place fluorescence detector after UV–Vis detector and monitor excitation at 296 nm and emission at 336 nm.
   - For a diode array detector (optional): Monitor 296 nm for tocopherols, 325 nm for retinol, and 450 nm for carotenoids.

7. Calculate the final concentrations as described above (see Sect. 4.2.1, step 7).

4.2.4 Normal-Phase Analysis of Xanthophylls

In normal-phase chromatography, polar components are more strongly retained than nonpolar components. Thus, hydrocarbon carotenes elute quickly while xanthophylls are retained and separated. This approach provides a more complete separation of polar carotenoids and their geometric isomers. This procedure is useful to the analyst that is specifically interested in the xanthophyll fraction of a sample.

HPLC system (see Sect. 4.2.1)

- Column: 5-μm × 250-mm × 4.6-mm Lichrosorb Si column (ES Industries, Phenomenex, or EM Science) and guard column containing similar packing material

Procedure:

1. Prepare mobile phase by mixing 800 ml hexane, 200 ml dioxane, 15 ml indole-3-propionic acid (IPA), and 2.0 ml TEA.
   Note: TEA serves as a modifier to prevent both nonspecific adsorption and oxidation.
2. Degas the mobile phase via vacuum filtration, ultrasonic agitation, or inline vacuum degasser.
3. Set the HPLC pump flow rate at 1.0 ml/min.
4. Set UV–Vis detector at 450 nm (436 nm if using filter photometer).
5. Inject 10–100 μl individual standards and the standard mixtures, including any optional internal standard as described to generate a standard curve (see Sect. "Standards Preparation and Calibration").
6. Inject 10–100 μl of standard (see Sect. "Standards Preparation and Calibration") or sample, including any optional internal standard, (see Sect. "Sample Preparation") dissolved in hexane.
   Note: The complete separation from β-carotene to violaxanthin requires ~35 min. Figure 111.8 illustrates the separation of carotenoids in a mixed natural products extract using this LC system. The hydrocarbon carotenes (β-carotene, α-carotene, lycopene) elute together at the solvent front. The elution order is: β-cryptoxanthin, α-cryptoxanthin, lutein, *cis*-lutein, zeaxanthin, *cis*-zeaxanthin, neoxanthin, and violaxanthin.
7. Calculate the final concentrations as described above (see Sect. 4.2.1, step 7).

4.3 Mass Spectrometry of Carotenoids

The high sensitivity and selectivity of mass spectrometry (MS) facilitates the identification and structural analysis of small quantities of carotenoids that are typically obtained from natural product samples such as plants, animals, or human serum and tissue. Structural information from the abundant fragmentation is provided by classical ionization methods, such as electron impact (EI; see Sect. 4.3.1) and chemical ionization (CI; see Sect. 4.3.1), but molecular ions are not always observed. Recent advances in soft ionization techniques, such as fast

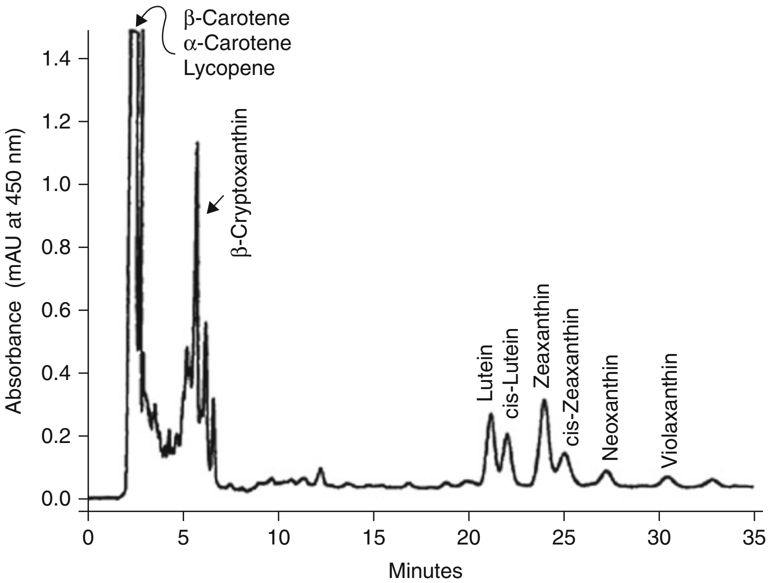

Fig. 111.8 Isocratic HPLC analysis of the natural products reference material carotenoids using procedure 4.2.4. Conditions: Lichosorb Si60, 5-μm × 250-mm × 4.6-mm column, hexane/dioxane/IPA/TEA (80:20:0.15:0.02) mobile phase, 1.0 ml/min flow rate, and visible detector at 450 nm [84]

atom bombardment (FAB; see Sect. 4.3.2), matrix-assisted laser desorption/ionization (MALDI; see Sect. 4.3.3), electrospray ionization (ESI; see Sect. 4.3.4), and atmospheric pressure chemical ionization (APCI; see Sect. 4.3.5), have facilitated the molecular weight determination of carotenoids by minimizing fragmentation that is typical of EI and CI. Once the molecular weight of a carotenoid has been established using one of these ionization techniques, collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS) can be used to augment fragmentation and obtain structurally significant fragment ions that may aid in the differentiation of structural isomers, such as differentiation of lutein from zeaxanthin or of α-carotene from β-carotene and lycopene. CID and MS/MS parameters are independent of the ionization step so that no modifications of the sample preparation and ionization procedures are needed. Although CID and MS/MS can be used with any ionization technique, the application of this approach is illustrated using FAB ionization in Sect. 4.3.2.

MS can be coupled to high-performance liquid chromatography (HPLC) to obtain separation of isomeric carotenoids or to remove interfering contaminants prior to ionization and detection. Except for MALDI, every ionization method discussed in this unit has been utilized during liquid chromatography MS (LC/MS; see Sects. 4.3.1–4.3.2 and Sect. 4.3.5). However, LC-APCI-MS is now the preferred approach due to its widespread availability and ease of use. Although LC/MS and LC/MS/MS (see Sect. 4.3.2) are routinely carried out, gas chromatography MS (GC/MS) is rarely used because carotenoids typically decompose when exposed to the high temperatures of the GC process. Reviews of LC/MS and MS of carotenoids have been published by van Breemen [106, 107]. A general introduction to mass spectrometry is given in Watson [108].

4.3.1 Electron Impact and Chemical Ionization Mass Spectrometry of Carotenoids

Most of the original structural elucidation studies of the >600 known carotenoids used El and CI [109]. Electron impact (EI) and chemical ionization (CI) MS have been used for carotenoid analysis for more than 30 years. Therefore, mass spectra of unknown carotenoids can be compared to a large number of published mass spectra to aid in identification. Unlike the newer "soft" ionization techniques in MS, EI and CI produce considerable fragmentation, and molecular ions are not always observed. Therefore, these techniques are most useful for obtaining fingerprints, or characteristic fragmentation patterns, instead of confirming or determining molecular weights of carotenoids. Alternatively, direct exposure EI (DEI) and/or direct exposure CI (DCI) MS can be used.

EI/CI-MS system:

- Reagent gas: methane or isobutane (for CI only)
- Mass spectrometer equipped with direct insertion probe and EI and/or CI ionization

Procedure:

1. Using a microsyringe, load 1 μl carotenoid sample onto a direct insertion probe of a mass spectrometer.
   Note: Carotenoid sample (1–100 mg/l; 2–200 μM) is dissolved in volatile organic solvent (e.g., hexane, THF, methyl-tert-butyl ether, acetone), stored in an airtight glass vial.
2. Let solvent evaporate and then insert probe into the ion source of the mass spectrometer.
3. Introduce reagent gas (for CI only) and then turn on the electron beam (typically 70 eV for EI and 200 eV for CI).
4. Heat probe to vaporize carotenoid.
5. Record mass spectrum over the range m/z (mass-to-charge ratio) 50–800 for EI and m/z 100–800 for CI.
   Note: All known carotenoids and their major fragment ions should be included in this range. By beginning the scan at m/z 100 during CI, reagent-gas ions can be avoided.

4.3.2 Fast Atom Bombardment, Liquid Secondary Ion Mass Spectrometry, and Continuous-Flow Fast Atom Bombardment of Carotenoids

Fast atom bombardment MS (FAB-MS) and liquid secondary ion MS (LSIMS) are matrix-mediated desorption techniques that use energetic particle bombardment to simultaneously ionize samples such as carotenoids and transfer them to the gas

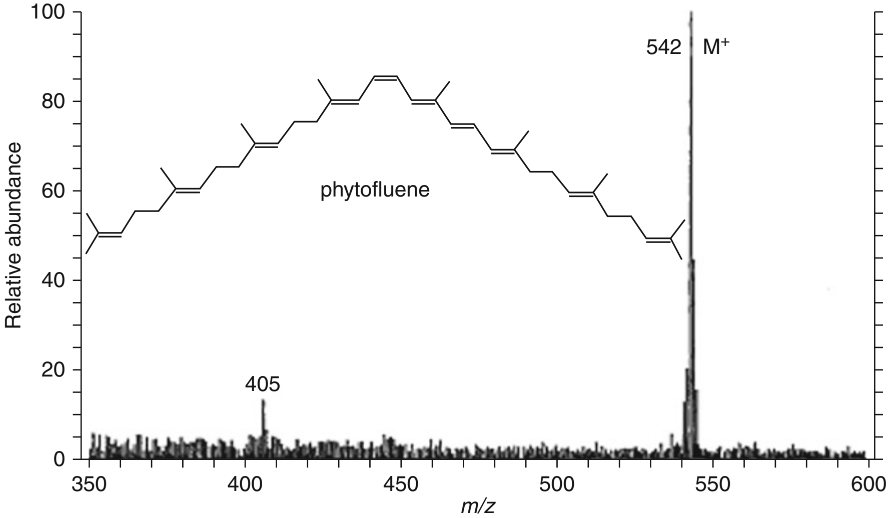

Fig. 111.9 Positive ion fast atom bombardment (FAB-MS) mass spectrum of phytofluene isolated from blueberries. The base peak of m/z 542 corresponds to the molecular ion. Characteristic of FAB-MS, background signals are observed at every m/z value. The mass spectrum was obtained during continuous-flow FAB-MS LC/MS using a magnetic sector mass spectrometer. Although the 16-*cis* isomer of phytofluene is shown, the FAB mass spectra of all-trans and other cis isomers are indistinguishable [84]

phase for mass spectrometric analysis. Unlike with the EI and CI techniques, molecular ions are usually abundant and fragmentation is minimal. Figure 111.9 shows the positive ion FAB-MS mass spectrum of carotene phytofluene. The base peak of m/z 542 corresponds to the molecular ion.

FAB-MS system:

- Mass spectrometer or tandem mass spectrometer equipped for fast atom bombardment (FAB)-MS or liquid secondary ion (LSI)-MS, with direct insertion probe and with continuous-flow ionization source (as needed)
- Syringe pump or HPLC pump capable of delivering flow rates of 1–10 μl/min (for continuous-flow only)
- Reversed-phase HPLC (typically C18 or C30) column (for continuous-flow FAB-MS or LSIMS)

Procedure:

For probe analysis:

1a. Load 1 μl of 3-nitrobenzyl alcohol onto a direct insertion probe and then use a microsyringe to load 1 μl carotenoid sample onto the surface of the liquid matrix. Note: Carotenoid sample (1–100 mg/l; 2–200 μM) is dissolved in volatile organic solvent (e.g., hexane, THF, methyl-tert-butyl ether, acetone), stored in an airtight glass vial.

2a. Let solvent evaporate and then insert probe through the vacuum interlock into the ion source of a mass spectrometer or tandem mass spectrometer.

3a. Turn on FAB-MS or LSIMS beam and record the positive ion mass spectrum over the range m/z (mass-to-charge ratio) 50–900.

Note: If desired, the peaks at m/z 154 and m/z 307 for the protonated monomer and dimer of the matrix, 3-nitrobenzyl alcohol, can be eliminated by scanning from m/z 310–900.

For continuous-flow analysis:

1b. Set up a syringe pump or an HPLC pump and equilibrate a reversed-phase HPLC column with appropriate eluents such as methanol and methyl-tert-butyl ether (e.g., 70:30, v/v).

Note: A reversed-phase HPLC column (typically C18 or C30) is required for HPLC separations. Because the flow rate into the continuous-flow FAB-MS or LSIMS source must be 10 μl/min, either a capillary column must be used or else the flow must be split postcolumn. For narrow-bore HPLC columns operated at 200 μl/min, the split ratio would be 30:1. Isocratic or gradient analysis may be used. A syringe pump is usually necessary for capillary columns, but standard HPLC pumps are suficient for applications using narrowbore columns.

2b. Add the matrix, ~0.1% (v/v) 3-nitrobenzyl alcohol prepared in appropriate mobile phase, postcolumn at a flow rate of ~1–3 μl/min.

3b. Interface the continuous-flow probe to a mass spectrometer. Tune the continuous-flow FAB-MS or LSIMS ion source on the 3-nitrobenzyl alcohol dimer ion at m/z 307.

4b. Inject a carotenoid sample onto the HPLC column, turn on the FAB-MS or LSIMS beam, and record the positive ion mass spectrum over the range m/z 300–1,000.

Note: An injection volume of 15 μl is typical for narrow-bore columns.

4.3.3 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry of Carotenoids

A matrix-mediated ionization technique, matrix-assisted laser desorption/ionization time-of-flight (MALDI/TOF) MS, uses an intense flash of laser light to vaporize a solid matrix containing the sample. Although usually regarded as an ionization method reserved for high-mass compounds such as proteins and polymers, MALDI has shown remarkable promise for the analysis of carotenoids. In particular, MALDI has been effective in the ionization of intact esterified carotenoids that would fragment too extensively using other ionization methods. Figure 111.10 shows the mass spectrum profile of nonpolar carotenoids extract from Tangela ripe tomato fruit. The spectrum revealed a collection of m/z ranged from 536 to 545. Although overlap between the isotopic species occurs, the monoisotopic peaks correspond to pro-lycopene ($C_{40}H_{56}$; m/z detection, 536.426; theoretical, 536.438), neurosporene ($C_{40}H_{58}$; m/z detection, 538.447; theoretical,

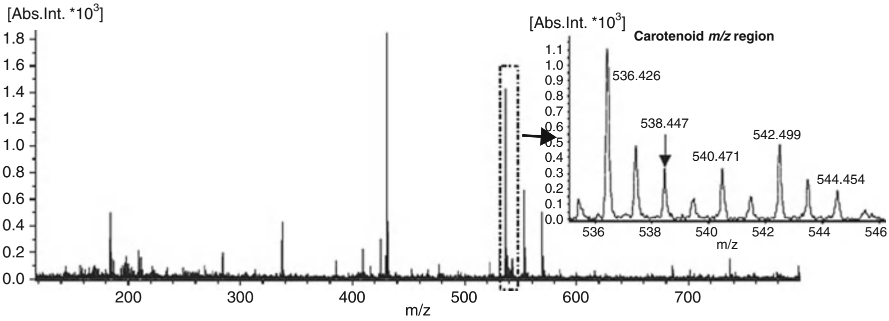

Fig. 111.10 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI/TOF-MS) profiles of nonpolar extracts in ethyl acetate (1 mg/ml) from Tangella ripe tomato fruit. MALDI/TOF-MS spectra was acquired with a Bruker MALDI/TOF-MS reflex III operated in the positive reflectron mode. A nitrogen laser emitting 337 nm was used to generate ions. A source voltage of 25 kV was used, and 20.7-kV extraction voltage and no ion suppression were employed. Scans were extended to 3,000 m/z [110]

538.453), ζ-carotene ($C_{40}H_{60}$; m/z detection, 540.471; theoretical, 540.469), phytofluene ($C_{40}H_{62}$; m/z detection, 542.499; theoretical, 542.485), and phytoene ($C_{40}H_{64}$; m/z detection, 544.454; theoretical, 544.500).

MALDI/TOF-MS system:

- Matrix-assisted laser desorption ionization time-of-flight (MALDI/TOF) mass spectrometer with UV laser (i.e., 337-nm nitrogen laser), MALDI probe, and optional delayed extraction and postsource decay

Procedure:

1. Mix 20 µl carotenoid sample with 10 µl acetone saturated with 2,5-dihydroxybenzoic acid as sample matrix.
2. Using a microsyringe, load 5–10 µ1 carotenoid/matrix sample onto the target of a MALDI probe.
3. Let solvent evaporate (only a few seconds are required) and then insert probe through the vacuum interlock into the ion source of a MALDI/TOF mass spectrometer.
4. Record MALDI/TOF mass spectra in positive ion mode. Look for molecules ions and protonated molecules in the range m/z 300–1,000.

4.3.4 Electrospray Ionization Liquid Chromatography/Mass Spectrometry of Carotenoids

Unlike other LC/MS techniques in which removal of the mobile phase and sample ionization are discrete steps, electrospray is both an ionization method and an interface between an HPLC system and a mass spectrometer. Electrospray is also one of the most universal ionization techniques for MS, as virtually every class of compound has been analyzed using this technique, including carotenoids.

For flow-injection analysis of carotenoids, ~1 μl of a 0.1–100 μM carotenoid sample can be injected into a solvent stream and carried into the electrospray source without chromatography. This approach is particularly useful for high-throughput analysis of pure samples and for tuning and optimizing the parameters of the electrospray source. The sample should be dissolved in a volatile organic solvent (e.g., hexane, THF, methyl-tert-butyl ether, acetone, methanol) but may contain water. The sample should be stored in an airtight glass vial. A narrow scan range (e.g., m/z 520–620 for compounds such as β-carotene and lutein) or selected ion monitoring of the molecular ions (e.g., m/z 536 and 568 for β-carotene and lutein, respectively) is usually used during flow-injection analysis.

When HPLC is used as part of the analysis, the mobile phase is typically a mixture of methanol and methyl-tert-butyl ether (i.e., 50:50, v/v), although other HPLC solvents for LC/MS using electrospray (e.g., water, THF) can be used. It is important to note that entirely organic solvent systems might pose a fire hazard for some home-built ion sources and some older commercial instruments if air leaks into the ionization chamber. Therefore, water or a halogenated solvent should be added to the mobile-phase postcolumn to suppress ignition. The electrospray source must always be vented outside the laboratory. The scan range is typically m/z 300–1,000 in order to include known carotenoids and this esters.

When tuning the electrospray source of a mass spectrometer or tandem mass spectrometer, optimum sensitivity for carotenoids will be obtained using the highest possible voltage on the electrospray needle before corona discharge occurs. For example, an electrospray needle voltage of −5,100 V provided excellent sensitivity in one published report [111]. Because not all electrospray ion sources support voltages this high, the sensitivity might be lower in other systems. A reversed-phase HPLC C18 or C30 column is typically used for LC/MS with electrospray ionization (ESI). The flow rate into the mass spectrometer, as controlled by a syringe pump or HPLC pump, should be matched to the design of the system, but in most current commercial systems, the flow rate can be varied between 1 μl/min and 1,000 μl/min. Microbore, narrow-bore, or analytical-scale columns at flow rates from 1 μl/min to 1,000 μl/min are compatible with most LC/MS electrospray interfaces.

2,2,3,4,4,4-Heptafluoro-1-butanol can be added postcolumn to give a final concentration of 0.1% (v/v) to enhance ionization efficiency during electrospray. Typically, a 2% (v/v) solution in mobile phase is added at 50 μl/min to the HPLC column effluent at 1 ml/min. Addition of this reagent is optional.

Because of the efficiency of the solvent removal in the LCMS interface and the flexibility of the ESI process, ESI is compatible with a wide range of HPLC flow rates (from 0.1 ml/min to 1 ml/min) and with a variety of mobile phases including the methanol/methyl-*tert*-butyl ether solvent system that is ideal for analysis using C30 carotenoid columns. A potential limitation of ESI for quantitation is its relatively narrow dynamic range (approximately two orders of magnitude). Aside from this narrow range of linear response during quantitative analysis, ESI shows excellent sensitivity and is compatible with HPLC using a wide range of solvents and flow rates. An example of the positive ion LC-ESI-MS analysis of carotenes in an extract of human plasma is shown in Fig. 111.11.

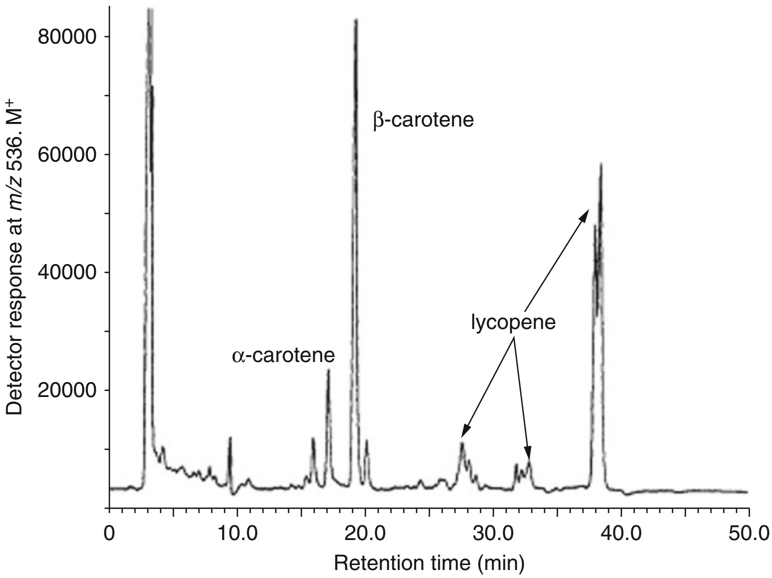

Fig. 111.11 Liquid chromatography/mass spectrometry (LC/MS) analysis of isomeric carotenes in a hexane extract from 0.5 ml human serum. Positive ion ESI-MS was used on a quadrupole mass spectrometer with selected ion monitoring to record the molecular ions of lycopene, β-carotene, and α-carotene at m/z (mass-to-charge ratio) 536. A C30 HPLC column was used for separation with a gradient from methanol to methyl-tert-butyl ether. The all-trans isomer of lycopene was detected at a retention time of 38.1 min, and various cis isomers of lycopene eluted between 27 min and 39 min. The all-trans isomers of α-carotene and β-carotene were detected at 17.3 min and 19.3 min, respectively [84]

4.3.5 Atmospheric Pressure Chemical Ionization Liquid Chromatography/Mass Spectrometry of Carotenoids

Most mass spectrometers equipped for electrospray ionization can be converted to atmospheric pressure chemical ionization (APCI), and many commercial LC-APCI-MS instruments are equipped with both ionization techniques. During APCI, ionization takes place in an atmospheric pressure chamber when the sample molecules collide with solvent ions formed in a continuous corona discharge. Unlike electrospray, the needle used to spray the HPLC effluent is not at high voltage.

For flow-injection analysis of carotenoids, ~1–5 μl of a 0.1–100 μM carotenoid sample, which may contain water, can be injected into a solvent stream and carried into the APCI source without chromatography. This approach is particularly useful for high-throughput analysis of pure samples and for tuning and optimizing the parameters of the APCI source. The sample should be dissolved in a volatile organic solvent (e.g., hexane, THF, methyl-tert-butyl ether, acetone, methanol) but may contain water. The sample should be stored in an airtight glass vial. A narrow scan range of m/z 520–620 is appropriate for most carotenoids such as

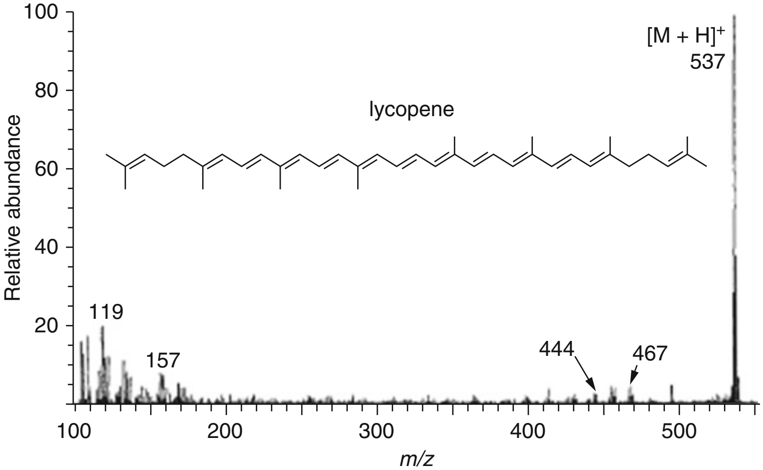

Fig. 111.12 Flow-injection positive ion atmospheric pressure chemical ionization (APCI) mass spectrum of −1 pmol lycopene. The carrier solvent for flow-injection analysis consisted of methanol/methyl-tert-butyl ether (5,050; v/v) at a flow rate of 200 μl/min. The lycopene standard was isolated from tomatoes. The all-trans isomer of lycopene is shown, which is the most abundant isomer found in the tomato. This carotene is the familiar red pigment of the tomato [112]

β-carotene and lutein. Alternatively, selected in monitoring of the molecular ions or protonated or deprotonated molucules may be used.

When HPLC is used as part of the analysis, the mobile phase is typically a mixture of methanol and methyl-tert-butyl ether (i.e., 50:50, v/v), although other HPLC solvents for LC/MS using APCI (e.g., water, THF) can be used. It is important to note that if combustible nonaqueous solvent systems are used, water or a halogenated solvent such as methylene chloride or chloroform should be added to the mobile-phase postcolumn to suppress ignition in the ion source. In addition, the APCI source must be vented outside the laboratory and should not allow air into the ionization chamber. A scan range of m/z 300–1,000 will include the known carotenoids and their most common esters.

A reversed-phase HPLC C18 or C30 narrow-bore column is typically used for LC/MS with APCI. For most APCI systems, the optimum flow rate into a mass spectrometer or tandem mass spectrometer equipped with APCI, as controlled by a syringe pump or HPLC pump, is usually between 100 μl/min and 300 μl/min, which is ideal for narrow-bore HPLC columns. Larger diameter columns should be used with a flow splitter postcolumn to reduce the solvent flow into the mass spectrometer. For example, if a 4.6-mm i.d. column was used at a flow rate of 1.0 ml/min, then the flow must be split postcolumn ~5:1 so that only 200 μl/min enters the mass spectrometer.

Carotenoids form both molecular ions and protonated molecules during positive ion APCI and molecular ions and deprotonated molecules during negative ion analysis. The relative abundances of molecular ions and protonated or deprotonated molecules vary with the mobile-phase composition [112]. For example, polar

Table 111.6 Elemental composition and exact masses[a] of common carotenoids [109]

Carotenoid	Elemental composition	Exact mass
β-Apo-8′-carotenal	$C_{30}H_{40}O$	416.3079
3-Hydroxy-β-apo-8′-carotenal	$C_{30}H_{40}O_2$	432.3028
α-Carotene	$C_{40}H_{56}$	536.4382
β-Carotene	$C_{40}H_{56}$	536.4382
γ-Carotene	$C_{40}H_{56}$	536.4382
Lycopene	$C_{40}H_{56}$	536.4382
Neurosporene	$C_{40}H_{58}$	538.4539
ζ-Carotene	$C_{40}H_{60}$	540.4695
Phytofluene	$C_{40}H_{62}$	542.4852
Phytoene	$C_{40}H_{64}$	544.5008
2′,3′-Anhydrolutein	$C_{40}H_{54}O$	550.4175
Echinenone	$C_{40}H_{54}O$	550.4175
α-Cryptoxanthin	$C_{40}H_{56}O$	552.4331
β-Cryptoxanthin	$C_{40}H_{56}O$	552.4331
Alloxanthin	$C_{40}H_{52}O_2$	564.3967
Cantaxanthin	$C_{40}H_{52}O_2$	564.3967
Diatoxanthin	$C_{40}H_{54}O_2$	566.4124
Lutein	$C_{40}H_{56}O_2$	568.4280
Isozeaxanthin	$C_{40}H_{56}O_2$	568.4280
Zeaxanthin	$C_{40}H_{56}O_2$	568.4280
Lycopene-16,16′-diol	$C_{40}H_{56}O_2$	568.4280
4-Ketoalloxanthin	$C_{40}H_{50}O_4$	578.3760
Pectenolone	$C_{40}H_{52}O_3$	580.3916
Phoenicoxanthin	$C_{40}H_{52}O_3$	580.3916
4-Ketozeaxanthin	$C_{40}H_{54}O_3$	582.4073
Antheraxanthin	$C_{40}H_{56}O_3$	584.4229
Lutein epoxide	$C_{40}H_{56}O_3$	584.4229
7,8,7′,8′-Tetradehydroastaxanthin	$C_{40}H_{48}O_4$	592.3553
7,8-Didehydroastaxanthin	$C_{40}H_{50}O_4$	594.3709
Astaxanthin	$C_{40}H_{52}O_4$	596.3866
Neoxanthin	$C_{40}H_{56}O_4$	600.4179
Isozeaxanthin bispelargonate	$C_{58}H_{88}O_4$	848.6683

[a] Exact mass is defined as the monoisotopic molecular weight of a molecule and is calculated using the mass of the most abundant isotope of each element

solvents such as alcohols lead to an increased abundance of protonated carotenoids, and nonpolar solvents such as methyl-tert-butyl ether facilitate the formation of molecular ions. Even though APCI tends to produce more fragmentation in the ion source than either ESI or FAB-MS, these fragment ions are often not abundant. For example, the positive ion APCI mass spectrum of lycopene is shown in Fig. 111.12. If additional fragmentation is desired for structure confirmation, then CID with MS/MS would be required. The elemental composition and exact mass of many common carotenoids is shown in Table 111.6.

5 Conclusions

A practical way of extracting, isolating, and purifying carotenoids from plant materials is described in this chapter. The method is based mainly on the natural form in which carotenoids are found (esterified or free) and to some extent on their polarity and/or solubility in the solvents used. Common and readily available solvents and laboratory equipment are suggested. Preparation and determination of carotenoids are described. Moreover, an advanced extraction and analytical technique of carotenoids is also explained.

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Part XIII

Terpenes: Pharmacology and Bioavailability

Cannabinoids: Chemistry and Medicine 112

Giovanni Appendino and Orazio Taglialatela-Scafati

Contents

1 Introduction 3417
2 Structural Classification of Cannabinoids 3418
2.1 Monocyclic Cannabinoids: CBG-Type 3420
2.2 Bicyclic Cannabinoids: CBC-, CBD-, CBM-Types 3420
2.3 Tricyclic Cannabinoids: CBE- and CBF-Types 3421
2.4 Tricyclic Cannabinoids: THC-Type 3421
2.5 Tetracyclic Cannabinoids: CBL- and CBX-Types 3422
2.6 Rearranged Cannabinoids 3423
3 The Cannabinoid (CB) Receptors and Endocannabinoids (EC) 3423
4 CB-Mediated Effects of Cannabinoids 3425
4.1 Psychotropic Effects 3425
4.2 Analgesic Effects 3425
4.3 Immunomodulation 3426
4.4 Cardiovascular Effects 3426
5 Structure-Activity Relationships of Cannabinoids 3426
6 Other Biological Activities of Cannabinoids 3428
6.1 Neuroprotective Effect 3428
6.2 Anti-inflammatory and Anticancer Activity 3429
6.3 Antibacterial Activity 3429
7 Conclusions 3430
References 3431

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Abstract

Cannabinoids, a class of meroterpenoids derived from the alkylation of an olivetol-like alkyl resorcinol with a monoterpene unit, are the most typical constituents of *Cannabis*. This class includes over a hundred members belonging to several structural types, mainly differing by the constitution of their terpenoid moiety. The biomedical relevance of cannabinoids transcends the psychotropic properties of Δ^9-THC since, because of their anti-inflammatory, analgesic, and antibacterial activity, they show potential in various fields of medicine, addressing unmet needs like the symptomatic mitigation of multiple sclerosis and the treatment of MRSA infections. Research on cannabinoid activities has yielded to the discovery of an entire new class of human receptors, called cannabinoid (CB) receptors, and of their endogenous agonists, collectively named endocannabinoids (EC). Since its discovery in the early 1990s, the EC system has increasingly emerged as a key signaling system involved in a plethora of physiological and pathological functions in mammals. This chapter will provide an overview on this multi-faceted class of natural products.

Keywords

Cannabinoids • Endocannabinoids • Δ^9-THC • Psychotropic effects • Pain treatment

Abbreviations

CB receptor	Cannabinoid receptor
CBC	Cannabichromene
CBD	Cannabidiol
CBE	Cannabielsoin
CBF	Cannabifuran
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBL	Cannabicyclol
CBM	Cannabimovone
CBN	Cannabinol
CBX	Cannabioxepane
CNS	Central nervous system
EC	Endocannabinoid
FAAH	Fatty acid amide hydrolase
GPP	Geranyl pyrophosphate
MAG	Monoacylglycerol
MAP	Mitogen-activated protein
MRSA	Methicillin-resistant *Staphylococcus aureus*
PEA	Palmitoylethanolamide
PPAR	Peroxisome proliferator-activated receptor

SAR	Structure-activity relationship
THC	Tetrahydrocannabinol
TRPV1	Vanilloid receptor type 1

1 Introduction

Cannabinoids are a large class of meroterpenoids (prenylated polyketides) isolated from *Cannabis sativa* L. (Cannabaceae). These secondary metabolites are accumulated in the epidermal resinous glands in the leaves and flowerheads of the plant, and are characterized by very specific and potent pharmacological activities, as exemplified by the well-known activity of Δ^9-tetrahydrocannabinol (Δ^9-THC) on central nervous system (CNS). At least two *Cannabis* chemical phenotypes can be identified: (a) a fiber-type *Cannabis*, rich in cannabidiol (CBD) and almost devoid of Δ^9-THC (generally $< 0.2\ \%$ dry weight), also called hemp and used for textile or seed oil purposes, and (b) the drug type *Cannabis*, a psychotropic, Δ^9-THC-rich plant (concentration up to 20 %), whose flowers and subtending leaves and stalks are known as marijuana and are used to obtain hashish. With the exception of cannabigerol (CBG), which also occurs in an African *Helichrysum* species [1], the different varieties of *C. sativa* represent the sole natural source of these compounds.

C. sativa and cannabinoids constitute one of the most paradigmatic examples of the deep impact that natural products can have on biochemistry, pharmacology, and toxicology. This plant has been domesticated thousands of years ago, and it is one of the oldest medicinal plants, already used in China about 6,000 years ago. Modern science has discovered a wealth of pharmacological activities for the secondary metabolites of this venerable plant: in addition to the psychotropic activity of Δ^9-THC, cannabinoids have been reported to possess pharmacological potential in several other fields of medicine, including analgesic, neuroprotective, anti-inflammatory, and antibacterial activities. Most importantly, some cannabinoids have shown the capacity to address unmet needs like the relief of nausea [2] and anorexia [3], especially those associated to radio- and chemotherapy, and very significantly to mediate a mitigation of multiple sclerosis symptoms [4]. Investigation on these activities has yielded to the discovery of an entire new class of human receptors, called cannabinoid (CB) receptors, and many of the potential therapeutic uses of cannabinoids seem to be related to interaction with these receptors. However, a number of activities, like the antibacterial or the antitumor properties, are largely independent of the interaction with CB receptors and, therefore, they could be potentially dissected from the mind-altering effects of *Cannabis*. The intense research activity on CB receptors resulted also in the discovery of their endogenous agonists (collectively called endocannabinoids, EC) and, since its discovery in the early 1990s, the EC system has increasingly emerged as a key signaling system involved in a plethora of physiological and pathological functions in mammals.

The Δ^9-THC-rich chemotype of *C. sativa* constitutes nowadays the most widely utilized illicit recreational drug in the world, and the cultivation and use of psychotropic *Cannabis*, even for medicinal purposes, is illegal in many countries due to the potentially harmful acute and short-lasting effects of this drug. Many interesting comments on the impact of *C. sativa* and cannabinoids on society, on criminology, and even on art (e.g., the "Club des Hashischins" of the French artistic community [5]) could be done, but they are beyond the scope of this chapter.

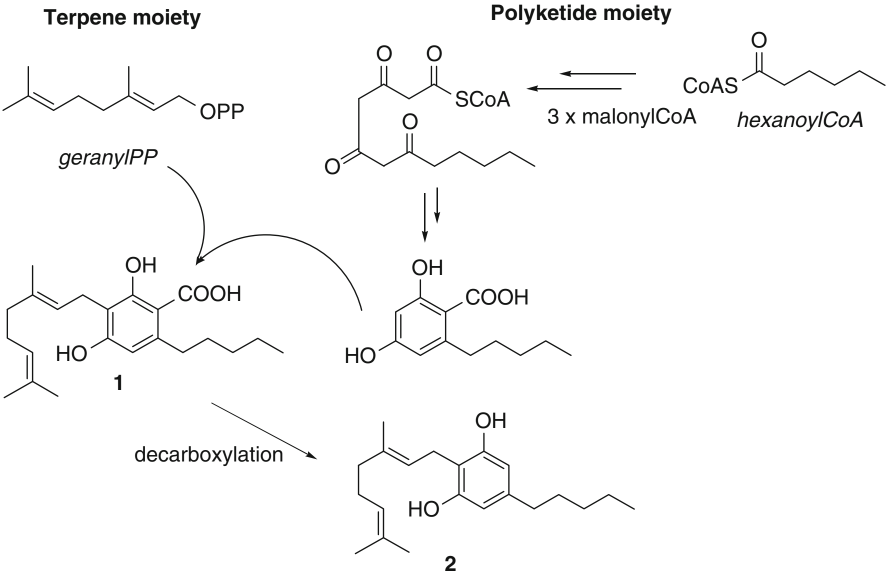
2 Structural Classification of Cannabinoids

The phytochemical diversity of *C. sativa* is well illustrated by more than 500 compounds isolated from this plant, encompassing all major classes of phytochemicals (polyketides, terpenoids, alkaloids, flavonoids, stilbenoids, oxylipins). Undoubtedly, the most important and peculiar secondary metabolites of *C. sativa* are cannabinoids, a class of mono- to tetracyclic C_{21} (or C_{22}) meroterpenoids encompassing more than 100 members. These compounds are synthesized in secretory cells of glandular trichomes, most concentrated in unfertilized female cannabis flowers prior to senescence. A number of detailed accounts on the cannabinoid chemistry have been reported in the literature [6–8], also recently by Appendino et al. [9]. In this paragraph, we will provide an updated, although not comprehensive, account of the chemistry of this fascinating class of secondary metabolites.

As shown in Scheme 112.1, cannabinoids are secondary metabolites of mixed biogenesis, derived from the assembly of a resorcinyl-type polyketide moiety (commonly C_{12}, starting from hexanoylCoA, elongated with three malonylCoA units and then cyclized) and a monoterpene moiety (geranyl pyrophosphate, GPP), in turn derived from the deoxyxylulose phosphate/methylerythritol phosphate pathway. The linking of these two moieties gives rise to the formation of cannabigerolic acid (CBGA), which should be considered as the parent compound of the entire class. All the other cannabinoids differ from CBGA for an extensive rearrangement of the terpene moiety which can give rise to the formation of up to three additional rings (Fig. 112.1). It has also been demonstrated that many rearranged cannabinoids derive directly from CBG through the intervention of a single enzyme. In this context, the case of Δ^9-THC, for which an FAD-dependent enzyme (THCA synthase) has been isolated [10], is emblematic.

CBGA and the products of its rearrangement are normally isolated in small amounts and they are commonly obtained as decarboxylated analogs. This decarboxylation step is, most likely, nonenzymatic and it should occur spontaneously during either storage or extraction/purification of the compounds. Light, temperature, and cofactors are believed to facilitate the decarboxylation of the 2-hydroxybenzoic moiety. Interestingly, the carboxylated version of Δ^9-THC is not psychoactive.

An important subfamily of cannabinoids differs from the major compounds for a shorter linear alkyl chain attached to the phenyl ring. The most frequently


Terpene moiety
Polyketide moiety
OPP
geranylPP
O
O
SCoA
O
O
3 x malonylCoA
CoAS
hexanoylCoA
OH
COOH
HO
1
OH
COOH
HO
decarboxylation
OH
HO
2

Scheme 112.1 Biogenesis of cannabinoids

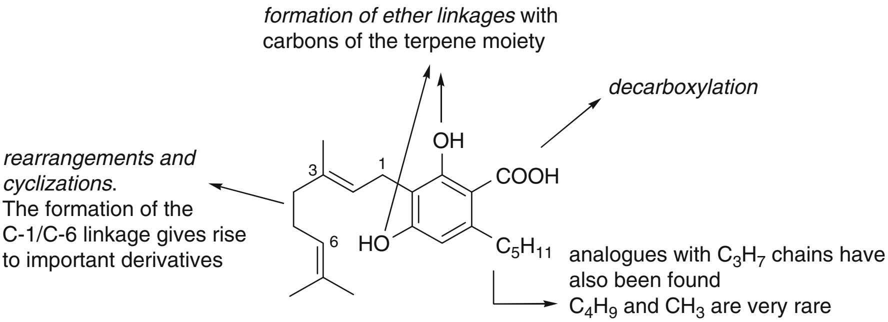
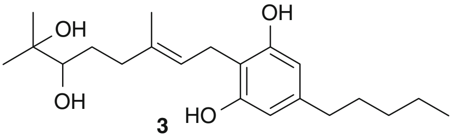

formation of ether linkages with carbons of the terpene moiety
decarboxylation
rearrangements and cyclizations. The formation of the C-1/C-6 linkage gives rise to important derivatives
3
1
6
OH
COOH
HO
C_5H_{11}
analogues with C_3H_7 chains have also been found
C_4H_9 and CH_3 are very rare

Fig. 112.1 Structural variability within the cannabinoid family

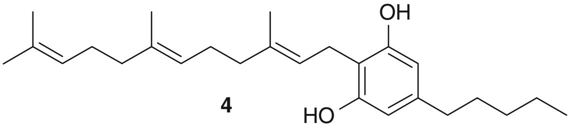
encountered are the C_3 counterparts (named cannabivarins) of the standard C_5 cannabinoids, believed to derive from a shorter starter unit (butanoylCoA) for the ketide homologation. For simplicity, in the next paragraphs, we will no longer mention cannabinoid acids and cannabivarins, but the reader should consider that practically for all the major cannabinoids also the corresponding acids and the corresponding cannabivarin analogs have been isolated. Figure 112.1 summarizes the structural variability of cannabinoids, starting from the structure of CBGA.

2.1 Monocyclic Cannabinoids: CBG-Type

A few analogs of CBG have been obtained and they differ in oxidation of either the side chain or the ring moiety, for example, carmagerol (**3**) [11]. Some quinone derivatives of cannabigerol have been recently reported from a high-potency Δ^9-THC-rich variety of *C. sativa* [12].



3



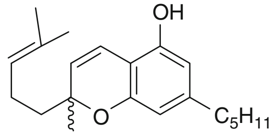
4

We have very recently reported the isolation of sesquicannabigerol (**4**) [13], the farnesyl prenylogue of CBG and the first example of cannabinoid including a terpene unit of the sesquiterpene (C_{15}) type. This molecule could be the result of a loss in the substrate specificity of the prenyltransferase-synthesizing CBG, which could occasionally accept farnesylPP in addition to the standard substrate geranylPP. Since CBG is the precursor of all the series of rearranged cannabinoids described in the next paragraphs, prenylogues of all major cannabinoids could be assumed to exist in Cannabis.

2.2 Bicyclic Cannabinoids: CBC-, CBD-, CBM-Types

An oxidative intramolecular cyclization on the CBG skeleton affords cannabichromene (CBC, **5**), found as the racemate and well represented in both psychotropic and fiber-type varieties of *C. sativa* [14]. Cannabidiol (CBD, **6**), characterized by the formation of a carbon-carbon linkage between C-1 and C-6 of the prenyl unit, is the main constituent of the non-psychotropic (fiber-type) varieties of *C. sativa*. The aromatized analog of CBD is named cannabinodiol (**7**) [15], and it is likely an artifact, since it has been observed that its concentration increases with the age of the stored plant.

Cannabimovone (CBM, **8**), isolated from a non-psychotropic variety of *C. sativa* (Carma) [16], is characterized by an unprecedented *abeo*-menthane terpenoid structure, which can be rationalized in terms of dihydroxylation of the endocyclic double bond of CBD, followed by oxidative cleavage of the glycol system and then aldolization of the resulting dicarbonyl. Attempts to mimic this pathway led only to the crotonized analog of the natural product.



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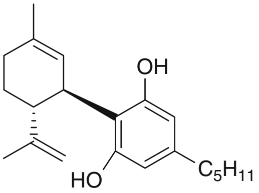

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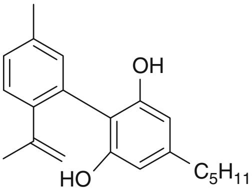

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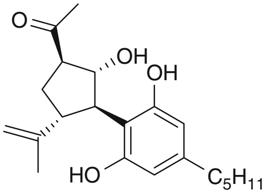

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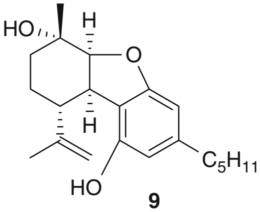
2.3 Tricyclic Cannabinoids: CBE- and CBF-Types

Cannabielsoin (CBE, **9**)-type compounds formally derive from attachment of one of the two phenolic oxygen atoms to the endocyclic double bond of the CBD monoterpene unit, thus generating a dihydrofuran-type ring [17]. Interestingly, compounds of the CBE class have also been detected during studies of the mammalian metabolism of CBD [18]. An aromatized analog of CBE, cannabifuran (CBF, **10**) [19], is characterized by a dibenzofuran skeleton.



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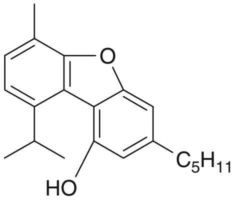

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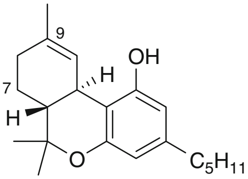
2.4 Tricyclic Cannabinoids: THC-Type

This is the structural class of Δ^9-THC (**11**), the most important cannabinoid and the main bioactive component of the psychotropic varieties of *C. sativa*. Δ^9-THC (**11**) is commonly accompanied by minor amounts of the isomer Δ^8-THC (**12**) and by the completely aromatized analog cannabinol (CBN, **13**). Similar to cannabinodiol, CBN is thought to be an artifact and the Δ^9-THC/CBN ratio is used as an indication of the duration for which the marijuana samples are stored.

Cannabiripsol (**14**) is the dihydroxylated analog of Δ^9-THC [20], while cannabitriol (**15**) [21] shows a double bond at the junction between the two nonaromatic rings. Cannabitetrol (**16**) [22] is a trihydroxylated analog of Δ^9-THC. The configuration at the three stereogenic carbons of this metabolite has never been investigated.

Most recently, ElSohly and coworkers [23] have reported the isolation of a Δ^9-THC dimer, called cannabisol (**17**) from high-potency marijuana samples. Authors hypothesized that the methylene bridge connecting the two THC units could derive from a Claisen-type reaction between two THCA units followed by decarboxylation and enzymatic reduction of the resulting ketone. A dicumarol-type biogenesis involving the reaction of two molecules of THC with one formaldehyde-equivalent seems also plausible. Unfortunately, the impact on the activity of this dimerization has not been reported.



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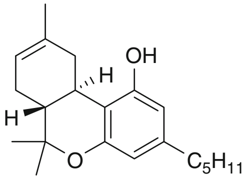

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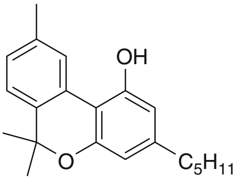

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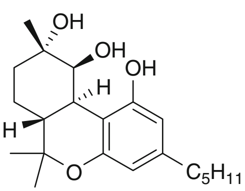

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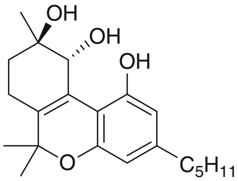

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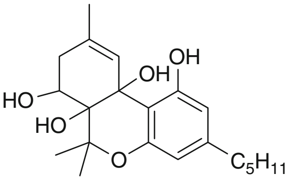

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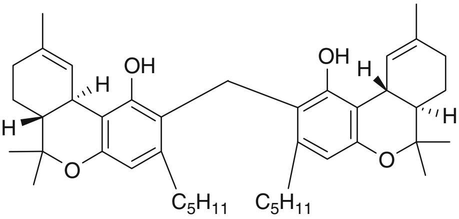

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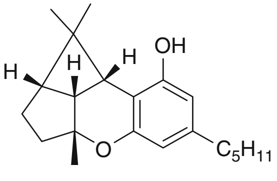
2.5 Tetracyclic Cannabinoids: CBL- and CBX-Types

The tetracyclic system of cannabicyclol (CBL, **18**) [24] seems to derive from a [2 + 2] intramolecular cycloaddition of CBC (**5**). It is still not clear whether the formation of CBL is the result of natural irradiation on the plant or this metabolite is an artifact formed during the extraction process. Cannabioxepane (CBX, **19**) is a novel bis-oxygen bridged diphenyl-type cannabinoid obtained by Pagani et al. [25] from the same Italian fiber hemp (cultivar Carmagnola) which yielded also CBM (**8**, see below). The skeleton of CBX bears some similarities with CBF (**10**), but it is the first cannabinoid to show a linkage between the oxygen atom at C-5 and C-8′, thus giving rise to an unprecedented seven-membered ring.



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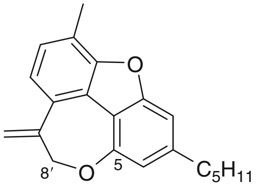

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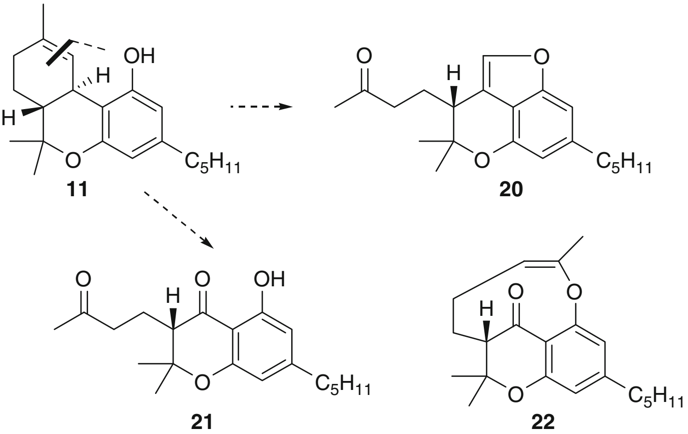
2.6 Rearranged Cannabinoids

A family of rearranged cannabinoids formally derive from Δ^9-THC through the cleavage of the tri-substituted Δ^9 double bond. In the case of cannabicoumaronone (**20**), this cleavage should be followed by hemiacetalization/dehydration yielding to the furan ring [26]. A further oxidative cleavage is the likely biogenetic origin of cannabichromanone (**21**), for which a series of derivatives, including the cyclized analog **22**, have been reported [27].



3 The Cannabinoid (CB) Receptors and Endocannabinoids (EC)

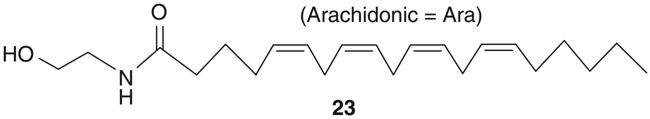
Although the psychotropic properties of Δ^9-THC were known since the middle of last century (Δ^9-THC was isolated in the pure form in 1964), investigations on the mechanism of this activity resulted in the discovery of the CB receptor family only in 1988 and their cloning in early 1990s [28].

Two cannabinoid (CB) receptors with about 40 % homology have been identified and cloned to date, namely CB_1 and CB_2. These receptors belong to the superfamily of G-protein coupled membrane receptors [29] and consequently they show a typical heptahelical structure. Interaction with cannabinoid receptors stimulates a cascade of signal transduction pathways, including interaction with potassium and calcium channels (for CB_1) and several kinases (e.g., MAP kinase). Since a number of cannabinoid-like effects persist in CB_1/CB_2 knockout mice, the existence of other cannabinoid receptors different from CB_1 and CB_2 has been suggested (e.g., GPR55) [[30], [31]], but their effective role still awaits to be clearly defined.

CB_1 is expressed in the brain but also in liver, kidneys, and in many other organs, while CB_2 is mainly localized on cells of the immune system (macrophages, T- and B-cells), hematopoietic cells but also on peripheral nerve terminals. The cannabinoid system has been identified as one of the most ubiquitous and pleiotropic pro-homeostatic system, whose role should be to help cells and organs to reestablish the physiological steady state after acute or chronic perturbations of their homeostasis [32]. Thus, cannabinoids participate in the regulation of practically all the biological and physiological systems of the organism.

The presence of CB receptors implies the existence of endogenous ligands. The first member of this family of neuromodulator lipids, collectively indicated as endocannabinoids, has been discovered in 1992 by Mechoulam and Devane [33], identified as *N*-arachidonylethanolamine and called anandamide (**23**). Endocannabinoids are amide (e.g., anandamide), ester (e.g., 2-arachidonoyl glycerol, **24**) [34], or ether (e.g., noladin) derivatives of arachidonic acid with small molecules, such as ethanolamine or glycerol.

Other endogenous fatty acid amides (such as palmitoylethanolamide, PEA) do not activate CB_1 and CB_2 receptors, but they exhibit anti-inflammatory and analgesic properties [35], due to the interaction with peroxisome proliferator-activated receptor alpha (PPAR-α) and possibly also with the postulated new cannabinoid receptors GPR55 and GPR119 [36].



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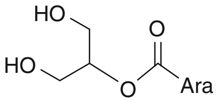

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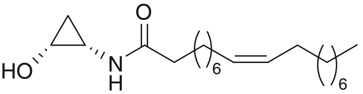

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PEA has been demonstrated to potentiate the effects of classical endocannabinoids as anandamide or arachidonoyl glycerol, and this has been called "entourage effect" [37]. We have recently shown [38] that introduction of a methylene lock on the ethanolamide head to generate a cyclopropane ring is able to trigger strong CB_1 affinity in oleoylethanolamide, as in compound **25**, but not in PEA.

The enzymes that degrade the endocannabinoids, such as fatty acid amide hydrolase (FAAH) or monoacylglycerol (MAG) lipase, are an obvious pharmacological target to modulate the effects of cannabinoid receptors (illustrated in the next paragraph). In particular, targeting FAAH activity has been identified as a promising new therapeutic strategy for the treatment of pain and other neurological-related or inflammatory disorders. FAAH belongs to the superfamily of serine hydrolases characterized by a Ser-Ser-Lys catalytic triad. Most of the FAAH inhibitors disclosed so far act through a covalent modification of the catalytic serine in the active site, but recently the first noncovalent inhibitor of FAAH, characterized by a keto-benzimidazole structure, has been reported [39].

4 CB-Mediated Effects of Cannabinoids

Given their ubiquitous distribution, CB receptors are involved in the regulation of many biological and physiological systems of the organism. However, the most important pharmacological effects triggered by stimulation of CB receptors can be identified as the: (a) *psychotropic effects*, (b) *analgesic effects*, (c) *immunomodulatory effects*, and (d) *cardiovascular effects*.

4.1 Psychotropic Effects

The psychoactive effects of cannabinoids are mediated by interaction with central CB_1 receptors [40]. Δ^9-THC shows the highest affinity to CB receptors (K_i ab. 40 nM for both CB_1 and CB_2) [41], while CBD and other non-psychoactive cannabinoids show a very low affinity for CB receptors, but some of them act as inverse agonist for CB_1 [42].

The euphoric feelings associated with *Cannabis* use are thus ascribable to the interaction of Δ^9-THC with CB_1 receptors on presynaptic nerve terminals. However, CB receptors are also present in other areas of CNS, and thus, the same cannabinoids also cause impairment of cognition and memory (hippocampus) [43], involuntary movements and partial loss of motor control (basal ganglia and cerebellum) [44], and a beneficial and potential therapeutically useful inhibition of emesis [45], especially of the chemotherapy-induced emesis. It is still unclear whether the CB receptors are responsible for all of the centrally mediated actions of the cannabinoids.

4.2 Analgesic Effects

CB_1 receptors play an important role in the transmission of the nociceptive information in several key tissues, and the interaction of some cannabinoids with CB_1

confers them a powerful analgesic effect. It has been estimated that Δ^9-THC is as potent as morphine in blocking nociceptive stimuli in many animal models [46], and, moreover, it can act synergistically with opioid-receptor agonists.

The analgesic effect of cannabinoids could be partly due also to the binding of PPAR-γ and of some thermo-TRPs, ion-channels characterized by an intracellular ligand-binding domain and co-involved in pain and inflammation [47].

4.3 Immunomodulation

The immunomodulatory properties of Δ^9-THC [48] are likely due to the interaction with CB_2 receptors, highly expressed in cells of the immune system (T-cells, B-cells, macrophages). The effect on the immune system of cannabinoid administration appears to be hormesic, and related to the dosage. Indeed, high doses of cannabinoids suppress immune responses, while more physiologically relevant concentrations of cannabinoids result in metabolic stimulation of lymphocytes and in an increase in pro-inflammatory cytokine production rather than immunosuppression. It has been proposed that the immunosuppressive properties of cannabinoids can find application in the treatment of multiple sclerosis.

4.4 Cardiovascular Effects

Δ^9-THC has a well-known effect on blood pressure (prolonged hypotension) and heart rate (initial bradycardic response) through interaction with CB_1 receptor [49], but also with TRPV1. FAAH inhibitors have revealed the antihypertensive effects of endogenously elevated anandamide levels and have been proposed as a strategy to prevent complications of cardiomyopathies.

5 Structure-Activity Relationships of Cannabinoids

Δ^9-THC is the single most potent natural cannabinoid interacting with CB receptors, and the isolation of analogs, coupled to the synthesis of several derivatives, have highlighted the most important pharmacophoric portions of this molecule.

The presence of an alkyl side chain on the aromatic ring is required for activity. The affinity for the CB receptor decreases in cannabivarins (C_3 alkyl chain) [50], while it has been demonstrated to increase for synthetic C_7 or C_8 chains. Synthetic cannabinoids with methyl branching at one or both the first two carbons of the side chain (or showing double or triple bonds at these positions) have also shown a greater affinity for both CB receptors [51]. For example, compound **26** (Fig. 112.2) showed high affinities for both CB_1 and CB_2 (ab. 0.4 nM).

The free hydroxyl group on the aromatic ring A is required for activity on CB_1 presumably due to the formation of hydrogen bonding with Lys12 in transmembrane helix 3 [52].

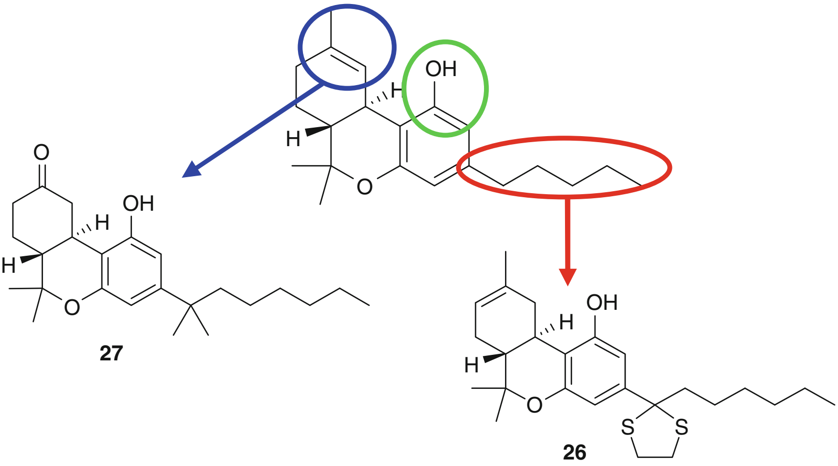
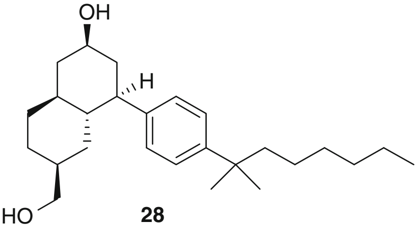


Fig. 112.2 Main sites of modification on the scaffold of Δ^9-THC

Hydroxylation or oxidative cleavage of the allylic methyl group present on the carbocyclic nonaromatic ring increases affinity for both CB receptors, as exemplified by the structure of nabilone (**27**), which has been introduced into the market as antiemetic agent for cancer supporting care and has also recently been approved in USA for the treatment of neuropathic pain [53]. The position of the double bond seems to be not crucial, indeed Δ^8-THC is almost equipotent with Δ^9-THC; however, aromatization of this ring, as occurs in CBN, causes a marked decrease in affinity.

A number of synthetic compounds, showing only vague similarities with Δ^9-THC and other natural cannabinoids, have been described. These compounds show potent affinity for both CB_1 and CB_2 in the one-digit nanomolar range (e.g., CP-55,244, **28**) [54]. The synthesis of some of these compounds is simple and has fueled the development of an illegal market for cannabinoid analogs, as exemplified by the infamous *spice* [55]. Interestingly, β-caryophyllene, a very common sesquiterpenoid also present in cannabis, has been found to be a selective full agonist of CB_2 [56], qualifying as the first phytocannabinoid of wide distribution in plants.

A number of synthetic compounds, completely unrelated to the structure of natural cannabinoids have been found to act as CB_1 and/or CB_2 antagonist/inverse agonist. The most important member of this class of compounds is rimonabant (**29**), a diarylpyrazole derivative [57], approved for the treatment of obesity and as an aid in the cessation of cigarette smoking, but later withdrawn from the market because of the severe depression it could induce in sensitive patients. A number of structural motifs significantly different from the pyrazole system have been proposed for CB_2 selective antagonists, as exemplified by the quinoline derivative **30**.



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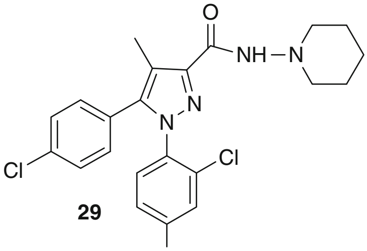

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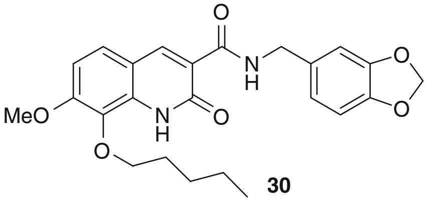

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6 Other Biological Activities of Cannabinoids

Owing to the presence of high concentrations of Δ^9-THC, biomedical attention on *C. sativa* has so far mainly focused on the psychotropic varieties of this plant, while the pharmacological potential of fiber hemp has been largely overlooked. However, certain biological properties of THC are shared also by non-psychotropic cannabinoids, and thus, some pharmacologically useful activities of *C. sativa*, such as antibacterial and anti-inflammatory actions, could be dissected from its psychotropic potential and an easier therapeutic application found [58].

6.1 Neuroprotective Effect

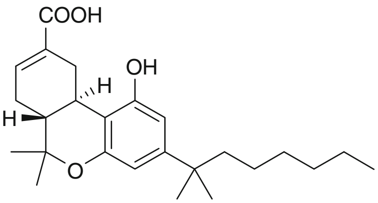
The neuroprotection exhibited by Δ^9-THC and CBD is likely a multicomponent effect, mediated by interaction with CB, PPAR-α [59], 5-HT$_1$a, and other receptors which are involved in a very complex neural web and whose manipulation is difficult to predict in terms of in vivo effects. Δ^9-THC likely acts mainly by activating CB$_1$ receptors on presynaptic terminals of glutamatergic and GABAergic synapses and suppressing the presynaptic release of these neurotransmitters [60], while CBD likely acts by interacting with 5-HT$_1$a receptors leading to an increased cerebral blood flow and neuroprotective effect [61].

Cannabinoids, particularly CBD, have been proposed for treatment of spasticity and pain symptoms associated with multiple sclerosis and amyotrophic lateral sclerosis, and some CBD analogs have been proposed for neuroprotection in glaucoma to delay the progressive damage of the optic nerve [62].

6.2 Anti-inflammatory and Anticancer Activity

Since non-psychoactive cannabinoids, such as CBD and CBN, are able to induce a marked decrease in the levels of interferons, pro-inflammatory cytokines, and chemokines after stimulation with LPS [63], this activity is unlikely to be mediated by interaction with CB receptors, while the involvement of the adenosine signaling has, rather, been postulated [64]. CBD and CBG have also demonstrated a non-CB-mediated inhibition of the proliferation of human keratinocytes, supporting a potential role for cannabinoids in the treatment of psoriasis [65]. A synthetic cannabinoid, ajulemic acid (**31**), has been proposed for treatment of arthritis and for the management of pain and inflammation in multiple sclerosis patients [66].

Owing to the connections existing between inflammation and cancer, it is not surprising that some cannabinoids have been associated with a reduction of the cancer proliferation. Δ^9-THC retards the growth of lung adenocarcinoma [67] and induces apoptosis in C6 glioma cells through activation of mitogen-activated protein (MAP) kinases and interaction with ERK-dependent pathways. CBD (or its quinone derivative) seems to act through an apoptotic mechanism and activation of caspase-3 [68, 69]. CBD has also been shown to reverse insensitivity of cancer cells to vinblastine by reducing expression of Pgp [70] and to inhibit BCRP and MRP1, the two additional transporters involved in chemoresistance [71, 72].



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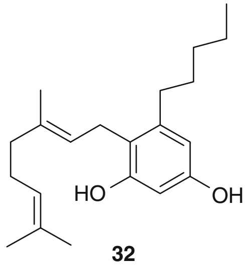

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In this context, it should be remarked that *Cannabis* contains also non-cannabinoid phenolics (cannaflavins, canniprene, cannabispirenone), endowed with potent inhibitory activity against some pro-inflammatory enzymes like COX [73, 74], with the potential to complement the activity of cannabinoids in terms of anti-inflammatory action.

6.3 Antibacterial Activity

Since cannabinoids are phenolics, their antibacterial activity is not surprising; however, their potency and the activity against multidrug-resistant bacterial strains are remarkable. Data on the antibiotic activity of CBC [75], CBG [76], CBD [77], and Δ^9-THC [77] have been obtained, and *C. sativa* preparations have been investigated as topical antiseptic agents. More recently, the potential of the major cannabinoids to address antibiotic resistance to methicillin-resistant

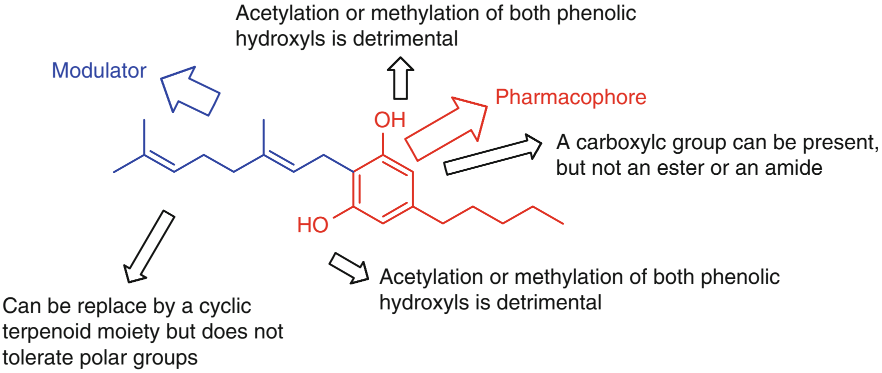

Acetylation or methylation of both phenolic hydroxyls is detrimental
Modulator
Pharmacophore
OH
A carboxylc group can be present, but not an ester or an amide
HO
Acetylation or methylation of both phenolic hydroxyls is detrimental
Can be replace by a cyclic terpenoid moiety but does not tolerate polar groups

Fig. 112.3 Structure-activity relationships for the antibacterial activity of cannabinoids

Staphylococcus aureus (MRSA) has been investigated [78]. MRSA infections are responsible for over 10 % of cases of septicemia cases, and CBD, CBC, CBG, Δ^9-THC, and CBN showed potent activity against a variety of MRSA strains of current clinical relevance (MIC values in the 0.5–2 μg/mL range), outperforming antibiotics currently used for the management of these infections.

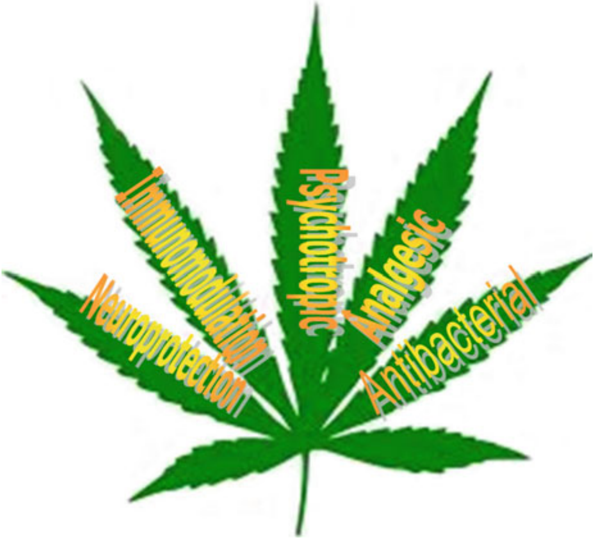
Structure-activity studies on the non-psychotropic CBD and CBG showed that the antibacterial activity was remarkably tolerant toward the nature of the prenyl moiety, its relative position compared to the *n*-pentyl moiety (abnormal cannabinoids, e.g., **32**), and toward carboxylation of the resorcinyl moiety (cannabinoid acids). Conversely, decrease in the polarity, obtained through derivatization of the phenolic hydroxyls, or introduction of a second prenyl moiety is proved to be detrimental for antibacterial activity. SAR for the antibacterial activity is summarized in Fig. 112.3.

It has been proposed that some monoterpenoid constituents of *Cannabis*, such as α-pinene or limonene, may synergize the antibacterial action of CBD, CBG, and CBN [79]. Interestingly, a potentiating effect of mono- or sesquiterpenoids present in *Cannabis* has been postulated also for other activities, and this observation supports arguments that whole plants and their complex preparations are better drugs than individual components isolated from them.

7 Conclusions

Cannabinoids and endocannabinoids are a hot topic of chemical and biomedical research, and more than one thousand research articles and a host of well-documented reviews have been written in the last three decades on this class of compounds. The debacle of the CB_1 inverse agonist rimonabant well exemplifies the difficulties of working with these pleiotropic compounds, while the successful development of Sativex^{TM} – a combination of natural cannabinoids approved in Canada, UK, and Spain for the symptomatic treatment of multiple sclerosis – is encouraging.

Fig. 112.4 A pictorial view of the most important pharmacological activities associated to cannabinoids



In Fig. 112.4, we have pictorially indicated the most important pharmacologic activities associated to cannabinoids; the contribution of novel researches in this area will, hopefully, foster the optimal exploitation of this ancient but still largely unexplored plant.

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Phytosterols: Beneficial Effects 113

Jonathan Santas, Rafael Codony, and Magda Rafecas

Contents

1 Introduction 3439
2 Metabolism and Physiological Effects 3439
2.1 Phytosterol Bioavailability 3439
2.2 Hypocholesterolemic Effect 3441
2.3 Combination with Other Therapies 3442
3 Mechanism of Action 3444
3.1 Competitive Incorporation into Mixed Micelles 3445
3.2 Effect of PS at Intestinal Level 3446
3.3 Effect of PS on the Liver 3447
4 Other Biological Activities 3447
4.1 Cancer Preventive Agents 3448
4.2 Modulation of the Immune System and Anti-inflammatory Properties 3449
4.3 Antidiabetic Effect 3450
5 Side Effects 3450
5.1 Toxicity 3450
5.2 Sitosterolemia (Phytosterolemia) 3451
5.3 Phytosterol Oxides 3451
5.4 Decrease in Plasma Levels of Carotenoids 3453
5.5 Other Safety Aspects 3454
6 Conclusion 3455
References 3455

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Abstract

Phytosterols (PS) are plant sterols and stanols widely distributed in plant sources that resemble cholesterol in terms of structure and physiological functions. The cholesterol-lowering capacity of PS is well documented in animal and human studies. However, recent studies suggest that the beneficial effects of PS are not only limited to their hypocholesterolemic capacity as they can also act as immunomodulatory, anti-inflammatory, and antidiabetic agents. Further, there is a growing body of evidence which supports that they play an important role in the prevention of other diseases such as cancer and atherosclerosis. Nevertheless, the mechanisms by which PS exert their beneficial functions, the physiological relevance of PS, and their potential adverse effects are not yet fully understood. Therefore, the main aim of this chapter is to provide a contemporaneous overview of the beneficial properties of PS, their mechanism of action, and safety.

Keywords

anticancer • antidiabetic • cardiovascular diseases • cholesterol • immunomodulation • inflammation • molecular mechanism • phytostanols • phytosterols • side effects

Abbreviations

ABC	ATP-binding cassette transporter
ACAT	Acyl-CoA: cholesterol *O*-acyltransferase
CHD	Cardiovascular heart disease
GST	Glutathione-*S*-transferase(s)
HDL	High-density lipoprotein
HMG-CoA	3-Hydroxy-3-methyl-glutaryl-coenzyme A
hs-CRP	High-sensitive C-reactive protein
IDL	Intermediate-density lipoprotein
IL	Interleukin
LDL	Low-density lipoprotein
LDL-c	Low-density lipoprotein cholesterol
LTB_4	Leukotriene B4
LXR	Liver X receptor
NPC1L1	Niemann-Pick C1-like 1 protein
PS	Phytosterol(s)
PSO	Phytosterol oxide(s)
TICE	Transintestinal cholesterol efflux
TNF-α	Tumor necrosis factor alpha
VLDL	Very-low-density lipoprotein

1 Introduction

Phytosterols (PS) are plant sterols or stanols found in plants. Plant sterols belong to the triterpene family and differ from cholesterol by having a methyl or ethyl group in C24. Plant stanols, on the other hand, are the saturated form of the plant sterols (Fig. 113.1). PS are present in free or conjugated form as fatty-acyl esters, hydroxycinnamate steryl esters, steryl glycosides, or acylated steryl glycosides. The main function of plant sterols/stanols is to stabilize plant membranes and serve as precursors in the synthesis of steroidal saponins, alkaloids, and other steroids [1].

PS are widely distributed in plants and plant-containing foods. The most abundant of which are β-sitosterol, campesterol, and stigmasterol [2]. Table 113.1 shows the most common sources of PS. Vegetable oils are considered to be the major sources of PS and their esters [2]. Other good dietary sources include legumes, plant seeds, cereals, and cereal-milling products [3–6]. It is estimated that the dietary intake of PS ranges between 150 mg day^{-1} in western-style diets to 500 mg day^{-1} in diets rich in vegetable dietary habits [7, 8].

The capacity of both plant sterols and stanols to reduce blood cholesterol is well documented [10–12]. However, their precise mechanism of action is not yet fully defined. Current research is providing new insights on the mechanisms of action of PS as well as potential new roles in other physiological benefits. In addition, the safety of these products when they are used at high doses has recently been challenged. Therefore, the main objective of the present review is to discuss current evidences regarding not only the bioactive properties of PS and their mechanism of action but also their potential undesirable effects.

2 Metabolism and Physiological Effects

A large number of studies have provided consistent evidence on the beneficial physiological effects of PS, especially their hypocholesterolemic capacity [10, 13–15]. Thus, the use of PS as functional food components or dietary supplements has become of great interest and led to the development of a wide variety of functional foods and nutraceutical products [16, 17].

2.1 Phytosterol Bioavailability

Like cholesterol, PS are absorbed in the proximal part of the small intestine after being incorporated into mixed micelles. Compared to cholesterol, the intestinal absorption of PS is low. While 40–60% of dietary cholesterol is absorbed, only about 5% of the PS are absorbed [18]. In addition, the efficiency of PS absorption is critically dependent on the structure of both sterol nucleus and side chain. For instance, the rate of plant sterol absorption was investigated in a human study

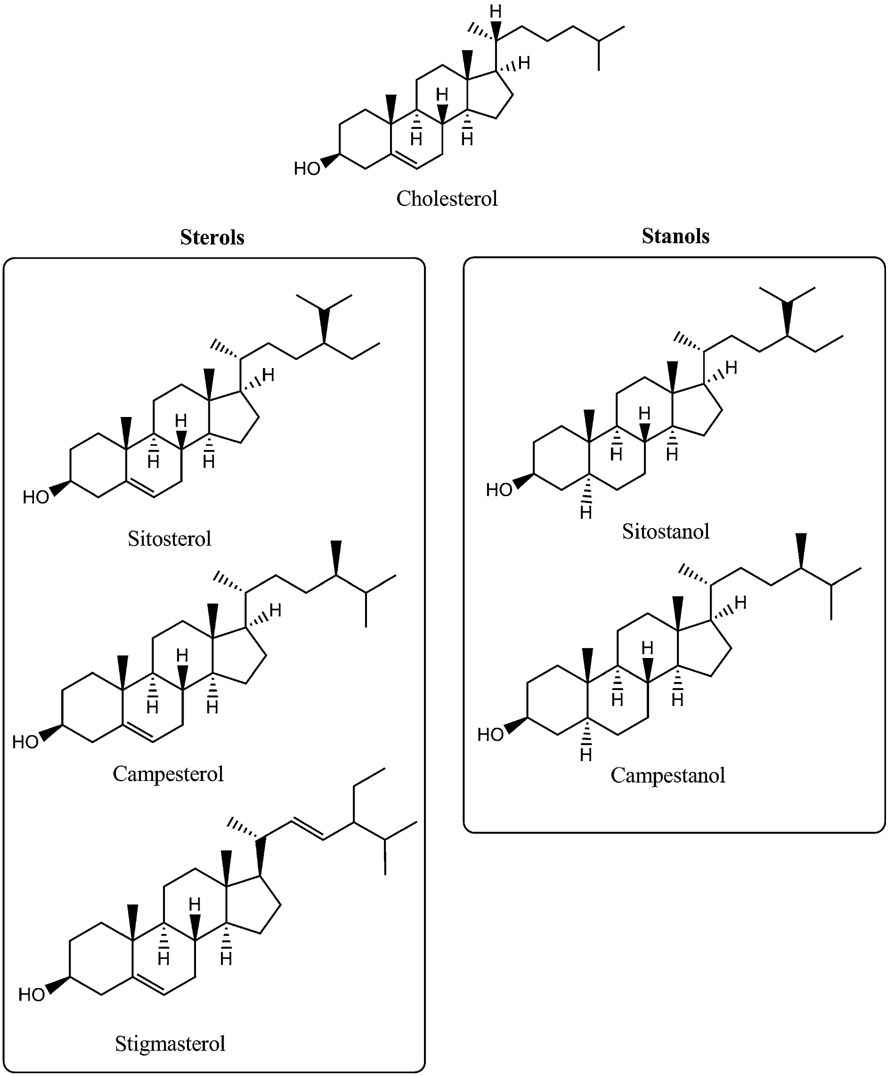

Cholesterol
Sterols
Stanols
Sitosterol
Campesterol
Stigmasterol
Sitostanol
Campestanol
HO
H

Fig. 113.1 Structure of cholesterol and representative sterols and stanols

using deuterium-labeled PS. The absorption rates of the different plant sterols were 1.9% and 0.5% for campesterol and β-sitosterol, respectively, whereas that of stanols were 0.16% for campestanol and 0.04% for sitostanol [19]. Plasma concentrations of PS are normally very low due to the low absorption of these compounds but it can be very variable among populations mainly due to the differences in dietary habits. Plasma concentrations of campesterol range from 6.9 to 27.9 μmol L^{-1}, whereas those of sitosterol range from 2.8 to 16.0 μmol L^{-1} [20].

Table 113.1 Phytosterol content from different food sources

	Total phytosterols (mg 100 g^{-1})
Vegetable oils[a]	
Corn oil	686–1,400
Rapeseed oil	250–878
Soybean oil	203–328
Olive oil	114–162
Palm oil	49–79
Grain and cereals	
Corn	66–178
Rye	77–113
Barley	59–83
Wheat	45–83
Nuts	
Peanuts	220
Almonds	143
Walnuts	108
Fruits	
Avocado	75
Orange	23–24
Grape	4–20
Apple	13–18
Banana	12–16
Vegetables	
Olives	50
Broccoli	4–50
Cauliflower	31–40
Carrots	16–30

[a] The range includes the content from crude and refined oils
Adapted from references [2, 3, 5, 6, 9]

2.2 Hypocholesterolemic Effect

An elevated concentration of plasma cholesterol is considered one of the most important risk factors for the development of coronary heart disease (CHD) [21]. The hypocholesterolemic effect of PS was first demonstrated in the 1950s [22]. Since then, the capacity of both plant sterols and stanols to reduce blood cholesterol has been well documented [10–12]. Moreover, a large number of clinical studies has confirmed their efficiency as cholesterol-lowering agents in humans (see Ref. [15] and [23] for exhaustive summary of clinical trials).

The beneficial effects of PS on cholesterol levels are usually shown after a period as short as 2–3 weeks of intervention and remain stable for at least 1 year of continuous treatment [15]. In humans, the absorption of cholesterol can be reduced by 30–40% after consumption of 1.5–2.0 g day^{-1} [15, 24]. Doses of 0.8–4.0 g of PS day^{-1} have been efficient in reducing LDL-cholesterol (LDL-c) concentration by 10–15% [10]. However, a dose of 2.0 g day^{-1}, which can result in a reduction of plasma LDL-c of 10%, has been proposed as optimal [25]. Higher doses than 2.0 g day^{-1} are in general not recommended as they do not show additional reductions in cholesterol levels and may lead to undesirable side effects [16], although this aspect remains controversial [26]. In addition, it has been suggested that in some cases, PS exert beneficial effects on other lipid variables, such as increasing HDL-cholesterol, decreasing triglycerides levels, and decreasing the ratio of apolipoprotein B/apolipoprotein A1 [27].

It is still a matter of controversy whether plant sterols and stanols are equally efficient in reducing cholesterol levels [28]. Some studies have shown that despite their different bioavailability, there is no clinical relevance with regard to their effect on total cholesterol, LDL-c, HDL-cholesterol, or triglyceride levels [25, 29]. Nevertheless, other authors have suggested that the differences in efficacy between plant sterols and plant stanols remain in the long-term interventions rather than in the short-term studies [30]. For instance, in a recent meta-analysis of randomized placebo-controlled trials, decreases in LDL-c concentrations were dose-dependent for plant stanols but not for sterols. Similarly, intakes of plant stanols higher than 2 g day^{-1} have been associated with additional and dose-dependent reductions in LDL-c [26]. Yet, this effect remains questionable [29]. It has been proposed that the difference in efficiency between these two compounds may be explained by the fact that plant stanols may reside longer in the intestine due to their lower absorption [31].

Several factors can influence the overall effect of plant sterols and stanols. Naumann et al. [32] reported that men are slightly more sensitive to PS intake than women, although the responsible mechanism for this difference is unknown. Another factor that may influence the efficiency of PS involves the baseline levels of plasma lipids [25, 32, 33]. For example, subjects with high and very high baseline levels showed stronger reductions in LDL-c levels than subjects with levels near optimal clinical concentrations [25, 33]. However, these effects could not be reproduced by other authors [34]. Similarly, controversial effects are seen on the improvement of other lipid parameters as HDL-cholesterol concentration increases in subjects with low baseline levels and decreases in those subjects with initially high levels [32]. These differences suggest that people with an unfavorable ratio of total to HDL-cholesterol would especially benefit from PS consumption.

Another beneficial effect of PS is based on their ability to decrease serum concentrations of triacylglycerols especially in people with high serum concentrations [32]. This effect may be attributed to a reduction in the synthesis of very-low-density lipoproteins (VLDL), which are the main transporters of this type of lipids [35].

2.3 Combination with Other Therapies

In order to increase the effectiveness of PS in the reduction of CHD-associated factors, PS can be used in combination with other drugs or bioactive substances [23]. For instance, PS have been used in combined therapies with statins (3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) inhibitors) [36], with

Table 113.2 Summary of intervention studies of PS combined with other therapies

Combined therapy	Study design	Time[a]	Conclusions	References
Stable statin treatment + plant sterols (2.0 g day^{-1})	Double-blind, randomized trial (55 patients on stable statin treatment)	6 weeks	No further cholesterol reduction observed	[40]
Stable statin treatment + plant sterols or plant stanol (2.5 g day^{-1} each)	Double-blind, randomized trial (54 patients on stable statin treatment)	85 weeks	Increased reduction of LDL-c for both plant sterols (8.7%) and plant stanols (13.1%) compared to statin treatment alone	[41]
Ezetimibe (10 mg day^{-1}) + phytosterols (2.0 g day^{-1})	Double-blind, randomized trial (40 mildly hypercholesterolemic subjects)	4 weeks	No therapeutic benefit over ezetimibe	[42]
Ezetimibe (10 mg day^{1}) + phytosterols (2.5 g day^{-1})	Double-blind, randomized, placebo-controlled, triple crossover study (21 mildly hypercholesterolemic subjects)	3 weeks	Enhanced reduction of LDL-c from 16% (ezetimibe alone) to 22% (combined therapy)	[43]
Saturated-fat dairy and whole wheat cereal diet + diet containing viscous fibers (5–10 g day^{-1}), soy foods (25 g soy protein day^{-1}), and almonds (estimated total PS intake of 1–3 g day^{-1})	Randomized crossover trial (34 hypercholesterolemic subjects)	4 weeks	LDL-c reduction of 29%, which was similar to that of statins	[44]
Omega-3 polyunsaturated fatty acids provided as sunola (1.4 g day^{-1}) or fish oils (1.4 g day^{-1}) + plant sterols (1.4 g day^{-1})	Randomized, double-blind, 2 × 2 factorial design (60 hyperlipidemic subjects)	3 weeks	Reduction of inflammatory markers: hs-CRP, TNF-α, IL-6, and LTB_4. Increased levels of adiponectin. Higher CHD risk reduction	[38]
Fish oil (2.0 g day^{-1}) + plant sterols (2.0 g day^{-1})	Randomized, double-blind, 2 × 2 factorial design (200 hypercholesterolemic subjects)	4 weeks	The combination lowered triglycerides by 15% compared to control but no significant interaction between PS and n-3 on plasma cholesterol	[45]

(*continued*)

Table 113.2 (continued)

Combined therapy	Study design	Time[a]	Conclusions	References
Oat β-glucan (5.0 g day^{-1}) + plant stanols (1.5 g day^{-1})	Randomized, controlled, 3-period crossover study (40 mildly hypercholesterolemic subjects)	4 weeks	Slightly further reduction of LDL-c of combined treatment (9.7%) compared to oat β-glucan alone (5.1%)	[39]

Abbreviations: *LDL-c* LDL-cholesterol, *hs-CRP* High-sensitive C-reactive protein, *TNF-α* tumor necrosis factor alpha, *IL-6* interleukin-6, *LTB$_4$* leukotriene B4
[a]Refers to the time of combined treatment

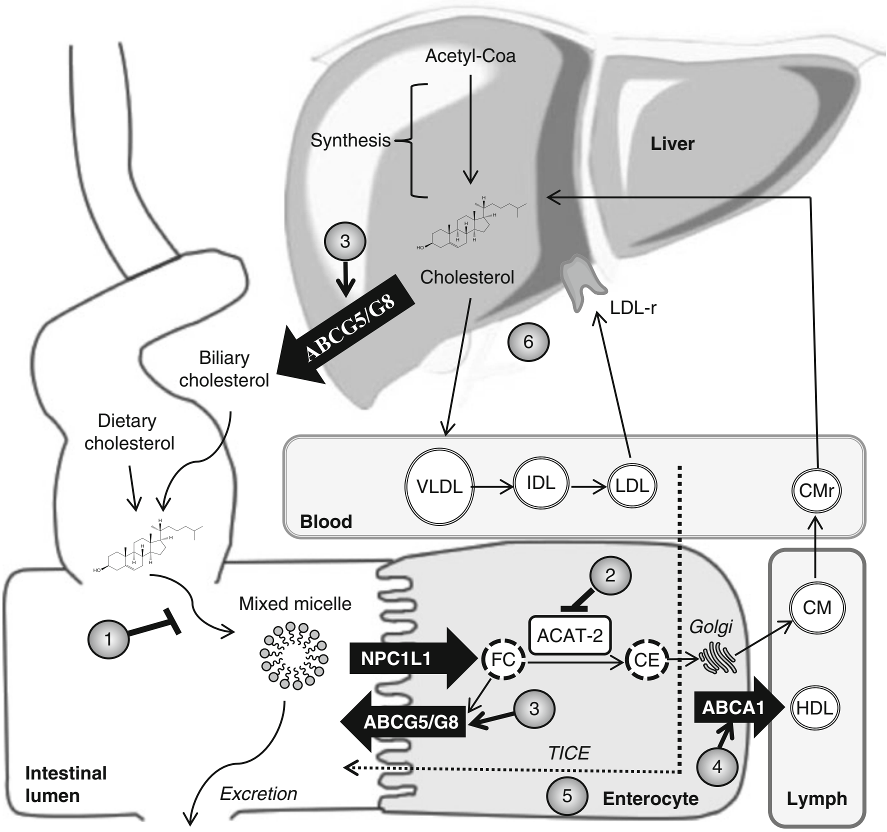
ezetimibe (inhibitor of cholesterol transporter of Niemann-Pick C1-like 1 (NPC1L1) that blocks the intestinal absorption of both biliary and dietary cholesterol) [37], with n-3 polyunsaturated fatty acids [38], and with different fibers [39], among others. A summary of clinical studies is shown in Table 113.2.

The combination of n-3 polyunsaturated fatty acids with PS is having an emerging interest; it has been reported that it is effective in reducing the levels of inflammation markers [38, 46] and cardiovascular risk factors including total cholesterol and triglyceride concentrations, pro-aggregatory factors, eicosanoid, and thromboxane A2 levels [47]. Moreover, Micallef et al. [38] estimated that sunola or fish oils (4.0 g day^{-1}), which are rich in n-3 fatty acids, were more efficient in reducing cancer risk when administered in combination with 2.0 g day^{-1} of PS than when administered alone.

Nevertheless, despite that the use of PS with other therapies seems promising, clinical evidence remains scarce and far from being conclusive.

3 Mechanism of Action

The cholesterol-lowering activity of PS has been attributed to several mechanisms which are mainly ascribed to their structural similarities with cholesterol. Traditionally, the main mechanism accounted for the cholesterol-lowering capacity of PS has been attributed to the competitive solubilization into mixed micelles between cholesterol and PS at the intestinal level [48]. However, several studies have provided evidence to support that the cholesterol-lowering activity is also related to mechanisms other than interference with cholesterol incorporation into micelles [15, 28, 49, 50]. Although the physiological relevance of all these mechanisms is not clear, the metabolic effects of PS are commonly attributed to lower absorption of cholesterol and changes in intestinal cholesterol efflux and lipoprotein homeostasis. These mechanisms are summarized in Scheme 113.1.


Acetyl-Coa
Synthesis
Liver
3
Cholesterol
ABCG5/G8
LDL-r
6
Biliary cholesterol
Dietary cholesterol
VLDL
IDL
LDL
CMr
Blood
2
ACAT-2
Mixed micelle
1
Golgi
CM
NPC1L1
FC
CE
ABCA1
HDL
3
ABCG5/G8
TICE
4
Intestinal lumen
Excretion
5
Enterocyte
Lymph

Scheme 113.1 *Schematic overview of cholesterol metabolism and main proposed mechanisms of action of phytosterols. 1*. The absorption of dietary and/or biliary cholesterol is reduced by competition with PS for incorporation into mixed micelles. *2*. Esterification of free cholesterol in the enterocyte is reduced by competition with PS for ACAT-2 enzyme. *3*. Upregulation of the heterodimer ABCG5/G8 by PS can increase intestinal and hepato-biliar secretion. *4*. Upregulation of ABCA1 by PS can increase the incorporation of sterols into nascent HDL. *5*. Increased cholesterol excretion via TICE. *6*. Although it is not directly mediated by PS, the lower levels of hepatic cholesterol can lead to a lower VLDL secretion and upregulation of LDL receptor, which improves the clearance of plasma cholesterol. Abbreviations: *FC* free cholesterol, *CE* cholesterol esters, *ACAT-2* Acyl-CoA: cholesterol O-acyltransferase 2, *CM* chylomicron, *CMR* chylomicron remnant, *TICE* transintestinal cholesterol efflux, *LDL* low-density lipoprotein, *IDL* intermediate-density lipoprotein, *HDL* high-density lipoprotein

3.1 Competitive Incorporation into Mixed Micelles

Intestinal cholesterol absorption begins with the incorporation of both dietary and biliary cholesterol into mixed micelles. A large number of studies have demonstrated the competition between cholesterol and PS for solubilization into micelles [48, 51–54], wherein the incorporation of PS into mixed micelles seems to be more favorable than that of cholesterol [48, 54]. However, it remains questionable whether sterols and stanols can compete for micellar incorporation differently. In vitro studies using micelle preparations have shown that cholesterol can be substituted in a similar extent by the most abundant phytosterols: campesterol, sitosterol, and sitostanol [54]. On the contrary, the effect of minor plant sterols (i.e., stigmasterol) is not clear. While some authors have reported that minor sterols have lower ability to decrease cholesterol solubility [53], others have not detected such differences [52]. Differences in the micellar system used may well be responsible for the discrepancies between studies.

3.2 Effect of PS at Intestinal Level

Once cholesterol is incorporated into the micelles, its uptake by enterocytes is actively mediated by transporters, mainly NPC1L1 protein, which is located in the brush border membrane [55, 56]. Cholesterol is then esterified with fatty acids by acyl-CoA: cholesterol *O*-acyltransferase 2 (ACAT-2), incorporated into chylomicrons and then secreted to the lymph through the basolateral membrane of the enterocyte [57]. Unesterified cholesterol can be secreted back to the intestinal lumen by ATP-binding cassette transporters G5 and G8 (ABCG5/ABCG8) in direct opposition to NPC1L1 [58]. Moreover, ABCA1 can mediate the incorporation of sterols into nascent high-density lipoproteins (HDL) which lead to their secretion into the lymph [59]. Alternatively, emerging evidence suggests that the proximal part of the small intestine is able to secrete cholesterol actively, a pathway called transintestinal cholesterol efflux (TICE), although its molecular mechanism has not yet been elucidated [60].

Therefore, cholesterol absorption is a complex process that involves different molecular targets. Recent studies have proposed that PS can play an important role in this process [49, 50], although the mechanisms are not yet fully understood and further investigation is necessary. It has been postulated that LXR induction could explain the hypocholesterolemic action of PS [51, 61]. However, this hypothesis is still controversial [62]. For instance, although some authors have reported that PS can act as LXR ligands [51, 61] and regulate the expression of NPC1L1 and ABC transporters [58, 63], others could not confirm this role [49, 50]. Moreover, it has been reported that dietary PS decrease intestinal cholesterol absorption independently of changes in gene expression of intestinal NPC1L1 and ABC transporters [64, 65]. These observations do not rule out the possibility that the activity of these transporters could be altered by PS through posttranscriptional mechanism. Thus, several studies have used genetically engineered mice to examine the involvement of these transporters [66, 67]. However, these studies have shown that the reduction in cholesterol induced by PS is not influenced by the absence of the heterodimer ABCG5/G8 [66] and ABCA1 transporter [67].

Other mechanisms independent to LXR have also been proposed [49]. For instance, it is plausible that PS could reduce plasma cholesterol levels by competing with cholesterol for esterification in the enterocyte by ACAT-2 enzyme, thus reducing its incorporation into chylomicrons [68]. In addition, in vitro experiments with CaCo-2 cells have demonstrated that PS can reduce endogenous cholesterol synthesis by inhibiting the expression of HMG-CoA, a rate-limiting enzyme in the synthesis of cholesterol [69]. However, other reports have reported contradictory results [70, 71]. Although its functional importance is less clear, it has been hypothesized that PS can also interfere with the expression of other mucosa proteins from the ANX family, such as ANXA2, which are involved in cholesteryl ester transport [49, 72]. Since ANXA2 mediates the internalization of cholesteryl esters from caveolae to internal membranes of the brush border [73], it has been postulated that PS may reduce cholesterol transport [49]. Nevertheless, there is yet no clinical evidence to confirm this hypothesis. Finally, it has been recently reported that PS can also increase cholesterol excretion via TICE [50], although further research is necessary to elucidate the molecular mechanisms.

In conclusion, although recent insights into the intestinal absorption of cholesterol have also provided new evidence regarding the potential action of PS, the molecular mechanisms of PS are still a field of debate and further research is necessary.

3.3 Effect of PS on the Liver

It is well known that the liver plays a critical role in cholesterol homeostasis. Hepatic cholesterol concentrations are a balance of its intestinal absorption, its synthesis, its degradation to bile acids, and its excretion with the bile or as VLDL [74]. Reduced cholesterol absorption results in multiple changes in lipid homeostasis in the liver.

Firstly, a decrease in hepatic cholesterol concentrations leads to an upregulation of LDL-receptor expression, ultimately leading to a decrease in plasma LDL-cholesterol [28]. In addition, lower hepatic cholesterol may lead to reduced liver secretion of VLDL [75, 76]. Like in the intestine, ABCG5 and ABCG8 transporters can also be upregulated by PS, which favors hepatic secretion of cholesterol into the intestinal lumen [49].

Finally, since the absorption of cholesterol is reduced by PS, this reduction would lead to a compensatory increase in de novo synthesis of cholesterol [71]. Indeed, cholesterol biosynthesis is upregulated after consumption of diets containing phytosterols, although this increment is insufficient to offset the beneficial effects of phytosterol [28, 49, 77].

4 Other Biological Activities

The beneficial effect of PS has been traditionally ascribed to their cholesterol-lowering properties. However, recent research reveals that their biological role has been underestimated. Several in vitro and in vivo studies have reported that PS can act as immunomodulatory and anti-inflammatory agents and reduce the risk of several diseases such as cancer [13, 78, 79].

4.1 Cancer Preventive Agents

The second major beneficial effect of PS is based on their role as cancer preventive agents [78, 80–82]. The activity of a large number of phytochemicals (alone or combined with other factors) as cancer preventives has been largely reviewed and discussed [80, 83]. Some authors have suggested that PS can reduce cancer risk, although there is still a lack of data in humans, similar to those manifested by other phytochemicals [81]. PS have been proposed to prevent cancer development through several mechanisms of action such as inhibition of carcinogen production, cancer-cell growth, angiogenesis, and promoting apoptosis [80]. Emerging evidence suggests that PS can consequently play an important role in the prevention of several types of cancer such as lung, stomach, prostate, ovarian, and breast cancer [80].

One of the first studies suggesting the preventive effect of PS on cancer showed that Seventh-Day Adventists, having a high dietary intake of PS, presented low rates of colon cancer [84]. The PS intake in this population could reach 344 mg day^{-1}, which was considerably high in comparison with the average intake of the USA population. This preventive effect was mainly attributed to the reduced bile acid excretion of this population after PS intake [85], as it is known that high levels of bile acids in the bowel can increase the risk of colon cancer [86]. In contrast, contradictory results have been reported with regard to decreased bile acid excretion due to PS intake [14]. Moreover, these findings are limited by the possible modulating effect coming from other components of the diet. Ileostomy studies are more accurate to determine their effect on reducing bile acid levels in the bowel as the variability attributed to side factors can be minimized or even eliminated [87]. Revision of the available studies on this subject reveals that the magnitude of the effect attributed to PS on the reduction of bile acid excretion can be highly dependent on other dietary factors that must be taken into account. In addition, the effect on bile acid excretion can vary according to the molecule of the PS studied [81].

Some authors have proposed that other mechanisms could be involved in the cancer preventive effect of PS. Awad and Fink [88] proposed a hypothesis based on the inhibition of cell growth through stimulation of apoptosis (programmed cell death). In vitro studies have also shown the inhibitory effect of certain PS on breast- and colon-cancer cell cultures and, in a lower extent, on prostate-cancer cells [88, 89]. Another proposed mechanism is based in the capacity of PS to stimulate the sphingomyelin cycle. For instance, sitosterol seems to have a clear in vitro modulatory effect on this cycle. The PS molecule can be incorporated into the cell wall, thus reducing sphingomyelin and increasing ceramide levels in the cell membrane, which can consequently increase cell apoptosis [89, 90]. Finally, changes in testosterone concentrations can also be accounted as an alternative mechanism involved in the prevention of prostate cancer [88]. It has been also reported that diets containing 2% PS reduce the activity of 5α-reductase in liver and prostate and thus the testosterone levels in plasma [91]. However, this hypothesis has not yet been confirmed in human studies.

Furthermore, PS have also been associated as agents capable to reduce angiogenesis and metastasis [80]. In vitro studies reveal that a reduction in the invasiveness and adhesiveness of cancer cells can be responsible, at least in part, of a lower metastasis capacity of cancer cells [92, 93]. However, further clinical studies are necessary to corroborate the anti-angiogenic properties of PS.

In a similar way than other phytochemicals, comparison between animal and human studies is controversial as it is difficult to extrapolate the effective doses necessary to reach clinical relevance. In general terms, extrapolation of doses from animals to human suggests that very high intakes would be necessary to reach significant effects.

4.2 Modulation of the Immune System and Anti-inflammatory Properties

Modulation of the immune system and reduction of inflammatory disorders have also been proposed as other beneficial effects of PS consumption [13, 79].

First evidences suggested that PS can modulate the immune system by improving the activity of T lymphocytes and natural killer cells [94]. Further clinical studies have investigated the immunomodulatory effect of PS under clinical trial situations [94, 95]. The findings reveal that PS consumption can improve the clinical recovery of pulmonary-tuberculosis patients and ameliorate the adverse effects caused by immune suppression induced by immunodeficiency viruses or stress [95]. It has been recently reported that β-sitosterol can enhance the action of vitamin D on the immune function of macrophages [96]. In addition, research conducted in human Jurkat T cells has revealed that campesterol, β-sitosterol, and β-sitostanol can suppress mitogen-induced IL-2 production in a dose-dependent manner [97]. This interaction with IL-2 could be useful for patients requiring immunosuppressive effects, although further research is needed to elucidate its clinical relevance.

The role of PS as anti-inflammatory agents is commonly ascribed to their capacity to modulate cytokine production. However, how this production is modulated remains unclear. Some authors have reported that PS can reduce the production of pro-inflammatory cytokines such as IL-6 or TNF-α [94, 98], whereas others have reported the opposite effect [96, 99]. Other results show that the induced production of cytokines IL-10, IL-4, and gamma interferon in Jurkan T cells is not altered by PS [97]; however, this observation is not devoid of controversy as this effect could not be confirmed in animals [100]. However, regardless of their mechanism, different animal and human studies provide a reasonable body of evidence supporting the anti-inflammatory properties of PS [13, 100–102].

Altogether, these beneficial effects on immune and inflammatory functions seem promising as they can also be involved in the development of other pathologies such as cancer or atherosclerosis. Nevertheless, current evidence is not consistent enough, and further research is necessary to elucidate the clinical implications of sterol supplementation.

4.3 Antidiabetic Effect

PS may play an important role in ameliorating obesity or diabetic-associated disorders [13], despite the evidence is scarce. Misawa et al. [103] reported that oral administration of two types of antidiabetic PS isolated from *Aloe vera* (lophenol and cycloartenol) improves hyperglycemia in Zucker diabetic fatty rats [103]. Moreover, these PS can downregulate the expression of hepatic genes involved in the expression of gluconeogenic enzymes (glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, Pepck) and upregulate that of β-oxidation enzymes such as peroxisome proliferator-activated receptor alpha (Ppar-α) [104]. Likewise, PS have been proposed to be one of the main compounds responsible of the antidiabetic effect of some plant extracts [13, 105], although further clinical evidence is necessary.

5 Side Effects

The hypocholesterolemic effect of PS at high doses is well known, and several nutraceutical products have been approved until now by the administrations of the EU, USA, and other countries. However, much less is known about the possible toxicity or undesirable side effects of this high intake.

5.1 Toxicity

It is generally accepted that consumption of PS is safe due to their negligible rate of absorption in the upper small intestine. However, it is still in debate whether toxicological effects could appear at very high and continuous intake of some plant sterols and stanols [16].

Several studies conducted in animals [106, 107] and humans [108, 109] did not find negative physiological effects at high and/or continuous intake of plant sterols and stanols. However, other reports have reported that some toxicological effects, mainly related to the possibility of anomalous accumulation of PS in some tissues, must be taken into account [110, 111]. For instance, the study of Lees et al. [111] on hypercholesterolemic patients treated with plant sterol preparations found high serum levels of campesterol (mean 16 mg dL^{-1}) in five patients, suggesting the possibility of iatrogenic atherosclerosis due to PS accumulation. However, another study conducted in rabbits fed with semi-purified diets containing sitosterol or sitostanol esters revealed that while serum cholesterol was present at levels of milligrams dL^{-1}, serum PS were present at levels of micrograms dL^{-1} [112]. In the same study, aortic cholesterol and PS were found in microgram and nanogram quantities, respectively, and no aortic lesions were observed. One of the major concerns regarding the PS accumulation in tissues is based on their accumulation in the brain. Jansen et al. [110] reported that increased circulating levels of plant sterols, as a result of intake of a plant sterol-enriched diet in wild-type mice or as a consequence of ABCG5 or ABCG8 deficiency, was associated with elevated levels of plant sterols in the brain. More recently, Vanmierlo et al. [113] reported that mice fed with a plant sterol ester-enriched diet for 6 weeks displayed increased concentrations of plant sterols in serum, liver, and brain. In addition, the authors observed that after stopping plant sterol intake for a period of 6 months, brain PS levels remained unaffected. Interestingly, this accumulation was not found when animals received plant stanols. However, the clinical implications of these are still to be established.

5.2 Sitosterolemia (Phytosterolemia)

It has been estimated that a maximum of 5% of the plant sterol intake is absorbed, resulting in very low levels in plasma (0.5 mg dL^{-1},representing less than 0.5% total neutral sterols in plasma) [16]. However, there is a very restricted group of patients presenting a rare autosomal recessive disease called phytosterolemia [114]. These subjects can absorb up to 60% of the dietary plant sterols whereas the rate of absorption of cholesterol seems to be normal [115, 116]. This disease is characterized by mutations occurring in ABCG genes controlling the efflux of PS at intestinal level and the delivery of PS by the liver [117, 118]. Several studies reported that these patients have PS plasma levels from 18 to 72 mg dL^{-1}, which represents 7–30% of total neutral sterols in plasma [119].

With this disease there is an accumulation of PS not only in plasma but also in adipose tissue, skin, aorta, and other tissues. As a result, main symptoms of this disease include xanthomatosis and atherosclerosis [120]. This accumulation is not only related by hyperabsorption of PS but also by impaired biliary secretion. Some authors have found that phytosterolemia patients present around 20% reduction of PS biliary excretion and around 50% reduction of the whole-body cholesterol synthesis [30, 121, 122]. It has been also found that in sitosterolemic patients, the hepatic conversion of cholesterol to bile acids is blocked, which can result in cholesterol accumulation and atherosclerosis [115].

New interest in the development of sitosterolemia has arisen from the fact that several studies found relationships between anomalous high levels of PS in plasma and CHD in non-sitosterolemic subjects [120]. For instance, Glueck et al. [123] found that plasma cholesterol levels of 7 mmol L^{-1} and 40 μmol L^{-1} of PS were associated with a higher deposition of PS in the aorta in seven subjects. Likewise, Stalen et al. [124] reported that lethal atherosclerosis is related to increases in plasma PS levels. Therefore, PS levels in phytosterolemia patients must be carefully controlled.

5.3 Phytosterol Oxides

Although cholesterol oxides and their biological effects have been studied for many years [125, 126], much less is known about the biological effects of phytosterol oxides (PSO). However, the approval to supplement several food products with high doses of PS has generated the need to determine in which extent PSO can be present or formed in these products and whether they can constitute a risk for the consumer. As PS are very closely related to cholesterol in their molecular structure, it seems logical to hypothesize that they can undergo similar oxidative reactions. Thus, hydroxy-, keto-, epoxy-, and triol-derivatives of sterols would be expected to be the most abundant oxides found in food products. In contrast, plant stanols are lacking the nucleus double bound, which indicates that their oxidative pathways would not be analogous to those of cholesterol when they are subjected to processes involving reactive oxygen and free radical species, as well as irradiation and heating [127]. For stanols, the main ways of oxidation involve enzymatic reactions, which can affect the side chain [128].

The attribution of biological effects to PSO is controversial as studies reporting reliable values of their concentrations in food products are scarce and very recent [126, 129]. In addition, the values reported for PSO from food, plasma, or tissues are not easy to compare between studies due to the different analytical methods used for quantification, the lack of availability of PSO standards, the similarity of their structure, and their presence in trace amounts [130]. Thus, a better validation and standardization of analytical procedures is the main concern for future research in this field as higher amount of reliable results would help to elucidate the biological effects of PSO.

In phytosterol-enriched spreads, the content of PSO ranges from 12 to 68 μg g^{-1} [131, 132]. This would correspond to a possible ingestion of less than 1.7 mg PSO day^{-1}, according to the recommended daily intake of 20–25 g of spread. Abramsson-Zetterberg et al. [133] estimated that in the Swedish adult population, the intake of PSO, which are originally from heated vegetable oil, should be less than 0.7 mg day^{-1}. This value would be clearly lower than that estimated for the intake of cholesterol oxides (3.0 mg per day) in the context of a low-cholesterol intake population (<300 mg of cholesterol day^{-1}) [127]. The total amount of PSO in the body is still controversial, and correlations between PSO intakes and PSO levels are difficult to establish as PSO levels in the body not only come from dietary sources but also from endogenous formation [134–136]. The first reliable data regarding plasma PSO levels were reported by Plat et al. [137]. These authors were able to detect sitostanetriol and 7-keto, 7α- and 7β-OH, and α-epoxy derivatives from β-sitosterol in phytosterolemia patients, although they did not correlate these levels with adverse health effects. Some other studies were able to measure PSO in plasma of healthy volunteers [132, 138]. In all cases, β-sitosterol oxides seem to be predominant, which suggests that the absorption of β-sitosterol oxides is higher than that of other PS oxides or that the oxidation of circulating β-sitosterol in plasma is higher than that of other PS [127].

Several studies have reviewed the biological effects of PSO [126, 127, 130], although it remains questionable whether PSO can exert undesirable or beneficial effects. Some authors have proposed that PSO can be pro-inflammatory, pro-atherosclerotic, and cytotoxic [139, 140]. In contrast, others have reported beneficial effects, including modulation of cholesterol homeostasis and anti-inflammatory, lipid-lowering, and antidiabetic properties [126, 127, 130]. Moreover, for a long time, oriental traditional folk medicine has been using extracts from plants belonging to the genera Euphorbia, Urtica, and Bombyx for the treatment of some cancer-type pathologies. Recently, it has been reported that, in these extracts, PSO are the most abundant steroid compounds [141]. It has been demonstrated that PSO can induce cell death by apoptosis in different type of cell models [126]. Some observations suggest that the biological effects of PSO are similar to that of cholesterol oxides, but being five times less active [142]. Therefore, although PSO can modulate the human metabolism in some extent, it is difficult to conclude whether they are responsible of any relevant toxic effect.

5.4 Decrease in Plasma Levels of Carotenoids

Several studies have reported associations between high doses of plant sterol and stanol ester intakes and decrease in plasma carotenoid concentrations. The first problem in understanding the relevance of this effect is that no standard levels of plasma carotenoids can be clearly established [16]. Several studies comparing high and low intakes of PS have reported a parallel decrease in plasma carotenoid levels when high amounts of PS are consumed. Kritchevsky [16] compiled nine different studies in humans that were administered with pure stanols (between 0.8 and 3.2 g day^{-1}), pure sterols (between 0.8 and 3.6 g day^{-1}), wood-derived stanols (2.3 g day^{-1}), or vegetable-derived stanols (2.6 g day^{-1}) during a variable period. Results of these studies showed that in all cases, relevant dose-dependent decreases in plasma total cholesterol and LDL-c were found, which was correlated with a parallel decrease in carotenoid concentrations. The reduction was up to 20% in some cases. However, results between studies are not easy to compare as the carotenoids determined are not always the same. Even though, most of these intervention studies showed that the decrease in plasma carotenoid levels, which was accompanied by α-tocopherol decreases, disappeared when values were corrected for total cholesterol [143]. In other cases, this decrease in carotenoid and tocopherol was not observed [34]. In addition, certain studies reported that basal plasma levels of these carotenoids can be maintained by supplementing with carotenoids a high-PS diet [144]. Given these evidences, Kritchevsky [16] explained the disparity of results from different studies by differences in the carotenoid and vitamin levels present in the basal diets.

It is worth mentioning that reductions in plasma carotenoids up to 10–20% of plasma carotenoids could not be associated with negative physiological effects [145, 146]. However, it has been hypothesized that a reduction on carotenoid plasma levels could induce some disturbance in the antioxidative pathways associated with the prevention of cancer development [81]. Glutathione-*S*-transferases (GST) are enzymes with antioxidant properties which can be partly involved in the prevention of some cancers [147]. In prostate cancer, a reduction of GST1 expression may promote the susceptibility to the carcinogenic effect of chemicals, while induction of GST (i.e., by carotenoid supplementation) in early-stage prostate cancer can be a useful as a protective strategy [148]. Data existing at this moment reveal that only a slight reduction of GTS activity can be observed after PS intake [81]. Moreover, no clinical data showing a direct relationship between PS intake and risk of prostate cancer is available.

Nonetheless, since there is a parallel reduction in plasma cholesterol and carotenoid levels, it is advised to supplement the diet with these microcomponents in order to avoid the risk of side effects.

5.5 Other Safety Aspects

Other unfavorable effects have been proposed for PS intake at high doses. For instance, it is well known that PS intake increases the excretion of coprostanol and cholesterol [149]. Some recent data have shown that this increased excretion can promote the development of colon cancer [150]. However, this association was found in studies where the population consumed low-quality, high-fat diets. Therefore, it is difficult to investigate the direct relationship between high levels of PS intake and a higher colon cancer risk [81]. Further, some authors have suggested that PS could also participate as promoters of colon cancer by a possible mutagenic effect on gut bacteria. Studies on the mutagenic potential effect of PS and their esters have been conducted in bacterial in vitro cell gene mutation and in vivo rat mutagenicity assays [151]. Negative results have been obtained in all cases for PS, phytosterol esters, and for several metabolites of cholesterol (4-cholesten-3-one and 5β-cholestan-3-one). Some authors have also suggested that a high intake of PS and their esters can lead to higher levels of oxides, which can accelerate lipid autoxidation [81]. However, there is not enough information to conclude on the relevance of this oxidation process in promoting mutagenicity.

On the other hand, some studies, mainly performed in animals, have shown certain interesting and noteworthy effects of high PS intakes. Studies conducted in stroke-prone spontaneously hypertensive rats (SHRSP) demonstrated that dietary cholesterol and PS clearly affect the development of stroke and the survival of rats after the stroke [152]. According to these authors, certain level of plasma cholesterol is needed after the stroke in order to maintain the integrity of red blood cells. In contrast, high levels of PS in plasma can lead to a higher fragility of these cells and a shorter life span.

In addition, another safety aspect of relevant interest is the existence of a population of hypercholesterolemic patients with normal diets that also show hyperphytosterolemia [153]. This fact was observed in a study conducted in the USA with 595 subjects that constituted the top serum cholesterol quintile among a total population of 3,472 subjects. Approximately a 3.5% of these subjects showed anomalous high campesterol and stigmasterol levels, being considered "hypercholesterolemic-hyperphytosterolemic." This fact was associated with a personal or familiar history of premature CHD (<55 years old). However, a relevant fact reported in a follow-up study was that the levels of phytosterolemia can be normalized in this type of patients by eating a low plant sterol diet [123].

Other studies have reported similar observations [154, 155], supporting the conclusion that subjects with low endogenous cholesterol synthesis are at increased risk for coronary heart disease if they do not restrict PS intake in their diets. For these subjects, a careful control of the ratio of cholesterol/plant sterols in the diet and in plasma is recommended.

6 Conclusion

CHD is one of the main causes of mortality in developed countries. Elevated blood cholesterol concentration is known to be a major risk in the development of CHD. LDL-c in plasma can be reduced by 10% by consuming 2 g day^{-1} of PS. Although it has been suggested that higher doses can lead to undesirable side effects, the consumption of PS is generally considered safe. Current research is providing valuable insights with regard to the pathways associated to the beneficial effects of PS, although their exact mechanisms are not yet fully understood. Recently, some studies have suggested that these compounds may be able to prevent diseases such as cancer, diabetes, and inflammatory and immune disorders. Nevertheless, more studies are necessary to confirm their clinical relevance. The combination of PS with other bioactive compounds, drugs, or therapies also seems promising as their beneficial effects can be complementary or synergic.

Therefore, the incorporation of PS into functional foods or nutraceutical products can be of relevant interest not only to reduce the risk of CHD but also to prevent the development of many other diseases.

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Withanolides: Chemistry and Antitumor Activity 114

M. Iqbal Choudhary, Sammer Yousuf, and Atta-ur-Rahman

Contents

1 Introduction 3467
2 Classes of Withanolides 3467
2.1 Withaphysalins 3467
2.2 Physalins and Neophysalins 3468
2.3 Ring D Aromatic Withanolides 3469
2.4 Ring A Aromatic Withanolides 3469
2.5 Acnistins 3470
2.6 Ixocarpalactones or Perulactones 3470
2.7 Withajardins 3471
2.8 Withametelins 3471
2.9 Norbornane-Type Withanolides 3472
2.10 Sativolides 3472
2.11 Spiranoid δ-Lactones 3472
3 Spectral Generalization of Withanolides 3472
3.1 Ultraviolet and Infrared Spectra 3472
3.2 Mass Spectra 3473
3.3 Nuclear Magnetic Resonance Spectra 3476

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4 Pharmacological Importance of Withanolides 3476
4.1 Antitumor Activity of Withanolides 3477
5 Other Pharmacological Activities of Withanolides 3488
6 Conclusion 3488
References 3489

Abstract

Withanolides are a group of naturally occurring C_{28} steroidal lactones with ergostane-based skeleton. They are highly oxygenated natural products, and the oxidation at various sites of skeleton is responsible for the structural variations in different classes of withanolides. Withanolides possess a wide range of biological activities including antitumor activity. The extracts of withanolide-containing plants have been used for the treatment of various types of cancers and tumors. However, the antitumor activity of a purified withanolide was first reported in 2004. Since then, many withanolides have been evaluated for their anticancer and antitumor activities by using a number of bioassays. The chapter deals with the structural classes of withanolides and their biological activities. The reported antitumor activity of withanolides (covering the period till early January 2012) is discussed in detail, along with other biological and pharmacological activities of withanolides.

Keywords

Anticancer • antitumor • cyclooxygenase • cytotoxic • ergostane • immunomodulatory • quinone reductase • steroidal lactones • withanolides

Abbreviations

ESI-QqTOF-MS/MS	Positive ion electrospray ionization quadrupole time-of-flight mass spectrometry
CID-MS/MS	Collision-induced dissociation tandem mass spectrometry
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
EI-MS	The electron impact mass spectrum
IC_{50}	Concentration which causes 50 % inhibition of activity
IR	Infrared spectrometry
UV	Ultraviolet spectrometry
M^+	Molecular ion
MS/MS	Mass-mass or tandem spectroscopy
m/*z*	Mass to charge ratio
NMR	Nuclear magnetic resonance spectroscopy
ROS	Reactive oxygen species
SAR	Structure-activity relationship

1 Introduction

The withanolides are a group of naturally occurring steroids with an ergostane-type of skeleton having δ- or γ-lactone-containing side chain of nine carbons, attached at C-17 of main steroidal skeleton [1–5]. The rearranged ergostane framework of withanolides (**1**) is commonly known as the "22-hydroxyergostan-26-oic acid 26,22-lactone." Withaferin A (**2**), the first member of this group, was isolated from *Withania somnifera* by Lavie and coworkers in 1965, and the name withanolide was accordingly given based on the name of the source genus [4, 6]. In the last three decades, a number of reviews focusing on the structural properties, biosynthesis, synthetic approaches, and biological properties have been published [2–5]. However, this chapter is the first comprehensive review on the antitumor and anticancer properties of withanolides reported in last three decades, covering the literature up to early January 2012.

Since 1965, a large number of withanolides were isolated from the plants of Solanaceae, Taccaceae, Leguminosae, Labiatae, and Myrtaceae, as well as from some marine organisms [1–5, 7, 8]. Among all these plant families, the Solanaceae is the richest source of withanolides, containing 14 genera, namely *Acnistus, Datura, Deprea, Dunalis, Discopodium, Exodeconus, Hyoscyamus, Iochroma, Jaborosa, Larnax, Lycium*, *Nicandra, Physalis, Salpichroa, Trechonaetes, Tubocapsicum, Vassobia, Withania*, and *Withanergia* [2].

In addition to the plants of Solanaceae family, certain withanolides named "minabeolides" have been reported from the sea soft coral belonging to the genus *Paraminabea*, representing the first class of marine withanolides [7, 9]. Minabeolides were reported three decades ago and found to be structurally similar to the simple withanolides such as withaferin A (**2**). However, recently they have attracted attention because of numerious reports of their cytotoxic properties.

2 Classes of Withanolides

Oxidation at different sites of the steroidal skeleton is responsible for the rearrangement of the basic skeleton of withanolides to the following classes:

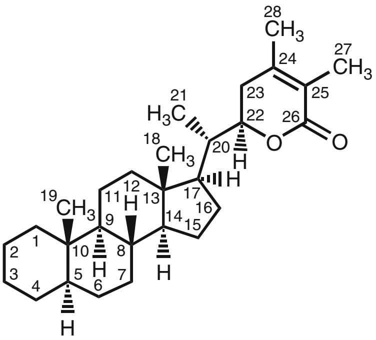
2.1 Withaphysalins

The oxidation of the C-18 methyl into carboxylic acid leads to the formation of an additional lactone ring by the linkage between C-18 carboxylic acid and C-20 hydroxyl group in the basic skeleton of withanolide [10, 11]. Such withanolides are known as “withaphysalins.” Examples include withaphysalins A (**3**) and E (**4**) [11–13].

2.2 Physalins and Neophysalins

Physalins are the steroidal lactone constituents of *Physalis minima* and other closely related genera, corresponding to 16,24-cyclo-13,14-*seco* steroidal structures [14, 15]. The highly oxidized skeleton of physalins is biogenetically related to the withanolides, with (a) an oxidized 13,14-bond ring cleavage yielding a nine-membered ring, (b) formation of a new six-membered carbocycle between C-16 and C-24, and oxidation of the C-13 methyl group into a carboxylic acid which results in 18,20-lactonization [11]. Examples include physalins A (**5**) [16, 17] and B (**6**) [17].

The further rearrangement of the skeleton of physalin due to the C-15/C-16 bond cleavage, followed by the C-14/C-16 bond formation, leads to the formation of neophysalin. Physalin P (**7**) was the first neophysalin isolated from *Physalis alkekengi* [18].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26
O
O
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CH3
20
H
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19
CH3
11
12
13
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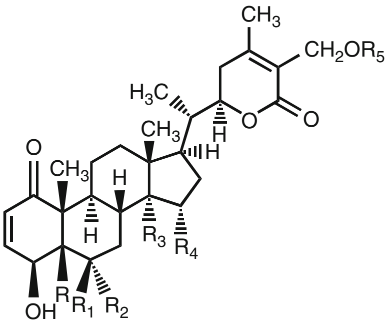
H
H

1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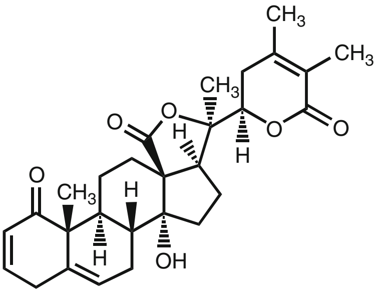
CH2OR5
H3C
O
O
H
CH3
H
O
CH3
H
H
R3
R4
OH
R
R1
R2

2 R=R$_1$=$\underset{O}{\bigtriangledown}$, R$_2$=H, R$_3$=R$_4$=R$_6$=H
45 R=Cl, R$_1$=OH, R$_2$=R$_3$=R$_4$=H, R$_5$=COCH$_3$
46 R=R$_1$=$\underset{O}{\bigtriangledown}$, R$_2$=H, R$_3$=R$_4$=$\underset{O}{\bigtriangledown}$, R$_6$=H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O
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H
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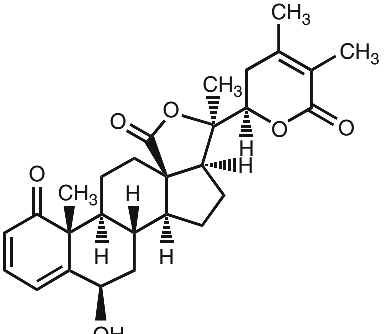
OH

3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O
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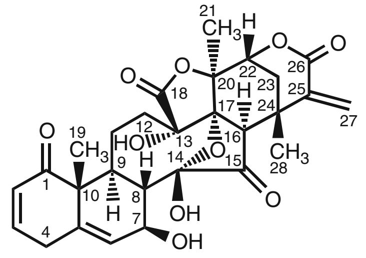
H
O
CH3
H
H
H
OH

4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27
O
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HO
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CH3
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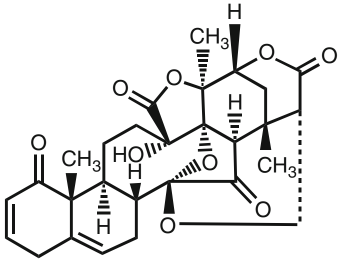
OH
O
4
OH

5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CH3
HO
H
O
CH3
H
O
O

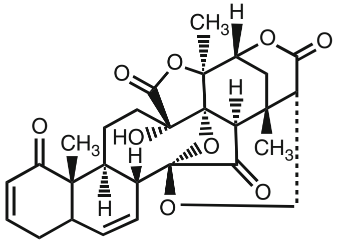
6

2.3 Ring D Aromatic Withanolides

The withanolide having unique structural feature, aromatic ring D, example is nicandrenone (**8**), only reported from *Nicandra physalodes* [19, 20].

2.4 Ring A Aromatic Withanolides

Many withanolides, such as jaborol (**9**), reported from plants of genus *Jaborosa*, have a skeleton with aromatic ring A [21].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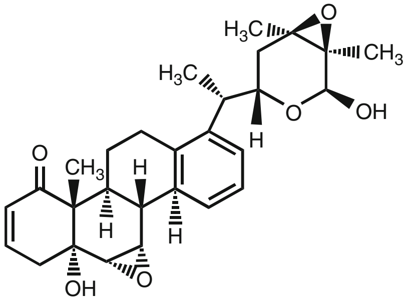
O

7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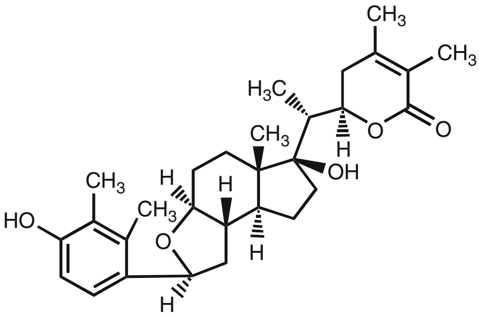
H
H
OH
O

8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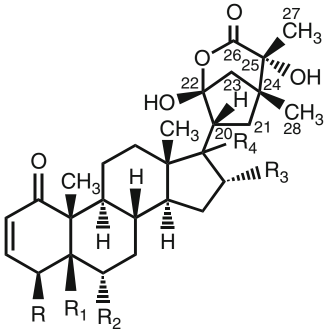
O
H
OH
CH3
H
HO
CH3
H
O
H
H

9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CH3
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26
25
OH
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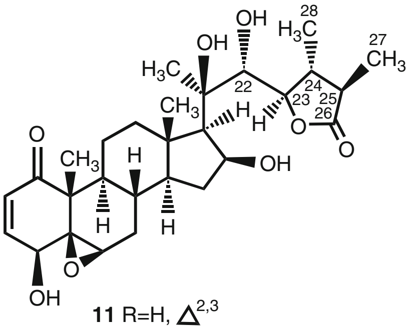
23
24
HO
H
CH3
CH3
28
21
20
R4
O
CH3
H
R3
H
H
R
R1
R2

10 R=R$_3$=H, R$_2$=R$_3$=$\underset{O}{\triangledown}$, R$_4$=$\beta$OH
41 R=R$_1$=R$_2$=R$_3$=OH, R$_4$=αOH
42 R=R$_1$=R$_3$=OH, R$_2$=Cl, R$_4$=αOH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H3C
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CH3
H3C
22
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26
CH3
H
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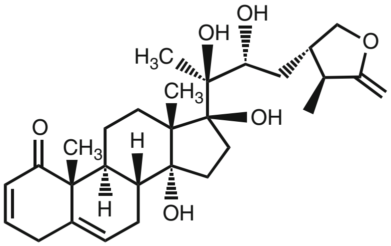
CH3
H
H
H
O
OH

11 R=H, $\Delta^{2,3}$
79 R=OCH_3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H3C
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CH3
OH
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CH3
H
H
OH

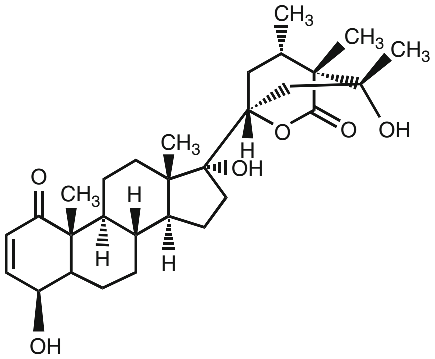
12

2.5 Acnistins

Acnistin-type withanolides have a bicyclic system, instead of a lactone ring at the C-17 side chain. The linkage between C-21 and C-24 of the lactone ring is responsible of the formation of a five-membered ring, attached to the lactone ring as in acnistin E (**10**), isolated from *Acnistus ramiflorus* [22].

2.6 Ixocarpalactones or Perulactones

These are the withanolides with a characteristic modification in the side chain to form 26,23-γ-lactone. Examples include ixocarpalactone A (**11**) [23, 24] and perulactone B (**12**) [25].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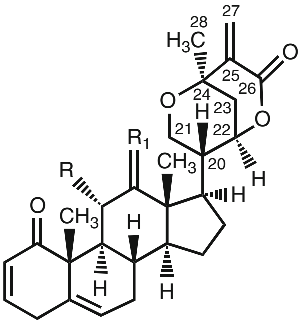
13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R1
CH3
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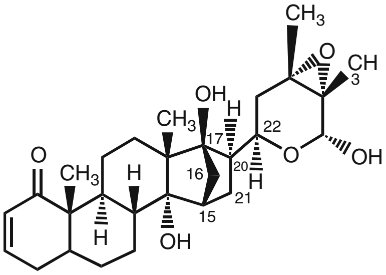
H
H

14 R = H, R_2= αOH, βH
15 R = H, R_2= O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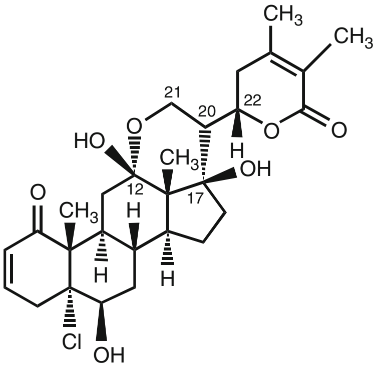
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OH

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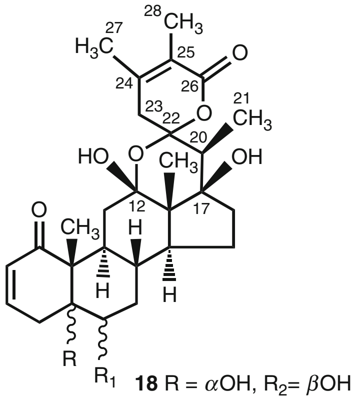
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CH3
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H
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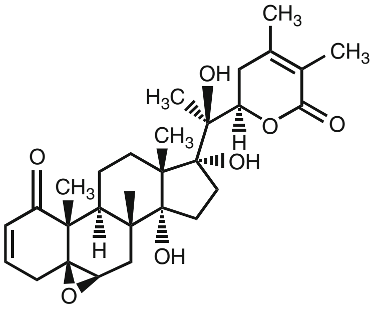
R
R1

18 R = αOH, R_2= βOH
19 R = αCl, R_2= βOH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CH3
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OH
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CH3
H
OH
O

20

2.7 Withajardins

Structurally, withajardins closely resemble the acnistin-type withanolides with the difference that the bicyclic side chain contains a six-membered ring, instead of a five-membered ring, obtained by the linkage between C-21 and C-25 of the lactone ring. Examples include withajardin A (**13**) isolated from *Deprea orinocensis* [26, 27].

2.8 Withametelins

These withanolides are only reported from plants of genus *Datura*. They are closely related to the withajardin- and acnistin-types with the difference that the C-21 and

C-24 of the lactone ring are linked through an ether bridge to form another six-membered ring, along with the presence of an exocyclic double bond between C-25 and C-27 [28]. Examples include withametelinol (**14)** and withametelinone (**15**) [29].

2.9 Norbornane-Type Withanolides

These withanolides have an additional ring that arises by the linkage of C-15 and C-17 of ring D. Jaborosalactol 18 (**16**) is among the first members of the norbornane-type of withanolides, isolated from the aerial parts of *Jaborosa bergii* [30].

2.10 Sativolides

Withanolides having six-membered hemiketal, obtained by the linkage between keto (oxidized C-12) and hydroxylated C-21, are known as sativolides [5]. Jaborosalactone 37 (**17**) is an example of this class of withanolides, isolated from the plants of genus *Jaborosa* [31].

2.11 Spiranoid δ-Lactones

These withanolides are formed by the double oxidation of C-22, leading to the formation of an ether linkage between C-12 and C-22 and a spiranoid δ-lactone at C-22. Examples include jaborosalactones 27 (**18**) and 28 (**19**), isolated from *Jaborosa rotacea* [31].

3 Spectral Generalization of Withanolides

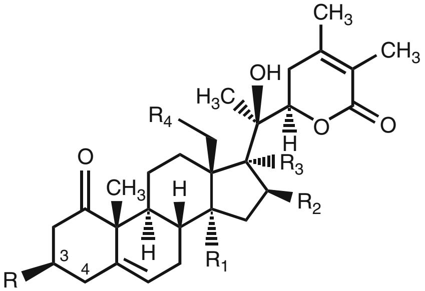
The withanolides exhibit characteristic spectroscopic behavior, which can help in their structural elucidation.

3.1 Ultraviolet and Infrared Spectra

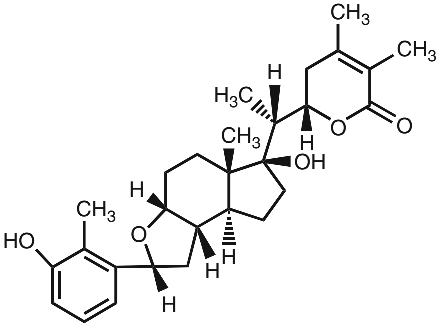
The majority of withanolides possess two types of chromophores:

1. An enone (2- or 5-ene-1-one) such as **2**, **14**, and **15**
2. An α,β-unsaturated δ-lactone, such as in withanolide E (**20**) and 3β-hydroxy-2,3-dihydrowithanolide F (**21**)

The UV absorptions due to these chromophores generally appear in the range of 218–230 nm [38]. The compounds have aromatic rings in their skeleton that generally absorb in the range of 224–301 nm, as in (+)-jaborol (**22**) [32] and salpichrolide G (**23**) [33].



21 $R=R_1=R_3=OH$, $R_2=R_4=H$
78 $R=R_1=R_3=H$, $R_2=R_4=OH$, $\Delta^{3,4}$



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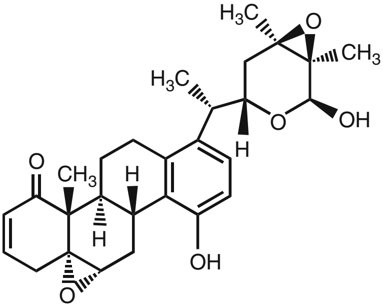
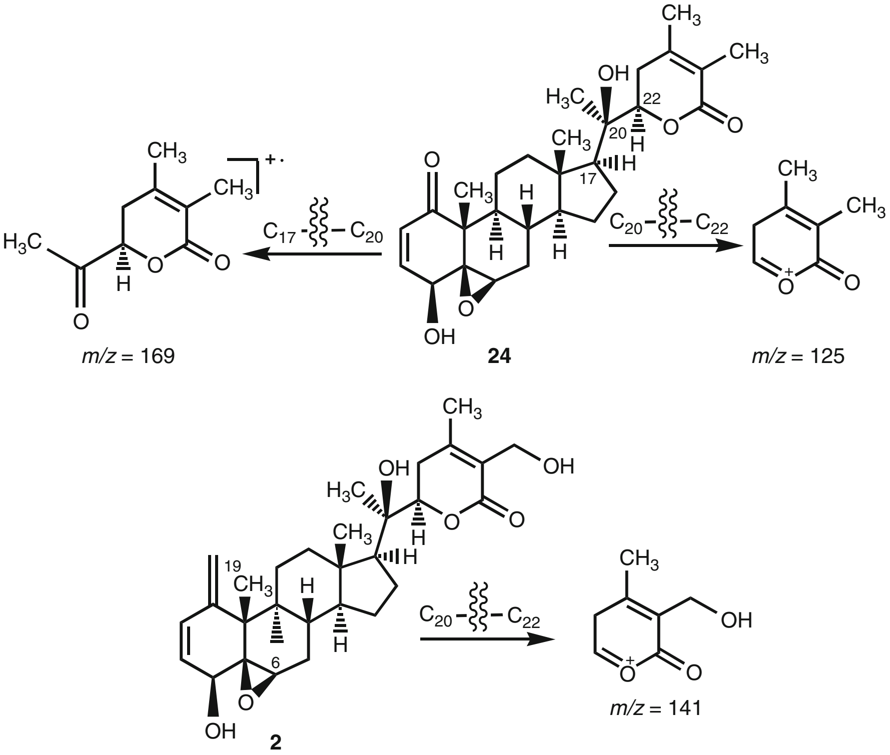


23

Almost all types of oxygen functionalities are present in this class of steroidal lactones, and the characteristic IR spectral bands for various functionalities appear in the spectra. The characteristic enone and *α,β*-unsaturated *δ*-lactone moieties of withanolides show absorptions at or near the 1,660 and 1,710 cm^{-1}, respectively. An IR absorption at 1,778 cm^{-1} is due to carbonyl stretching vibrations in five-membered lactone ring, found in all physalins and other classes of withanolides with modified skeleton [34–36].

3.2 Mass Spectra

Mass spectrometry is a powerful tool in structure determination of withanolides. The M^+ in electron impact mass spectra (EI-MS) is either weak or absent in various types of withanolides. The EI-MS spectra of withanolides are more informative, as compared to those having modified withanolide skeleton. A base peak at *m*/*z* 125 is formed by fission of the C-20/C-22 bond and is considered to be a diagnostic feature for withanolides with an unsubstituted *α,β*-unsaturated *δ*-lactone moiety [37] (Scheme 114.1). However, the presence of a hydroxyl group at C-20 facilitates the cleavage of the C-17/C-20 bond and gives rise to a peak at *m*/*z* 169

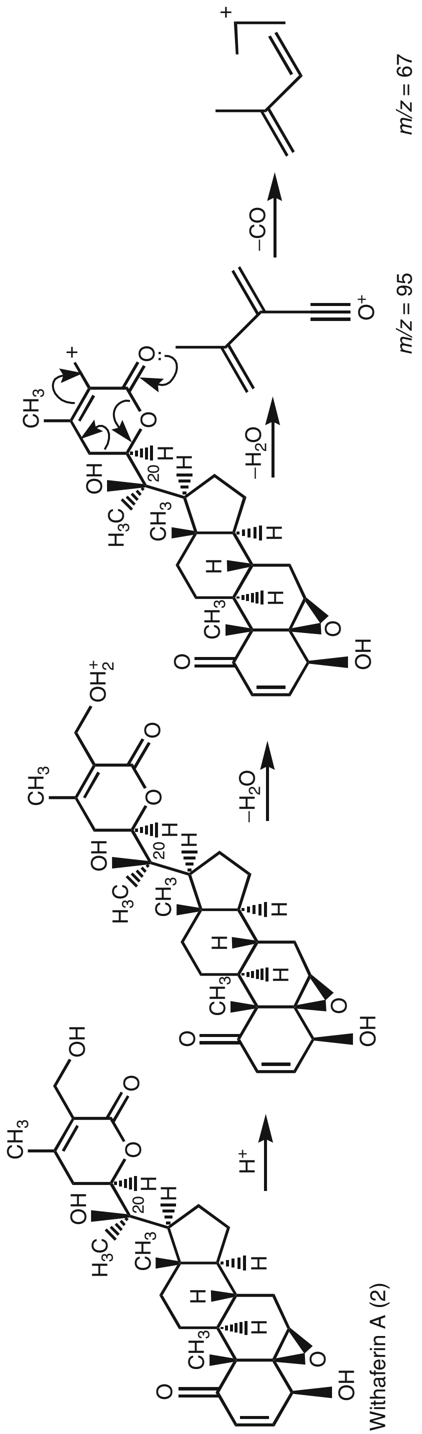


Scheme 114.1 The characteristic EI-MS fragmentation pattern of withanolides

(Scheme 114.1) [38]. The presence of a hydroxyl group in the lactone part of the molecule shifts the peak from *m*/*z* 125 to *m*/*z* 141 [39–41].

Gas-phase fragmentation of withanolides studied by ESI-QqTOF-MS/MS established a rapid method for the identification of low quantities of withanolides in complex plant extracts, herbal formulations, and pharmaceutical products [42]. Structure-fragmentation relationship (SFR) of ten standard withanolides was studied. The withanolides with hydroxyl groups at C-4, C-5, C-17, C-20, and C-27 and an epoxy group between C-5 and C-6 were evaluated for their characteristic fragmentation pattern. The CID-MS/MS analysis indicates the multiple removal of water molecules and the removal of C-17 lactone moiety as a major pathway. However, C-24 hydroxylated withanolides, such as withaferin A (**2**), exhibit diagnostic ions at *m*/*z* 95 and 67, generated due to the cleavage of *δ*-lactone moiety (Scheme 114.2). A generalization based on MS/MS fragmentation was developed for the rapid identification of withanolide in the complex plant extract by employing LC-MS/MS analysis.

The mass spectra of other types of withanolides exhibit no systemic fragmentation pattern, which may be due to their highly complex and oxygenated skeletons.


Withaferin A (2)
H⁺
−H₂O
−H₂O
−CO
m/z = 95
m/z = 67

Scheme 114.2 Proposed CID-MS/MS fragmentation of the precursor ion at *m/z* 471.2714, which yields the product ions *m/z* 67 and 95

3.3 Nuclear Magnetic Resonance Spectra

3.3.1 ^{1}H-NMR

The C-22 proton of a simple withanolide shows a characteristic pattern, resonating in the range of δ 4.00–4.90 and referred as the withanolide "fingerprint." It is a diagnostic doublet of triplets in typical withanolides, which have unsubstituted C-20 and C-23 [43], as in withaferin A (**2**), and as a double doublet when either C-20 or C-23 is substituted [44–46], as in **24**.

A typical withanolide exhibits characteristic singlets for five methyl groups in the range of δ 0.99–2.00, two angular methyls (C-18 and C-19), two vinylic methyls (C-27 and C-28), and one secondary methyl (C-21). In the case of unsubstituted C-20 compounds, the C-21 methyl appears as a doublet between δ 1.00 and 1.02, with the coupling constant (J) of 6.4–6.7 Hz. The characteristic one proton doublet, appearing at δ 3.22 ($J_{6\alpha,7\beta} = 2.1$ to 2.4 Hz), is due to an oxirane ring between C-5 and C-6, commonly found in withanolides of diverse classes [47, 48].

The ^{1}H-NMR spectral data of physalins display several characteristic signals. These include three characteristic methyl singlets at δ 1.25, 1.35, and 1.99 Hz, which are due to C-28, C-19, and C-21 tertiary methyls, respectively [34, 49]. The presence of a C–O bridge between C-14 and C-27 is inferred from the appearance of a pair of characteristic signals for the C-27 methylene protons, resonating at δ 3.75 as a doublet ($J_{AB} = 13.5$ Hz) and at δ 4.53 as a double doublet ($J_{AB} = 13.5$ Hz, $J_{27,25} = 4.4$ Hz) [34, 50, 51].

3.3.2 ^{13}C-NMR

The ^{13}C-NMR spectral data of withanolides has been found to be very useful in the structural and conformational analysis of withanolides. The downfield singlets between δ 202–216 and 166–168 are attributed to the carbonyl group at C-1 and the lactone carbonyl at C-26, respectively. Two signals around δ 149 and 121 correspond to the olefinic C-24 and C-25 of the lactone ring. The C-22 bearing the δ-lactone oxygen, appears between δ 74 and 78 in all members of this class. Three methyl carbon signals at δ 26.5, 21.2, and 14.5 are due to the C-21, C-19, and C-28 quaternary methyl carbons of physalins, respectively. The C-27 methylene carbon appears at δ 60.7, as in other members of physalins [34, 49–51].

4 Pharmacological Importance of Withanolides

The withanolides containing plants of the family Solanaceae possess a wide range of pharmacological properties and are well-known sources of medicinal agents, stimulants, and poisons [52].

Withanolide-containing plants are widely used for hair care. *Datura metel*, or Datura, is a widely recommended remedy for the treatment of dandruff. *Withania somnifera*, also known as ashwagandha or Indian "ginseng," is the most widely used withanolide-containing plant in the Ayurvedic medicinal system for therapeutic purposes. The flowers of the *Withania somnifera* are recommended in the Siddha system of medicine as an exclusive hair remedy [53–55].

The methanolic extracts of different parts of *W. somnifera* exhibit therapeutic potential against various types of cardiovascular problems and are also effective against hyperlipidemia, obesity [56], aging, and copper-induced pathophysiological conditions [57]. This plant is reputed to promote vitality during recovery from chronic illnesses [58] and is also useful for the pain management in arthritic conditions [59].

Withania somnifera has a calming effect on mind and thus promotes sound sleep. In bronchial asthma and spasmodic cough, the ash of its burnt roots is given with honey and oil to liquify the phlegm. The black root ash of the plant, mixed with sesame oil, helps in removing skin depigmentation and minor skin ailments [54]. Ashwagandha also helps to maintain the heart in good condition and is reputed to be a blood purifier. It is considered to be beneficial in blood diseases [54].

Similarly, *Physalis minima* is widely used in folk medicines for the treatment of bronchitis, inflammation, enlargement of spleen, urinary disorders, abdominal troubles, and headache [53, 60]. The roots are used as a vermifuge and febrifuge and for the treatment of diabetes. In Guatemala, the plant is popularly used for the treatment of gonorrhea [61]. The fruits of *Physalis minima* are said to be an appetizer and have also been recorded as an ingredient of medicinal oil for spleen disorders. The fruits are also considered as a tonic, diuretic, and purgative in different areas of the Punjab, Pakistan [12, 53, 62, 63].

The Mundas (a tribe) of Nagpur (India) mix the juice of the leaves with water and mustard oil and use it as a remedy against earache [60]. Similarly, *Physalis philadelphica* is used by the people of Guatemala for the treatment of gastrointestinal disorders and in Mexico for the treatment of leprosy [64].

A wide range of the biological activities of the plants having withanolides as principle constituents have attracted the attention of the researchers and led to the isolation of the therapeutically active constituents, the withanolides, followed by the pharmacological evaluation of these compounds to validate their traditional uses.

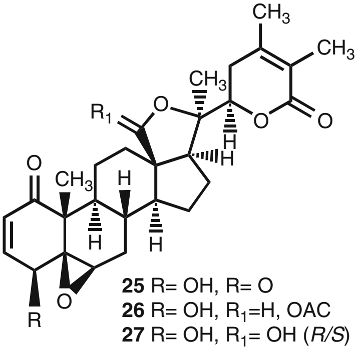
4.1 Antitumor Activity of Withanolides

The antitumor potential of withanolides has been extensively investigated by the researchers all around the world, which led to the identification of diverse properties such as cytotoxicity, cell differentiation induction, cancer chemoprevention, and COX-2 and quinine reductase enzymes inhibition potential of withanolides. Recently, over 80,000 natural and synthetic compounds were evaluated by Santagat and coworkers for their anticancer activity, targeting protein homeostasis. They concluded that many active compounds were natural products belonging to the five classes, limonoids, celastraloids, colletofragarones, curvularins, and withanolides [65].

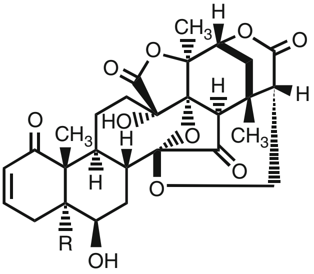
4.1.1 Cytotoxicity of Withanolides

The cytotoxicity of withaphysalin-types of withanolides was first reported in 2004 by Veras and his coworkers. They evaluated the cytotoxic potential of ethanolic extract of leaves of *Acnistus arborescens* and isolated withaphysalins M (**25**) and O (**26**), active against human lung cancer (Lu1), hormone-dependent human prostate cancer (LNCaP), and estrogen-dependent human breast cancer (MCF7) cell lines, and showed the potent cytotoxic abilities [66, 67]. They further studied the incubation of withaphysalin F (**27**) on the leukemia cells and found a reduction in 5-bromo-2′-deoxyuridine incorporation after 24 h of incubation. Furthermore, studies have also showed that withaphysalins M and O are cytotoxic and seemed to induce apoptosis and necrosis in tumor cells [68]. In vitro cytotoxic activity of physalin D (**28**) against human cancer lines was reported by Cordero et al. in 2009. The compound showed weak cytotoxicity against normal human fibroblast cultures (Fib04) [69]. The cytotoxic activities of withaphysalins M and O and physalin D thus validate the folk uses of *A. arborescens* as a remedy to suppress the growth of cancer.

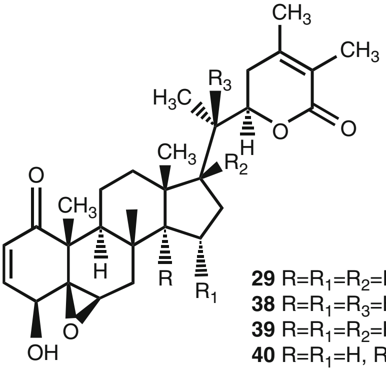
The cytotoxic activity of a series of withanolides, isolated from *Physalis angulata*, was evaluated against various human cancer cell lines. Strong cytotoxicity was exhibited by withangulutin B (**29**) and physalins B (**6**), D (**28**), F (**27**), and U (**30**) [67, 68] against DU-45, 1A9, HCT116, LNCAP, KB, KB-VIN, A431, A549, HCT-8, PC-3, and ZR751 human tumor cell lines [70, 71].



25 R= OH, R= O
26 R= OH, R$_1$=H, OAC
27 R= OH, R$_1$= OH (*R/S*)



28 R=OH
44 R=Cl

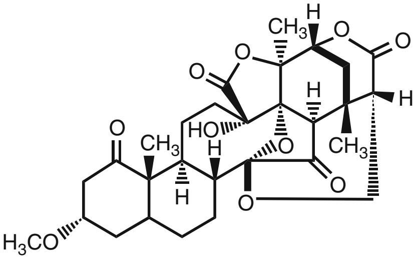


29 R=R$_1$=R$_2$=R$_3$=OH
38 R=R$_1$=R$_3$=H, R$_2$=OH
39 R=R$_1$=R$_2$=H, R$_3$=OH
40 R=R$_1$=H, R$_2$=R$_3$=OH

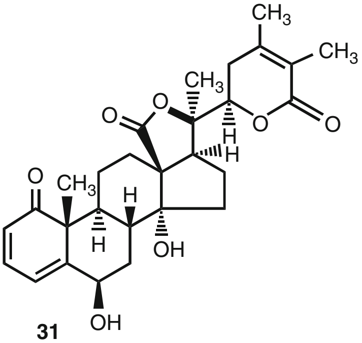
A series of ten withanolides, isolated from *Physalis minima*, were evaluated for their cytotoxic properties against HCT-116 and NCI-H460 cell lines. It was found that withaphysalin C (**31**) was active against both the cell lines [72]. The structure-activity relationship (SAR) studies of the cytotoxic withanolides from *Physalis minima* and *Physalis angulata* indicate that the α,*β*-unsaturated ketone moiety in ring A and 5*β*-6*β* oxirane moiety in ring B are important contributors in the cytotoxicity of withanolides. The absence of these two structural features may result in a loss or reduction of the cytotoxicity of withanolide [4].

The evaluation of cytotoxic activity of 17 withanolides against various human cancer cell lines, such as hepatocellular carcinoma (Hep G2, Hep 3B), breast carcinoma (MDA-MB-231, MCF-7), lung carcinoma (A-549), and embryonic lung (MRC-5) cell lines, disclosed the significant cytotoxic potential of withanolides **32–42** [73]. The SAR studies showed that the C-4 hydroxyl group apparently has no contribution to their activity. 6-Chloro withanolides **34** and **42** are found to be less cytotoxic, as compared to their hydroxyl analogues **33** and **41**. This initial inference is further supported by the significant cytotoxic potential of 5-chloro substituted physagulin B (**43**) [74] and physalin H (**44**) [21, 75, 76]. The cytotoxic potential of the chlorinated (**45**) and diepoxide-containing withanolide (**46**) was also evaluated by our group against the human lung cancer cell line (NCI-H460), along with withaferin A (**2**). All compounds exhibited cytotoxicity and growth inhibition activity which further support the role of C-5 Cl group in antitumor activity of withanolides. Withaferin A (**2**) was found to be the most potent withanolide against lung cancer cell line [77].

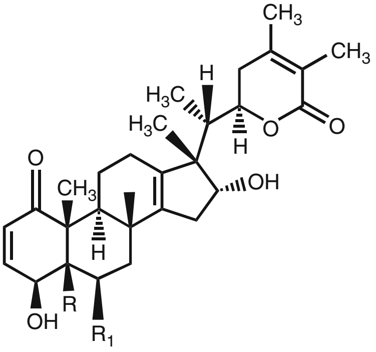
The cytotoxic behavior of the withanolides, having 7*β* or 16α acetoxy group, was evaluated by Minguzzi in 2002. They observed that above-mentioned functionalities have no contributions in the cytotoxicity of the withanolides [78]. However, the C-17-oxygenated withanolides with C-16α hydroxyl or acetoxy group showed enhanced the cytotoxicity [79].



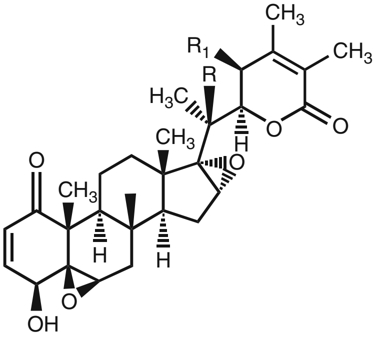
30



31



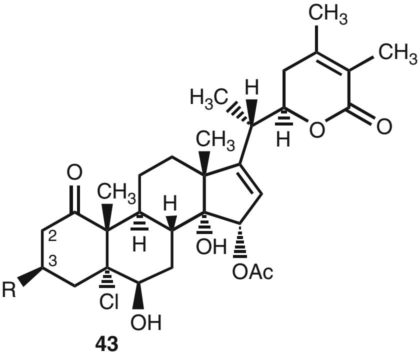
32 R=R$_1$=▽O
33 R=R$_1$=OH
34 R=OH, R$_1$=Cl



35 R=R$_1$=H
36 R=OH, R$_2$=H
37 R=H, R$_2$=OH

The cytotoxic potential of a series of 14 withalongolides was evaluated by using a MTS viability assay, and it was found that eight withanolides **47**–**54** and four acetylated derivatives **47a**, **47b**, **48a**, and **48b** showed potent cytotoxicity against human head and neck squamous cell carcinoma (JMAR and MDA-1986), melanoma (B16F10 and SKMEL-28), and normal fetal fibroblast (MRC-5) cells. The results further support the role of an acetyl group in the antitumor activity of **47a**, **47b**, **48a**, and **48b**. However, the contribution of the acetoxy functionalities to the cytotoxic potential of withanolides needs to be further evaluated [80]. The cytotoxicity of withanolide-type steroids, isolated from Tunisian *Datura metel*, against human lung carcinoma cells (A549) and human colorectal adenocarcinoma cells (DLD-1) [81] has been observed. Discorolides A (**55**) and B (**56**) showed promising results against tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT15) for further studies [82].

Cytotoxicity of six new withanolides was evaluated against human liver carcinoma Hep G2 cell lines, and it was found that peramenolide A (**57**) and minabolides-1 (**58**) were cytotoxic withanolides [6].



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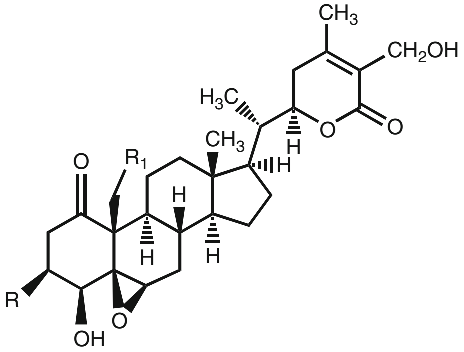
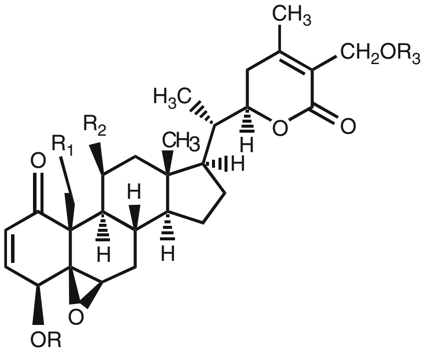

CH3
CH2OH
H3C
O
O
H
CH3
H
O
R1
H
H
H
R
O
OH

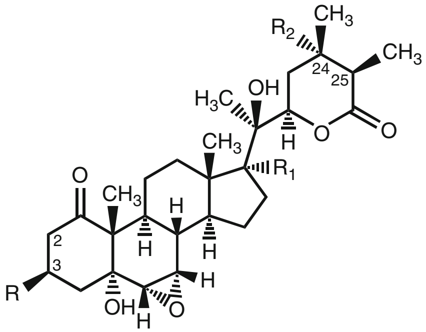
53 R=OCH$_3$, R$_1$=OH
54 R=OSO$_3$H, R$_1$=H


CH3
CH2OR3
H3C
R2
O
O
R1
CH3
H
O
H
H
H
H
O
OR

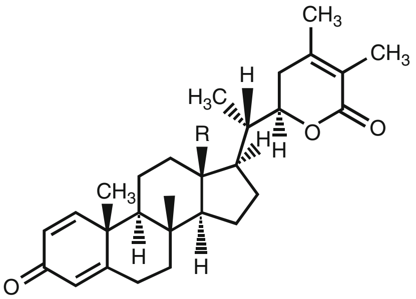
47 R=R$_2$=R$_3$=H, R$_1$=OH
47a R=R$_3$=Ac, R2=H, R$_1$=OH
47b R=R$_3$=Ac, R$_1$=OAc, R$_4$=H
48 R=R$_2$=R$_3$=H, R$_1$=OH
48a R=R$_2$=R$_3$=H, R$_1$=OAc
48b R=Ac, R$_1$=OAc, R$_2$=R$_3$=H
49 R=H, R$_1$=R$_3$=H, R$_2$=OH
50 R=R$_1$=R$_2$=H, R$_3$=Glc-Rah
51 R=R$_1$=R$_2$=H, R$_3$=Glc-Rah
52 R=R$_1$=R$_2$=H, R$_3$=OH

4.1.2 Cell Differentiation Inducing Activity

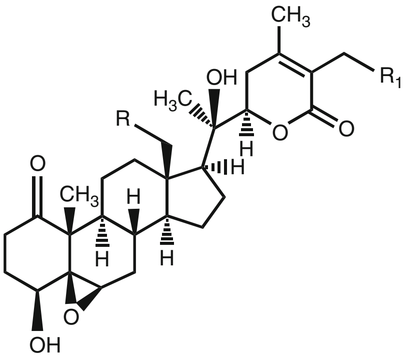
Kuroyanagi and his coworkers evaluated a series of sixteen withanolides, isolated from *Withania somnifera*, for their cell differentiation inducing ability and found that withaferin A (**2**), withanolide D (**24**), dihydrowithanolide D (**59**), and 27-hydroxywithanolide D (**60**) were potent cell differentiation inducers against the MI cells [83]. It was further concluded that all these withanolides have a similar 4*β*-hydroxy-5*β*-6*β*-epoxy-2-ene-1-one moiety in ring A, apparently responsible for their cell differentiation inducing activity.


CH3
R2
CH3
24
25
OH
H3C
H
O
O
CH3
R1
O
CH3
H
H
H
2
3
R
H
OH
O
H

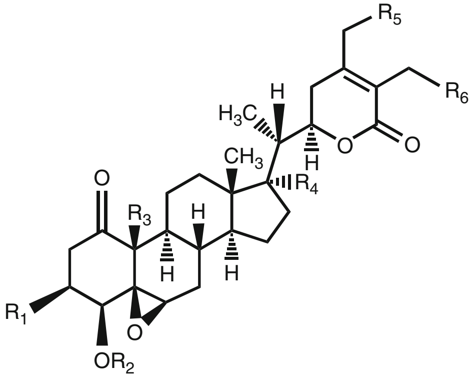
55 R=R$_1$=H, R$_2$=OH, $\Delta^{2,3}$
56 R=OAc, R$_1$=H, R$_2$=OH
73 R=R$_2$=H, R$_1$=OH, $\Delta^{2,3}$, $\Delta^{24.25}$

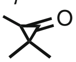

CH3
CH3
H
H3C
O
O
R
H
H
CH3
H
H
O

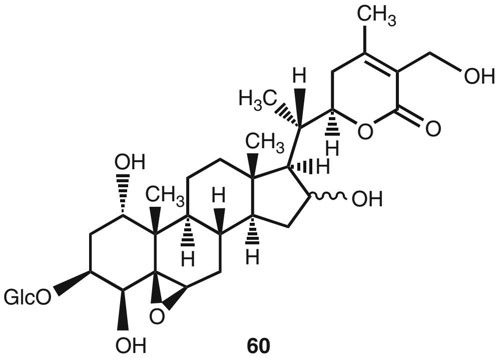
57 R= CHO
58 R= H



59 R=R$_1$=H,
60 R=H, R$_1$=OH, $\Delta^{2,3}$
72 R=OH, R$_1$=H, $\Delta^{2,3}$



61 R$_1$=OH, R$_2$=R$_4$=R$_5$=H, R$_3$=CH$_3$, R$_6$=Oβ-D-Glc
63 R$_1$=R$_4$=R$_5$=H, R$_4$=CH$_3$, R$_6$=OH, R$_2$=  O



60

4.1.3 COX-2 Inhibition and COX-1 Non-inhibition Activity

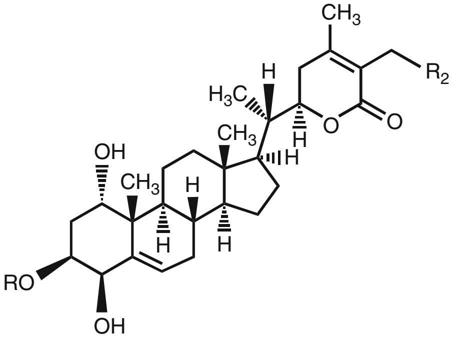
The overexpression of COX-2 enzyme in various types of tumor cells indicates the role of this enzyme in the onset of tumors. On the other hand, the inhibition of COX-1 enzyme is responsible of the formation of ulcers. The COX-2 inhibition ability of twelve withanolides from *Withania somnifera* was evaluated, along with their lipid peroxidation missing ability. Compounds **61, 62, 63**, and **64** and **65** were found to be the inhibitors of COX-2 enzyme at 50 μg mL^{-1} concentration. These withanolides were found to be noninhibitors of COX-1 even at a concentration of 500 μg mL^{-1}. The selective inhibition of COX-1 by the sulfur-containing dimeric withanolides, ashwagandhanolides (**66**) and thiowithanolide (**67**), was also investigated by the same group, along with the growth inhibition potential of both compounds against various cancer cell lines [84–86]. The results of the COX-2 inhibition assay by withanolides provided the scientific validation for the traditional uses of *Withania somnifera* leaves as remedy for cancers.

4.1.4 Radiosensitizing Activity

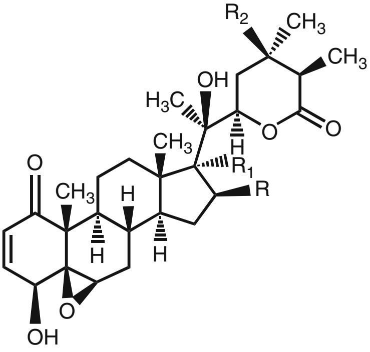
Effective tumor control can be achieved by the use of substances that can increase the sensitivity of the tumor cells for chemotherapeutic agents and therefore reduce the side effects for the normal cells. Withaferin A (**2**), the major constituent of the *Withania somnifera*, was found to be a potent radiosensitizing agent when administered before radiotherapy. The effect was studied on B16F1 melanoma and fibrosarcoma mouse tumors grown in C57BL and Swiss albino mice, respectively. The study concluded that the radiotherapy response of both tumors increased substantially on administration of withaferin A (**2**) [87].

4.1.5 Quinone Reductase Inducing Activity

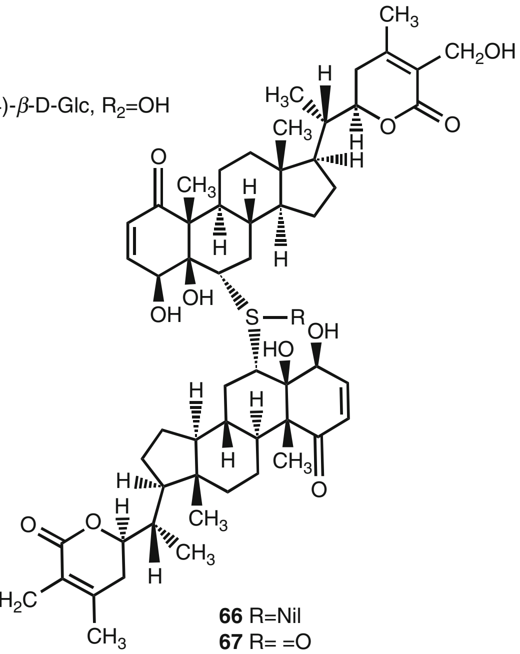
Induction of quinine reductase enzymes is considered as an important mechanism to study the protection against toxic effects of many carcinogens. Therefore, the quinine reductase inducers are important chemoprotective agents against cancer.


CH3
R2
H
H3C
O
O
H
CH3
H
OH
CH3
H
H
H
RO
OH

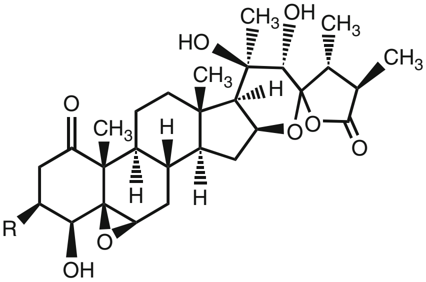
64 R=β-D-Glc-(1-6)-β-D-Glc-(1-4)-β-D-Glc, R_2=OH
65 R=β-D-Glc, R_1=Oβ-D-Glc


R2
CH3
CH3
OH
H3C
O
O
H
CH3
R1
R
O
CH3
H
H
H
O
OH

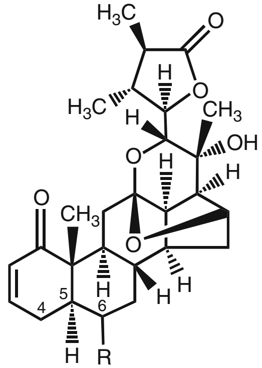
68 R=R_2=H, R_1=OH
69 R_1=R_2=OH, R=H
71 R_1=OH, R_1=R_2=H


CH3
CH2OH
H
H3C
CH3
H
O
O
H
O
CH3
H
H
H
OH
OH
S—R
OH
HO
H
H
H
H
CH3
O
O
O
H
CH3
CH3
H
HOH2C
CH3

66 R=Nil
67 R= =O

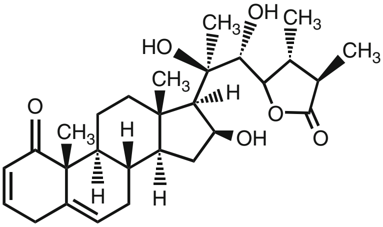


70 R=H, $\Delta^{2,3}$
80 R= OCH_3
81 R= H

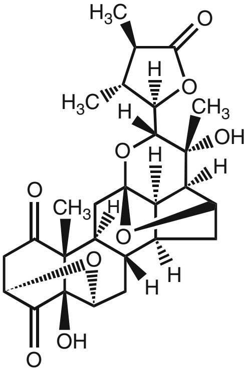


74 R=H, $\Delta^{5,6}$
77 R=βOH, $\Delta^{4,5}$

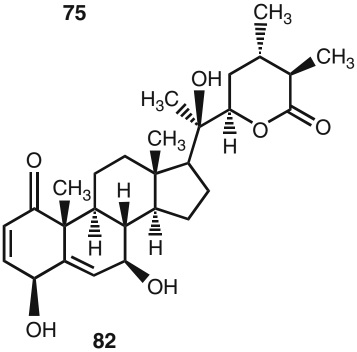
A series of withanolides was evaluated by Su and coworkers for their ability to stimulate quinine reductase induction with cultured mouse hepatoma, Hepa 1c1c7 cells. It was found that philadelphicalactones A (**68**) and B (**69**), ixocarpalactones A (**11**) and B (**70**), withaphysacarpin (**71**), 18-hydroxywithanolide D (**72**) and withanone (**73**), subtrifloralactones A (**74**) and F (**75**), subtrifloralactones K (**76**) and L (**77**), and 3*β*-hydroxymethylsubtrifloralactone E (**78**) are effective inducers of quinine reductase enzyme [64, 88–90]. Gu and coworkers also evaluated the significant quinine reductase potential of 2,3-dihydro-3*β*-methoxyixocarpalactone A (**79**), 2,3-dihydro-3*β*-methoxyixocarpalactone B (**80**), 2,3-dihydroixocarpalactone-B (**81**), and 4*β*,7*β*,20*R*-trihydroxy-1-oxo-with a-2,5 diene-22,26-olide (**82**) for their ability to induce quinine reductase in Hepa 1c1c7 cells [23].



75



76



82

4.1.6 Mechanism of Antitumor Activities of Withanolides

The mechanism of antitumor activity of three withanolides, withaferin A (**2**), physalin B (**6**), and tubocapsanolide A (**35**), was studied by different research groups. Withaferin A, the major constituent of *Withania somnifera*, was found to be able to bind with and inactivate Hsp90 (heat shock protein 90) through ATP-dependent mechanism, and it results in HsP90 protein degradation which contributes to its anticancer activity against pancreatic cancer [91]. The induction of the ROS generation and mitochondrial dysfunction of cancer cells are responsible for both mitochondrial-dependent and mitochondrial-independent apoptosis by withaferin A as reported by Singh and coworkers [92]. The inhibition of Notch-1 signaling and downregulation of Akt/NF-kB/Bcl-2 pathway in HCT-116, 5 W-480, and Sw-620 colon cancer cell lines have also been reported. In addition to this, the downregulation of pS6K and p4E-BPI and activation of c-Jun-NH_2-kinase-mediated apoptosis of colon cancer cells by withaferin A further supports the potential of withaferin A as a useful chemotherapeutic agent to treat colon cancer [93]. Withaferin A is also known to induce the aggregation of vimentin filaments through inhibition of F-actin, required for vimentin expression. The capillary growth induction in corneal neovascularized mouse model is also known to be reduced by the use of withaferin A in vimentin-deficient mice. Thus withaferin A has therapeutic potential to treat angioproliferative and malignant health disorders [94].

Ubiquitin-proteasome pathway, involved in the degradation of tumor growth proteins, plays an important role in the treatment of cancer. Physalin B (**6**), the major steroidal lactone of *Physalis angulata*, is known to produce the ubiquitinated protein accumulation and inhibit the TNFa-induced activation of NF-KB in DLD-1 4Ub-Luc cell assay. Physalin B does not inhibit purified proteasome catalytic activities but interferes with the cellular catalytic activities of the proteasome proteins at 4–8-fold high concentration, which is required to produce significant rise in bioluminescence and ubiquitinated protein accumulation in DLD-1 4Ub-Luc cells. The results indicate that physalin B is cytotoxic and works as an apoptotic triggering agent in DLD-1 4Ub-Luc cells [95].

The cancer-preventing ability of the tubocapsanolide A (**35**) against Skp2 overexpressed cells was checked by the immunoprecipitation assay. The results suggest that the mechanism involves the Skp2 suppression by blocking the Rel A binding site of nuclear factor-kB of Skp2 gene promoter by tubocapsanolide A [96]. The inhibitory potential of tubocapsanolide A against Hsp90-Hsp70 chaperone complex was investigated by Wu and coworkers. The mechanism proceeds through the oxidation of the thiol group which leads to the depletion of Hsp90 client proteins and thus arrest apoptosis in MDA-MB-231 cells [97]. The inhibition potential of tubocapsanolide A against TAK1 to suppress NF-kB-induced CCR7 breast cancer cell expression further supported the potential of tubocapsanolide A to prevent lymphocyte invasion of breast cancer cells [98].

The antitumor and anticancer potential of withanolides against various cancer cell lines and in different biological assays has been summarized in Table 114.1.

Table 114.1 The antitumor/anticancer potential of withanolides

S. no.	Withanolides	Anticancer activity	References no.
		Cytotoxicity	
	25, **26**	Cytotoxic against human lung cancer (Lu1) cell line	[66–68]
	28	Weak cytotoxicity against normal human fibroblast cultures (Fib04)	[69]
	27, 28, 29, 30	A strong cytotoxicity was exhibited against DU-45, 1A9, HCT116, LNCAP, KB, KB-VIN, A431, A549, HCT-8, PC-3, and ZR751 human tumor cell lines	[70, 71]
	31	Cytotoxic against HCT-116 and NCI-H460 cell lines	[72]
	32-42	Cytotoxic against hepatocellular carcinoma (Hep G2, Hep 3B), breast carcinoma (MDA-MB-231, MCF-7), lung carcinoma (A-549), and embryonic lung (MRC-5) cell lines	[73]
	43 and **44**	Cytotoxic against the human lung cancer cell line (NCI-H460)	[21, 75, 76]
	45 and **46**	Cytotoxic against the human lung cancer cell line (NCI-H460)	[77]
	47-**54** and **47a**, **47b**, **48a**, and **48b**	Cytotoxic against human head and neck squamous cell carcinoma (JMAR and MDA-1986), melanoma (B16F10 and SKMEL-28), and normal fetal fibroblast (MRC-5) cells	[80]
	55 and **56**	Cytotoxic against tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT15)	[82]
	57 and **58**	Cytotoxic against human liver carcinoma Hep G2 cell lines	[6]
		Cell differentiation inducing activity	
	2, 24, 59 and **60**	Potent cell differentiation inducers against MI cells	[83]
		COX inhibitors	
	61, 62, 63, **64** and **65**	COX-2 inhibitors	[84, 85]
		COX-1 non-inhibitors	
	66 and **67**	COX-1 non-inhibitors	[86]
		Radiosensitivity inducers	
	2	Increase the radiosensitivity of B16F1 melanoma and fibrosarcoma mouse tumor grown in C57BL and Swiss albino mice	[87]
		Quinine reductase inducers	
	11, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82	Effective inducers of quinine reductase enzyme in cultured mouse hepatoma, Hepa 1c1c7 cells	[64, 88–90]

(*continued*)

Table 114.1 (continued)

S. no.	Withanolides	Anticancer activity	References no.
		Study of mechanism of antitumor activities of withanolides	
	2		[91–94]
	6		[95]
	35		[96–98]

Table 114.2 A list of pharmacologically active withanolides, other than antitumor/anticancer activity

S. no.	Withanolides	Pharmacological activity	References no.
	Salphichloride A-C and G	Antifeedent and insecticidal	[114, 115]
	Physalin F	Trypanocidal activity	[116]
	Withaphysalins M, N, and O		
	Physagulins A, C, and H		
	Withangulutin A		
	11*β*-Hydroxy-physalin D	Antileishmanial activity	[50, 51, 99]
	2,3-Dihydro-5*β*,6*β* − epoxy-3*β*-methoxy- physalin D		
	6-Deoxyphysalin D		
	6-Oxo-11*β*-hydroxy-physalin D		
	6-Deoxy-11*β*-hydroxy-physalin		
	27-Hydroxy-1-oxo-witha-5,14,2-trienolide		
	5*β*-6*β* -Epoxy physalin B		
	6,7-Dehydrophysalin H		
	6-Deoxyphysalin H		
	Physalin H, F		
	Isophysalin B		
	Withanolide Z		
	Physalin B and D	Antimicrobial activity	[49]
	Physalin A, B, F, I, O, Y and Z	Inhibition of nitric oxide production in lipopolysaccharide-activated macrophages	[107, 117]
	Coagulin H	Significant antistress and immunomodulatory potential	[106, 107, 109]
	Withanolide A		
	2,3-Dihydro-3-sulfonylwithanone		
	Physalin B, F, and G		
	Withanoside IV	Significant neurite growth and synapses reconstruction activity	[110, 113]
	Withanoside VI		
	Coagulin Q		
	Withanolide A		
	3*α*,6*α* -Epoxy-4*β*,5*β*, 27-trihydroxy-1-oxo-witha-24-enolide		
	4*β*,5*β*,6*α*, 27-Tetrahydroxy-1-oxo-witha-2,24-dienolide		

(*continued*)

Table 114.2 (continued)

S. no.	Withanolides	Pharmacological activity	References no.
	Bracteosin A, B, and C Withaferin A 2,3-Dihydrowithaferin A 6α,7α -Epoxy-5α,20β-dihydroxy-1- oxo-witha-2,24-dienolide	Acetyl- and butyrylcholinesterase inhibitors	[1, 113]
	4β,7β,20α-Trihydroxy-1-oxo-witha-2,5,24-trienolide Jaborosalactol 18 Jaborosalactones 29, 30, 31, 33, 38 12-*O*-Methyljaborosalactone 38 12-*O*-Ethyljaborosalactone 42	Phytotoxic activity	[30, 31, 103, 118]
	Withaferin A Witharistatin	Diuretic activity	[119]
	Coagulanolin C and L 17β-Hydroxywithanolide K Withanolide F	Significant inhibition of postprandial rise in hyperglycemia	[4, 120]

5 Other Pharmacological Activities of Withanolides

In addition to antitumor activities, withanolides exhibited a wide range of other biological activities, including antifeedent, insecticidal (such as **23**) [84, 85], trypanocidal (such as **25, 26**) [29, 87, 88], antileishmanial [51, 99, 100], antimicrobial [98, 101], phytotoxic [31, 102, 103], and diuretic [4]. Anti-inflammatory [104] and immunomodulatory properties [104–109] of steroidal lactones have been extensively investigated by different groups, along with the study of their mechanism of action. The significant inhibition of postprandial rise of hyperglycemia and fall in fasting blood glucose level in diabetes rate models has also been studied [4].

Withanolides have also been evaluated by a number of groups, including us, for their potential to treat various neurodegenerative disorders, such as Alzheimer's, Parkinson's, and Huntington's diseases [1, 110–112]. Our group has reported for the first time the anti-Alzheimer's potential of withanolides as acetyl- and butyrylcholinesterase inhibitors [1]. Docking studies on these withanolides by us further supported their drug-like potency to treat Alzheimer's and associated complications [113].

A list of pharmacologically active withanolides, other than antitumor/anticancer potential has been presented in the Table 114.2.

6 Conclusion

Withanolides are among the most common classes of natural products with tremendous potential to treat various health disorders including tumors and cancers.

The purification, structure elucidation, and pharmacological studies on different classes of bioactive withanolides further validate the traditional uses of withanolide-producing plants to treat various ailments by local and traditional people. Diverse structural features, low toxicity, and novel and diverse mechanism of action make them suitable drug candidates for the treatment of various cancers and tumors. However, there is a need of extensive studies at the interface of chemistry and biology to fully explore the potent anticancer activity of withanolides, an essential step toward anticancer drug development with enhanced activity and lower toxicity, as compared to the available chemotherapeutic agents.

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Pharmacology of Ginsenosides

115

Kar Wah Leung

Contents

1 Introduction 3498
2 Chemical Constituents of Ginseng 3501
2.1 Polysaccharides 3501
2.2 Phenolics and Flavonoids 3501
2.3 Saponins 3501
3 Ginsenosides Are Part of the Defense Mechanisms in Ginseng 3503
4 The "Yin and Yang" Actions of Ginseng 3503
5 Standardized Ginseng Extracts 3504
6 Biotransformation of Ginsenosides 3504
7 Bioavailability of Ginsenosides 3505
8 Physiological Effects of Ginseng 3506
8.1 Pro- and Anti-angiogenesis 3506
8.2 Anticancer 3506
8.3 Combat Diabetes 3507
8.4 Immune System Enhancement 3507
9 Cellular Signal Transduction Pathways 3508
10 Conclusion 3508
References 3509

Abstract

Ginseng is also known as "The King of All Herbs." It not only possesses superior status in the field of traditional Chinese medicine and being extensively used in Chinese communities for thousands of years, it is one of the most popular herbs in the world and accounts for over 800 million US dollar of international market. Many scientific approaches (e.g., bioassays and omics studies) have been used to

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unlock the mechanisms behind the biological effects of ginseng. Frequently, ginsenosides are being said to be the most pharmacologically active constituents in ginseng. In this chapter, we will cover the basic biochemistry and pharmacology of ginsenosides, as well highlight how ginsenosides work in human body with respect to various pathological conditions, including cancer and age-related disorders. Lastly, we will discuss the possibilities of developing ginsenosides into targeted therapeutic agents to benefit the human society.

Keywords

Ginseng • ginsenosides • steroidal hormone receptors

Abbreviations

ER	Estrogen receptor
GR	Glucocorticoid receptor

1 Introduction

The word “ginseng” is translated from the pronunciation of the Chinese words “人蔘” (Renshen) that means “essence of men,” and is conventionally referring to the Asian ginseng (*Panax ginseng*). The botanical name *Panax* means “all-healing” in Greek, which is first coined by the Russian botanist Carl A. Meyer in 1843 (Image 115.1). The *Panax* family consists of at least nine species, and are mostly named by their geographic origins, although they could be cultivated and processed elsewhere. For example, American ginseng (*Panax quinquefolium*) was discovered by a Jesuit priest Father Joseph Francois Lafitau near Montreal/Ottawa, Canada, in 1716. Later, he started to export wild American ginseng to China and experiment on ginseng cultivation technique. As time goes by, China now becomes the second largest producer and exporter of the American ginseng. Other members of the family include *Panax notoginseng* (Sanqi 三七/ Tian-qi 田七), *Panax japonicus* (Japanese ginseng), *Panax vietnamensis* (Vietnamese ginseng), and *Panax trifolius* (Dwarf ginseng).

Siberian ginseng (*Eleutherococcus senticosus*) is also frequently found in the market. However, Siberian ginseng is only distantly related to the Panax family and can be considered as an entirely different plant. It contains a different set of active components that leads to distinct biological activities. Therefore, Siberian ginseng is now more commonly known as Eleuthero to avoid confusion.

Ginseng is a slow-growing perennial herbaceous plant that grows to about a half meter tall. Wild ginseng could be found in cold (optimal growing temperature is 8–15 °C) and well-shaded areas of moist hardwood forests. It takes the plant approximately 10 years to grow into maturity (has 4–5 long leaves with red berries) (Image 115.2). Because of the increasing popularity of this powerful herb, wild ginseng is almost being ripe off. Both wild Asian and American ginsengs are now protected under the Convention on International Trade in Endangered Species of

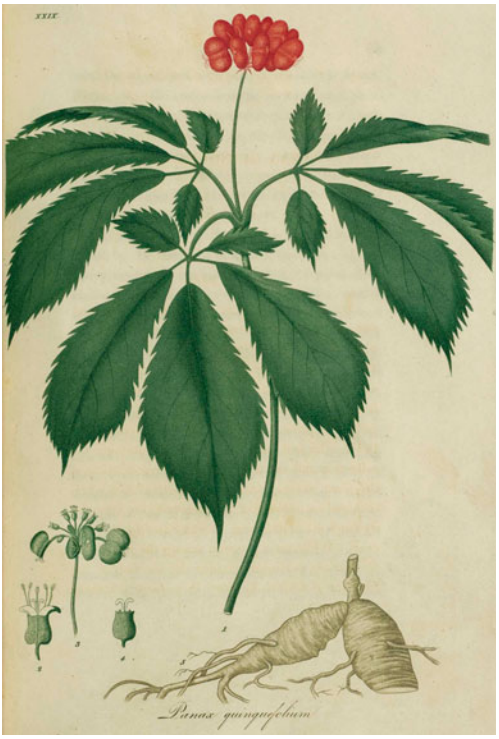


Image 115.1 The drawing depicts American ginseng adopted from the book of American Medical Botany: (1817) [1]

Image 115.2 A mature ginseng plant. (The image is adopted from http://www.herbs.org/)

Wild Fauna and Flora (CITES), an international trade agreement that is signed by 135 nations in 1973 and went into effect in 1975 (Image 115.3).

To meet demand of trade needs from the whole world, many countries start growing ginseng using systematic farm cultivation and wood grown wild-simulation approaches. Farm cultivated ginsengs usually grow faster (4–6 years to maturity) and

Image 115.3 The image shows a typical ginseng root. The shape of the root resembles a "little human" with a head, a body and four limbs. A wild Panax ginseng roots grown in a desirable shape can worth tens of thousands of dollars

lost the "man-like" appearance compared to the wild-simulated ginsengs (8–10 years to maturity). China is the main producer (50 %) of the cultivated ginseng, followed by South Korea (32 %), the United States (7.5 %), Japan (2.3 %), Canada (2.2 %), North Korea (1.2 %), and all others (4.6 %). Annual world production of cultivated ginseng is over 5,000 t [2], and accounts for over half a billion US dollar of international market value. Hong Kong SAR, China, acts as the world's clearing-house for ginseng, which imports 3,895 t in 1990 [2]. Apart from a substantial consumption within Hong Kong, significant amount of ginseng is redistributed to China, Taiwan, Japan, Malaysia, Singapore, North America, and Europe.

The ginseng species share more or less the same set of active components, although each has a unique composition prolife to give its unique biological properties. The constituent composition of a ginseng species can also be affected by a range of factors, such as age and part of the plant, cultivation method, harvesting season, and preservation method [3, 4]. The ginseng roots air-dried after harvested appear to be white, while the darker-colored red roots are produced by steaming at 98–100 °C for 2–3 h before drying [5]. The steaming process transforms the major medicinal constituents of ginseng into novel compounds that adds unique therapeutic values to these red roots [6–10]. In 2001, some roots are steamed at an even higher temperature (120 °C for 4 h) to enhance the antitumor properties of the ginseng, and this new type of ginseng is known as Sun ginseng [11–16]. The Sun ginseng is also found to possess enhanced anxiolytic effects compared to white and red ginsengs [17].

2 Chemical Constituents of Ginseng

Ginseng has been widely used in Chinese community for thousands of years. It is generally believed that consuming ginseng or ginseng extracts can support overall health and boost the immune system. It is accepted by the European countries and the United States to be used in complementary and alternative therapies [18, 19]. However, the mechanisms of ginseng's biological actions are still not fully understood. Identifying and characterizing the pharmacological active components of ginseng is a key area in ginseng researches. To date, majority of the effects of ginseng points to three categories of constituents, they are polysaccharides, phenolics, and flavonoids, and saponins.

2.1 Polysaccharides

The polysaccharides of the ginseng comprised 40 % (by weight) of the root. This class of compound was first isolated and documented in 1966 [20]. Most biologically active carbohydrates in ginseng are acidic polysaccharides, known as ginsan, which have the typical structure of pectin [21, 22].

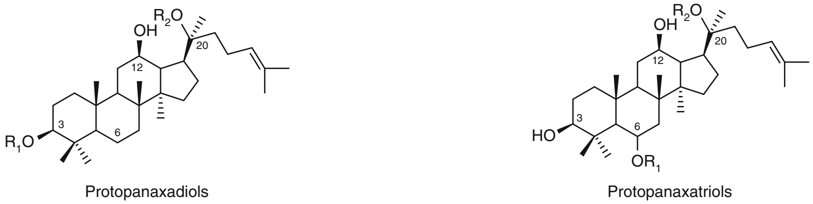
Ginseng polysaccharides have both stimulating and suppressive actions on the immune system. It was found that ginseng polysaccharides increases serum IgG levels, activates the reticuloendothelial system (RES), and anti-complementary and alkaline-phosphatase-inducing activities [21]. At the same time, ginseng polysaccharides are reported to calm down enteric immune responses by activating Peyer's patch lymphocyte [23]. Ginseng polysaccharides also possess anticancer, antimutagenic, and radioprotective effects [22, 24, 25].

2.2 Phenolics and Flavonoids

Phenolics and flavonoids are powerful plant-derived antioxidants. These compounds demonstrate radical-scavenging and ferrous ion–chelating activities. Ginseng's phenolics and flavonoids comprised 2–7 % (by weight) of the root [26], in which quercetin and kaempferol are the most abundant ones [27]. Quercetin and kaempferol can also be found in *Ginkgo biloba* and St. John's wort, and ginseng-specific phenolics and flavonoids have not been identified.

2.3 Saponins

Ginseng triterpene saponins are also known as Ginsenosides. This class of compounds accounts for most of the ginseng's biological activities. Ginsenosides



	R_1	R_2	Formula	T_{melt} (°C)
Rb_1	*-glc²-glc*	*-glc⁶-glc*	$C_{54}H_{92}O_{23}$	*198–200.5*
Rb_2	*-glc²-glc*	*-glc⁶-ara(p)*	$C_{53}H_{90}O_{22}$	*200–202*
Rc	*-glc²-glc*	*-glc⁶-ara(f)*	$C_{53}H_{90}O_{22}$	*198–201*
Rd	*-glc²-glc*	*-glc*	$C_{48}H_{82}O_{18}$	*206–209*
Rg_3	*-glc²-glc*	-H	$C_{42}H_{72}O_{13}$	*298–303*
Rh_2	*-glc*	-H	$C_{36}H_{62}O_8$	*218–220*

	R_1	R_2	Formula	T_{melt} (°C)
Re	*-glc²-rha*	*-glc*	$C_{48}H_{82}O_{18}$	*199–201*
Rf	*-glc²-glc*	-H	$C_{42}H_{72}O_{14}$	*197–198*
Rg_1	*-glc*	*-glc*	$C_{42}H_{72}O_{14}$	*194–195*
Rg_2	*-glc²-rha*	-H	$C_{42}H_{72}O_{13}$	*183–184*
Rh_1	*-glc*	-H	$C_{36}H_{62}O_9$	*248–252*

Fig. 115.1 Images depicted the structures of protopanaxadiol (*PPD*) and protopanaxatriol (*PPT*) (Figure adopted from Leung et al. [82]. *glc* glucosyl, *ara*(*p*) pyranosyl arabinosyl, *ara*(*f*) furanosyl arabinosyl, *rha* rhamnose

can be found in all parts of the plants – the roots, stems, leaves, as well as flowers. Each part of the plant contains distinct ginsenoside profiles [28]. Being the most concentrated region, the root contains 3–6 % (by weight) of ginsenosides, and the overall saponin content is directly proportional to the age. On average, the root saponin content reaches peak levels at around 6 years of age for cultivated ginseng, and at least 10 years for the wild ones [29, 30].

To date, more than a 100 naturally occurring ginsenosides have been isolated from roots, leaves, stems, fruits, and flower heads of ginseng [31]. The triterpenoid core of ginsenosides is a four-ring structure with various sugar moieties (e.g., glucose, rhamnose, xylose, and arabinose) attached to the C-3, C-6, and C-20 positions. Ginsenosides are named according to the chromatographic polarity in alphabetical order with a prefix "R" that stands for the root [32]. Therefore, Ra is the least polar ginsenoside and Rg3 is more polar than Rg1. The presence or absence of the C-6 carboxyl group categorize ginsenosides into two groups – the 20(S)-protopanaxatriol (PPT) (Re, Rf, Rg1, Rg2, Rh1) and the 20(S)-protopanaxadiol (PPD) (Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, Rs1), respectively [33] (Fig. 115.1).

Besides the common ginsenosides (Rb1, Re, Rg1, Rg3), ginsenoside composite varies among ginseng species. For example, ginsenoside Rf is unique to Asian ginseng and F11 is found exclusively in American ginseng. Thus, the Rf/F11 ratio is used as a phytochemical marker to distinguish American ginseng from Asian ginseng [34, 35]. Such differences can generally correlate with the physiological properties of the ginseng – American ginseng is described to be cooling and soothing to body conditions, while Asian varieties are thought to be "hot" and stimulating – although detailed ginsenoside property identification is still under investigation. The heat-processed red ginseng contains multiple unique ginsenosides, including Rg3, Rg5, Rk1, Rk2, Rk3, Rs4, Rs5, Rs6, and Rs7 [36]. It is these unique ginsenosides to produce the signature effects of the red ginseng.

3 Ginsenosides Are Part of the Defense Mechanisms in Ginseng

Over evolution, plants eliminate most of the non-necessary biological pathways and reserve those benefit to its own survival. Thus, we ask what is the purpose for ginseng to make the saponins called ginsenosides?

Plants produce antibiotic substances to defense themselves from insect and microbial attack, such as nicotine from tobacco leaves [37], rotenone from derris tree roots [38], pyrethroids from chrysanthemum flowers [39], and triterpenoids from neem tress [40]. Evidence indicated that ginsenosides are one of the phytoanticipins to protect the ginseng plant.

Ginsenosides are constantly synthesized by the ginseng plant prior to pathogenic invasion, and defensive stress signals, such as methyl jasmonate and salicylic acid, can enhance ginsenoside production and accumulation by 3–4 times, as illustrated in ginseng root cells' in vitro culture [41–44]. Ginsenosides are found to possess antimicrobial and fungitoxic properties [45–47], and the bitter taste keeps insects and animals from feeding on them [48]. Ginsenosides share high degree of molecular structure similarity with the insect molting and metamorphosis hormone ecdysteroids, suggesting ginsenosides may be an agonist to the ecdysteroid receptor. Thus, ginsenosides may protect the plant by interfering with the life cycle of herbivorous insects [49] (Fig. 115.2). These lines also verify the folk belief that wild ginsengs grown in a more challenged environment contain larger amount of ginsenosides and have greater biological effects compared to the cultured ginseng, thus are sold at a much high price range. To reproduce the quality of wild ginseng, ginseng growers developed the "wild simulating cultivation" approach to provide a more natural environment for the plants.

4 The "Yin and Yang" Actions of Ginseng

Ginsengs are described as an "adaptogens." The concept of "adaptogen" was first proposed by a Soviet pharmacologist and toxicologist, Dr Nikolai Vasilievich Lazarev (1895–1974). At that time, adaptogen is a substance that can be administrated to individuals of any health condition to increase nonspecific resistance to various kinds of biological stresses. However, adaptogenicity can hardly be proved by scientific evidence. Here I reframe it and elaborate this property of ginseng in the yin and yang concept.

The "yin and yang" concept in Chinese is the coexistence and balance of opposing forces, such as light and dark, good and evil, calm and irritation. Unlike most of the other herbs, ginseng is commonly taken by its own. This would be explained by the coexistence of ginsenosides of various properties, and consumption of ginseng alone can lead to a combination of effects, which resemble "Fufang" (複方) in Chinese medicinal concept, or the cocktail therapy in the Western approach. Taking angiomodulation effects as an example, angiogenic properties of ginseng are related to the compositional ratio between ginsenosides Rg1 and Rb1. Asian ginseng with

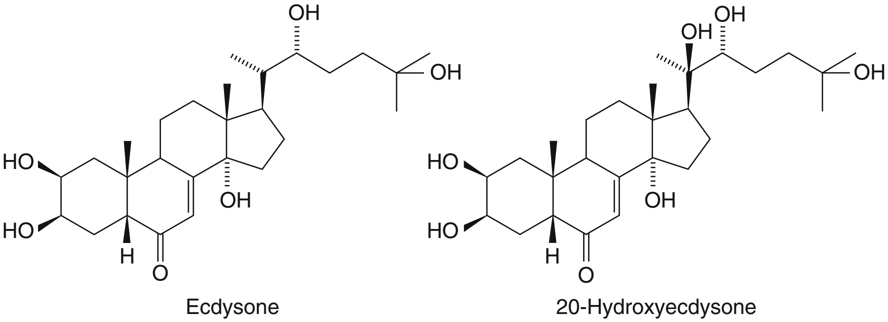


Fig. 115.2 Molecular structures of the insect molding hormones ecdysone and 20-Hydroxyecdyson. Structural similarities between ginsenosides and the ecdysone suggest ginsenosides maybe analogs to ecdysone receptors

higher Rg1 levels is found to promote growth of blood vessels and consumption of Asian ginseng can reduce hypertension. In contrary, American ginseng that contains more Rb1 is found to suppress angiogenesis, and is used in cancer treatment. The coexisting Rg1 and Rb1 interact with different receptors and give ginseng the ability to balance the dynamic equilibrium of human physiological processes. We will discuss the ginsenoside-receptor interactions later in this chapter.

5 Standardized Ginseng Extracts

Because of complex composition of the ginseng and some of the ingredients are having contrasting actions, variations in composition will lead to inconsistent experimental results. For this reason, standardized extracts are made and are commercially available to minimize variability among preparations. Two commonly used standardized extracts are G115 from *P. ginseng* (total ginsenoside adjusted to 4 %) (Pharmaton SA, Switzerland) and NAGE from *P. quinquefolius* (total ginsenoside content adjusted to 10 %) (Canadian Phytopharmaceuticals Corporation, Canada). Studies on these two ginseng extracts using high-performance liquid chromatography (HPLC) found ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1 in both G115 and NAGE, and ginsenoside Rg2 in G115 only. Comparing G115 and NAGE: G115 has higher Rg1, but NAGE has higher in Rb1 and Re [50–52].

Moreover, the butanol-soluble fraction of Sun ginseng is formulated into KG-135 which contains Rk3 Rs3, Rs4, Rs5, Rs6, and Rs7 in addition to the major antitumor ginsenosides [16, 53].

6 Biotransformation of Ginsenosides

As a group of defensive phyto-compounds, ginsenosides may require certain degree of transformation to gain its full activity in mammalian system. In fact, major

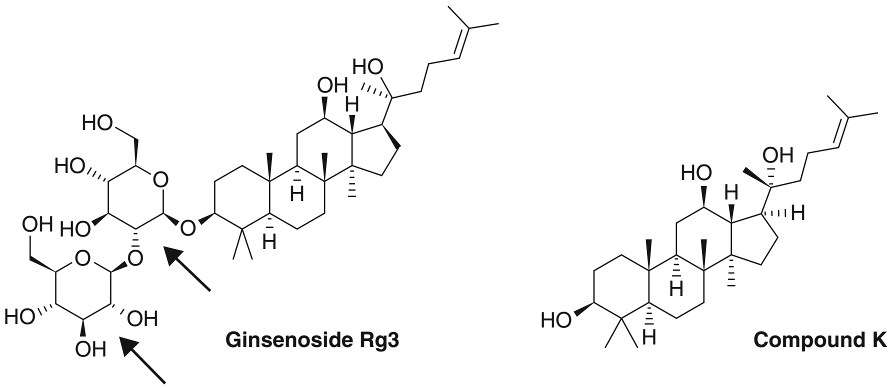


Fig. 115.3 Molecular structures of ginsenoside Rg3 and its metabolite Compound K. *Arrows* indicate the C3 glucoses to be deglycosylated by the microflora

ginsenosides, such as Rg1, Rg3, Rb1, Re, and Rc, are bulky molecules that are poorly membrane permeable. Moreover, these ginsenosides are antigenic in the circulatory system. Antibodies against these ginsenosides can be purified from immunized animals and are commercially available [54–57].

Ginsenosides in orally consumed ginseng preparations are subjected to acid hydrolysis, glycosyl elimination, and intestinal microflora-driven sugar moiety cleavage [58–60]. Following biodegradation, PPDs are converted to compound K (also known as M1 or IH901) and panaxadiol, while ginsenoside F1 and panaxatriol are the major metabolites of PPTs. Several bacterial strains are identified and selected transforming enzymes are overexpressed using recombinant DNA technique for in vitro ginsenoside modification [61].

Studies have shown that ginsenoside metabolites indeed have greater biological effects compared to the naturally occurring ginsenosides [62–64]. Ginsenoside Rh2, compound K, and panaxadiol, the metabolites of Rg3, are found to have greater antitumor activities than ginsenoside Rg3 itself [62, 65]. Similarly, compound K, panaxatriol, and panaxadiol possess the human liver enzyme cytochrome P450 inhibitory effects that are not found in the bulk ginsenosides Rb1, Rb2, Rg1, and Re [64] (Fig. 115.3).

7 Bioavailability of Ginsenosides

Multiple systematic actions of ginsenosides are reported and intensively investigated, but how ginsenosides are absorbed from the digestive system and reach the systemic organs remains largely unknown.

Although ginsenosides have the basic structure of a steroid, the sugar side chains increase the hydrogen bond count and polar surface area that hurdle effective permeability of these compounds across the membranes. The side chains are prone to degradation via hydrolytic cleavages and deglycosylations. Thus, availability of intact ginsenosides from the intestine is extremely low [58, 66, 67]. To detect the ginsenosides in the blood samples after consumption using high-performance liquid chromatography, only 3.29–18.4 % Rg1 and 0.64–4.35 % Rb1 are detected in rat serum after oral administration of the ginsenosides [67–71].

Ginsenosides are shown to be transported across the intestinal mucosa in an energy-dependent and non-saturable manner [68, 69, 72]. The sodium-dependent glucose co-transporter-1 may be involved in this process [73]. Several approaches has been tested to increase the bioavailability of ginsenosides, including coadministration of ginsenosides with adrenaline [74], emulsification of ginsenosides into lipid-based formulation [75, 76], micronization of the ginsenoside particle and consequently increase the dissolution rate [77], and suppression of p-glycoprotein efflux system [78]. However, not a single approach is proved to enhance the bioavailability of all ginsenoside.

After absorption, ginsenosides continue to be biologically modified, such as oxidation and small degree of deglucosylation at the tissue levels [79]. As well, circulating ginsenosides and their deglycosylated products were subject to rapid and extensive biliary excretion through active transport, resulting in short biological half-lives and low systemic exposure levels [79].

8 Physiological Effects of Ginseng

Being crowned as the King of Herbs, ginseng has diverse activities on multiple organ systems in human regardless of the low bioavailability. In this chapter, biological effects on cardiovascular system, cancer, diabetes, immune system, and neurological system will be reviewed.

8.1 Pro- and Anti-angiogenesis

Ginsenosides are found to be able to modulate angiogenesis in both directions via altering genes involved in cell architectural dynamics, adhesion, and migration. For example, ginsenoside Rg1 interacts with glucocorticoid receptor (GR) and stimulates angiogenesis through augmenting the production of nitric oxide (NO) and vascular endothelial growth factor (VEGF) in endothelial cells [80, 81]. Angiogenesis promotion can accelerate wound healing and support stroke recovery. At the same time, ginsenoside Rb1 interacts with estrogen receptor-beta (ER-β) and enhances the production of anti-angiogenic pigment epithelium-derived factor (PEDF) from the endothelial cells [82]. Since tumor mass attracts blood vessels, suppressing angiogenesis ginsenosides is then exploited as part of the anticancer treatment.

8.2 Anticancer

American ginseng has been shown to have powerful anticancer properties, and heat-processed red ginseng has more potent inhibition on tumor growth compared to the untreated white ginseng. Patients with stage-3 gastric cancer taking red ginseng were observed to have a higher 5-year disease-free survival rate and better restoration of immune functions during adjuvant chemotherapy compared to control patients [83]. Regular consumption of ginseng also demonstrates a dose-dependent decrease in risk of cancer in Korea [84, 85]. Besides the anti-angiogenic properties of certain class of ginsenosides that have been exploited as an anticancer modality, ginsenosides Rg3 and Rh2 are shown to inhibit growth of several cancer cell lines [86, 87] and capable in reversing the multidrug resistance properties of cancer cells by inhibiting the efflux transporter P-glycoprotein (P-gp) [88–90]. Inhibition of P-gp leads to the improvement of bioavailability of several orally administrated anticancer drugs and could be taken to assist cancer chemotherapy [91, 92]. “Anticancer capsules” of Rg3 alone or in combination with ginsenoside Rh2 are available in Mainland China as over-the-counter drugs.

8.3 Combat Diabetes

In genetically obese diabetic KK-CA model, intraperitoneal injection of ginseng root extract can significantly lower blood glucose levels [93, 94]. As well, total ginseng berry extract reduces body weight and improves glucose homeostasis in type-2 diabetic ob/ob mice [95]. Particularly, treatment with ginsenoside Re lowers the elevated fasting blood glucose to normal levels and enhances glucose tolerance capacity in diabetic mice, but has no effects in the nondiabetic littermates [95]. Yet the mechanism of such effects is unclear. In human studies, consumption of 3 g of American ginseng root 40 min before the test meal significantly lowered blood glucose level in both nondiabetic subjects and diabetic patients [96].

8.4 Immune System Enhancement

Ginseng is shown to be an immunostimulant. It activates macrophages of healthy and fungal-infected mice [97, 98], as well as in mice exposed to the cold-water swim stress [99]. Ginseng also assists recovery of natural killer (NK) cells function in immunosuppressed mice [100, 101]. In a randomized, placebo-controlled double-blind trial, volunteers were treated with an influenza vaccine plus either placebo or a standard ginseng extract G115 over a 3-month period [102]. The frequency of upper respiratory infections (i.e., colds and flus) was significantly reduced by three times in the ginseng group compared to the placebo. In addition, antibody titers and NK activities were significantly higher in the ginseng group [102]. In a study, hot-water wild ginseng extracts resulted in increased lymphocyte proliferation both in vitro and in mouse model, while extracts from cultured ginseng had no effect [103]. This finding paralleled to the folk belief that wild ginseng has stronger immunomodulating effects compared to the cultured or domesticated ones.

Besides the cancer-combating potential, ginseng is often taken by patients in advance stage tumor for its immune stimulation properties. Cancer patients are inherently immunosuppressive due to tumor-derived factors and the standard treatments, such as chemotherapy [104, 105]. Thus, many patients choose ginseng preparations to complement their standard cancer treatments.

9 Cellular Signal Transduction Pathways

Given the steroidal structure of ginsenosides, it has been shown to interact with the ligand binding sites of various steroid hormone receptors – ginsenosides Rg1 [82, 106] and Re [107] are functional ligands of the glucocorticoid receptor (GR), whereas ginsenosides Rh1 [108] and Rb1 [82] are functional ligands of the estrogen receptor (ER). However, the effects elicited by the ginsenoside-receptor complex are not as prominent as the native agonist of the steroidal receptors, i.e., the glucocorticoid and estrogen for the GR and ER, the ginsenosides may therefore function as partial agonists, which ginsenoside compensate for the insufficient steroidal activities when the intrinsic ligand is absent or inadequate in the system, while reversibly occupying the steroidal receptor with low affinity to modulate the steroidal effects when large amount of intrinsic ligand is present. This hypothesis also explained the adaptogenic properties of ginseng to bring extreme physiological conditions back to balance.

Moreover, each ginsenoside is able to bind to multiple steroid hormone receptors with different affinity. In addition to GR, ginsenosides Rg1 acts through ER and elicits cross-talking with insulin-like growth factor-1 receptor (IGF-IR) in neuronal cells [109]. Effects of ginsenoside Re on cardiac myocytes are related to ER alpha isoform, androgen receptor, and progesterone receptor [110]. The end metabolites PD and PT bind and activate both GR and ER in endothelial cells [111]. The multi-target properties of ginsenosides may explain why ginseng has a wide range of beneficial effects.

On the other hand, taking ginseng preparation enhances the mood and health-related quality of life in menopausal women with no change in female hormone–related physiological parameters, e.g., follicle-stimulating hormone (FSH) and estradiol levels, endometrial thickness, maturity index, and vaginal pH were not affected by the treatment [112], indicating some of the beneficial effects of ginseng are not mediated via steroidal hormone receptors. Other studies showed that ginsenosides can modulate expressions and functions of receptors, such as receptor tyrosine kinases (RTK) [113], serotonin receptors (5-HT) [114], NMDA receptors [115], and nicotinic acetylcholine receptors (AChR) [116]. Thus, ginsenosides are involved in a very complicated network of actions and more research is required to acquire thorough understanding.

10 Conclusion

Although ginsengs come in many different varieties and different molecular composition, the active effective compound is ginsenosides. The structural analyses, binding studies, and functional investigations demonstrated the steroidal hormone receptor partial agonist properties of ginsenosides and explained the multiple therapeutic effects of this group of molecules. At the same time, ginsenosides modulate the activities of other cellular signaling pathways that awaits further investigations. This allelopathic property of ginseng attracts growing attention since many diseases, such as cancer, neurodegenerative disorders, and metabolic syndromes, are not isolated conditions of a single organ, and promising cure are currently unavailable. Therefore, unraveling the action mechanisms of the natural occurring systemic modulatory compounds, like ginsenosides, would be beneficial to the overall community welfare.

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Sesquiterpenes and Cytotoxicity 116

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Contents

1 Introduction 3517
2 Terpenes and their Classification 3519
2.1 Classification of Terpenes 3519
3 Sesquiterpenes and their Classification 3519
3.1 Acyclic 3520
3.2 Monocyclic 3521
3.3 Bicyclic 3521
3.4 Tricyclic 3521
3.5 Tetracyclic 3521
3.6 Pentacyclic 3521
3.7 Functions of Sesquiterpenes in Plants 3521
4 Cytotoxicity and Apoptosis 3522
4.1 Morphological and Biochemical Alterations 3523
4.2 Caspases 3523
4.3 TNF Pathway 3524
4.4 Fas Pathway 3524
4.5 Other Components 3524
5 Cancer 3525
5.1 Approaches for Cancer Inhibition 3526
5.2 Natural Compounds as Inhibitors of Cell Proliferation 3527
6 Description of Cytotoxicity of Sesquiterpenes and their Derivatives 3528
6.1 Sesquiterpene Coumarins 3532
6.2 Sesquiterpene Umbelliprenin 3533
6.3 Sesquiterpene Ethers 3533
6.4 Prenylated 4-Hydroxycoumarins 3533

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7 Allelopathic Potential of Sesquiterpenes 3533
8 Specificity of Sesquiterpenes 3540
9 Synergism and Antagonism Among Sesquiterpenes 3541
10 Regulatory Issues 3541
11 Therapeutic Potential and Future Prospects 3542
References 3543

Abstract

Plants have a long history as therapeutic tools in the treatment of human diseases and have been used as a source of medicines for ages. In addition, plants play a role in disease prevention. For example, reduced risk of cancer is associated with high consumption of vegetables and fruits. Thus, the cancer chemopreventive cytotoxic potential of naturally occurring phytochemicals is of immense interest. There is an urgent need to develop new anticancer chemotherapeutic agents to overcome the development of multidrug resistance tumors. Essential oils exhibit many potential activities such as cytotoxicity, antimicrobial, anti-inflammatory, analgesic, and insect repellant. Most of the essential oils are extracted from plants by hydrodistillation so they contain a variety of volatile molecules such as terpenes and terpenoids, phenol-derived aromatic components, and aliphatic components. Essential oils act on via apoptotic pathway, affecting mitochondrial functions. This may impart free radical scavenging potential which could be controlled and targeted. This property makes the terpenoids favorable anticancer agents. Depending on type and concentration, they exhibit cytotoxic effects on living cells but are usually nonmutagenic and nongenotoxic. In some cases, changes in intracellular redox potential and mitochondrial dysfunction induced by essential oils can be associated with their ability to exert antigenotoxic, antimutagenic, and anticancer effects. These findings suggest that the encountered beneficial effects of essential oils are due to pro-oxidant effects on the cellular level. This chapter focuses on the cytotoxic activity of sesquiterpenes and their derivatives.

Keywords

Apoptosis • cytotoxicity • essential oil • sesquiterpenes

Abbreviations

3-HMGCoA	3-Hydroxy-3-methylglutaryl-coenzyme A
DAG	Diacyl glycerol
DMAPP	Dimethylallyl pyrophosphate
EPA	Environmental protection agency
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain
FPP	Farnesyl pyrophosphate
FTase	Farnesyltransferase
GC-MS	Gas chromatography-mass spectrometry

GGPP	Geranylgeranyl pyrophosphate
IPP	Isopentenyl pyrophosphate
MDR	Multidrug resistance
NFkB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
PARP	Poly (ADP-ribose) polymerase
PKC	Protein kinase C
ROS	Reactive oxygen species
SLs	Sesquiterpene lactones
TNF	Tumor necrosis factor

1 Introduction

Plants produce primary and secondary metabolites which encompass a wide array of functions. Primary metabolites are the compounds which play an important role in the regulation of cellular and physiological processes, for example, carbohydrates, lipids, and proteins. Secondary metabolites are the compounds produced in response to stress, such as the case when acting as a deterrent against herbivores [1]. Plants can manufacture many different types of secondary metabolites, which have been subsequently exploited by humans for their beneficial role in a diverse array of applications [2]. Often, plant secondary metabolites may be referred to as plant natural products, in which case they elicit effects on other organisms. Although this review focuses on plant terpenes, it should be realized that other organisms are able to synthesize terpenes. For example, the endophytic fungus isolated from *Hypericum perforatum* has been producing hypericin and emodin, terpene lactones [3]. There are three broad categories of plant secondary metabolites as natural products: terpenes and terpenoids (~25,000 types), alkaloids, and phenolic compounds [4].

Essential oils are secondary metabolites produced in the plants as volatile aromatic principles. They are colorless and rarely colored and soluble in organic solvents. It is found in all part of the plants and is stored in epidermal cells, trichomes, secretory cells, canals, and cavities. They are extracted by the use of liquid carbon dioxide or microwaves, expression and mainly steam or hydrodistillation process. Due to their biological, nutritive, and pharmaceutical properties, they are more attractive alternatives to synthetic chemical products to protect the equilibrium.

The extraction process, collection of drug material, plant parts, age of the plant, and geographical and climatic conditions affect chemical profile and stereochemistry of the essential oil products [5, 6]. Most of the commercialized essential oils are analyzed by gas chromatography (GC), mass spectrometry (MS), or a combination of GC-MS technique [7].

Essential oils have been largely employed for their properties already observed in nature, that is, for their cytotoxicity, antimicrobial, anti-inflammatory, analgesic, and insect repellant activities. At present, approximately 3,000 essential oils are known, out of which 300 are commercially important especially for the agriculture, food, pharmaceutical, cosmetic, and perfume industries. Moreover, essential oils are used in massages as mixtures with other oils (aromatherapy). Some essential oils exhibit particular medicinal and therapeutic properties and have been used in the prophylaxis and treatment of acute and chronic disorders.

Terpenes are a large class of natural hydrocarbon secondary metabolites built up from five-carbon isoprene units linked together most commonly in a head to tail arrangement, but can be constructed in other configurations with varying degree of unsaturation, oxidation, reduction, functional groups, and ring closure, giving rise to a rich diversity of structural classes, with novel skeleton being continuously discovered. These modified hydrocarbons are referred to as terpenoids, which are primarily found to occur in a wide variety of higher plants. They can also be found in insects and marine organisms. The name terpene is derived from the word turpentine, a product of coniferous oleoresins.

The terpenes or terpenoids are classified or grouped according to the number of isoprene units found in parent nucleus ranging from one to many. Terpenoids are the chemically modified products of terpenes. Terpenes and terpenoids are made up of isoprene unit $(C_5H_8)n$. Chemically isoprene is 2-methylbuta-1,3-diene and commercially used for the preparation of rubber. As with any class of compounds, not all terpenoids contain even number of intact isoprene units, with a few being degraded isoprene moieties with missing carbons such as C_{19} diterpenoids. The simplest type of terpenoids is hemiterpenes consisting of single five-carbon isoprene unit. Their occurrence is rare and is not biologically significant. Monoterpenes consist of two isoprene units that can be found in acyclic, monocyclic, and bicyclic forms and in various state of oxidation. Sesquiterpenes are C_{15} compounds containing three isoprene units, occurring in simple acyclic to macromonocyclic rings as well as simple and complex bicyclic and tricyclic forms. The structural diversity of sesquiterpenes is such that the number of carbon skeletons having been reported far exceeds 100. C_{20} terpenoids are the diterpenes which contain four isoprene units. Their structural diversity ranges from simple acyclic to complex polycyclic rings. Sesquiterpenes are rare C_{25} compounds. Triterpenes are C_{30} compounds numbering more than 4,000 distributed in more than 40 different carbon skeletons, arising from the cyclization of an oxidized form of squalene, the linear parent triterpene and carotene. Natural rubber which contains long chain of many cis isoprene units, and gutta-percha which contains many trans isoprene units. These natural products are the members of polyterpenoids [8].

Biosynthetically, majority of terpenoids are formed via the mevalonic acid, but they may also be formed through methyl-erythritol-4-phosphate (MEP) pathway. The C_5 isoprene unit which can be linked together “head to tail” to form linear chains or cyclized to form rings is considered the building blocks of terpenes. Rather the C_5 units exist as isopentenyl pyrophosphate or its isomer dimethylallyl pyrophosphate by enzymatic conversion and phosphorylation from mevalonic acid. The IPP may be considered as the precursor of hemiterpenes. In the biosynthesis of mono- and higher terpenes/terpenoids, the starting molecule is DMAPP, which condenses with an IPP unit to form geranyl pyrophosphate, leading to the formation of monoterpenoids. Condensation of GPP with and IPP leads to the formation of farnesyl pyrophosphate (FPP), the precursor of sesquiterpenoids. FPP and IPP form geranylgeranyl pyrophosphate, the precursor of diterpenes. GGPP and IPP lead to the formation of sesquiterpenoids. Dimerization of two molecules of FPP gives rise to squalene, precursor of triterpenes and steroids. Condensation of intermediate precursor and IPP leads to tetra- and polyterpenoids. Chemical and biological studies have shown that the terpenoids possess a variety of chemical, physical, and biological activities. Biologically, the terpenoids possess anticancer, antimicrobial, cytotoxic, anti-inflammatory, and analgesic activities [8].

2 Terpenes and their Classification

Terpenes (turpeen) are a large and diverse group of organic compounds, produced by a variety of plants, particularly conifers, though also by some insects such as termites or swallowtail butterflies. They have characteristic odor and thus may have had a protective function [9]. Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. Essential oils are used widely as natural flavor in food additives, as fragrances in perfumery, and in traditional and alternative medicines such as aromatherapy. Terpenes are released by trees more actively in warmer weather, acting as a natural form of cloud seeding. The clouds reflect sunlight, allowing the forest to regulate its temperature.

2.1 Classification of Terpenes

Classes	No. of isoprene unit	No. of carbon atoms
Hemiterpenes	1	5
Monoterpenes	2	10
Sesquiterpenes	3	15
Diterpenes	4	20
Triterpenes	6	30
Tetraterpenes	8	40
Polyterpenes	n	Cn

3 Sesquiterpenes and their Classification

Sesquiterpenes are a class of terpenes that consist of three isoprene units and have the molecular formula $C_{15}H_{24}$. Sesquiterpenes may be acyclic or contain rings, including many unique combinations. Biochemical modifications such as

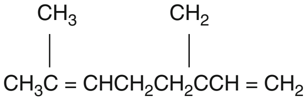


Fig. 116.1 Structure of isoprene unit

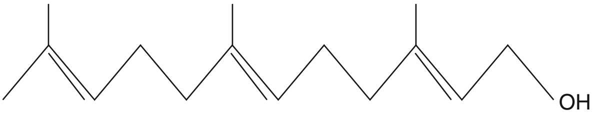


Fig. 116.2 Farnesol

oxidation or rearrangement produce the related sesquiterpenoids which are naturally found in plants and insects, as defensive agents or pheromones (Fig. 116.1).

Sesquiterpene lactones are secondary metabolites that belong to the group of C_{15} terpenoids. They are formed from three isoprene units. One of their methanol groups, a part of the isoprene group, was oxidized to lactones [10]. It constitutes an important group of secondary metabolites which play an important role in plant defense, as antimicrobials and insecticides. This group of secondary metabolites shows allelopathic prospective.

In recent years, there is an increasing demand of sesquiterpene lactones, mostly because of their high therapeutic potential as cytotoxic and anticancer agent. This is a large group of secondary metabolites. More than 90% of lactones were isolated from the members of Asteraceae family, while members of Magnoliaceae, Lauraceae, and Apiaceae family were the more primitive representatives of sesquiterpene lactones. Lipophilic solvents or supercritical fluid technology are used for the extraction of sesquiterpene lactones from plant material. The purification and structure elucidation was performed using chromatographic techniques and NMR and mass spectroscopy [11]. The presence of α,β-unsaturated γ-lactonic ring in sesquiterpene lactones has distinguishing therapeutic activity. Some sesquiterpene shows an allelopathic potential. The sesquiterpene shows an antibacterial, antimicrobial, antiviral, antiprotozoal, cytotoxic, and anticancer activity.

3.1 Acyclic

When geranyl pyrophosphate reacts with isopentenyl pyrophosphate, the result is the 15-carbon farnesyl pyrophosphate, which is an intermediate in the biosynthesis of sesquiterpenes such as farnesene. Oxidation can then provide sesquiterpenoids such as farnesol and juvenile hormone (Fig. 116.2).

3.2 Monocyclic

With the increased chain length and additional double bond, the number of possible ways that cyclization can occur is also increased, and there exists a wide variety of cyclic sesquiterpenes, for example, abscisic acid, fumagillin, germacrene, dendrolasin, and trans-y-monocyclo farnesol. In addition to common six-membered ring systems such as in zingiberene, a constituent of the oil from ginger, cyclization of one end of the chain to the other end can lead to macrocyclic rings such as humulene.

3.3 Bicyclic

In addition to common six-membered rings such as in the cadinenes, one classic bicyclic sesquiterpene is caryophyllene, which has a nine-membered ring and cyclobutane ring. Additional unsaturation provides aromatic bicyclic sesquiterpenoids such as vetivazulene and guaiazulene. Examples are caryophyllene, y-muurolene, petasin, lporneamarone, carotol, helminthosporal, avocettin, alkaloids of Nuphar, and mycophenolic acid.

3.4 Tricyclic

With the addition of a third ring, the possible structures become increasingly varied. Examples are longifolene, copaene, patchoulol, illudins, hirsutic acid, corlolins, trichothecin, helicobasidin, picrotoxinin, dendrobine, sativene, and santonin.

3.5 Tetracyclic

These compounds have four-membered rings. Examples are gossypol and marasmic acid.

3.6 Pentacyclic

These compounds have four-membered rings, for example, siccanochromenes (Fig. 116.3).

3.7 Functions of Sesquiterpenes in Plants

Sesquiterpene lactones have been isolated from all plant parts. However, most commonly they are obtained from glandular trichomes in leaves. They are bitter

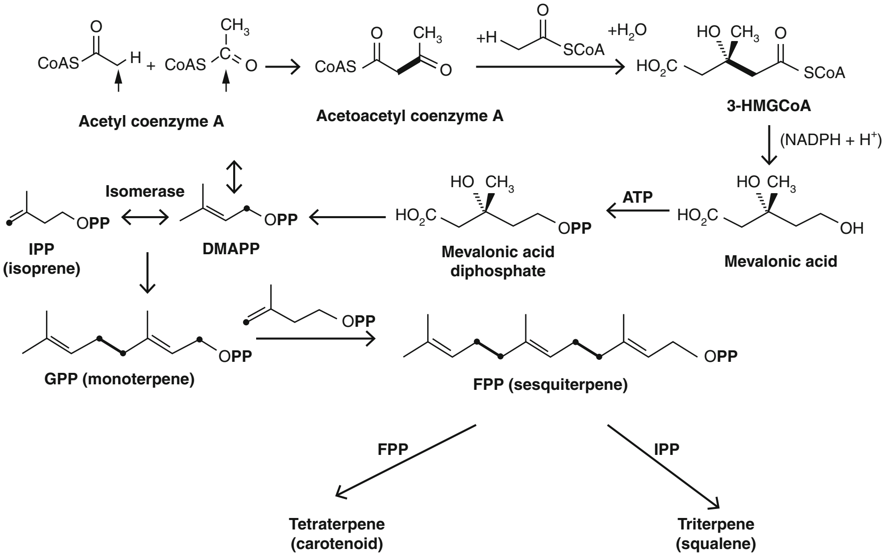

CoAS
H
CoAS
CH3
C
O
Acetyl coenzyme A
CoAS
O
CH3
O
Acetoacetyl coenzyme A
+H
SCoA
+H2O
HO2C
HO CH3 O
SCoA
3-HMGCoA
(NADPH + H+)
HO2C
HO CH3
OH
Mevalonic acid
ATP
HO2C
HO CH3
OPP
Mevalonic acid diphosphate
OPP
DMAPP
Isomerase
OPP
IPP (isoprene)
OPP
GPP (monoterpene)
OPP
OPP
FPP (sesquiterpene)
FPP
IPP
Tetraterpene (carotenoid)
Triterpene (squalene)

Fig. 116.3 Biosynthesis of sesquiterpenes

in taste and are not a good food candidate for herbivorous animals. These animals include herbivorous insects as well as mammals [10]. It protects plant material and makes its defense system stronger.

4 Cytotoxicity and Apoptosis

Cytotoxicity is the quality of cellular toxicity and the causative agents are cytotoxic agents. Cytotoxic agents are chemical substances, cells of immune system, and some venom (e.g., spider toxin). Treating cells with the cytotoxic compounds may undergo necrosis, in which they lose their membrane integrity and die rapidly as a result of cell lysis. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of apoptosis (controlled cell death).

Cell death occurs by two alternative and basically different modes: necrosis and apoptosis. Necrosis, the “ordinary” cell death with the characteristics of a passive process, is traditionally associated with inflammation, occurs in response to severe forms of physical or chemical types of injury, or results from severe depletion of cell energy and nutrition stores [12]. Apoptosis (programmed cell death) is a fundamental and complex biological process that enables an organism to kill and remove unwanted cells during any stage of development, therefore maintaining normal homeostasis and eliminating infected or malignant cells. In the development of new anticancer agents, apoptosis is considered the paramount form of pathophysiological cell death [13].

Apoptosis is one of the most important regulatory functions whereby a living organism maintains homeostasis. In some diseases, this regulation is disturbed, and the rate of apoptosis is increased (e.g., neurodegenerative diseases) or decreased (e.g., cancer).

4.1 Morphological and Biochemical Alterations

The onset of apoptosis is characterized by the shrinkage of cell and nucleus and by the condensation of nuclear chromatin into sharply delineated masses. Later, the nucleus progressively condenses and breaks up. The term budding has been coined for a process whereby the extensions separate and the plasma membrane seals to form a separate membrane around the detached solid cellular material. These apoptotic bodies are crowded with closely packed cellular organelles and fragments of the nucleus. The apoptotic bodies are rapidly phagocytosed into neighboring cells, including macrophages and parenchymal cells. Apoptotic bodies can be recognized inside these cells, but eventually they become degraded. If the fragmented cell is not phagocytosed, it will undergo degradation, which resembles necrosis, in a process called secondary necrosis. Apoptotic shrinkage, disassembly into apoptotic bodies, and engulfment of individual cells characteristically occur without associated inflammation [14].

Biochemical events lead to characteristic cell changes (morphology) and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Unlike necrosis, apoptosis produces cell fragments called apoptotic bodies that phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out onto surrounding cells and cause damage [15–17]. Activated nucleases are responsible for DNA degradation. As a consequence, some of the cells contain a decreased amount of DNA and can be detected as a subdiploid population in the cell cycle distribution by flow cytometry.

In the early stages of apoptosis, plasma membrane alterations occur at the cell surface, and PS translocates from the inner side to the outer layer of the plasma membrane. PS and phosphatidylethanolamine are actively confined to the inner cytofacial leaflet of the plasma membrane by the aminophospholipid translocase. This has been identified as a trigger for stimulation of the phagocytosis of apoptotic cells by macrophages, thus preventing secondary necrosis and inflammation of the surrounding tissue.

4.2 Caspases

Caspases are a family of specific proteases, present as inactive precursors in growing cells which trigger apoptosis [18]. These proteases use a cysteine residue for catalysis and only cleave substrates at Asp-Xxx bonds [19]. Caspases are synthesized as zymogens with an N-terminal prodomain, followed by a large and a small subunit. The mature enzyme contains two heterodimers and two active sites [20]. In apoptosis, caspases function in both cell disassembly and in initiating this disassembly in response to proapoptotic signals. More than ten different caspases have been identified in mammalian tissues [21]. Caspase 3 is activated by other caspases, and it can cleave many cellular targets, resulting in the attainment of apoptotic morphology. Activation of this caspase generally results in an irreversible commitment to cell death. Caspases are responsible for cleaving numerous cellular targets, including structural elements, nuclear proteins, and signaling proteins, leading to the morphological hallmarks of apoptosis [20].

4.3 TNF Pathway

TNF is a cytokine produced mainly by activated macrophages and is the major extrinsic mediator of apoptosis. Most cells in the human body have two receptors for TNF: TNF-R1 and TNF-R2. The binding of TNF to TNF-R1 has been shown to initiate the pathway that leads to caspase activation via membrane proteins, TNF receptor-associated death domain and Fas-associated death domain (FADD.) proteins [22]. Binding of this receptor can also indirectly lead to the activation of transcription factors involved in cell survival and inflammatory responses [23].

4.4 Fas Pathway

The Fas receptor (CD95) binds the Fas ligand (FasL), a transmembrane protein part of TNF family. The interaction between Fas and FasL results in the formation of death-inducing signaling complex (DISC), which contains the FADD., caspase-8, and caspase-10. In some types of cells, processed caspase-8 directly activates other members of the caspase family and triggers the execution of apoptosis of the cell. In other types of cells (type II), the Fas-DISC triggers the release of proapoptotic factors from mitochondria and the amplified activation of caspase-8 [24].

4.5 Other Components

A balance between proapoptotic (BAX, BID, BAK, or BAD) and anti-apoptotic (Bcl-Xl and Bcl-2) members of the Bcl-2 family is recognized. The proapoptotic members are required to make the mitochondrial membrane permeable for the release of caspase activators such as cytochrome c. Control of proapoptotic proteins under normal cell conditions of non-apoptotic cells is incompletely understood, generally Bax or Bak are activated by the activation of BH3 proteins [23].

5 Cancer

Cancer, neoplasm, involves uncontrolled and unregulated cell division. In cancer, cells divide and grow uncontrollably, forming malignant tumors, and invade nearby parts of the body. The cancer may also spread to more distant parts of the body through the lymphatic system or blood stream (metastasis).

The causes of cancer are complex. Many things are known to increase the risk of cancer, including tobacco use, certain infections, radiation, lack of physical activity, poor diet and obesity, and environmental pollutants [25]. These can directly damage genes or are entirely hereditary [26].

Cancer can be detected symptomatically and through screening tests or medical imaging. Once a possible cancer is detected, it is diagnosed by histological examination. Cancer is usually treated with chemotherapy, radiation therapy, and surgery. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment. While cancer can affect people of all ages, and a few types of cancer are more common in children, the risk of developing cancer generally increases with age. In 2007, cancer caused about 13% of all human deaths worldwide (7.9 million). Rates are rising as more people live to an old age and as mass lifestyle changes occur in the developing world [27]. The vast majority of cancer risk factors are due to environmental (including lifestyle) factors, and many of these factors are controllable. Thus, cancer is largely considered a preventable disease [28]. Greater than 30% of cancer is considered preventable by avoiding risk factors including tobacco, overweight/obesity, insufficient diet, physical inactivity, alcohol, sexually transmitted infections, and air pollution. Not all environmental causes can be prevented completely such as naturally occurring background radiation.

Cancer cells are the descendants of a normal cell in which something has gone wrong. In this normal cell, some kind of internal or external stress causes a mix-up in its genetic code. This event is said to “initiate” the cell to a precancerous state. After its DNA has been damaged, the cell withdraws from close communication with its neighboring cells. Interrupted cell-to-cell communication is a common result of DNA damage or other forms of cellular damage. Separated from the regulatory controls of its community, it is now at the mercy of its environment. The environment around this cell contains a promoting agent that stimulates cell proliferation. In response to the promoting agent, this precancerous cell divides to produce daughter cells, and these daughter cells divide to produce more daughter cells, and so on. All are proliferating only in response to the promoting agent. The promoting agent may be a chemical foreign to the body, or it could come from a natural process such as inflammation. The genetic instabilities passed down through the generations finally result in one cell that becomes capable of self-stimulation, and on this day an autonomous cancer cell is born. This cell no longer requires the promoting agent to stimulate its proliferation. The role of the promoting agent is made obsolete by the cell’s ability to make proteins such as growth factors that stimulate proliferation. This original cancer cell divides to produce daughter cells; these cells also divide, and soon there is a population of cancer cells. As they divide, they develop malignant characteristics, such as the ability to invade and metastasize. They also develop other characteristics that help assure survival, for example, the ability to evade the immune system, to mutate when faced with adverse conditions, and to induce the growth of new blood vessels through the process called angiogenesis. The development of these characteristics marks the third stage in carcinogenesis, the first two stages being initiation and promotion, respectively.

5.1 Approaches for Cancer Inhibition

5.1.1 Reduction of Genetic Instability

Genetic instability is provoked by oxidative stress (free radicals). High oxidative stress results in mutation and more destructive cancers. Thus, one way of reducing genetic instability is by reducing oxidative stress.

5.1.2 Inhibit Irregular Gene Expression

Gene expression can be normalized through modifying the activity of transcription factors. Genes that inhibit cancer progression are underexpressed and genes that facilitate cancer are commonly overexpressed in cancer cells. Therefore, cancer can be inhibited by normalizing the activity of those transcription factors that control gene expression.

5.1.3 Inhibit Abnormal Signal Transduction

The signal transduction from outside the cell to the nucleus depends on several proteins (kinase enzymes and ras proteins). Signal transduction is a normal process needed by healthy cells, but in cancer cells excessive signal transduction favors proliferation and metastasis. So cancer can be inhibited by normalizing signal transduction.

5.1.4 Cell-to-Cell Communication

Normal cell-to-cell communication can be promoted by improving gap junction communication and by normalizing CAM activity.

5.1.5 Inhibit Tumor Angiogenesis

Angiogenesis is a normal process that is required during wound healing and in other situations. Angiogenesis in tumors is uncontrolled which can be regularized by regularizing the factors that control it. Angiogenesis is most successful in presence of angiogenic factors and certain environmental conditions, such as hypoxia. Cancer can be inhibited by inhibiting the release of angiogenic factors or altering the local environment to inhibit tumor angiogenesis.

5.1.6 Inhibit Invasion and Metastasis

Invasion requires enzymatic digestion of the healthy tissue surrounding the tumor and can be reduced by inhibiting tissue digestive enzymes, by protecting normal tissues from the enzymes, and by reducing the ability of tumor cells to migrate.

Metastasis requires that cells detach from the primary tumor, enzymatically digest blood vessel walls to gain access to and exit from the blood circulation, and evade the immune system while in the circulation.

5.1.7 Increase the Immune Response

Cancer is an immune disorder. The immune response against cancer cells can be increased by stimulating the immune system and by reducing the ability of cancer cells to evade immune attack. The healthy, fundamental immune cells can destroy cancer cells, if it can be recognized antigen in the body [29]. Nowadays, immunomodulator is an emerging concept to combat against cancer.

5.2 Natural Compounds as Inhibitors of Cell Proliferation

The flow of information leading to cell proliferation is mediated through proteins. Abnormal gene expression is the most prominent feature, which results in four types of protein signals that support proliferation or malignant behavior. All four of these primary protein signals can be inhibited by natural compounds (herbal drugs).

1. Fault in the p53, Bax, and Bcl-2 gene can produce proteins that fail to induce apoptosis in cancer cells and protect cancer cells from apoptosis.
2. Abnormalities in some genes can produce excessive amounts of proteins that support angiogenesis, metastasis, or elusion of the immune system. It affects proliferation and apoptosis.
3. Overexpression of oncogenes such as *fos*, *jun*, and *myc* can produce large amounts of *fos* proteins which act as transcription factors to induce the expression of cyclin genes, whose proteins make the cell cycle proper.
4. Abnormal genes produce proteins like growth factors, growth factor receptors, kinase enzymes, ras proteins, and others several proteins that facilitate signal transduction. Overproduction of these proteins results in increased signal transduction, which stimulates abnormal activity of transcription factors such as NF-kB. Abnormal transcription factor activity stimulates gene expression, resulting in the overproduction of cyclin proteins that drive the cell cycle and the overproduction of other proteins that assist angiogenesis, invasion, and metastasis. Their actions include lowering mutation rates by scavenging free radicals, normalizing p53 activity, or both; inhibiting abnormal transcription factor activity; inhibiting kinases or other proteins involved in signal transduction; inhibiting the activity of cyclin proteins, which drive the cell cycle; and increasing cell-to-cell communication, which sends signals that normalize gene expression. Natural compounds that inhibit signal transduction can play a dual role in inhibiting cancer. In addition to proliferation signals, cancer cells also require signals to prevent apoptosis. In cancer cells, these signals come in part from both growth factors and increased signal transduction. Therefore, natural compounds that reduce signal transduction not only inhibit proliferation but also induce apoptosis [29].

6 Description of Cytotoxicity of Sesquiterpenes and their Derivatives

The plants of the genus *Taraxacum* contain a number of sesquiterpenes including eudesmanolides, taraxacolide, guaianolides, ixerin D, and germacranolide esters, taraxinic acid derivatives, and ainslioside [30, 31]. Furthermore, the two germacranolides and guaianolide glycosides sonchuside and vernoflexuoside were isolated from roots of *Taraxacum bicorne* and *Taraxacum hondoens* [32]. Eudesmanolides derivatives and taraxinic acid were isolated from *Taraxacum linearisquameum; Taraxacum coreanum* shows significant cytotoxicity against human leukemia-derived cell lines [33]. The recently discovered cytotoxic sesquiterpene lactones deacetylmatricarin and 2-oxo-guaianolides were present in *Taraxacum platycarpum*, *Taraxacum obovatum*, *Taraxacum hondoense*, and *Taraxacum bessarabicum* [34]. The taraxasterol and taraxerol from *Taraxacum japonicum* exhibited strong inhibitory effects in the carcinogenesis of mouse skin tumors [35]. The increased amounts of TNF-α and IL-1 contributed to dandelion (*Taraxacum officinale*)-induced apoptosis in hepatoma cell lines [36].

Eupatorium perfoliatum L. (Asteraceae), boneset also known as Indian sage, produces a number of sesquiterpene lactones possessing cytotoxic and immunostimulant activities [37, 38]. The sesquiterpenes lactones are guaianolides such as euperfolid, 11,13-dihydroeuperfolid, eufoliatin, eufoliatorin, or germacranolides such as euperfolitin and euperfolin [39].

Great burdock (*Arctium Lappa* L.) is widely used in folk medicine. The root extracts of Burdock shows antitumor properties against colon, ovarian, prostate, and renal cancer. The burdock seeds contain sesquiterpenes, lactucopicrin [40].

Telekin from the roots of *Telekia speciosa*, helenin from the roots of *Inula helenium* L., and sesquiterpene alcohol ledol from the aerial parts of *Ledum palustre* L. show cytotoxicity [41]. Bisabolol, a nontoxic sesquiterpene alcohol, was isolated from *Matricaria chamomilla*, *Carthamus lanatus*, and *Matricaria recutita* and exhibits a cytotoxic apoptosis-inducing effect [42] against human glioblastoma and pancreatic carcinoma cell lines.

A new guaianolide derivative, which possesses cytotoxic activity, was obtained from leaves and stems of *Chrysanthemum boreale*. It is effective in kidney carcinoma, melanoma, colon adenocarcinoma, prostate adenocarcinoma, and lung carcinoma. *Chrysanthemum morifolium* effectively inhibited the cytotoxicity and improved cell viability. It also stimulates the elevation of reactive oxygen species (ROS) level, increase in Bax/Bcl-2 ratio, cleavage of caspase-3, and PARP proteolysis [43, 44].

Xanthium strumarium L. (Asteraceae), locally called Bandaa, exhibits in vitro cytotoxic activities against various cancer cell lines like adenocarcinoma. Several other species of the genus *Xanthium* produce sesquiterpene lactones termed xanthanolides, xanthatin, 8-epi xanthatin, and 8-epi tomentosin, which are responsible for most of the biological activities. Regarding lung cancer, a methanolic extract of *X. strumarium* leaves exhibited a strong inhibition of tumor cell proliferation. The active constituents responsible for cytotoxic effect include

8-epi-xanthatin and its epoxide, two xanthanolides, and sesquiterpene lactones. The two xantholides show promising farnesyltransferase (FTase) inhibitory effect. Farnesylation of certain oncoproteins (especially Ras proteins) is required for their oncogenic activity. Moreover *X. strumarium* extracts effectively inhibit tubulin polymerization in mammalian tissues [45]. The maximum caspases 3/7 activity was observed with xanthatin. It seems that the induction of apoptosis was effected by activation of caspase-3. DNA fragmentation was also observed in human leukemia cell line. Sesquiterpene lactones trigger mitochondrial membrane transition, loss of mitochondrial membrane potential, and release of proapoptotic mitochondrial proteins leading to caspase activation and apoptotic cell death [46]. Xanthatin also caused a programmed cell death like in trypanosomes as evidenced by a reduction in mitochondrial membrane potential [47].

Farnesol, a sesquiterpene present in orange peel, lemon grass oil, and strawberries, has been considered as promising anticancer drug by affecting apoptotic pathway [48–50]. The extrinsic apoptotic pathway involves the activation of death domain receptors and downregulation and activation of caspase-3, while the intrinsic apoptotic pathway enhances the degradation of mitochondria and activation of caspase-9 pathway, which induces caspase-3 activation. The HSP27 protein which is associated with cancers and resistance to therapy found to be downregulated [51]. Farnesol induces an inhibition of cell growth and promotes apoptosis in squamous cell carcinoma through the induction of caspases, inhibition of survivin, and downregulation of various cellular proteins, for example, keratin 5A, RAN, heat shock protein, and glutathione S-transferase [52–54]. It also interferes with a phosphatidylinositol-type signaling pathway, leading to decreased levels of intracellular diacyl glycerol (DAG), and results in the activation of caspases and inhibition of survivin, leading to cell disassembly and growth inhibition. Its effect on mevalonic acid metabolism may account for antitumor activities in pancreas, colon, and cervix and cancer of lymphatic gland [55].

Parthenolide (PRT) is a sesquiterpene lactone obtained from feverfew (*Tanacetum parthenium*). It inhibits NF-kB and induces ROS production [56, 57]. The cytotoxicity of PRT inhibits DNA binding of NF-kB, signal transducer, and transcription activators, as well as reduction in MAPK activity and the generation of reactive oxygen [58, 59]. Its anticancer activity was established in multiple myeloma, lymphocytic leukemia, and stem cells [60]. The proapoptotic activity of PRT includes stimulation of intrinsic apoptotic pathway with the higher level of intracellular ROS, conformational changes of Bcl-2 family proteins, and poly (ADP-ribose) polymerase degradation. Treatment with parthenolide led to G1 phase cell cycle arrest by modulation of cyclin D1 and phosphorylated cyclin-dependent kinase. Parthenolide also inhibited the invasive ability of bladder cancer cells. PRT amplifies the apoptotic signal through the sensitization of cancer cells to extrinsic apoptosis, induced by TNF-α. PRT is a promising metabolic inhibitor to retard tumor formation and suppress tumor growth.

The members of genus Vernonia (*V. scorpioides*, *V. cinerea*, *V. lasiopus*, *V. amygdalina*) are good sources of sesquiterpene lactones (SLs). It contains highly oxygenated germacranolides, such as glaucolides and hirsutinolides, which possess cytotoxic property [61, 62]. The cytotoxic SLs, pulchellin E and gaillardin, from Inula oculus-christi [63] and guaianolides were also obtained from the leaves of *Achillea depressa* [64]. The crude extracts, chloroform and hexane-derived fractions of *Vernonia scorpioides*, Asteraceae, are a good source of sesquiterpene lactones (SLs), highly oxygenated germacranolides, such as glaucolides, hirsutinolides, and cadinanolides [65]. Hirsutinolides and glaucolides show cytotoxic property [66]. Phytochemical analysis of the leaves of *Vernonia amygdalina* yielded two cytotoxic sesquiterpene lactones: vernolide and vernodalol [67].

Ginkgo biloba L., living fossil, is among the most sold medicinal plants in the world. A number of secondary metabolites have been isolated from the plant. However, are terpenes and flavonoid glycosides considered the main bioactives [68]? The leaf extract may have cytotoxicity, related to their antioxidant, antiangiogenic, and gene regulation property. The extract changes gene expression that regulates cell proliferation, cell differentiation, or apoptosis; enhances antioxidant status; and inhibits DNA damage. It inhibits the formation of radiation-induced chromosome-damaging factors and ultraviolet light–induced oxidative stress effects that may also be associated with anticancer activity. Synergism is found between terpenoid and other constituents to elicit optimum effects [69, 70]. In vivo study suggests that Ginkgo may promote apoptosis of cancer cells by caspase-3 activation in mouth and stomach cancer [71, 72].

Guaiazulene is a bicyclic sesquiterpene obtained from guaiac wood oil, *Callis intratropica* Blue, and *Matricaria chamomilla* [73]. Guaiazulene showed cytotoxicity against cancer and normal cells by inducing an intrinsic apoptotic pathway and caspase-3 activation [74].

Rolandra fruticosa (L.) Kuntze (Asteraceae) has been placed in the subtribe Rolandrinae. Bioactivity-guided fractionation of methanol extract *Rolandra fruticosa* results in the isolation of sesquiterpene lactones which was active against colon cancer cell [75].

Laurus nobilis L. (Lauraceae) is a native to southern Mediterranean region and is widely cultivated mainly in Europe and the USA. The plant contains sesquiterpenes lauroxepine, costunolide, gazaniolide, and four sesquiterpene lactones including santamarine, reynosin, 11,13-dehydrosantonin, and spirafolide as a cytotoxic principle against leukemia and ovarian and gastric cancers [76].

The genus *Lactuca* (*L. perennis*, *L. tatarica*, *L. indica*, *L. capensis*, *L. tenerrima*) has been shown to produce sesquiterpene lactones including guaianolides, germacranolides, and some eudesmanolides. Two new eudesmane sesquiterpene lactones show anticancer activity against epithelial carcinoma and colon carcinoma cell lines [77].

The leaves of *Helianthus annuus* are the source of sesquiterpene annuolide E, leptocarpin, sesquiterpene heliannuols A–I, bisnorsesquiterpene, annuionone E, heliannuol L, helibisabonol A, and helibisabonol B. Sesamol seems to be an almost equally potent chemopreventive agent. Sunflower oil offered protection in skin tumor [78, 79].

Achillea species have been widely applied in folk medicine for the treatment of different cancers. The cytotoxic or cytostatic effects of *A. alexandri-regis* [80], *A. clavennae* [81], *A. ageratum* [82], and *A. millefolium* [83] extracts have been established against breast, lung, and colon cancer. The SLs, guaianolide, 1,10-seco-guaiane, seco-pseudoguaianolides (paulitin and isopaulitin) responsible for the antitumor activity [84].

The acetone extract of *Ambrosia* species *A. artemisiifolia*, *A. psilostachya*, *A. trifida*, and *A. tenuifolia* contains cytotoxic sesquiterpene lactone, ambrosin, isabelin, psilostachyn [85, 86], cumanin, and peruvin [87]. The steroidal lactone blocks cells in mitosis and acts as a novel checkpoint inhibitor of the G_2/DNA damage [88], suggesting that these compounds can easily bind covalently to the target proteins [89]. The Compositae family is known to possess antitumor, cytotoxic, antimicrobial, and phytotoxic activities due to the presence of α, β-unsaturated-γ-sesquiterpene lactones (ridentin and santonin). These compounds may explain many of the observed pharmacologic activities of *Artemisia tridentata* (sagewood). The antitumor and cytotoxic activities may be due to the alkylation of enzymes that regulate cell division [90]. Many sesquiterpene lactones bearing methylene-7-lactone group include germacranolides, elephantin, elephantopin, costunolide, tulipinolide, guaianolides, gaillardin, euparotin acetate, and its companions, the pseudoguaianolide, damsin, elemanolide, and vernolepin show significant antitumor or cytotoxic activity [91]. *Artemisia vulgaris*, commonly known as mugwort, is a perennial weed native to Asia, Europe, and North America [92]. It shows anticancer effect against sarcoma cell lines. The extract of *Artemisia* species shows antimutagenic and antitumor effect [93].

Artemisia annua (annual wormwood) is the source of 20 known sesquiterpenes including artemisinin (arteannuin A), arteannuin B, artemisitene, and artemisinin acid. Artemisinin (qinghaosu) is an important natural sesquiterpene lactone with antimalarial effect against susceptible and multidrug resistant *Plasmodium* spp. Current research also shows that artemisinin drugs are effective against cancer [94, 95]. It inhibits cancer proliferation, metastasis, and angiogenesis, also reduces tumor volume and progression. Interestingly, the potent anticancer action of artemisinin can also be ascribed to the endoperoxide bond [96]. Generally, iron and heme or heme-bound proteins activate artemisinin [97]. Preloading of cancer cells with iron activates artemisinin cytotoxicity [98]. Moreover, artemisinins tagged to iron-carrying compounds exhibit greater activity compared to artemisinin alone [99]. Induction of heme oxidase and downregulation of the heme synthesis genes may also inhibit cytotoxicity of artemisinin. Continued proliferation and growth of malignant cells require higher iron metabolism which can be fulfilled by increase in transferring receptors (TfR). Blocking the TfR increases artemisinin activity [100]. It is reported that the cytotoxicity of iron-activated artemisinin due to the release of highly alkylating carbon-centered radicals and radical oxygen species (ROS). In artemisinin-treated cancer cells, radicals cause apoptosis, arrest growth, inhibit angiogenesis, and cause DNA damage. Artemisinin cytotoxicity associated with impaired cytokinesis enhanced levels of oxidative stress, inhibition of tumor invasion, migration, and metastasis [101]. Hence, increasing oxidative stress is a common anticancer mechanism of antitumor agents [102].

The sesquiterpene lactone, helenalin, is a component found in the aerial part of *Arnica montana* L (sneezeweed). Helenalin induces apoptosis in leukemia cells by the cleavage of caspase-3-like substrate as well as proteolysis of procaspase-3 and procaspase-8. It also induces the release of cytochrome c from mitochondria which leads to caspase activation. Overexpression of Bcl-xL or Bcl-2 proteins failed to give resistance to helenalin-induced apoptosis. It is reported that helenalin induces a mitochondria-dependent pathway. Thus, helenalin is a promising cytotoxic agent that overcomes apoptosis resistance attributable to overexpression of anti-apoptotic Bcl-2 proteins [103]. Furthermore, helenalin dramatically inhibits periodic Skp2 protein accumulation, p27 degradation, and S phase progression resulting in cell cycle arrest at G1 phase [104]. Helenalin inhibits nuclear factor kappa B (NF-kB) DNA-binding activity. Protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) inhibitors significantly inhibited HL-60 cell differentiation induced by helenalin. Moreover, helenalin enhanced PKC activity and also increased the level of ERK. The enhanced levels of cell differentiation closely correlated with the decreased levels of NF-kB binding activity by helenalin. These results indicate that PKC, ERK, and NF-kB may be involved in HL-60 cell differentiation induced by helenalin [105].

Ixeris sonchifolia (Asteraceae) contains 20 sesquiterpene lactones and shows potential chemopreventive and chemotherapeutic application of sesquiterpene lactones [106]. Methanolic extract inhibits the proliferation of Ehrlich carcinoma cells [107].

Centaurea deflexa is a perennial plant belonging to the Asteraceae family. In vitro cytotoxic activity against different pancreatic and colon cancer cell lines has been also reported for several *Centaurea* species [108]. The major constituents identified in *Centaurea* L. species, responsible for most of their pharmacological properties, are sesquiterpene lactones which induce apoptosis in cancer cells [109].

Ginger, the rhizome of *Zingiber officinalis*, one of the most widely used species of the Zingiberaceae family, is a common condiment for various foods and beverages [110]. The volatile oil components in ginger consist mainly of sesquiterpene hydrocarbons, predominantly zingeberene (35%), curcumene (18%), and farnesene (10%), with lesser amounts of bisabolene and β-sesquiphellandrene [111]. Zerumbone exhibits antiproliferative and anti-inflammatory activities [112]. It inhibits the activation of NF-kB and NF-kB-regulated gene expression, which may provide a molecular basis for cancer treatment by zerumbone. The ethanol extracts of ginger have antitumor-promoting effects in mouse skin tumorigenesis model [113]. The prevention of tumor initiation and promotion process occurs through the induction of antioxidant, metabolizing enzymes and suppression of proinflammatory signaling pathways. Antitumor promoter activity of gingerol and paradol was established by inhibition of cell transformation and protein-1 activation [114, 115].

6.1 Sesquiterpene Coumarins

Sesquiterpene coumarins constitute an interesting family of natural products. They are a group of molecules whose structures are based on a C_{15} terpene moiety linked through either an ether linkage with the 7-hydroxy group of umbelliferone, scopoletin, or isofraxidin or through a C–C bond with carbon 3 of 4-hydroxycoumarin. These are found in some plants of the families Umbelliferae, Asteraceae, and Rutaceae [116].

6.2 Sesquiterpene Umbelliprenin

Umbelliprenin is found in the plants of Apiaceae and Rutaceae family. It includes genera Angelica, Ferula, Heptaptera, Heracleum, Peucedanum, and Haplophyllum [117]. Umbelliprenin shows cytotoxicity against fibrosarcoma cell line [118], and its effect can be increased in combination with Fe_3O_4 nanoparticles [119]. The extract of the aerial parts of *Ferula vesceritensis* contains two sesquiterpene coumarins: farnesiferol and feselol having potential role in multidrug resistance cancer. Umbelliprenin isolated from the flowers of *Magydaris tomentosa* was reported to inhibit growth of pathogenic bacterial strains [120].

6.3 Sesquiterpene Ethers

Sesquiterpene ethers (scopoletin) scopofarnol and scopodrimol were also isolated from *Achillea* and *Artemisia* species and are potential modulators of MDR in tumor cells [121].

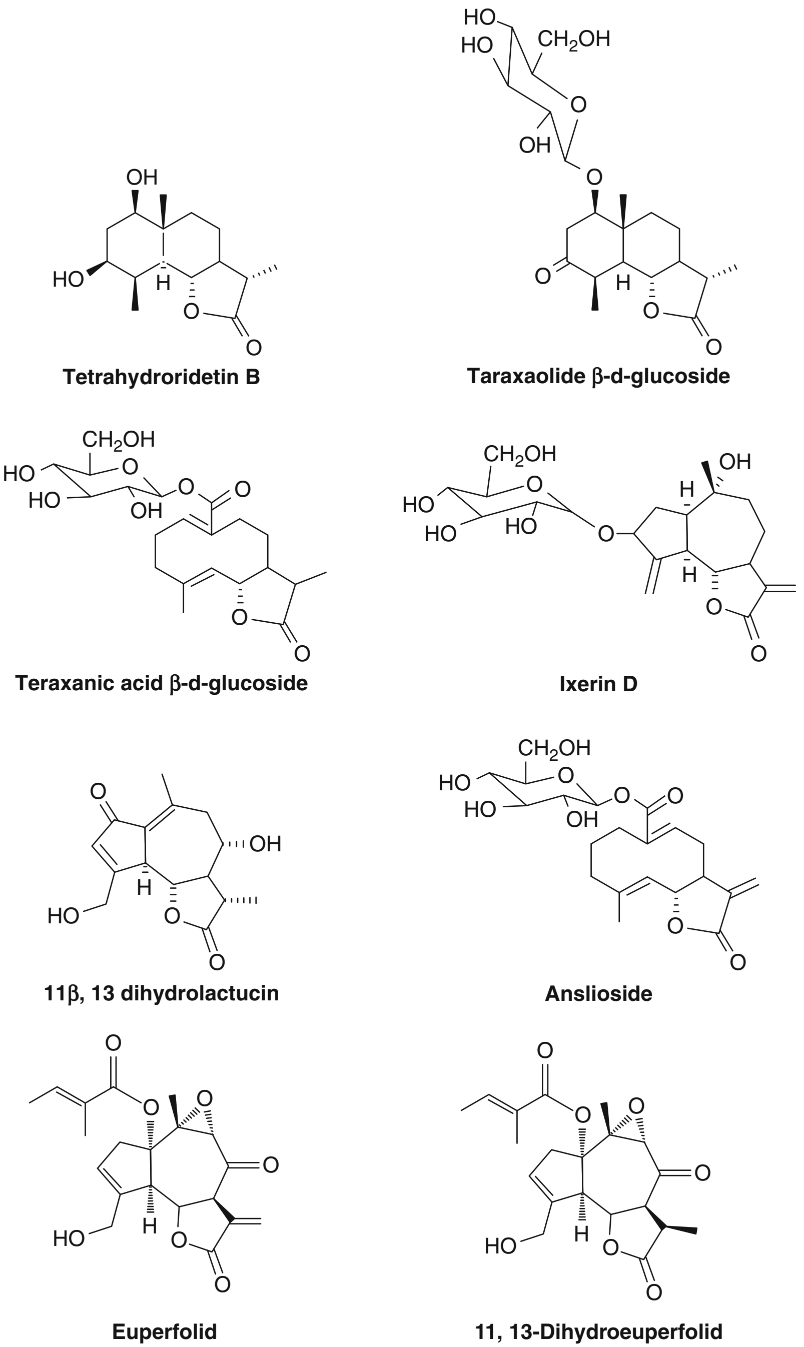
6.4 Prenylated 4-Hydroxycoumarins

Ferulenol was isolated from the extract of *F. communis*. Ferulenol displays microtubule interacting properties as indicated by its cytotoxic effects toward breast, colon, and ovarian cancers and leukemia. The cytotoxic effect of ferulenol may be related to the impairment of microtubule dynamic and mitochondrial function [122] (Fig. 116.4).

7 Allelopathic Potential of Sesquiterpenes

Allelopathy is a biological phenomenon by which an organism produces one or more biochemicals that influence the growth, survival, and reproduction of other organisms. These biochemicals are known as allelochemicals and can have beneficial (positive allelopathy) or detrimental (negative allelopathy) effects on the target organisms. Allelochemicals are a subset of secondary metabolites, which are not required for metabolism of the allelopathic organism. Allelochemicals with negative allelopathic effects are an important part of plant defense against herbivory [123, 124].

Allelopathy is characteristic of certain plants, algae, bacteria, coral, and fungi. Allelopathic interactions are an important factor in determining species distribution



Tetrahydroridetin B

Taraxaolide β-d-glucoside

Teraxanic acid β-d-glucoside

Ixerin D

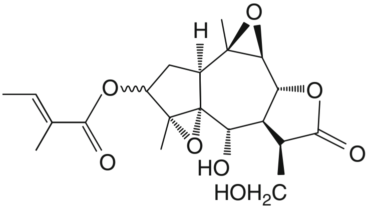
11β, 13 dihydrolactucin

Anslioside

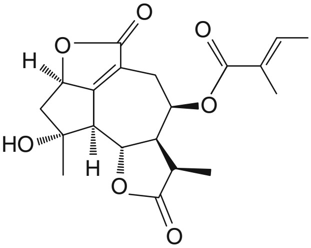
Euperfolid

11, 13-Dihydroeuperfolid

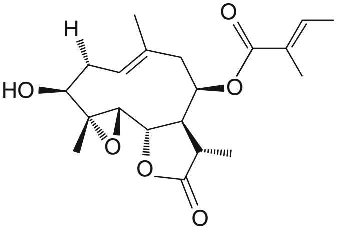
Fig. 116.4 (continued)



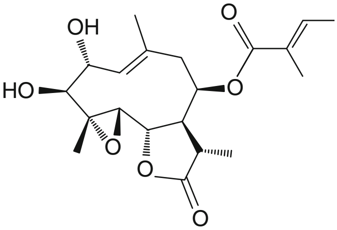
Eufoliatin



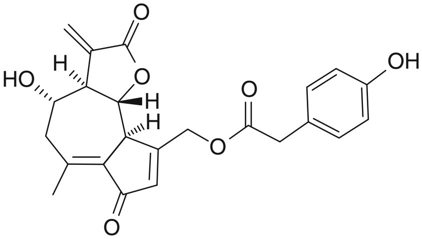
Eufoliatrin



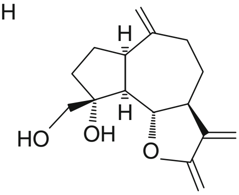
Euperfolin



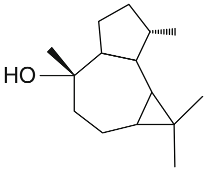
Euperfolitin



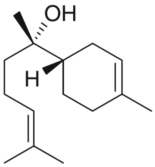
Lactucopicrin



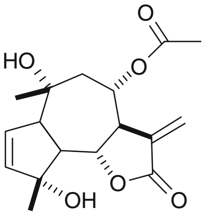
Telekin



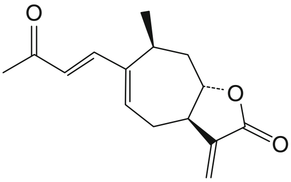
Ledol



Bisabolol

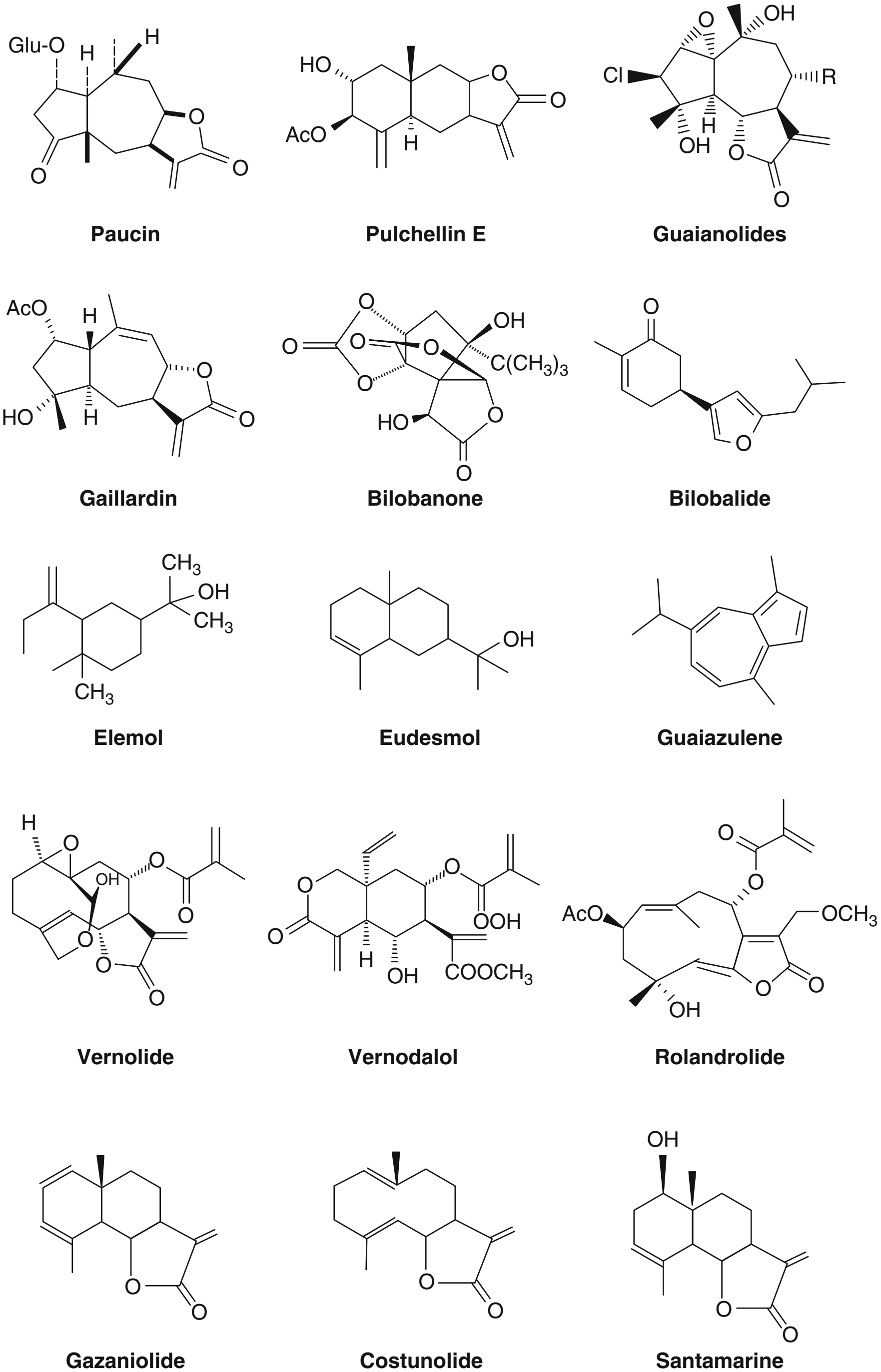


Guaianolide



8-Epixanthatin

Fig. 116.4 (continued)



Paucin

Pulchellin E

Guaianolides

Gaillardin

Bilobanone

Bilobalide

Elemol

Eudesmol

Guaiazulene

Vernolide

Vernodalol

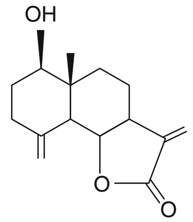
Rolandrolide

Gazaniolide

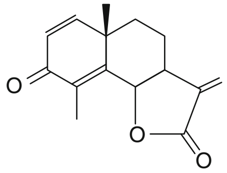
Costunolide

Santamarine

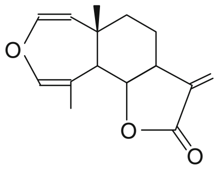
Fig. 116.4 (continued)



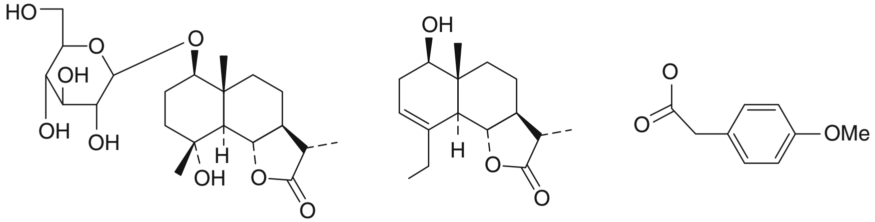
Reynosin



11,13-dehydrosantonin



Spirafolide


HO
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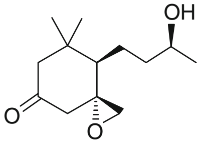
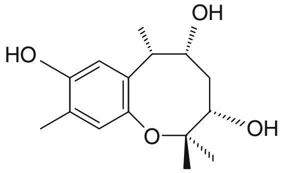
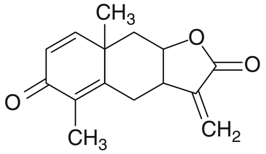
Eudesmanolides



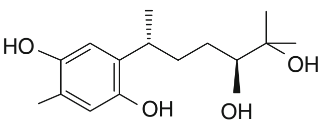
Annuinonone



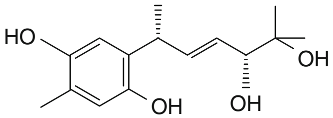
Heliannuol


CH_3
O
O
O
CH_3
CH_2

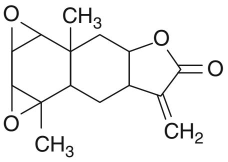
Yomogin



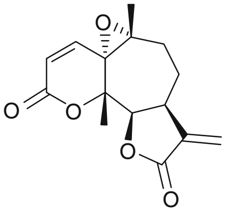
Helibisabonol A



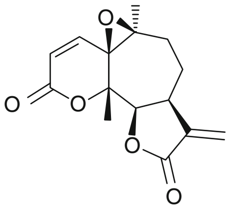
Helibisabonol B


O
CH_3
O
O
O
CH_3
CH_2

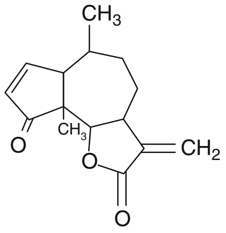
Diepodyeudesmenolide



Paulitin



Isopaulitin


CH_3
O
CH_3
O
CH_2
O

Ambrosin

Fig. 116.4 (continued)

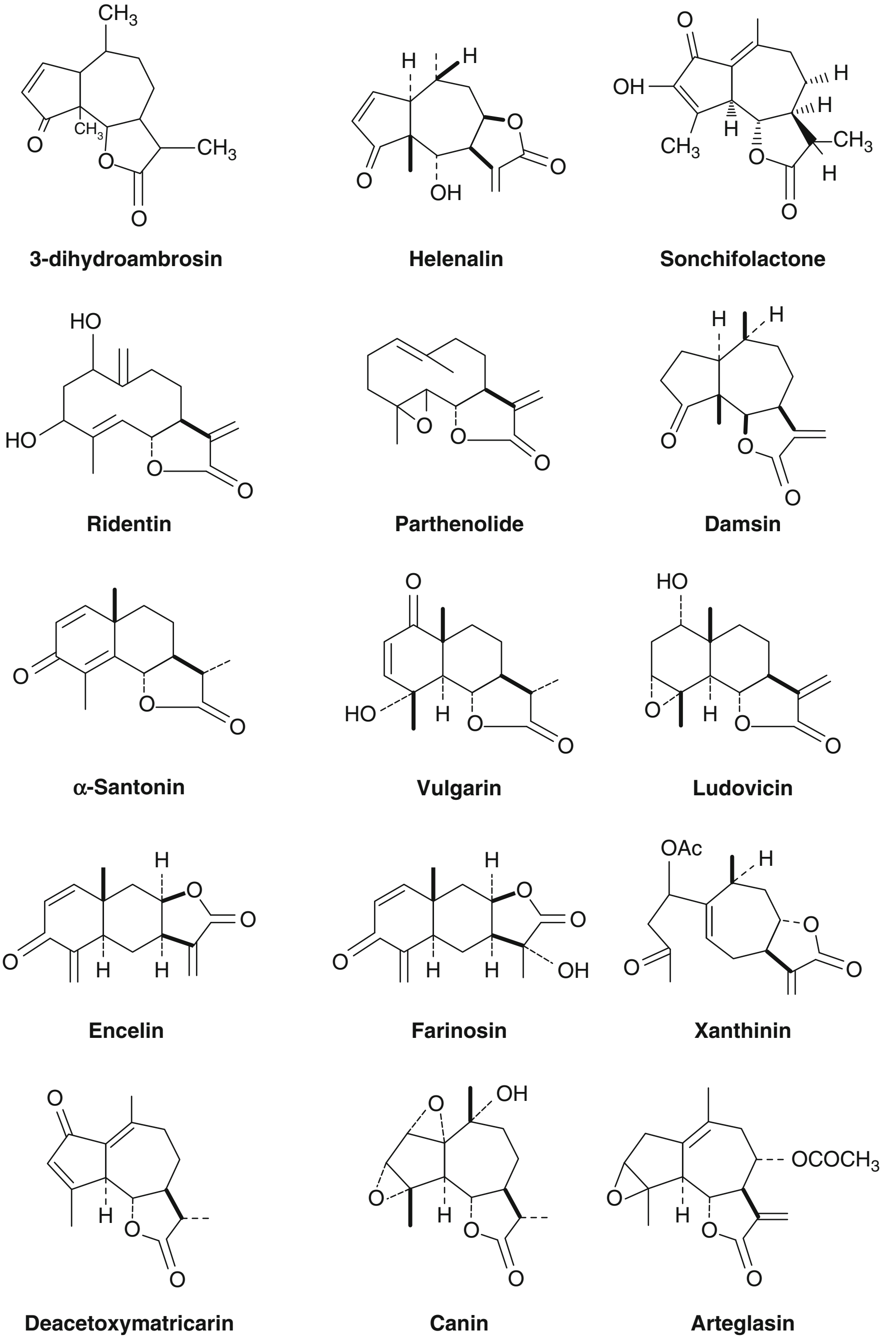

3-dihydroambrosin
Helenalin
Sonchifolactone
Ridentin
Parthenolide
Damsin
α-Santonin
Vulgarin
Ludovicin
Encelin
Farinosin
Xanthinin
Deacetoxymatricarin
Canin
Arteglasin

Fig. 116.4 (continued)

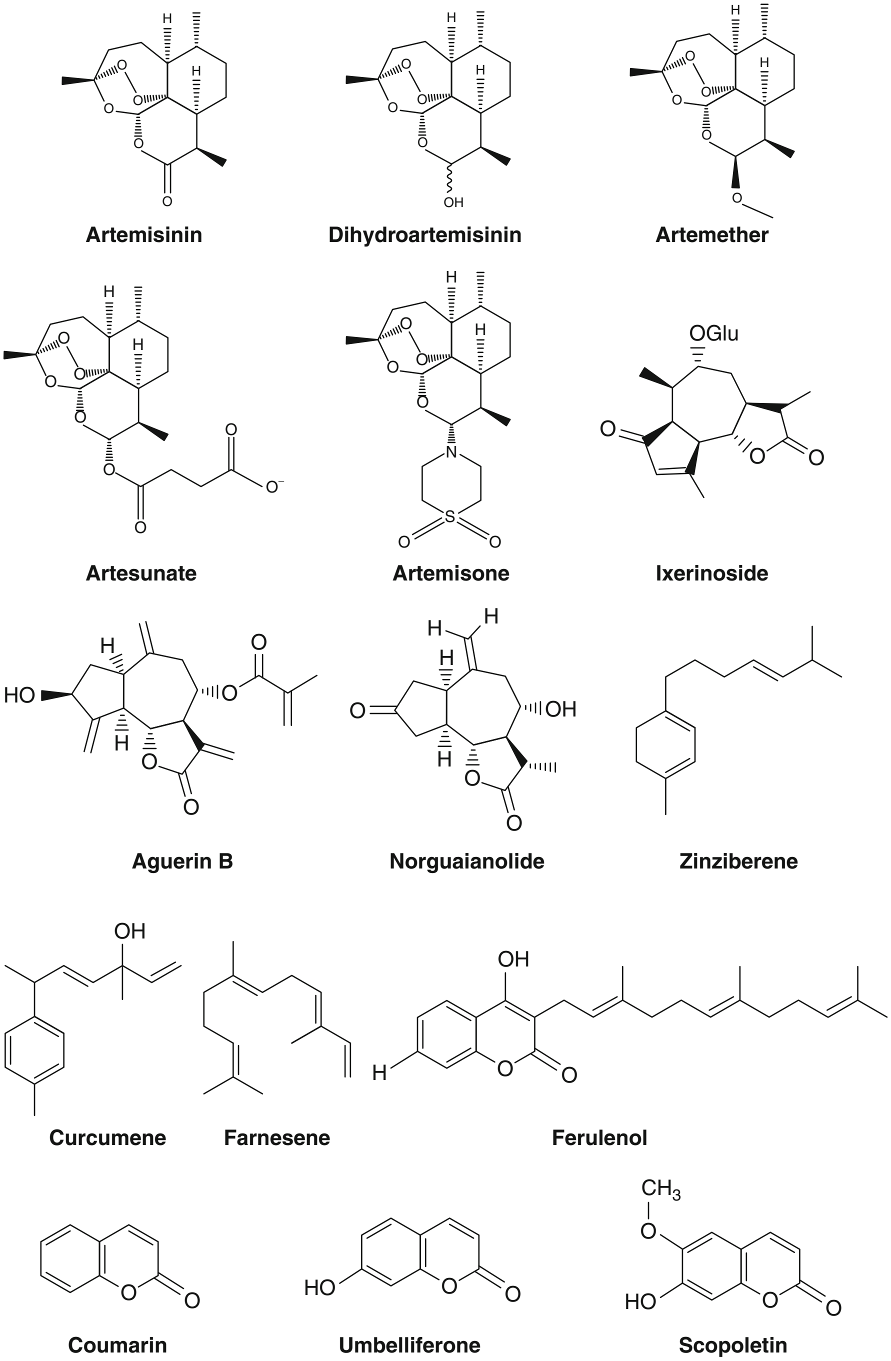

Artemisinin
Dihydroartemisinin
Artemether
Artesunate
Artemisone
Ixerinoside
OGlu
Aguerin B
Norguaianolide
Zinziberene
Curcumene
Farnesene
Ferulenol
Coumarin
Umbelliferone
Scopoletin
CH3
HO
OH

Fig. 116.4 (continued)

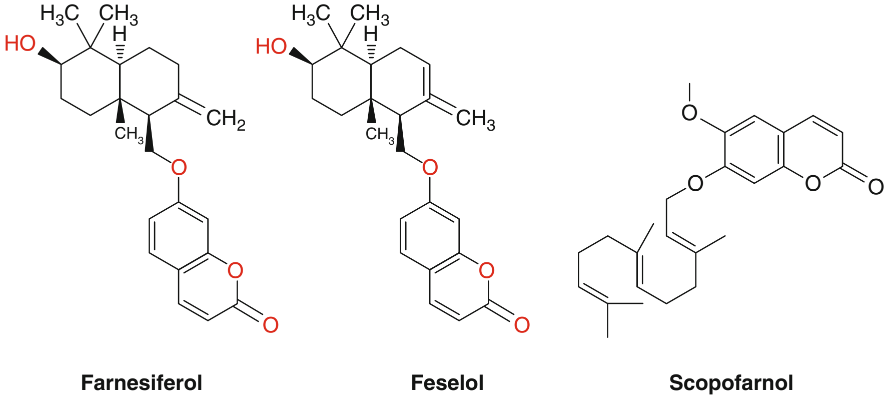


Fig. 116.4 Structures of cytotoxic sesquiterpenes and their derivatives

and abundance within plant communities and are also thought to be important in the success of many invasive plants. Allelopathic potential was found in the members of Asteraceae family [125]. The sesquiterpene lactones and a flavonoid compound isolated from this plant inhibit seedling growth in *Brassica parachinensis*. The isolated lactones deoxymikanolide, dihydromikanolide, mikamicranolide, and tetrahdroxyflavone derivatives show a great phytotoxicity on *B. parachinensis* seedlings. These compounds reduce the root and shoot growth in seedlings. The invasive effect of lactones is more than that of flavonoids.

8 Specificity of Sesquiterpenes

A particular sesquiterpene exhibits a particular biological activity, that is, cytotoxicity, genotoxicity, mutagenicity, stimulation, and suppression of genes. They are also specific in their mode of action relating to production of free radicals. This change in biological activity depends on the composition of sesquiterpenes, which are affected by endogenous and exogenous factors. The endogenous factors are related to anatomical and physiological properties of the plant material as well as their biosynthetic pathways, which might change in either the different tissues of the plants or in different seasons but also could be influenced by DNA adaptation. The exogenous factors might affect genes responsible for the formation of essential oil. Moreover, methods of drying, size reduction, and extraction methods affect the yield of volatile components. The concept of chemotypes in the same plant species is also gaining popularity. Chemotaxonomy classifies essential oils containing plant material characterized by species-specific chemical polymorphism. With reference to antigenotoxic and mutagenic effects, the sesquiterpenes and their oxygenated derivatives show similar type of protection, while the mechanism of protection does not depend on the composition of oil, but it depends on the type and nature of mutagens, type of enzymatic reactions, and programmed cell death or necrosis [126–128].

9 Synergism and Antagonism Among Sesquiterpenes

Chemically essential oil is terpenoids and their oxygenated derivatives. It contains a large number of molecules in their composition. We know that the presence or absence of other molecules in combination with the main molecules affects the biological and therapeutic property of essential oil. So it is very mandatory to study the effect of all constituents of essential oil. Sometimes, the other less available or less important constituents improve the biological properties of main molecule (synergism) so that the net effect will be more than the individual effect or vice versa (antagonism). On the basis of literature survey in most cases, generally, the main constituents of essential oil bisabolol, carvacrol, carvone, cinnamaldehyde, citronellol, eucalyptol, eugenol, farnesol, farnesene, geraniol, limonene, linalool, nerol, safrole, santalol, terpineol, and thymol were analyzed. Usually, the main components reveal good enough biochemical, biophysical, and therapeutic potential of the essential oils from which they were isolated [129], but the therapeutic effectiveness depends on the concentration of main component alone and in combination with the essential oil [130–132]. Furthermore, it is likely that several components, the cell penetration, cell distribution, hydrophilic and lipophilic magnetism, fixation on cell walls and membranes, color, density, fragrance, texture, of the essential oils were also affected by other constituents [133]. The cell penetration and cell distribution is very important because the distribution of the oil in cell compartment determines the intensity of the therapeutic effects. So for biological and therapeutic purposes, it is more meaningful to study entire oil rather than some of its individual components because the concept of synergism and antagonism seems to be more significant.

10 Regulatory Issues

The essential oil industry adopted the latest legislation from Europe on the safety aspect of essential oils. Any industry which deals with the selling of essential oils must now match to the latest CHIP-2 regulations, and this is a significant change in essential oils sold. At the current time, few companies adhere to even putting the “best before” and “batch number,” the most notable being the band of aromatherapy companies. However, it is likely to be enforced as the time goes. Most “trading standards officers” are overworked with other things to worry too much about the new legislation. However, once something hits the press or media, then they tend to swing into action. It also affects anyone selling bottles of essential oils to “the public.” Estimating the “best before” date is far more complex than may be seen at first examination. Some years ago, the “NORA program” researchers spent a lot of time for testing the oxidization of essential oils and gave the concept of decay time. It is not feasible to give a standard time to oils because of production date, oil composition, and storage condition. Some oils such as the citrus have a short shelf life especially in hot weather.

The most important part of the new CHIP regulations concerns the labeling of essential oils, and this will affect the insight of oils. The new definition of R65 (Risk Phrase) affects some oils and is the latest piece of legislation to emerge from the European Union. Briefly, it means that oils have to be labeled as “harmful and may cause lung damage if swallowed” followed by the S62 (Safety Phrase) “If swallowed do not induce vomiting; seek medical advice immediately and show this container or label.” It would appear to end the “internal uses” school of thought once bottles have got the orange and black harmful logo. One important point from the legislation is the all pure essential oils sold to the public must be in child-resistant closures. The warning labels need to be rewritten to cover the “harmful” aspect. There are some moves afoot to try and challenge this new legislation, but it depends upon aromatherapy organizations. A lesson can be learned from the MCA’s attempt to dragoon the Health Food Industry recently where public reaction forced it to abandon and rethink its legislation. No opposition and it becomes law. The EPA (environmental protection agency) regulates the use of pesticides and insect repellents. Even if they approve an essential oil such as lemon eucalyptus or citronella for use as an insect repellent, products making such claims must be registered and approved by the EPA before they can be sold. Many cosmetic and aromatherapy companies have tried to adopt structure/function claims in their labeling. However, structure/function claims are only applicable to dietary supplements and do not apply to cosmetics. So claims such as “provides support during cold and flu season” are not allowed for aroma therapy or cosmetic products [134].

11 Therapeutic Potential and Future Prospects

The cytotoxicity property of terpenoids can make them exceptional antioxidant, antiseptic, and antimicrobial agents. This property can be used for aromatherapy, purification of air, food and crop preservation, antiaging, as well as for various ailments like cancers. The main advantages associated with the use of terpenoids are as follows: generally free from long-term genotoxic and mutagenic risks, environmentally friendly, target specificity, ability to overcome resistance, low cost, and easy accessibility. Additionally, some terpenoid shows a very clear antimutagenic effect may be one of the mechanisms of anticancer activity of the drug. The antioxidant activity of terpenes and their derivatives, as well as polyphenols, is very efficient in reducing tumor weight, tumor volume, or tumor cell proliferation by apoptotic and necrotic effects [135–142].

The cytotoxic activity of sesquiterpene lactones in species *Ajania przewalskii Poljakov* and *Ligularia platyglossa* was also reported [143, 144]. The volatile principles of *Citrus aurantifolia* inhibit proliferation inhibition of human colon cancer cells by apoptosis. The volatile sesquiterpenes showed DNA fragmentation and induction of caspase-3 [145]. The antiproliferative and anticancer activity of *Nigella sativa* was established [146]. Sesquiterpene lactone from *Schistochila glaucescens*, *Laurencia majuscula*, *Curcuma parviflora*, and Liverwort shows cytotoxicity [147–150]. The antiproliferative activity of Thai medicinal plants and eugenol against murine leukemia and skin carcinoma cell lines was established [141]. Geraniol inhibits DNA synthesis and reduces the tumor volume [151]. The in vitro and in vivo antiangiogenic potential of β-eudesmol from *Atractylodes lancea* was reported [152].

Essential oils act via apoptotic pathway, affecting mitochondrial functions. This may impart free radical scavenging potential which could be controlled and targeted. This property makes the terpenoids favorable anticancer agents. The controlled-release formulations like liposome, nanosome, neosome, and nanoparticle are becoming popular day by day. These formulations not only control the release rate of essential oil but also target drug to the specific body tissue or organs. These formulations enhance the duration of action and minimize the side effect of drug [153, 154]. Thus, terpenes and their oxygenated derivatives could build their approach from the traditional system of medicine to the modern system of medicine. The therapeutic use of essential oils in treating several diseases like cancers, inflammation, neurodegenerative disorders, cardiovascular diseases, and bacterial and fungal diseases seems to be ignored. Very less information regarding pharmacokinetics, pharmacodynamics, and nutritional properties of essential oil is known. Therefore, more studies should be performed in this area. The possible relationship between therapeutic effectiveness and most active constituent of essential oil should also be performed. The essential oils used for food preservation, nutritional and therapeutic purposes, and issues of safety and toxicity should also be considered.

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Tanshinones Against Cancer and Cardiovascular Diseases and their Biosynthesis

117

Lixin Zhang and Xueting Liu

Contents

1 Introduction 3553
2 Tanshinones Isolated from Natural Sources 3553
3 Biological Activity of Tanshinones and their Mode of Action 3557
3.1 Antitumor Activity and their Mode of Action 3557
3.2 Anticardiovascular Activity of Tanshinones and their Mode of Action 3567
4 Biosynthesis of Tanshinones 3571
5 Conclusion 3574
References 3575

Abstract

Tanshinones are a series of abietane-type norditerpenoid quinones isolated from the roots of *Salvia miltiorrhiza* ("*tanshen*"), a well-known traditional Chinese medicine (TCM) with varied pharmacological activities such as antibacterial, antioxidant, anti-inflammatory, and antineoplastic. This chapter summarized about the biosynthesis and structural properties of tanshinones and the bioactivities against a variety of tumors and cardiovascular diseases and also provides our views on future trends.

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Keywords

Anticardiovascular • antitumor • biosynthesis • tanshinone

Abbreviations

5-HT	5-hydroxytryptamine
ABO	4-amino-2*H*-benzo[h]chromen-2-one
ADPRTL1	ADP-ribosyltransferase
Ang II	Angiotensin II
APL	Acute promyelocytic leukemia
ATBO	4-amino-7,8,9,10-tetrahydro-2*H*-benzo[h]chromen-2-one analogs
CRC	Human colon carcinoma
DMAPP	Dimethylallyl diphosphate
DXP	1-deoxy-D-xylulose-5-phosphate
DXR	DXP reductoisomerase
DXS	DXP synthase
ECM	Extracellular matrix
FNO	2-(fura-2-yl) naphthalen-1-ol
GA3P	Glyceraldehyde-3-phosphate
HMGB1	High-mobility group box 1
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	HMGR-CoA reductase
ICAM-1	Intercellular adhesion molecule-1
IPP	Isopentenyl diphosphate
MAPK	Mitogen-activated protein kinases
MMP	Mitochondrial membrane potential
MMP-2	Matrix metalloproteinases-2
MMP-9	Matrix metalloproteinases-9
MoA	Mode of action
NCAECs	Newborn cattle aortic endothelial cells
NQO1	NAD(P)H:quinone oxidoreductase
PARP	Poly(ADP-ribose) polymerase
ROS	Reactive oxygen species
STS	Tanshinone IIA sulfonate
Tan-I	Tanshinone I
Tan-IIA	Tanshinone IIA
TCM	Traditional Chinese medicine
TIMP	Matrix metalloproteinase protein
TNF-alpha	Tumor necrosis factor-alpha
TNO	Tetrahydronaphthalene-1-ol
TNT	Tetrahydroneotanshinlactone
UPA	Urokinase plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells

1 Introduction

Tanshinones are a series of abietane-type norditerpenoid quinones isolated from the roots of *Salvia miltiorrhiza* (“*tanshen*”), a well-known traditional Chinese medicine (TCM), by Nakano and Fukushima for the first time in 1930 [1, 2]. As its Chinese name implies, the *S. miltiorrhiza* root *tanshen* is characterized by the abundance of red pigments, which are largely ascribed to the tanshinones and related quinones. In other words, tanshinones are named after the source plant for “the quinone of *tanshen*.” *Tanshen* is an annual sage plant and has been used in TCM for the treatment of coronary heart diseases, particularly angina pectoris and myocardial infarction [3]. It has been used for hemorrhage, dysmenorrhea, miscarriage, swelling, insomnia, and inflammatory diseases such as edema, arthritis, and endangitis [4–6]. *Fu Fang Dan Shen* (a mixture of *S. miltiorrhiza*, *Panax notoginseng*, and *Cinnamomum camphora*) is registered as a drug in several countries outside China including Vietnam, Russia, Cuba, Korea, and Saudi Arabia. In 2010, *tanshen* was the first traditional Chinese medicine to pass US phase II clinical trials for cardiovascular indications.

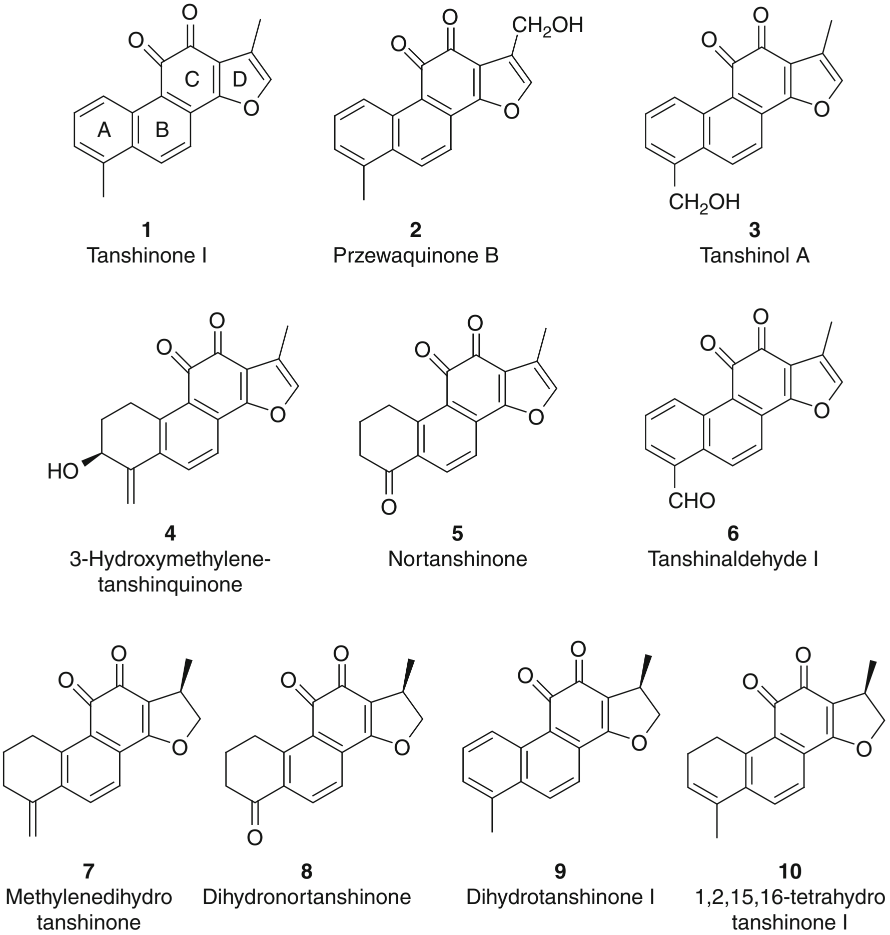
Diterpenoid tanshinones were found exclusively in the genus *Salvia* and attracted particular attention from many researchers for their wide-ranging pharmacological activities such as antibacterial, antioxidant, anti-inflammatory, and antineoplastic [3]. Tanshinone I (Tan-I) (**1**), tanshinone IIA (Tan-IIA) (**11**), and cryptotanshinone (1**3**) are the major constituents of *S. miltiorrhiza*. A related plant *S. columbariae*, which has been used by California Indians in the treatment of strokes, also contains tanshinones, especially cryptotanshinone [7, 8]. This chapter will summarize about the structural properties, the activities against a variety of tumors and cardiovascular diseases, and biosynthesis of tanshinones.

2 Tanshinones Isolated from Natural Sources

Tanshinones are generally composed of four rings, including naphthalene or tetrahydronaphthalene rings A and B, an ortho- or para-quinone or lactone ring C, and a furan or dihydrofuran ring D. Based on their structural properties, tanshinones documented in this chapter were divided to five classes, types I–V.

Type I tanshinones (Fig. 117.1) contain four rings of naphthalene rings A and B, ortho-quinone ring C, and furan or dihydrofuran ring D. Tan-I (**1**) was isolated and identified by Nakano and Fukushima from *S. miltiorrhiza* Bunge [1, 2]. Przewaquinone B (**2**) [9], tanshinol I (**3**) [10], 3-hydroxymethylenetanshinone (**4**) [11], nortanshinone (**5**) [12], tanshinaldehyde I (**6**) [13], methylenedihydrotanshinone (**7**) [13], dihydronortanshinone (**8**), dihydrotanshinone I (**9**) [14], and 1,2,15,16-tetrahydrotanshinone I (**10**) [13, 15] are serial type I tanshinones isolated from *S. miltiorrhiza* in the last two decades.

Type II tanshinones (Fig. 117.2) are structurally similar to type I tanshinones, except that the naphthalene rings A and B in type I tanshinones were instead of



1 Tanshinone I

2 Przewaquinone B

3 Tanshinol A

4 3-Hydroxymethylene-tanshinquinone

5 Nortanshinone

6 Tanshinaldehyde I

7 Methylenedihydro tanshinone

8 Dihydronortanshinone

9 Dihydrotanshinone I

10 1,2,15,16-tetrahydro tanshinone I

Fig. 117.1 Type I tanshinones

tetrahydronaphthalene in type II tanshinones. The representative compounds of type II tanshinone are Tan-IIA (**11**), tanshinone IIB (**12**), and cryptotanshinone (**13**), which are the major constituents of *S. miltiorrhiza*. The oxygenation of the ring A produces a series of analogs such as 1-hydroxytanshinone IIA (**14**) [16], 3-hydroxytanshinone IIA (**15**), tanshindiol A (**16**), tanshindiol B (**17**), tanshindiol C (**18**) [12], przewaquinone C (**19**), przewaquinone F (**20**), methyl tanshinate (**22**), and methyldihydrotanshinate (**23**) [17]. The oxygenation occurs in ring D of tanshinone IIA to afford przewaquinone A (**24**), which was found in TCM *S. przewalskii* Maxim only [9]. Dehydrogenation of tanshinone IIA results in two other analogs: dehydrotanshinone IIA (**25**) and dimethoxyltanshinone IIA (**21**) [18].

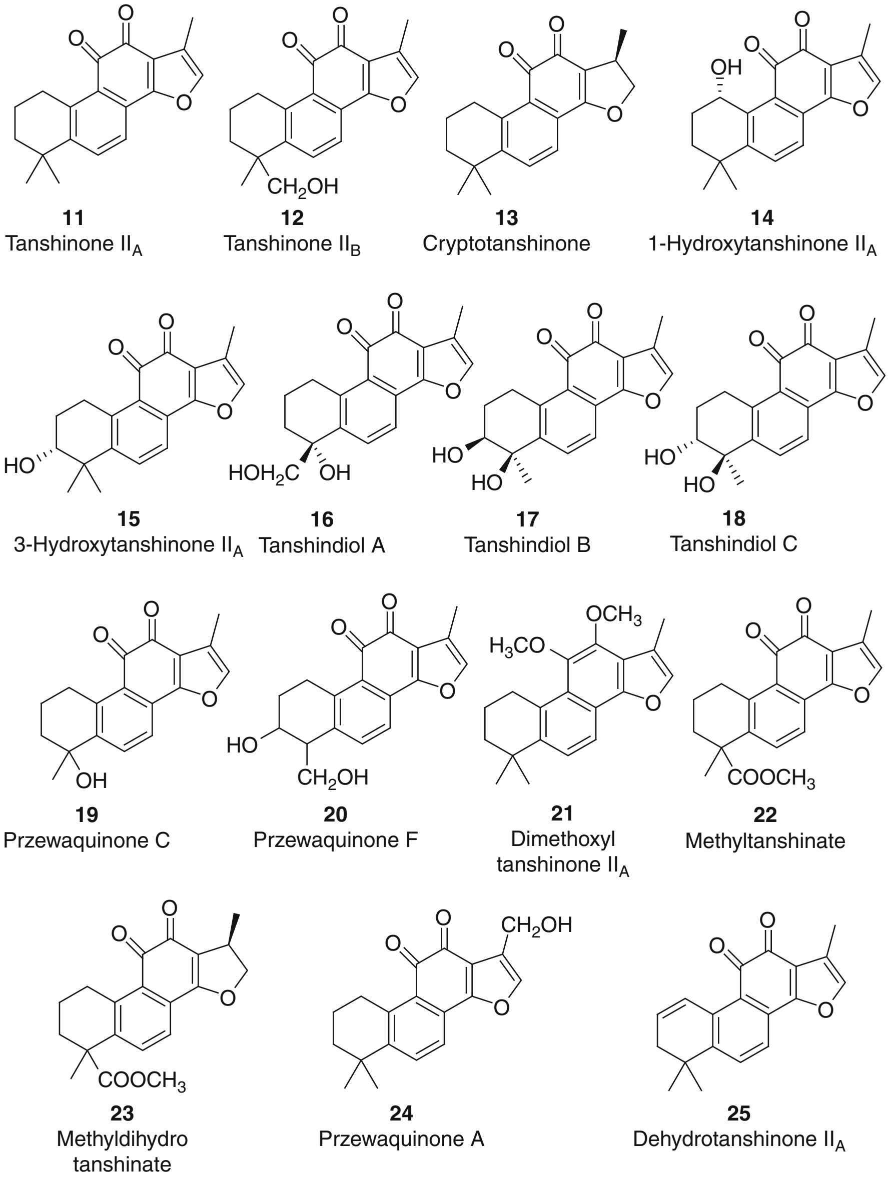
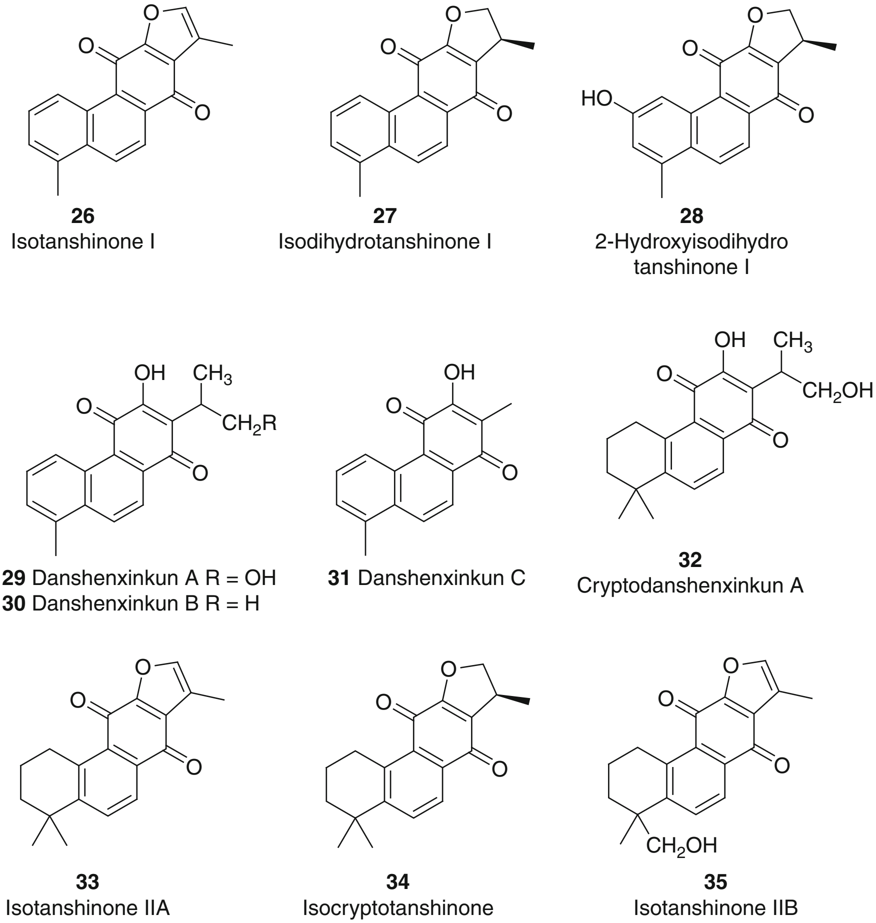

11
Tanshinone II$_A$
12
Tanshinone II$_B$
CH$_2$OH
13
Cryptotanshinone
14
1-Hydroxytanshinone II$_A$
OH
15
3-Hydroxytanshinone II$_A$
HO
16
Tanshindiol A
HOH$_2$C
OH
17
Tanshindiol B
HO
HO
18
Tanshindiol C
HO
HO
19
Przewaquinone C
OH
20
Przewaquinone F
HO
CH$_2$OH
21
Dimethoxyl
tanshinone II$_A$
H$_3$CO
OCH$_3$
22
Methyltanshinate
COOCH$_3$
23
Methyldihydro
tanshinate
COOCH$_3$
24
Przewaquinone A
CH$_2$OH
25
Dehydrotanshinone II$_A$
O

Fig. 117.2 Type II tanshinones

The significant property of type III tanshinones (Fig. 117.3) is the 1,4-quinone moiety, which is different from the 1,2-quinone moiety in the structures of types I and II tanshinones. These serial tanshinones are named “isotanshinone” in general, including isotanshinone I (**26**), isodihydrotanshinone I (**27**), 2-hydroxy



26 Isotanshinone I

27 Isodihydrotanshinone I

28 2-Hydroxyisodihydro tanshinone I

29 Danshenxinkun A R = OH
30 Danshenxinkun B R = H

31 Danshenxinkun C

32 Cryptodanshenxinkun A

33 Isotanshinone IIA

34 Isocryptotanshinone

35 Isotanshinone IIB

Fig. 117.3 Type III tanshinones

isodihydrotanshinone I (**28**), isotanshinone IIA (**33**), isocryptotanshinone (**34**), and isotanshinone IIB (**35**) [19–21]. The concentration of most of these analogs is low in plants, and few studies have been done on them.

Type IV tanshinones (Fig. 117.4) are a type of tricyclic diterpenoid quinones, and they share the isopropyl substitution C-13 of the structure in common. Among these compounds, miltirone I (**33**, Ro-09-0680) and miltirone (**38**) showed significant antiplatelet and antioxidant activity [22–25].

The other tanshinone-like compounds were classified as type V tanshinones (Fig. 117.5) which are the intermediates or postmodified products in the biosynthetic pathway [26–37]. Among these compounds, neo-tanshinlactone (**65**) and its analogs show the most promising potentials for antitumor drug discovery and will be discussed later in this chapter.

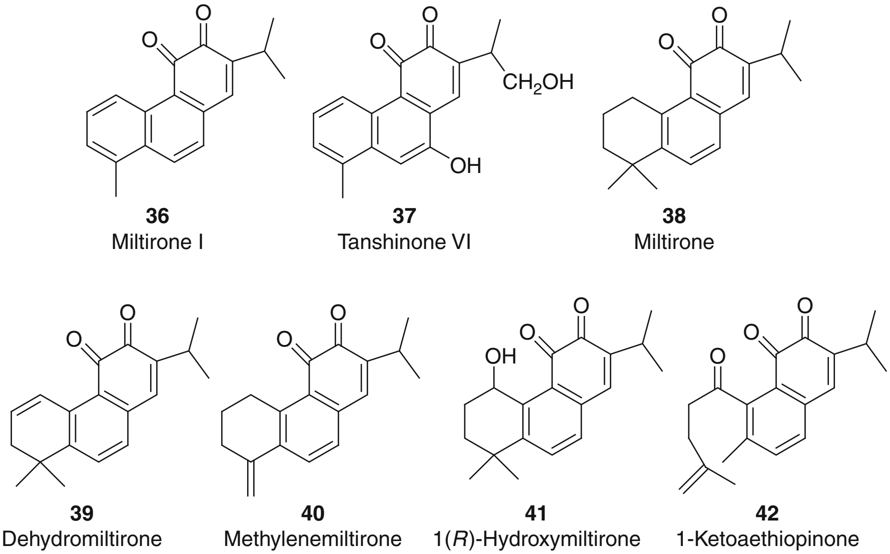


Fig. 117.4 Type IV tanshinones (tricyclic diterpenoid quinones)

3 Biological Activity of Tanshinones and their Mode of Action

3.1 Antitumor Activity and their Mode of Action

Tanshinones showed a broad spectrum of cytotoxic effects on cell lines derived from human carcinomas of the colon, ovary, lung, mouth, and breast [3, 38, 39] (Table 117.1). The mode of action (MoA) for antitumor activity has been studied for different tanshinones. Tan-I (**1**) and IIA (**11**) have been studied efficiently for their cytotoxic activity and induced differentiation and apoptosis [45, 46]. Przewaquinones A (**24**) and B (**2**) showed promising antitumor activity in mice [47]. This section will outline antitumor activity of tanshinones, especially Tan-I, Tan-IIA, and neo-tanshinlactones in vitro and in vivo, and their MoA.

Tan-I (**1**) was discovered; the activities on several cancer cell lines and their MoA were listed in Table 117.2.

Tan-I inhibited cell growth dose dependently at concentrations ranging from 0.5 to 25 μM and induced apoptosis in activated T-HSC/C-6 hepatic stellate cells. Tan-I increased caspase-3 activation via cytochrome c release and loss of mitochondrial membrane potential [48]. Nizamutdinova and coworkers investigated the effect of Tan-I on the induction of apoptosis in human breast cancer cells (MCF-7 and MDA-MB-231) in vitro. Tan-I inhibited cell proliferation of MCF-7 and MDA-MB-231 cells in a dose- and time-dependent manner and significantly induced apoptosis in MCF-7 and MDA-MB-231 cells. The induction of apoptotic cell death was mediated by the activation of caspase-3; the downregulation in the level of the

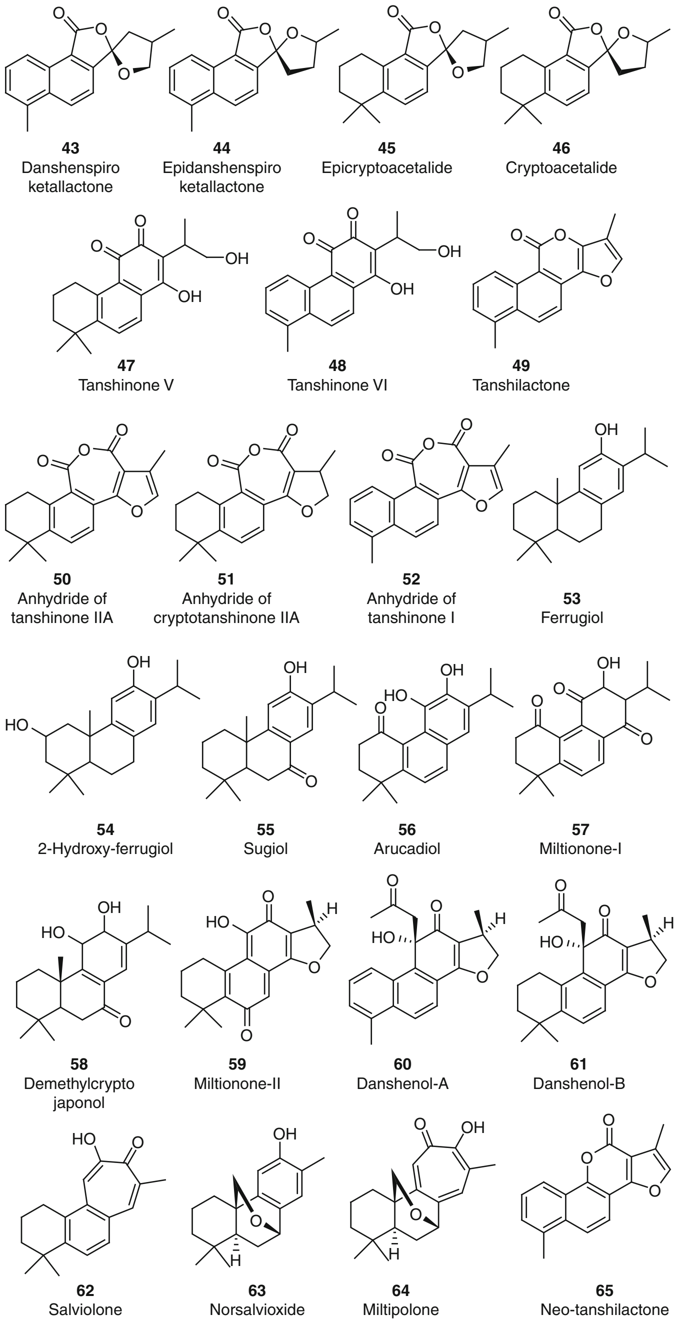

43
Danshenspiro ketallactone
44
Epidanshenspiro ketallactone
45
Epicryptoacetalide
46
Cryptoacetalide
47
Tanshinone V
48
Tanshinone VI
49
Tanshilactone
50
Anhydride of tanshinone IIA
51
Anhydride of cryptotanshinone IIA
52
Anhydride of tanshinone I
53
Ferrugiol
54
2-Hydroxy-ferrugiol
55
Sugiol
56
Arucadiol
57
Miltionone-I
58
Demethylcrypto japonol
59
Miltionone-II
60
Danshenol-A
61
Danshenol-B
62
Salviolone
63
Norsalvioxide
64
Miltipolone
65
Neo-tanshilactone

Fig. 117.5 Structure of type V tanshinones

Table 117.1 Cytotoxicity of tanshinones [4, 5, 40–44]

	Cell line IC_{50} (μg/mL)								
Compound	KB	HeLa	Colo 205	HepG2	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
Tanshinone I	2.8	2.3	3.0	3.4	4.3	4.1	2.8	1.6	4.2
Tanshinone IIA	4.1	>4	>4	>4	0.9	1.3	1.2	0.4	0.8
Tanshinone IIB	1.1	>4	>4	>4	5.2	9.2	2.9	3.2	1.7
Cryptotanshinone	2.00	2.50	0.91	3.8	0.7	2.5	1.6	0.5	1.2
Dihydrotanshinone I	2.70	0.79	0.86	0.97	3.0	1.6	1.2	0.9	1.4
Methyl tanshinate	0.6	4.0	1.5	4.0	0.8	5.0	6.9	1.5	1.0
1,2-dihydrotanshinone I	2.40	1.40	0.89	3.4	0.8	1.5	3.4	0.6	0.2
Methylene tanshiquinone	0.58	0.72	1.00	0.62	0.4	0.9	2.2	0.3	0.2
Nortanshinone	0.90	0.92	0.85	>4	NA	NA	NA	NA	NA
Didydronortanshinone	0.97	2.10	3.40	>4	NA	NA	NA	NA	NA
Methyldihydrotanshinonate	3.00	>4	3.80	4.0	NA	NA	NA	NA	NA
Hydroxyl tanshinone IIA	0.75	1.90	>4	1.7	NA	NA	NA	NA	NA
Tanshinol A	NA	NA	NA	NA	3.4	3.7	1.4	1.8	0.8
Tanshinol B	NA	NA	NA	NA	1.4	3.0	0.7	1.3	0.8
Przewaquinone A	NA	NA	NA	NA	2.3	1.7	1.9	1.6	0.8
Przewaquinone C	2.40	3.70	2.80	3.6	NA	NA	NA	NA	NA
3-dihydroxyltanshinone IIA	NA	NA	NA	NA	2.5	4.1	7.0	2.7	1.2
1,2-dihydro-methylene Tanshiquinone	NA	NA	NA	NA	1.5	3.4	1.0	1.0	0.9
Tanshinaldehyde	2.00	0.92	>4	3.6	NA	NA	NA	NA	NA
Tanshidiol A	0.74	1.60	2.00	0.62	0.7	0.8	0.2	0.2	0.4
Tanshidiol B	0.60	0.64	1.70	0.53	0.9	1.0	0.4	0.5	0.7
Tanshidiol C	0.70	2.50	3.39	0.90	0.7	0.9	0.3	0.5	0.4
Dihydroisotanshinone I	1.8	4.0	>4	4.0	NA	NA	NA	NA	NA
Miltirone	NA	NA	NA	NA	8.1	6.6	3.4	4.7	5.9
Dehydromiltirone	NA	NA	NA	NA	4.6	3.4	2.3	3.9	2.8

NA not tested or not available

antiapoptotic protein, Bcl-2; and the upregulation of the level of the proapoptotic protein, Bax [39]. Further investigation by Nizamutdinova et al. showed the effect of Tan-I on cancer growth, invasion, and angiogenesis on human breast cancer cells MDA-MB-231, both in vitro and in vivo. Tan-I exhibited the strongest inhibitory effect on TNF-α-induced adhesion molecules in endothelial cells. The role of cell adhesion molecules has been studied extensively in the process of inflammation, and these molecules are critical components of carcinogenesis and cancer metastasis. Tan-I regulated adhesion molecules in human MDA-MB-231 breast cancer cells, with a concentration ranging from 1 to 50 μM, on the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in tumor necrosis factor-alpha (TNF-alpha)-stimulated endothelial cells. Tan-I can significantly reduce adhesion of either monocyte

Table 117.2 Antitumor activities of tanshinone I and their MoA

Cell lines	IC_{50}	MoA	Reference
T-HSC/C-6 hepatic stellate cells	0.5–25 μM	Increased caspase-3 activation	[48]
Human breast cancer MCF-7 and MDA-MB-231	1–50 μM	Increased caspase-3 activation, downregulation of Bcl-2, and upregulation of Bax	[39]
Human breast cancer MDA-MB-231	5–50 μM	Regulated adhesion molecules	[49]
Human colon cancer Colo 205 cells	1–10 μg/mL	Increased activation of caspase-3, bax, p53, and p21	[50]
Human nonsmall-cell lung cancer CL1-5 cells	10 μg/mL	Reduced metastasis and tumorigenesis by inhibiting NF-κB and AP-1, suppressing the expression of the angiogenic factor IL-8	[51]
Monocytic leukemia cells (U937, THP-1, and SHI 1)	10–50 μg/mL	Activation of caspase-3, reduction of hTERT mRNA expression and telomerase activity, and downregulation of survivin expression	[52]

U937 or MDA-MB-231 cells to HUVECs. Furthermore, Tan-I effectively inhibited TNF-alpha-induced production of vascular endothelial growth factor (VEGF) and VEGF-mediated tube formation in HUVECs. At 10 and 50 μM, Tan-I inhibited more than 80% invasion of cancer cells. Additionally, reduction of tumor mass volume and decrease of metastasis incidents of MDA-MB-231 breast cancer cells to lung tissue by Tan-I were observed in vivo [49].

Su and coworkers investigated the induction of apoptosis by Tan-I at 1–10 μg/mL in human colon cancer Colo 205 cells. Tan-I reduced cell growth in a concentration-dependent manner, inducing apoptosis accompanied by an increase in TUNEL-stained cells in the sub-G1 fraction. The treatment with Tan-I at 0, 1, 2.5, 5, and 10 μg/mL for 72 h increased the percentage of cells in sub-G1 phase from 3.83% to 7.22%, 8.68%, 14.4%, and 32.98%, respectively. The expression of p53, p21, bax, and caspase-3 increased in Tan-I-treated cells. The authors suggested that Tan-I induces apoptosis in Colo 205 cells through both mitochondrial-mediated intrinsic cell-death pathways and p21-mediated G0/G1 cell cycle arrest [50].

Lee and coworkers investigated the anticancer effects of tanshinones on highly invasive human lung adenocarcinoma cell line, CL1-5. The results revealed that Tan-I significantly inhibited migration, invasion, and gelatinase activity in macrophage-conditioned medium-stimulated CL1-5 cells in vitro and also reduced the tumorigenesis and metastasis in CL1-5-bearing severe combined immunodeficient mice. They indicated that Tan-I did not have direct cytotoxicity, which is unlike Tan-IIA that induces cell apoptosis. Further investigation suggested that the anticancer effects of Tan-I might be mediated through the interleukin-8, Ras-mitogen-activated protein kinase, and Rac1 signaling pathways [51].

However, Liu et al. discovered the growth inhibition and apoptosis-inducing effects of Tan-I on three kinds of monocytic leukemia cells (U937, THP-1, and SHI 1) and found that Tan-I could inhibit the growth of these three kinds of leukemia cells and cause apoptosis in a time- and dose-dependent manner. They concluded that the induction of apoptosis by Tan-I in monocytic leukemia U937 THP-1 and SHI 1 cells was highly correlated with activation of caspase-3 and decreasing of hTERT mRNA expression and telomerase activity as well as downregulation of survivin expression [52].

The antitumor activity of Tan-IIA (**11**) has been studied a lot on many cells, tissues, and animal models. These studies revealed that Tan-IIA showed antitumor activity on inducing apoptosis, inhibiting invasion and metastasis, inhibiting angiogenesis, and downregulating epidermal growth factor receptors both in vitro and in vivo [53]. Different human cancer cell lines, glioma cells, THP-1 leukemia cell lines, MDA-MB-231 breast cancer cell lines, HL60 promyelocytic cell lines, K562 erythroleukemia cell lines, MKN-45 gastric carcinoma cell lines, human colon carcinoma (CRC) cell lines HT29 and SW480, and hepatocellular cancer (HCC) cell lines, were used [54–59].

Apoptosis is a new therapeutic target of cancer research, and recently, Tan-IIA has been reported to have apoptosis-inducing effects on a large variety of cancer cells. There are various MoA reported and revealed that Tan-IIA could induce apoptosis through activation of caspase-3, downregulation of the antiapoptotic protein bcl-2, and upregulation of the proapoptotic protein bax.

Wang and coworkers evaluated the inhibitory effect of Tan-IIA on cell growth of human glioma cells, and it showed an IC_{50} value of 100 ng/mL. After the treatment with 25–100 ng/mL of Tan-IIA, the apoptotic cells increased significantly ($P < 0.01$), the cells in G(0)/G(1) phase increased ($P < 0.01$) and decreased in S phase, and ADP-ribosyltransferase (ADPRTL1) and CYP1A1 mRNA expression increased one- to twofold. Their findings suggested that Tan-IIA exhibited strong effects on growth inhibition and induction of apoptosis and differentiation in human glioma cells [56]. Further study on Tan-IIA demonstrated a dose- and time-dependent inhibitory effect on human breast cancer cell growth ($IC_{50} = 0.25$ μg/mL) and suggested that ADPRTL1 might be the main target at which Tan-IIA acted [60]. Tan-IIA was observed to induce apoptosis in HL60 human premyelocytic leukemia cell line and human breast cancer. Tan-IIA-induced apoptosis in HL60 human premyelocytic leukemia cell line was accompanied by the specific proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) and the activation of caspase-3, a major component in apoptotic cell-death mechanism [55]. Zhou et al. revealed that Tan-IIA could arrest HeLa cells in mitosis by disrupting the mitotic spindle and subsequently triggered cells to enter apoptosis through the mitochondria-dependent apoptotic pathway. Tan-IIA destroyed only the mitotic spindle during the M phase but not the microtubule structure in interphase cells. Tan-IIA could trigger the mitotic-arrested cells to enter apoptosis faster than vincristine or Taxol [61].

Pan et al. reported Tan-IIA exhibited strong growth inhibition against human cervical cancer cells (HeLa cells) in dose- and time-dependent manners with a 50% cell growth inhibition with 2.5 μg/mL (8.49 μM). The authors executed a bioinformatics analysis of proteins to survey global protein changes induced by Tan-IIA treatment on HeLa cells. Significant changes in the levels of cytoskeleton proteins as well as stress-associated proteins were observed. Their further analysis suggested that Tan-IIA treatment regulated the expressions of proteins involved in apoptotic processes, spindle assembly, and p53 activation including vimentin, maspin, alpha- and beta-tubulins, and GRP75. The authors concluded that Tan-IIA might strongly inhibit the growth of cervical cancer cells through interfering in the process of microtubule assembly, leading to G(2)/M phase arrest and sequent apoptosis [62].

Dong and coauthors revealed that Tan-IIA upregulated expression of p53 gene and downregulated expression of bcl-2 gene in human gastric carcinoma MKN-45 cells. A significant inhibition of Tan-IIA on the growth and proliferation of MKN-45 cells was observed in a dose- and time-dependent manner ($P < 0.05$). Tanshinone A arrested MKN-45 cells in G(2)/M phase which resulted in apoptosis of MKN-45 cells, and the apoptosis rate was as high as 43.91% after treatment with 2.0 μg/mL Tan-II A for 96 h [63].

Liu and coworkers studied the antiproliferation and apoptosis-inducing effect of Tan-IIA as well as its influence on cell adhesion to and invasion through the extracellular matrix (ECM) on acute promyelocytic leukemia (APL) NB4 cells in vitro. Their investigation showed that Tan-IIA exhibited induction of apoptosis by activation of caspase-3, downregulation of antiapoptotic proteins bcl-2 and bcl-xl, and upregulation of proapoptotic protein bax, as well as disruption of the mitochondrial membrane potential. They also revealed that Tan-IIA could reduce cell adhesion to and invasion through ECM in leukemia NB4 cells [57]. Shan et al. found that Tan-IIA significantly inhibited tumor invasion and metastasis in human colon carcinoma (CRC) cell lines HT29 and SW480121 in vitro (0.5–2.5 mg/mL) [64]. In addition, Xu and coworkers reported that Tan-IIA inhibited both the invasion and metastasis of HCC cells in vitro (0–2 mg/mL) [65].

Since antineovascularization has generally been regarded as an effective strategy for anticancer therapy, researchers showed great interests on investigation of the activity of Tan-IIA on human endothelial cells. Fu et al. studied the effects of Tan-IIA on apoptosis and the expression of VEGF in the SMMC-7721 HCC cell line and revealed that Tan-IIA not only induced apoptosis but also reduced the expression of VEGF [66]. Yang and coworkers demonstrated that Tan-IIA elicits human endothelial cell death independent of oxidative stress and suggested that Tan-IIA-mediated cytotoxicity might occur through activation of NAD(P)H: quinone oxidoreductase (NQO1), which induces a calcium imbalance and mitochondrial dysfunction, thus stimulating caspase activity [67] (Fig. 117.6).

Chiu et al. studied the effects of Tan-IIA on the cell cycle, mitochondrial membrane potential (MMP), and calcium and reactive oxygen species (ROS) released in human lung cancer; A549 cells were detected by flow cytometry. They revealed that Tan-IIA inhibited the proliferation of nonsmall-cell lung cancer A549 cells, possibly by decreasing the MMP and inducing apoptosis due to the induction of a higher ratio of Bax/Bcl-2 [68].

Many Chinese researchers have studied the antiangiogenic, antiproliferation, and antimigration effects of Tan-IIA and resulted in the conclusion of multitarget interactions of mechanism. Xu et al. reported that Tan-IIA inhibited the

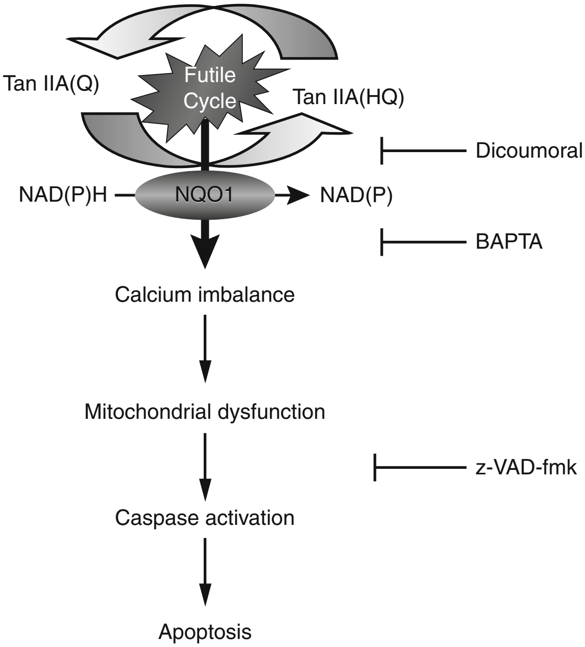


Fig. 117.6 A proposed model for tanshinone IIA-induced human endothelial EAhy926 cell apoptosis. Tanshinone IIA is reduced from quinone to hydroquinone in the presence of NQO1. The hydroquinone form of tanshinone IIA is quite unstable and autooxidizes to its original parent form. This futile cycle causes the rapid and severe loss of cellular NADP (H) and induces the sequestration of Ca^{2+} from ER causing a rise in cytosolic Ca^{2+} levels. The perturbation of the Ca^{2+} pools leads to cytochrome c release and mitochondrial membrane depolarization, which then stimulates the activation of caspases, ultimately resulting in apoptosis [67]

proliferation of the human MDA-MB-435 breast cancer cell line with an IC_{50} value of 21 nM and prevented breast cancer cell migration at 5 and 6 nM in a wound-healing assay and a Transwell migration assay, respectively. Further studies revealed that Tan-IIA inhibited the tube formation of newborn cattle aortic endothelial cells (NCAECs) after 2-h coincubation with MDA-MB-435 and mRNA expression of VEGF and two transcription factors (HIF-1a/c-Myc) in a dose-dependent manner [69]. More studies have been done and revealed that the downregulation of HIF-1a expression could be observed in SGC-7901 gastric cancer cells treated with Tan-IIA. In addition, the expression of c-Myc protein was inhibited by Tan-IIA in SGC-7901 gastric cancer and MDAMB-231 breast carcinoma cells [70–72].

Tan-IIA not only showed strong activity on different tumor cell lines in vitro but also showed promising activities in vivo. Seven nude mice bearing human breast infiltrating ductal carcinoma orthotopically were tested for anticancer activity and expression of caspase-3 in vivo by s.c. injection of Tan-IIA at a dose of 30 mg/kg, 3 times/week for 10 weeks. A 44.91% tumor mass volume reduction and significant increase of casepase-3 protein expression were observed in vivo [60].

Shan et al. found two significantly inhibited tumor invasions and metastasis in CRC cell lines HT29 and SW480 in vivo (0–80 mg/kg/day for 4 weeks). The results revealed that Tan-IIA showed the activity by reducing levels of urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMP)-2 and MMP-9 and by increasing levels of tissue inhibitor of matrix metalloproteinase protein (TIMP)-1 and TIMP-2 [64]. Xu and coworkers also reported that Tan-IIA inhibited the invasion and metastasis of HCC in vivo (0–13.5 g/kg/day for 5 consecutive weeks in male athymic BALB/c nu/nu mice) [65]. Su et al. found that Tan-IIA caused upregulation of TNF-α and caspase-3 and downregulation of ErbB-2 protein expression as compared to the controls in male SCID mice xenografted with Colo 205 cells [73].

Lee research group identified a new compound, neo-tanshinlactone (**65**), from the root of *S. miltiorrhiza* [74]. Serial neo-tanshinlactone analogs have been synthesized, and their antibreast cancer activities have been fully evaluated [75–81].

Neo-tanshinlactone (**65**), with strong and selective antibreast cancer activity, was identified and synthesized by Lee et al. [74]. Structure **65** showed strong activity against estrogen receptor positive (ER+) human breast cancer cell lines (MCF-7 and ZR-75-1) with ED50 values of 0.6 and 0.3 μg/mL, respectively. Otherwise, **65** was inactive against two ER− cell lines (MDA MB-231 and HS587-1) with ED_{50} values beyond 10 μg/mL (Table 117.3). Structure **65** also showed good activity against an HER-2-overexpressing breast cancer cell line (SK-BR-3 HER++) [74]. Since **65** showed significant inhibition against the two ER+ human breast cancer cell lines and was tenfold more potent and 20-fold more selective as compared to tamoxifen citrate, serial analogs have been studied in Lee's lab aimed at developing novel and promising antibreast cancer drug candidates from neo-tanshinlactone. Wang et al. developed serial strategies to provide different types of neo-tanshinlactone analogs with potent activity against different breast tumor cell lines. These strategies included the following modification of the structure **65**: (1) substitution of rings A and B and/or ring D, (2) modification of ring D, (3) cleavage of each ring [75, 80], (4) preparation of nonaromatic ring A [76], (5) structural simplification like getting rid of rings B or D [77, 78], and (6) bioisosteric replacement of the oxygens [78].

Synthesis of different compounds with variously substituted rings A and B or a modified ring D of the structure **65** was done, and **66** with an ethyl group at position-4 showed the most potent activity and selectivity with ED50 values of 0.45 and 0.18 μg/mL against MCF-7 and ZR-75-1 (ER+) and 13.5 and 10.0 μg/mL against MDA MB-231 and HS 587-1 (ER−), respectively. In addition, **66** showed potent activity against SK-BR-3 (ER−, HER2++) with an ED50 value of 0.10 μg/mL.

In general, a complex lead compound may have a simpler pharmacophoric moiety buried within its structure. If this pharmacophore can be clearly defined, the resulting biologically active, simpler molecules may improve synthetic tractability and be more useful as a scaffold for further drug development. In the other hand, the structural complexity of natural products, such as intricate ring systems and numerous chiral centers, may lead to limited supplies and hamper mechanism of action studies and clinical applications [82]. For this reason, structural simplification of natural products is a powerful and highly productive tool for lead development and analog design [83]. Dong et al. studied the individual contribution of the rings A, B, C, and D of **65** to the selective activity against breast cancer cells. Only compound **67**, with an opened ring C, showed primary antibreast cancer

Table 117.3 Cytotoxicity of neo-tanshinlactone analogs against breast cancer cell lines

Cmpd	ED_{50} (μg/mL or *μM), cell line							
	MCF-7(ER+)	ZR-75-1 (ER+)	MDA MB-231 (ER−)	HS587-1 (ER−)	SK-BR-3 (ER−, HER2+)	A431	SW620	KB
62	0.60	0.25	10.0	16.0	0.18	>10	>10	>10
63	0.45	0.18	13.5	10.0	0.10	>10	>10	>10
64	6.0	NA	NA	NA	7.0	NA	NA	NA
65	3.3	0.3	>10	NA	1.0	NA	NA	9.1
66	2.5	1.3	2.3	NA	1.2	NA	NA	NA
67	>20	0.6	>10	NA	3.5	NA	NA	9.7
68	NA	3.9 ± 0.2	4.3 ± 0.3	NA	2.5 ± 0.2	NA	NA	2.2 ± 0.3
69	NA	1.2 ± 0.1	1.1 ± 0.1	NA	1.9 ± 0.06	NA	NA	1.8 ± 0.08
70	NA	>10	>10	NA	0.73 ± 0.05	NA	NA	7.1 ± 0.3
71	NA	1.4 ± 0.1	1.7 ± 0.1	NA	8.8 ± 0.7	NA	NA	>10
72	NA	1.7 ± 0.1	0.85 ± 0.04	NA	>10	NA	NA	>10
73*	NA	1.7	24.7	NA	0.7	NA	NA	4.7
74*	NA	30.8 ± 1.7	>69.9	NA	0.28 ± 0.01	NA	NA	>69.9
75*	NA	29.1 ± 0.8	>31.6	NA	0.44 ± 0.01	NA	NA	>63.3
76*	NA	0.01	0.02	NA	0.13	NA	NA	0.11
77*	NA	0.08	0.76	NA	0.15	NA	NA	0.14
78*	NA	0.024 ± 0.002		NA	0.064 ± 0.027	NA	NA	0.037 ± 0.005
79*	NA	0.32 ± 0.06	0.23 ± 0.02	NA	0.037 ± 0.02	NA	NA	0.035 ± 0.02
80*	NA	NA	NA	NA	0.079	NA	NA	0.093
81*	NA	NA	NA	NA	0.090	NA	NA	0.067
TAM	5.0	3.6	8.5	7.0	5.0	7.0	4.0	9.0

TAM tamoxifen citrate, *NA* not tested or not available activity in vitro. Further optimization led to a novel class of antibreast cancer agents, 2-(fura-2-yl) naphthalen-1-ol (**FNO**) derivatives. Interestingly, compounds **68** and **70** showed decreased cytotoxic potency but better selectivity than neo-tanshinlactone analog **66**. Compound **69** showed broad cytotoxicity against human cancer cell lines, which suggested a different MoA from its structural derivatives. The authors suggested that intramolecular hydrogen bonding was important to form a rigid conformation and improved the in vitro anticancer selectivity of **68** [75]. The C11-esters **71** and **72** showed broad antitumor activity (ED50 1.1–4.3 μg/mL against several cancer cell lines), and C11-hydroxymethyl **73** showed unique selectivity against the SK-BR-3 breast cancer cell line (ED50 0.73 μg/mL). **74** and **75** displayed potent and selective antibreast cancer cell line with ED50 values of 1.7 and 0.85 μg/mL, respectively. In in vivo experiments, **68** significantly inhibited the proliferation of mammary epithelial cells and branching of mammary glands in both wild-type and *Brca1*/p53-deficient mice [80].

Neo-tanshinlactone analogs with nonaromatic ring A, tetrahydroneotanshinlactone (**TNT**) and tetrahydronaphthalene-1-ol (**TNO**), have been designed, and their antibreast cancer activity has been evaluated as well. Cyclohexene ring A could dramatically affect the antitumor activity and selectivity. Among the obtained analogs, compound **76** showed the highest potency with ED50 values of 0.7 and 1.7 μM against SK-BR-3 and ZR-75-1 breast cancer cell lines, respectively. Neo-tanshinlactone analogs with nonaromatic ring A provided the promising new lead compounds with a novel skeleton for further development toward a new potential clinical trial candidate [76]. A serial simplified neo-tanshinlactone analog has been discovered by Dong et al.; **77** and **78** showed the highest cancer cell line selectivity, being approximately 100–250-fold more potent against SK-BR-3 (ED50 0.28 and 0.44 μM, respectively) compared with other cancer cell lines tested. In addition, **77** displayed low cytotoxicity against normal breast cell lines 184A1 and MCF10A. The results indicated that the nonaromatic ring A of these structures is critical to both potency and cancer cell line selectivity. This case revealed two promising compounds (**77** and **78**) for further development as clinical trial candidates for antibreast cancer treatment, and the skeletal planarity is not indispensable for the entire molecule [77].

Further discovery revealed a new class of neo-tanshinlactone analogs, 4-amino-2*H*-benzo[h]chromen-2-one (**ABO**) and 4-amino-7,8,9,10-tetrahydro-2*H*-benzo[h] chromen-2-one analogs (**ATBO**), which were designed by combined structural simplification and bioisosteric replacement strategies [78, 79, 81]. Among all 4-substituted **ABO** analogs, the 4′-methoxyphenyl derivative (**79**) and 3′-methylphenyl derivative (**80**) showed the most potent antitumor activity against a broad range of cancer cell lines with ED50 values of 0.01–76 μM [79]. Among those **ATBO** analogs, compounds **81** and **82** with a 4-methoxyphenyl group at position C-4 were extremely potent with ED50 values of 0.008–0.064 and 0.035–0.32 μM, respectively [81]. Based on SAR study results on **ABO** and **ATBO** analogs, which displayed much higher potency than **65** analogs, Dong et al. designed a new class of **APO** analogs. Among these analogs, **83** and **84** were the most potent compounds with an ED50 range between 0.059 and 0.163 μM [84].

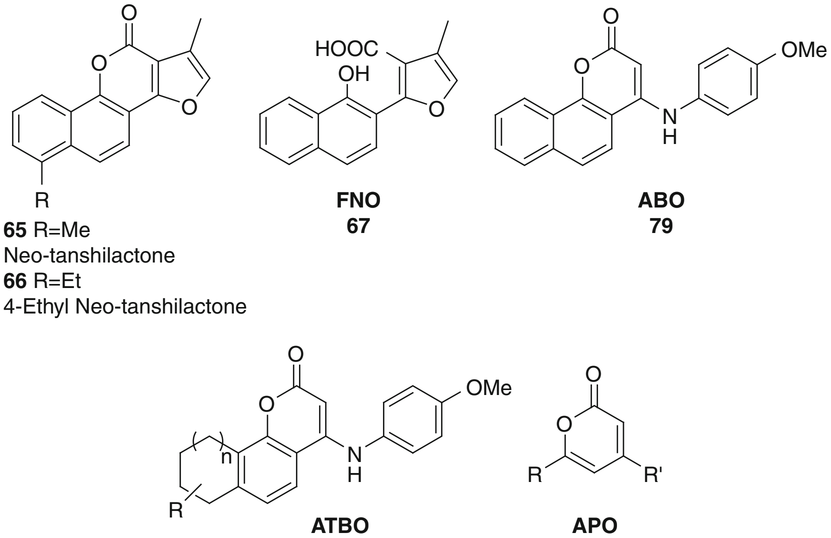


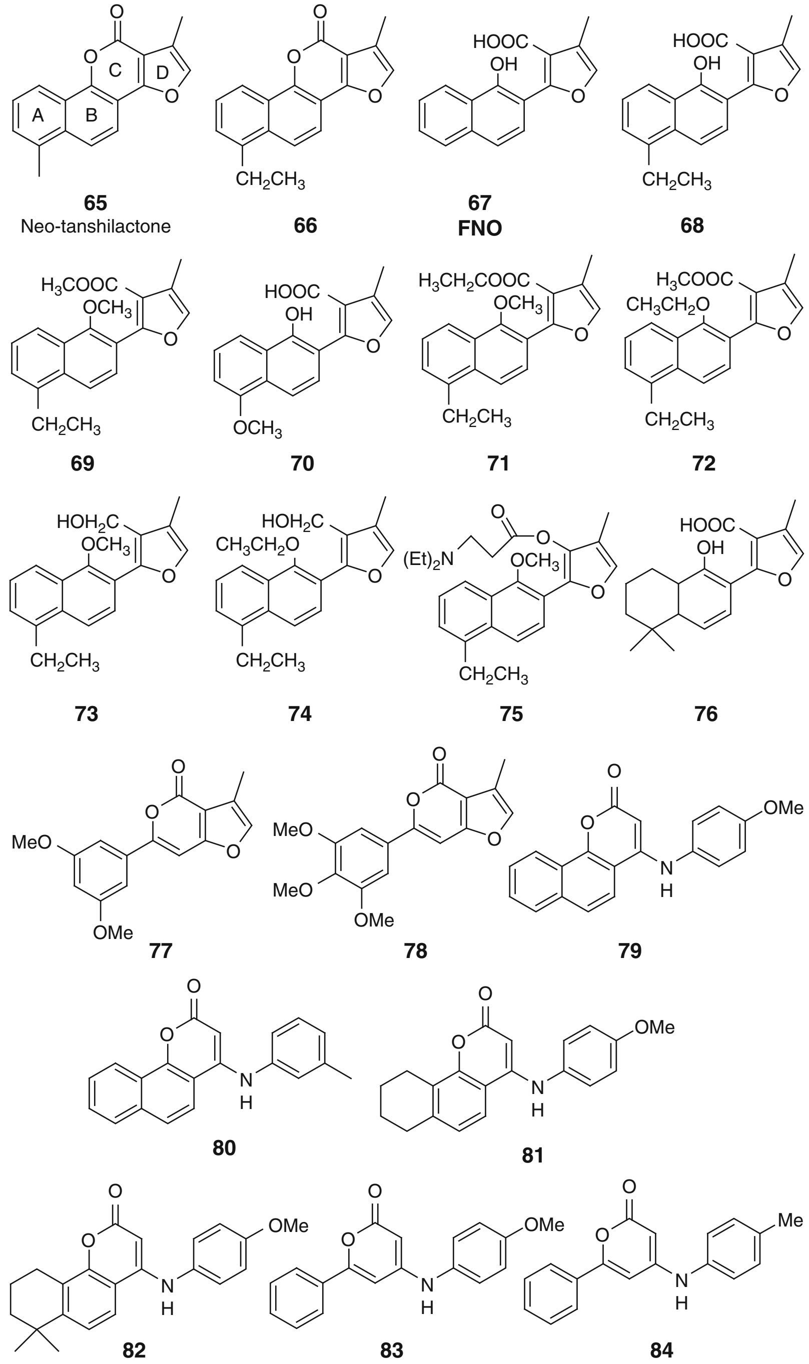
Fig. 117.7 Structure of different scaffolds of synthetic neo-tanshinlactone analogs

In conclusion, Lee research group discovered serial antibreast cancer neo-tanshinlactone analogs, and SAR studies have been fully addressed. The aromatic rings A and D were found to be important for antibreast cancer activity, and certain ring C-opened analogs retained activity and had increased selectivity toward specific breast cancer subtypes. The lead compound **66** was also tested in vivo against cancer cell xenografts in mice. At 10 mg/Kg, it remarkably delayed tumor growth compared to control against the human ZR-75-1 breast ductal carcinoma xenograft [38] (Figs. 117.7 and 117.8).

3.2 Anticardiovascular Activity of Tanshinones and their Mode of Action

The root of *Salvia miltiorrhiza* has been used as Chinese folk medicine for the treatment of cardiovascular diseases, such as ischemia, angina pectoris, coronary heart disease, myocardial infarction, and hypertension. Many studies on the secondary metabolites from *Salvia miltiorrhiza* revealed that tanshinones were a group of compounds responsible for these biological activities, especially for the treatment of coronary artery disease and hypertension.

Park et al. revealed that 15,16-dihydrotanshinone I inhibited collagen-induced aggregation of rabbit washed platelets with IC_{50} value of 8.7 ± 5.6 μM, which is sevenfold greater of potency than EGCG, an active green tea catechin component (IC_{50} 56.6 ± 48.7 μM). The authors revealed that 15,16-dihydrotanshinone I exerted potent antiplatelet activity via suppression of intracellular calcium mobilization and arachidonic acid liberation [85]. Tan-IIA was found to inhibit the


O
O
C
D
O
A
B
65
Neo-tanshilactone
O
O
O
CH2CH3
66
HOOC
OH
O
67
FNO
HOOC
OH
O
CH2CH3
68
H3COOC
OCH3
O
CH2CH3
69
HOOC
OH
O
OCH3
70
H3CH2COOC
OCH3
O
CH2CH3
71
H3COOC
CH3CH2O
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CH2CH3
72
HOH2C
OCH3
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CH2CH3
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HOH2C
CH3CH2O
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81
O
O
OMe
N
H
82
O
O
OMe
N
H
83
O
O
Me
N
H
84

Fig. 117.8 Structure of neo-tanshinlactone analogs

migration of human aortic smooth muscle cells through an AKT and matrix metalloprotease-9 pathway [86].

The in vivo experiments have been studied on Tan-IIA as well. A similar rat coronary artery ligation experiment found that Tan-IIA reduced monocyte chemoattractant protein I expression, thereby blocking inflammation. Macrophage infiltration and transforming growth factor (TGF)-beta secretion were also blocked [87]. The previous study has demonstrated that p38 MAPK played an important role in ischemia-induced signaling in mammalian cells, and an increase in phosphorylation of p38 MAPK contributed to myocyte injury. Zhang and coworkers revealed that Tan-IIA decreased p38 MAPK activation in MI rats. Tan-IIA could reduce the transcription factor SRF and MEF2 levels which could promote the miR-1 expression. This explains the reasons why Tan-IIA protects cardiac myocytes from ischemic injury [88].

Clinical evidence has shown that Tan-IIA increases coronary blood flow and protects heart against ischemia/reperfusion (I/R) injury. In order to explore the mechanism of its therapeutic action, Zhang and coworkers investigated the protective effects of Tan-IIA on I/R injury in diabetic rats and the underlying mechanism. Their study indicated that Tan-IIA pretreatment reduces infarct size and improves cardiac dysfunction after I/R injury. This was accompanied with decreased cardiac apoptosis and inflammation. They also found that Tan-IIA could inhibit inflammatory responses by inhibiting inflammatory factors including TNF-α and IL-6 production. Moreover, the specific PI3K inhibitor wortmannin can abolish the cardiac protective effects of Tan-IIA. In summary, the mechanism responsible for the effects of Tan-IIA is mediated, at least in part, by the PI3K/Akt-dependent pathway [89].

Liu and coworkers explored the protective effects of Tan-IIA in the acute stage of ischemic stroke and revealed that TORC1 nuclear accumulation and BDNF transcription are implicated in the early stage of ischemia. They concluded that TORC1 signal pathway, which controls the dynamics of CRE-target gene transcription, might be one of the Tan-IIA's effective therapeutic targets for cerebral ischemia [90].

Inflammation has been confirmed to play an important role in the pathogenesis of brain injury secondary to ischemia. Wang's studies evaluated the Tan-IIA's protective role in cerebral ischemia and its potential mechanism. After systemic administration of Tan-IIA, the infarct volume, neurologic deficits, and brain water content could be diminished in a dose-dependent manner, and the upregulation of HMGB1, NF-κB, RAGE, and TLR4 was significantly suppressed. They concluded that HMGB1 associated with NF-κB activation pathway might be one of the Tan-IIA's effective therapeutic targets for cerebral ischemia [91].

Hei and coworkers found that Tan-IIA reduced the hypoxic ischemic brain damage (HIBD)-caused downregulation of phosphor-NR1 S879 and the HIBD-caused [Ca^{2+}]. The neuroprotective effect of Tan-IIA may be related to influencing NMDA receptor expression and decreasing intracellular free-calcium aggregation [92].

Tan and coworkers discovered that Tan-IIA could protect cardiac hypertrophy induced by ISO. It is well known that the Ca^{2+}-activated Ser/Thr protein phosphatase calcineurin and the downstream transcriptional effectors of calcineurin, NFATcs, have been implicated as a key-signaling pathway in the hypertrophic response of the myocardium. In their study, they demonstrated that Tan-IIA inhibited the increased expression of calcineurin and NFATc3 induced by ISO, which indicates that Tan-IIA protects against cardiac hypertrophy in a calcineurin/NFATc3-dependent pathway [93].

Intimal hyperplasia is a key event of atherosclerosis and restenosis after PCI; smooth muscle cell migration and proliferation are thought to be central to the development of intimal hyperplasia. Li and coworkers demonstrated that Tan-IIA could significantly decrease intimal thickening, suppress cell proliferation and BrdU incorporation into DNA, and block cell cycle in G0/G1 phase. Mitogen-activated protein kinases (MAPK) cascade is one of the well-known signal transduction pathways for vascular smooth muscle cell (VSMC) proliferation and migration. Tan-IIA-decreased ERK1/2 activity and downregulation of c-fos expression in a dose-dependent manner were found, suggesting that TA inhibits VSMC proliferation by decreasing MAPK signaling pathway and downregulation of c-fos expression [94].

Yagi and coworkers demonstrated that tanshinone VI has two effects on heart. The first is that tanshinone VI can protect the heart against hypoxia/reoxygenation injury and improve the posthypoxic cardiac function, and the second one is about the decrease in the fibrosis of cardiac fibroblasts [95].

Yang and coworkers found that sodium Tan-IIA sulfonate (STS) can inhibit cardiomyocyte hypertrophy induced by angiotensin II (Ang II). The results indicated STS inhibited Ang II-induced increases in myocyte diameter and decreased the LVW/BW (left ventricular weight/body weight) ratio independent of decreasing systolic blood pressure. Therefore, STS may have some pharmacological properties similar to angiotensin-converting enzyme inhibitors or Ang II receptor antagonists, which need further research. It is also found that MEK inhibitor U0126 (20 μM) markedly enhanced STS-induced effects on cardiomyocyte. So we can conclude that MEK/ERK pathway plays a significant role in the antihypertrophic activities of STS [96].

The pathogenesis of sepsis is mediated in part by bacterial endotoxin, which stimulates macrophages/monocytes to sequentially release early (e.g., TNF, IL-1, and IFN-γ) and late (e.g., high-mobility group box 1 (HMGB1) protein) proinflammatory cytokines. Li and coworkers demonstrated that STS could selectively abrogate endotoxin-induced HMGB1 cytoplasmic translocation and release in a glucocorticoid receptor-independent manner. Interestingly, STS does not affect the release of most other cytokines, distinguishing itself from all previously known HMGB1 inhibitors [97].

Dihydrotanshinone relaxed isolated rat coronary arteries stimulated with 5-hydroxytryptamine (5-HT). The effect was blocked by the guanylate cyclase inhibitor ODQ, indicating a mechanism involving inhibition of calcium influx in smooth muscle cells of the vasculature [59].

With intact spontaneously hypertensive rats, Tan-IIA lowered systolic blood pressure when given *i.p.* at 10 mg/kg. The same dose had no effect on blood pressure in normotensive rats. Oral administration had the same effects, and in vitro experiments with isolated arteries implicated ATP-sensitive calcium channels in the mechanism [98].

4 Biosynthesis of Tanshinones

The extraction of diterpenoids from plants has been tedious and inefficient and requires substantial sacrifice of natural resources. Tanshinones show a broad spectrum of activities; the studies on biosynthesis of tanshinones are helpful to understand the biosynthetic pathway and the relationship between the properties of their scaffold and activities and produce new active analogs via genetic engineering. The biosynthetic pathways of the tanshinones have been explored for more than two decades, and work is still ongoing to determine the exact transformations and related enzymes.

Terpenes or isoprenoids are synthesized via at least two different pathways in higher plants, the mevalonate (MVA) pathway occurring in the cytosol and the non-MVA, 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway in the plastids of cell (Fig. 117.9) [99–101]. The MVA pathway is responsible for the synthesis of sesquiterpenes, triterpenes, sterol, and the side chain of ubiquinone. The MEP pathway takes part in the production of monoterpenes, diterpenes, carotenoids, and the side chains of chlorophylls and plastoquinone. These terpenoids are all derived from two common precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAP) [99]. In the MVA pathway, HMGR catalyzes the formation of MVA from 3-hydroxy-3-methylglutaryl-CoA, an initial and important step in the MVA pathway, followed by the formations of IPP and DMAP [102]. In the MEP pathway, DXP synthase (DXS) and DXP reductoisomerase (DXR) are the first two enzymes. Pyruvate and glyceraldehyde-3-phosphate (GA3P) initially form 1-deoxy-D-xylulose-5-phosphate (DXP) through the action of DXS. DXP is subsequently catalyzed by DXR to form 2-*C*-methyl-D-erythritol 4-phosphate (MEP). MEP then undergoes several steps catalyzed by a series of enzymes to afford IPP and DMAP [100, 103]. Chain elongation starts after the synthesis of IPP and DMAP via MVA or MEP pathway (Fig. 117.9). The carbonium ion is a potent alkylating agent that can react with IPP to produce GPP. GPP has the active allylic phosphate group to further react with IPP, giving FPP. GGPPS catalyzes the consecutive condensation of three IPP molecules with DMAPP to give C_{20} compound GGPP, a precursor of diterpenes [99, 104–108].

The biosynthesis and biotechnological production of tanshinones have been studied over two decades. Diterpenes in plants are presumably derived from the MEP pathway. In the MEP pathway, DXS plays an important role and is a rate-limiting enzyme for the synthesis of IPP and DMAP [109–111]. A full-length cDNA encoding DXR has been isolated from *S. miltiorrhiza* hairy roots [112]. Tissue expression pattern analysis revealed that the expression level of a cDNA encoding DXR (*Sm*DXR) was the highest in leaves, followed by roots and stems subsequently in *S. miltiorrhiza* plants [113]. The above studies provide the evidences for the biosynthesis of tanshinones via the MEP pathway.

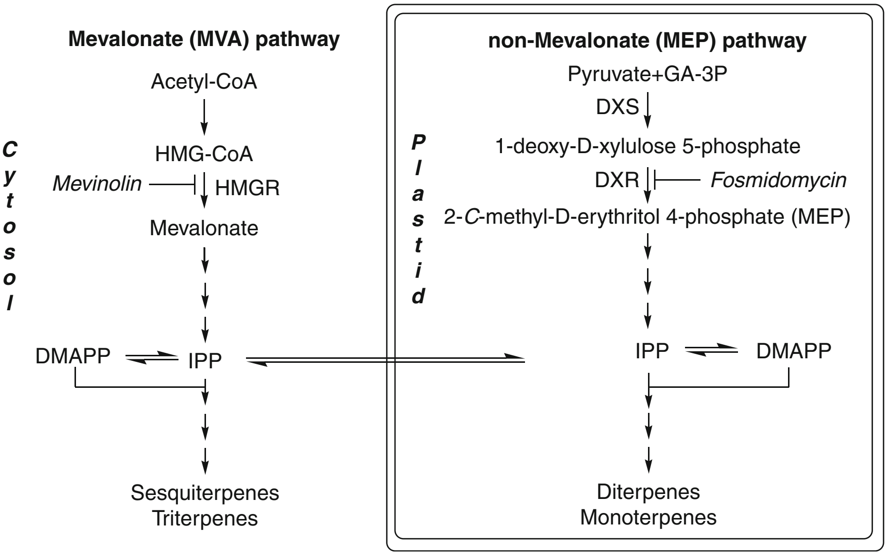
Mevalonate (MVA) pathway
Acetyl-CoA
HMG-CoA
Mevinolin
HMGR
Mevalonate
DMAPP
IPP
Sesquiterpenes
Triterpenes
Cytosol
non-Mevalonate (MEP) pathway
Pyruvate+GA-3P
DXS
1-deoxy-D-xylulose 5-phosphate
DXR
Fosmidomycin
2-C-methyl-D-erythritol 4-phosphate (MEP)
IPP
DMAPP
Diterpenes
Monoterpenes
Plastid

Fig. 117.9 An overview of the cytosolic mevalonate pathway and plastidial mevalonate-independent pathway for the biosynthesis of terpenoids (isoprenoids) in plant cells [99] (DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; DXS, DXP synthase; DXR, DXP reductoisomerase; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; GA3P, glyceraldehyde-3-phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMGR-CoA reductase; IPP, isopentenyl diphosphate. Mevinolin and fosmidomycin are inhibitors of HMGR and DXR, respectively)

Although there are some evidences that supported the biosynthesis of tanshinone in *S. miltiorrhiza* mainly via MEP pathway, this process may also depend on cross talk between the MVA and MEP pathways, based on some key enzymes and genes detected in the early steps of these pathways. Ge et al. revealed that elicitor-induced tanshinone accumulation in *S. miltiorrhiza* hairy roots was mainly synthesized through the MEP pathway (involving DXS) but also depended on a cross talk with the MVA pathway involving HMGR [100]. Hao et al. also detected an EST fragment of HMGR from a full-length cDNA library constructed with *S. miltiorrhiza* roots [114].

Diterpene synthases or cyclases are responsible for the cyclization of GGPP. Tanshinones belong to the abietane-type diterpenoids, and a sequential pair of cyclization reactions uniquely initiates the biosynthesis. The characteristic fused bicyclic hydrocarbon structure is formed from the diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP) in an initial carbon-carbon double-bond protonation-initiated reaction catalyzed by class II diterpene cyclases such as *syn*- and *ent*-copalyl diphosphate synthases (CPS). At this step, the initial stereochemistry is established, which is designated by comparison to that of the analogs A/B ring substructure in sterol biosynthesis [115]. Additional stereocenters are generally formed in the subsequent cyclization and/or rearrangement reaction catalyzed by CPP-specific class I diterpene synthase, kaurene synthase like (KSL), which is

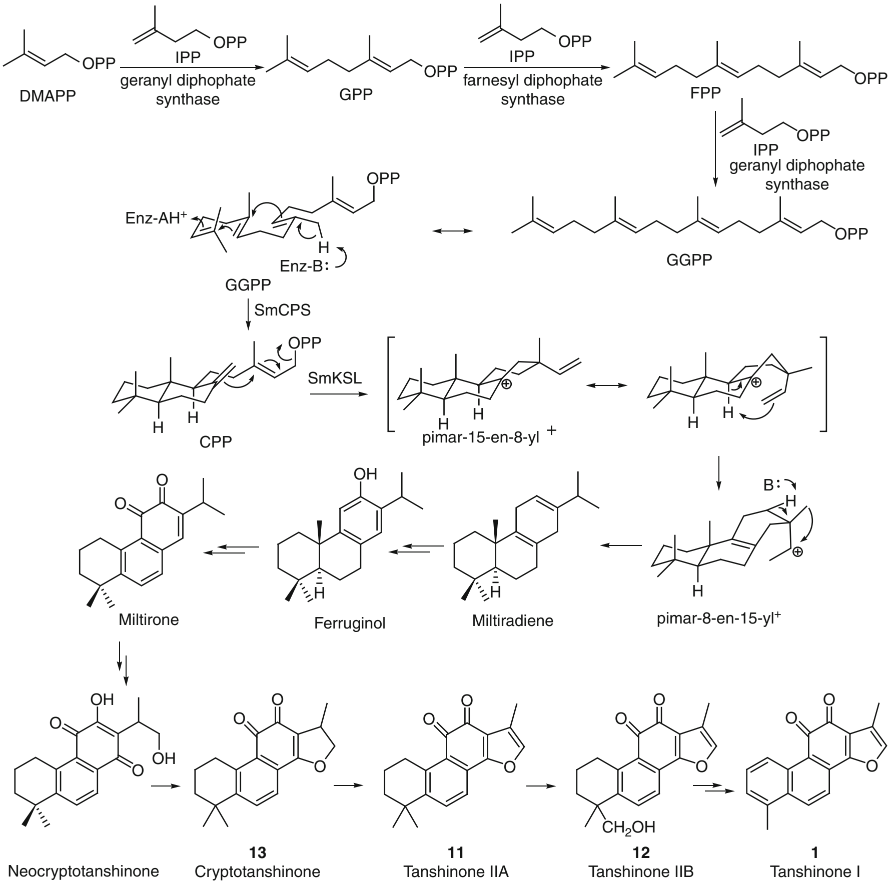
DMAPP
OPP
IPP
geranyl diphophate synthase
GPP
farnesyl diphophate synthase
FPP
GGPP
Enz-AH+
Enz-B:
SmCPS
CPP
SmKSL
pimar-15-en-8-yl +
pimar-8-en-15-yl+
B:
Miltiradiene
Ferruginol
Miltirone
Neocryptotanshinone
13
Cryptotanshinone
11
Tanshinone IIA
12
Tanshinone IIB
1
Tanshinone I
CH2OH
OH
H

Fig. 117.10 Biosynthetic pathway of tanshinones [53, 104, 105, 117]

similar to the kaurene synthase found in all higher plants for the requisite biosynthesis of gibberellin phytohormones [116]. As a subsequent of this step, miltiradiene is formed. The structure of miltiradiene has been determined by means of different NMR experiments. Because the cyclohexan-1,4-diene in the structure of miltiradiene is relatively unstable, the authors proposed that miltiradiene may undergo aromatization to ferruginol, followed by further introduction of different groups to give miltirone and neocryptotanshinone. The authors revealed that miltiradiene existed only transiently and its content decreased in correlation to an increase of Tan-IIA, suggesting that miltiradiene is an intermediate in the biosynthetic route to tanshinones. Based on the above evidences, Gao et al. proposed a biosynthetic pathway for tanshinones (Fig. 117.10) [53, 104, 105, 117]. This research has provided various methods to increase the production of tanshinones in *S. miltiorrhiza*, such as by elicitation with a yeast elicitor and transformations of cells [118–120].

Although a number of microorganisms have been engineered to produce isoprenoids as well as their intermediates [121–123], the overall efficiency remains low. Previous strategies commonly focus on the mevalonic acid (MVA) or methyl-D-erythritol phosphate (MEP) pathways that are at the early stage of terpenoid biosynthesis [122–124]. The conservation and interactions of diterpenoids synthases attracted little attention. Recently, Zhou et al. described the modular pathway engineering (MOPE) strategy and its application for rapid assembling synthetic miltiradiene pathways in the yeast *Saccharomyces cerevisiae*. They analyzed the molecular interactions between SmCPS and SmKSL and engineered their active sites into close proximity for enhanced metabolic flux channeling to miltiradiene biosynthesis by constructing protein fusions. The results revealed that the fusion of SmCPS and SmKSL, as well as the fusion of BTS1 (GGPP synthase) and ERG20 (farnesyl diphosphate synthase), led to significantly improved miltiradiene production and reduced by-product accumulation. The MOPE strategy facilitated a comprehensive evaluation of pathway variants involving multiple genes, and the diploid strain YJ2X reached miltiradiene titer of 365 mg/L in a 15-L bioreactor culture. Zhou's findings provided that the strategies of pathway assembling could be applied to engineering microbial hosts for the production of other valuable metabolites [125].

5 Conclusion

Tanshinones are a series of abietane-type norditerpenoid quinones isolated from TCM *S. miltiorrhiza* with wide-ranging pharmacological activities such as antibacterial, antioxidant, anti-inflammatory, and antineoplastic. Antitumor activities of tanshinones, especially Tan-I, Tan-IIA, and neo-tanshinlactone, have been studied both in vitro and in vivo against a variety of different human cancer cells such as glioma cancer, leukemia tumor, breast cancer, promyelocytic cancer, erythroleukemia cancer, gastric carcinoma, human colon carcinoma, and hepatocellular cancer. Their molecular mechanisms have been investigated in inducing apoptosis, inhibiting invasion and metastasis, inhibiting angiogenesis, and downregulating epidermal growth factor receptors. Tanshinones were reported as a group of compounds isolated from *S. miltiorrhiza* responsible in the treatment of coronary artery disease and hypertension.

Several tanshinones, such as Tan-I, Tan-IIA, and neo-tanshinlactone, might serve as the leads for the development of promising anticancer and anticardiovascular diseases, and further research needs to be focused on the preclinical evaluations. In addition, recent studies revealed tanshinones as the promising leads for anti-TB pharmaceutics development (Data not published, Liu XT et al.). These findings will extend the clinical uses of tanshinones, and further MoA against TB is in progress.

Chemical modifications of tanshinone analogs will improve the pharmacological profiles for preclinical testing. And considerable research efforts have been made in the last two decades to understand the tanshinone biosynthesis in *S. miltiorrhiza* at the protein and molecular levels which will help to develop biotechnological processes for tanshinone production. Our lab is striving in systematics-guided investigation of anti-infectious microbial natural products, such as anti-MRSA, anti-TB, and synergic antifungal, and led to a series of active natural products [69, 126–128]. Zhuo et al. adopted a successful proprietary approach to improve the yield of avermectin B1a (with 52% production improvement) and provide a route to optimize production in these complex regulatory systems. The engineered strain A56 was cultured in a 180-m^3 fermentor, in which the production has reached to 6,382 μg/mL [129]. These findings shed light on genetic engineering aimed at producing more secondary metabolites and increasing the yields of specific products, which provides an example for further study of tanshinones on synthetic biology.

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Strigolactones and Biological Activity 118

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Contents

1 Introduction 3584
2 Chemistry of Strigolactones 3585
3 Biosynthetic Pathway and Regulation 3588
3.1 Biosynthetic Pathway 3588
3.2 Regulation of Biosynthesis 3590
4 Analytical Methods for Strigolactones 3591
4.1 Bioassays 3591
4.2 UV Absorption 3592
4.3 GC–MS 3592
4.4 LC–MS 3592
5 Biological Activities 3593
5.1 Strigolactones as Rhizosphere Signaling Molecules 3593
5.2 Strigolactones as Plant Hormones 3596
6 Agronomical Uses 3597
7 Conclusion 3598
References 3598

Abstract

Strigolactones (SLs) are carotenoid-derived plant terpenes which function in the rhizosphere and in planta. In the rhizosphere, SLs induce seed germination in root parasitic plants and hyphal branching in arbuscular mycorrhizal (AM) fungi, therefore mediating both parasitic and symbiotic interactions with SL-producing plants and root parasitic plants and AM fungi, respectively. In planta, SLs are a group of plant hormones regulating shoot as well as root architecture. In this

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chapter, chemistry, biosynthesis and its regulation, analytical methods, and biological functions of SLs are discussed along with their potential agronomical uses.

Keywords

Arbuscular mycorrhizal fungi • parasitic plant • plant hormone • seed germination • shoot branching • Strigolactone

Abbreviations

AM	Arbuscular mycorrhizal
CCD	Carotenoid cleavage dioxygenase
CID	Collision-induced dissociation
GC–MS	Gas chromatography–mass spectrometry
HPLC	High-performance liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
M	Molecular ion
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
UPLC	Ultrahigh-performance liquid chromatography
SL	Strigolactone

1 Introduction

Among plant secondary metabolites, strigolactones (SLs) discussed in this chapter are unique as their new but probably original biological functions have been unveiled nearly 40 years after the isolation and structural determination of the first SL, strigol, from cotton root exudate as a seed germination stimulant for a root parasitic weed witchweed, *Striga lutea* (syn. *S. asiatica*), in 1966 [1]. Following this discovery and structural determination [2], strigol analogs collectively called “strigolactones” [3] have been isolated from root exudates of various plant species [4–6]. So far, more than 15 natural SLs have been characterized, and the total number of natural SLs may exceed 100 as they exist not only as structural isomers but also as stereoisomers. In addition, all plant species examined to date were found to produce and exude not a single SL but mixtures of several SLs. Compositions of these SL mixtures vary with plant species and among cultivars within the same genus, change with growth stage, and seem to be affected by growth conditions [4].

Since SLs induce seed germination of root parasitic weeds, SLs had been considered as wastes or harmful metabolites until their function as a host-recognition signal for arbuscular mycorrhizal (AM) fungi with which more than 80% of land plants form symbiotic relationship was uncovered in 2005 [7]. However, non-mycotrophic plant species such as Arabidopsis (*Arabidopsis thaliana* L.) [8], white lupin (*Lupinus albus* L.), and spinach (*Spinacia oleracea* L.) were found to produce SLs [9], indicating that SLs have important biological functions in SL-producing plants themselves. Within a few years, SLs or their further metabolites were identified as a novel class of plant hormones inhibiting shoot branching [10, 11]. Accordingly, SLs released from plant roots to the rhizosphere function as host-recognition signals for root parasitic plants and AM fungi and in plants as a plant hormone regulating aboveground architecture. In addition to these functions, SLs have been shown to be involved in the regulation of root architecture [12–14], photomorphogenesis [15], secondary growth [16], and rhizobia colonization [17, 18]. Furthermore, SL production by the moss *Physcomitrella patens* [19] and liverwort [20] indicates that SLs evolved before the first colonization of land by plants. Ubiquitous presence of SLs in the rhizosphere implies that any organisms in the rhizosphere are exposed to SLs, and thus, it is likely that SLs have other hidden roles in the interactions between SL-producing plants and other organisms in the rhizosphere.

In this chapter, diverse chemical structures of natural SLs, biosynthetic pathway and its regulation, analytical methods, biological activities, and potential agronomical uses are discussed.

2 Chemistry of Strigolactones

Most of SLs have been characterized as germination stimulants for root parasitic plants (weeds) of the family Orobanchaceae, including witchweeds (*Striga* spp.), broomrapes (*Orobanche* and *Phelipanche* spp.), and *Alectra* spp. *Striga* spp. are hemiparasites as they have functional chloroplasts but cannot survive without parasitizing to their hosts and thus regarded as obligate parasites. They parasitize important food crops including sorghum, maize, millet, and rice. *Orobanche* and *Phelipanche* spp. are holoparasites lacking chlorophylls and depend entirely on their hosts for the supply of water and nutrients. *Alectra* spp. are hemi- and obligate parasites like *Striga* spp. These root parasites are causing significant crop losses all over the world [21–23]. The seeds of these root parasites will not germinate unless they are exposed to chemical stimuli germination stimulants released from plant roots [24]. Majority of germination stimulants so far characterized are SLs [4, 25, 26].

All natural SLs identified to date contain a tricyclic lactone (ABC-part) which is connected to a butenolide moiety (the D ring) via a vinyl ether linkage [4–6]. These SLs have different substituents on the A and B rings but the same CD moiety (Figs. 118.1 and 118.2). Strigol **1**, along with its acetate (strigyl acetate), was isolated from root exudate of cotton (*Gossypium hirsutum* L.), nonhost of *Striga* [1, 2], and then its presence was confirmed in root exudates from *Striga* hosts, sorghum [*Sorghum bicolor* (L.) Moench], common millet (*Panicum miliaceum* L.), and maize (*Zea mays* L.) [27]. Sorgolactone **2** and alectrol **3** were identified as *Striga* germination stimulants from genuine hosts of *Striga* sorghum [28] and cowpea [*Vigna unguiculata* (L.) Walp.] [29], respectively. The structure originally proposed for alectrol **3** was disproved by comparison of spectral data with those of synthetic standard [30] and then identified as orobanchyl acetate [31–33]. Orobanchol **4a**, the first *Orobanche* germination stimulant, was isolated from red

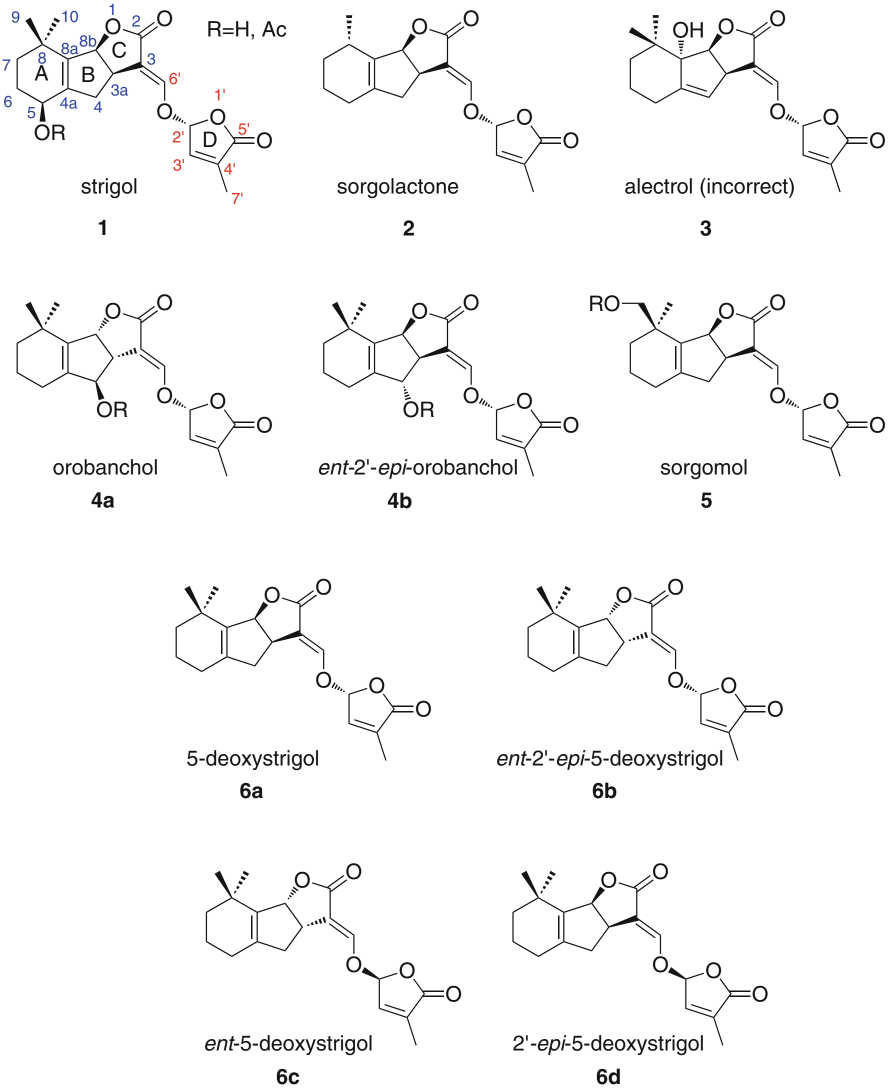

R=H, Ac
9
10
1
2
8a
8b
C
8
7
A
B
3
6'
3a
6
4a
4
1'
5
OR
2'
D
5'
3'
4'
7'
strigol
1
sorgolactone
2
OH
alectrol (incorrect)
3
orobanchol
4a
OR
ent-2'-*epi*-orobanchol
4b
RO
sorgomol
5
5-deoxystrigol
6a
ent-2'-*epi*-5-deoxystrigol
6b
ent-5-deoxystrigol
6c
2'-*epi*-5-deoxystrigol
6d

Fig. 118.1 Natural strigolactones and four stereoisomers of 5-deoxystrigol

clover (*Trifolium pratense* L.) root exudate [34, 35]. Structure of orobanchol was revised recently [33]. Red clover plants also produce orobanchyl acetate and an isomer of didehydro-orobanchol [36]. Sorgomol **5** was isolated from sorghum root exudate [37, 38] and then detected in some Fabaceae plants such as Chinese milk vetch (*Astragalus sinicus* L.) and white lupin (*Lupinus albus* L.) [9]. Sorgomol appears to be the biosynthetic precursor of sorgolactone because further oxidation of the hydroxymethylene group to carboxylic acid and the subsequent decarboxylation would afford sorgolactone [38, 39]. 5-Deoxystrigol **6a** was first identified

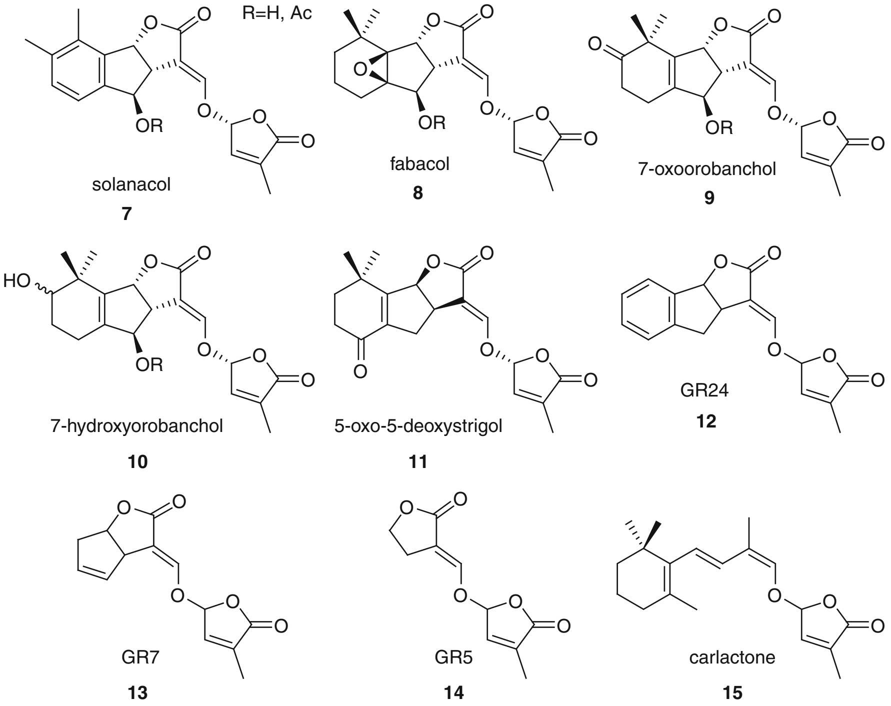


Fig. 118.2 Natural strigolactones, synthetic analogs, GR24, GR7 and GR5, and carlactone

from *Lotus japonicus* L. root exudates as a branching factor for AM fungi [7] and then as a germination stimulant in various plant species. One of its diastereomers, *ent*-2′-*epi*-5-deoxystrigol **6b**, was identified as a major SL in rice (*Oryza sativa* L.) plants [11] (Xie et al. unpublished data). Solanacol **7**, a unique SL containing a phenyl group, was purified from tobacco *(Nicotiana tabacum* L.) root exudates [40] and identified in tomato (*Solanum lycopersicum* L.) root exudates [41]. The structure of **7** was established by synthesis [42]. Tobacco plants also produce orobanchol **6a**, *ent*-2′-*epi*-orobanchol **4b**, orobanchyl acetate, *ent*-2′-*epi*-orobanchyl acetate, solanacyl acetate, 5-deoxystrigol **6a**, *ent*-2′-*epi*-5-deoxystrigol **6b**, and three isomers of putative didehydro-orobanchol whose structures have not yet been identified (Xie et al. unpublished data). Fabacol **8** and fabacyl acetate were originally purified from pea (*Pisum sativum* L.) root exudates [43]. 7-Oxoorobanchol **9** and 7-hydroxyorobanchol **10** and their acetates were found in root exudates of flax (*Linum usitatissimum* L.) and cucumber (*Cucumis sativus* L.) [4, 44]. 5-Oxo-5-deoxystrigol **11** was purified from root exudates of dokudami (*Houttuynia cordata* Thunb.) and detected in strawberry (*Fragaria grandiflora* Ehrh.) (Kisugi et al. unpublished data). This SL is one of the didehydro-orobanchol(-strigol) isomers but different from those detected in red clover [36], tobacco [40], tomato [41], and *Medicago truncatula* L. [45].

The CD moiety in the SL molecules has been suggested to be essential for the three representative biological activities – stimulation of seed germination in root parasitic plants [46, 47], induction of hyphal branching in AM fungi [48], and inhibition of shoot branching [4]. In fact, in the stimulation of parasite seed germination and hyphal branching of AM fungi, removal of the A ring of GR24 **12** as in GR7 **13** resulted in a reduction of the activity, and further removal of the B ring as in GR5 **14** brought about a significant (parasite seed germination) or complete loss of the activity (hyphal branching) [4, 47, 48]. For the inhibition of shoot branching (tillering) in rice plants, GR5 and GR7 were more active than GR24 when applied to growth media (Umehara et al. unpublished data). Therefore, natural SLs whose structures have not yet been identified would contain the ABC-part and the D ring. However, since carlactone **15** lacking the B and C rings, a possible intermediate in the SL biosynthetic pathway, could induce *Striga* seed germination [49], novel SLs without the B or C ring or carlactone analogs may exist.

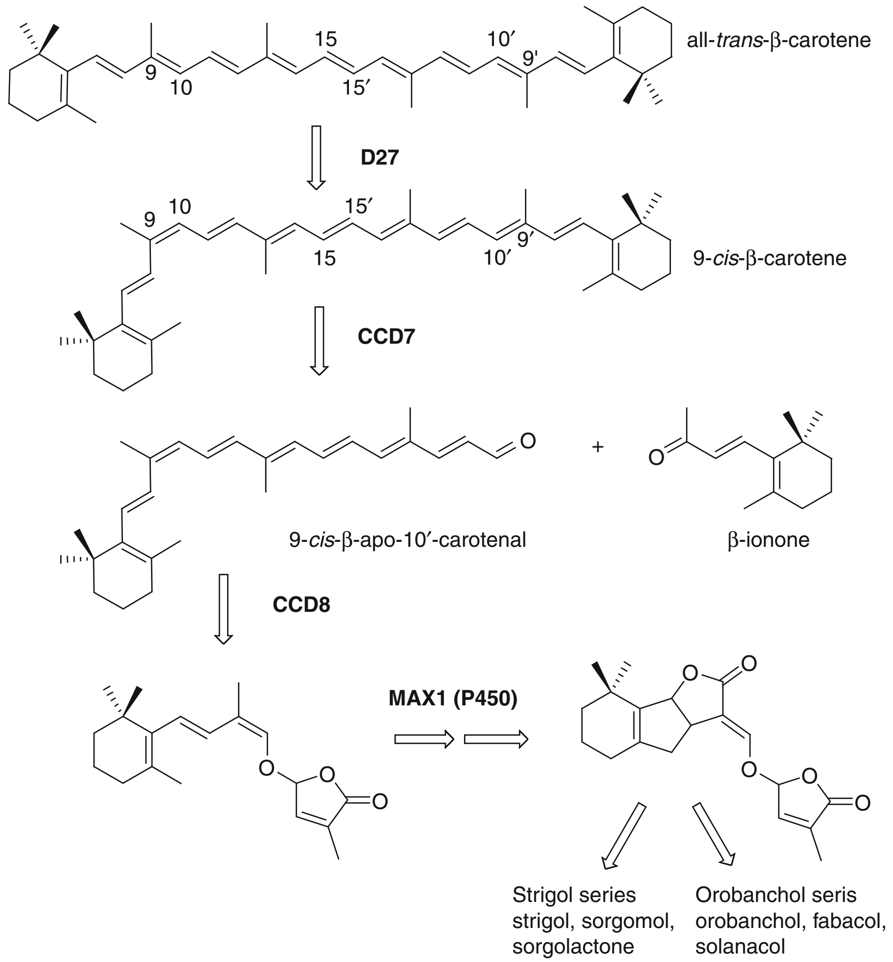
To date, only acetates of hydroxy-SLs have been identified. Conjugation with amino acids and sugars may also occur and may be important in regulation of endogenous levels of biologically active SLs [4].

3 Biosynthetic Pathway and Regulation

3.1 Biosynthetic Pathway

SLs had long been recognized as sesquiterpene lactones until Matusova et al. suggested that SLs would be derived from carotenoids [50]. Although they did not quantify SLs, the reduction in *Striga* seed germination stimulation activity of root exudates from carotenoid-deficient maize mutants and from maize, cowpea, and sorghum plants treated with carotenoid biosynthesis inhibitors well supported their hypothesis. This was then confirmed by the finding that SLs are a long-sought, carotenoid-derived, novel class of plant hormones inhibiting shoot branching [10, 11].

To date, four enzymes – D27, an iron-containing isomerase [49]; two carotenoid cleavage dioxygenases CCD7 and CCD8; and a cytochrome P450 monooxygenase, MAX1 in Arabidopsis – have been identified in the SL biosynthetic pathway (Scheme 118.1) [51, 52]. These enzymes are distributed widely in the plant kingdom, and even the moss *Physcomitrella patens*, although it lacks *MAX1* ortholog, was found to produce SLs [19]. In the biosynthetic pathway proposed recently, the C9–C10 double bond in all-*trans*-β-carotene is converted by the isomerase D27 to 9-*cis*-β-carotene [49]. This is the key step in the SL biosynthesis, since CCD7 is stereospecific for the 9-*cis* configuration and cleaves the C9′–C10′ double bond of 9-*cis*-β-carotene to afford 9-*cis*-β-apo-10′-carotenal and β-ionone. Then, CCD8 converts 9-*cis*-β-apo-10′-carotenal to carlactone [49]. These reactions occur in plastids since these enzymes and the starting materials – carotenoids – are located in plastids. MAX 1 and probably some other proteins in endoplasmic reticulum then catalyze the conversion of carlactone to 5-deoxystrigol and its


all-*trans*-β-carotene
9
10
15
15′
10′
9′
D27
9-*cis*-β-carotene
CCD7
9-*cis*-β-apo-10′-carotenal
+
β-ionone
CCD8
MAX1 (P450)
O
Strigol series
strigol, sorgomol,
sorgolactone
Orobanchol seris
orobanchol, fabacol,
solanacol

Scheme 118.1 Proposed biosynthetic pathway of strigolactones and enzymes

stereoisomers, which includes dioxygenation, dehydrogenation, and closure of the B and C rings [49]. These reactions may proceed without very strict stereochemical control and thus may afford all four stereoisomers of 5-deoxystrigol **6a–d**. In *L. japonicus*, 5-deoxystrigol **6a** is the major isomer but *ent*-2′-*epi*-5-deoxystrigol **6b** in rice plants [4]. Although *ent*-5-deoxystrigol **6c** and 2′-*epi*-5-deoxystrigol **6d** have not yet been detected so far, it is likely that plants produce all stereoisomers at different levels so that only major and/or accumulated ones have been detected [4].

Based on the stereochemistry or orientation of the C ring, natural SLs identified to date are divided into two groups [4]. One is "strigol series" starting from 5-deoxystrigol and including strigol, sorgomol, 5-oxo-5-deoxystrigol, and sorgolactone. The other is "orobanchol series" starting from *ent*-2′-*epi*-5-deoxystrigol, to which orobanchol, 7-hydroxyorobanchol, 7-oxoorobanchol, fabacol, solanacol, and probably most of didehydro-orobanchol isomers belong. In both SL series, an *R*-configuration at the C-2′ seems to enhance biological activities [4, 47]. Plants appear to produce both SL series.

3.2 Regulation of Biosynthesis

Various environmental factors have been shown to influence production and exudation of SLs. In particular, nutrient availability has profound effects on SL production and exudation. It has long been known that root parasitic weeds prevail on nutrient-deficient soils and fertilizer applications often alleviate crop damages caused by these parasites [53]. In addition, root exudates from plants grown under phosphate starvation are more active in AM hyphal branching than those from plants grown with sufficient phosphate supply [54]. Therefore, nutrient starvation, especially phosphate deficiency, was expected to enhance or stimulate SL production and exudation. Indeed, in red clover (*Trifolium pratense* L.), a host of *O. minor*, reduced supply of phosphorus (P) but not of other mineral elements (N, K, Ca, Mg) greatly promoted production of orobanchol, the major SL in red clover [55]. In the case of sorghum, a host of *S. hermonthica*, N deficiency as well as P deficiency profoundly increased the exudation of 5-deoxystrigol, the major SL in sorghum [56]. In both cases, SL contents in the roots were comparable to the increase in the root exudates, indicating that SLs may be rapidly secreted after their production in the roots. Similar results have been observed with tomato [41]. In petunia (*Petunia hybrida* L.), involvement of ATP-binding cassette (ABC) transporter PDR1 in SL exudation from roots and root-to-shoot transportation has been reported [57]. By contrast to these mycotrophic plant species, in non-mycotrophic white lupin (*Lupinus albus* L.), SL production responded to neither N nor P deficiency [9]. These results indicate that the regulation of SL production and/or exudation appears to be closely related to the nutrient acquisition strategy of the plants. More detailed analyses of the effects of N and P deficiencies on SL exudation in Fabaceae, Asteraceae, Solanaceae, and Poaceae plants suggested that the P status of the shoot regulates SL exudation [58, 59].

Major SLs produced by Chinese milk vetch (*Astragalus sinicus* L.) are sorgomol, 5-deoxystrigol, and orobanchyl acetate. Sorgomol is derived from 5-deoxystrigol but orobanchyl acetate (orobanchol) from *ent*-2′-*epi*-5-deoxystrigol. Exudation of sorgomol and 5-deoxystrigol was significantly enhanced by N and P deficiencies, whereas neither P nor N deficiency promoted orobanchyl acetate exudation [59]. These results imply that plants produce all SLs but may promote exudation of particular SLs in response to environmental stresses including nutrient starvation. In addition, the two different biosynthetic pathways for SLs starting from either 5-deoxystrigol to strigol series SLs or *ent*-2′-*epi*-5-deoxystrigol to orobanchol series SLs appear to be regulated somewhat independently under N and P deficiency, and the former pathway seems to be more sensitive than the latter to N and P deficiency in this plant [59].

Deficiency of other elements also affects SL production and exudation but to a lesser extent. Of course, enhancement of SL production and/or exudation under nutrient deficiency would be temporal and would last within several days to a few weeks when the plants exhaust their stocks. These responses, however, may vary with plant species and their growth stages.

Light is one of the environmental factors suggested to influence SL production and exudation in plants [60]. Light quality, intensity, and irradiation period seem to affect SL production and exudation directly or indirectly through its effects on biosynthesis, transport, and signaling of the other plant hormones, auxin, cytokinins (CKs), ethylene, gibberellins (GAs), abscisic acid (ABA), and brassinosteroids (BR). Other environmental factors including nutrient availability would also influence SL production and exudation indirectly in a similar manner. In general, auxin promotes but CKs and GAs suppress SL biosynthesis. ABA which antagonizes with SL in seed germination of parasitic plants [61] was suggested to regulate SL biosynthesis [62].

Seeds of root parasitic plants need to be kept in a warm–wet environment for several days prior to the exposure to germination stimulants including SLs. During this period – called conditioning period – the seeds become responsive to the stimulants [24]. Although it has not yet been confirmed by chemical analysis, root parasitic plants appear to have ability to produce their own SLs as genes for SL biosynthesis and signal transduction are preserved in their genome [63]. Expression analysis of genes for SL biosynthesis during seed conditioning and germination suggests that SL biosynthetic activity decreases to the minimum level toward the end of conditioning period, creating SL starvation in the seeds, and thus renders the seeds sensitive to exogenous SLs (Westwood et al. unpublished data). Inclusion of GAs in the conditioning media has been known to increase sensitivity of parasite seeds to germination stimulants [64, 65]. This may be due to the inhibition of SL biosynthesis by GAs. GA treatment further reduces endogenous SLs during the conditioning period and therefore enhances the sensitive to exogenous SLs.

4 Analytical Methods for Strigolactones

4.1 Bioassays

The most sensitive and selective detection methods for SLs are germination test with seeds of root parasitic plants and hyphal branching assays with AM fungi. For example, SLs are active at as low as 10^{-12} M in *Orobanche minor* seed germination [66] and at 0.1 pg/disc in hyphal branching of *Gigaspora margarita* [48]. Of course, only bioactive SLs and related compounds (precursors and metabolites) can be detected by these bioassays. Since these bioassays are very sensitive and specific, it is possible to detect SLs even in crude extracts without any purification. Semiquantitative analysis may be possible with these bioassays especially by the seed germination test, but inhibitors present in the crude extracts may mask the activity of SLs [24, 67]. This may be avoided by conducting bioassays using fractions after HPLC separation of crude extracts. By comparing retention times of germination stimulants with those of synthetic and/or natural SL standards, SLs present in the sample may be estimated. However, in the case of parasite seed germination, not only SLs but also other plant metabolites have been shown to be active to some parasite species. For example, *P. ramosa* germination stimulants produced by rapeseed (*Brassica napus* L.) are isothiocyanates [68], and dehydrocostuslactone produced by sunflower (*Helianthus annuus* L.) is the germination stimulant for *O. cumana* [69].

4.2 UV Absorption

All the natural SLs have weak to moderate UV absorption with a maximum at around 240–250 nm [1] and thus can be detected by UV or PDA (photodiode array) detector only when their concentration ($>10^{-6}$ M) and purity are high enough. In the case of plant root exudates collected and thus concentrated by absorption on activated charcoal, it is sometimes possible to detect SLs without any purification by HPLC equipped with a UV detector. Of course, various compounds have rather strong absorption in this UV region, and concentrations of SLs in plant root exudates are generally quite low. Consequently, it is quite difficult to analyze SLs in crude samples with HPLC–UV detector system.

4.3 GC–MS

In the first isolation of orobanchol, purified samples were analyzed by GC–MS after conversion to their TMS ethers [34]. Since decomposition of SLs and their TMS ethers occurs in the capillary column, column length should be shorter than 5m (DB-5). Most of natural SLs have been analyzed by GC–MS without derivatization, but sensitivity for hydroxy-SLs is rather low. Although detection limit of GC–MS (~ ng) is more than 1,000 times higher than that of LC–MS/MS (~ pg), fragment ions and fragmentation patterns obtained from GC–MS analysis provide more rich information on structural features of SLs than LC–MS/MS. In addition, SL isomers that cannot be differentiated by LC–MS/MS often have distinct retention times in GC–MS analysis. Unfortunately, crude samples cannot be analyzed by GC–MS, and extensive purification steps are required.

4.4 LC–MS

The most simple and highly sensitive analytical method for SLs is LC–MS/MS with multiple reaction monitoring (MRM) mode [36, 70]. Even crude samples can be directly analyzed by LC–MS/MS for the detection of known and unknown SLs with known or estimated molecular formulae. This is possible because all natural SLs contain the essential structure, the CD part. Electrospray ionization (ESI) of SLs results in the formation of their $[M + H]^+$ and $[M + Na]^+$ ions, and these ions give rise to characteristic product ions on collision-induced dissociation (CID) with argon due to the loss of the D-ring moiety. In general, reversed-phase HPLC equipped with a C_{18} column with solvent system of methanol–water or acetonitrile–water is used for the separation of SLs. In the first report on SL analysis by LC–MS/MS, only the transitions of sodium adduct ions $[M + Na]^+$ to the fragment ions losing the D-ring moiety $[M + Na - 97]^+$ were selected [36, 70]. Then it has become possible to set two or more MRM transitions for each SL for more accurate identification of known SLs. Development of ultrahigh-performance liquid chromatography (UPLC) has enabled clearer separations of SL isomers in shorter analytical time. For example, a single peak of monomethoxy-SL was detected in rice root exudates by a conventional HPLC (C_{18} column, 3 μm) connected to MS/MS, whereas the same sample gave four peaks in UPLC–MS/MS (C_{18} column, 1.8 μm) (Xie et al. unpublished data).

Although crude samples can be analyzed by LC–MS/MS, it is better to remove acidic compounds which strongly interfere with electrospray ionization of SLs. Therefore, direct comparison of peak areas in MRM chromatograms among different samples which contain different levels of matrices may lead to over- and/or underestimation of SLs in the samples. In particular, root exudates from plants grown under nutrient deficiency contain relatively large amounts of organic acids. In addition, for extracts of plant tissues, it is preferable to subject them to a simple purification step using a silica gel or C_{18} cartridge. For accurate quantifications of SLs, internal standards should be employed.

Instability of natural SLs in particular under basic conditions has been described repeatedly. In fact, natural SLs may decompose within a day in aqueous solutions [48]. In general, hydroxy-SLs are less stable than their acetates and SLs without hydroxyl substituents. Furthermore, it is better not to store root exudate or plant material in a freezer for a long period. Lyophilization (freeze drying) of these samples results in a partial or a complete decomposition of SLs (Kisugi et al. unpublished data). These samples should be extracted with ethyl acetate or other appropriate organic solvents immediately after collection.

5 Biological Activities

SLs exhibit biological activities in the rhizosphere and in plants. In the rhizosphere, SLs are signaling compounds involved in the communications between the SL-producing plants and other organisms including root parasitic plants and AM fungi. In plants, SLs work as a class of plant hormones regulating shoot branching. Hence, biological activities of SLs in the rhizosphere and in plants are discussed separately.

5.1 Strigolactones as Rhizosphere Signaling Molecules

Once released into the rhizosphere, SLs induce seed germination of root parasitic plants and hyphal branching of AM fungi. Since plant roots continuously release SLs into the rhizosphere, all soilborne organisms are exposed to SLs when they are in close vicinity to the root. SLs appear to diffuse only to a limited area as they decompose rapidly in soil, and thus, SLs are good cue of the presence of living plant roots nearby.

5.1.1 Germination Stimulants for Root Parasitic Plants

Natural SLs identified to date contain the same CD moiety but different substituents on the A and B rings. These substituents affect biological activity of SLs. In the case of *O. minor* seed germination, approximately 1,000-fold differences in activity were observed among 11 natural SLs [66]. Monohydroxy-SLs including orobanchol are most active and induced >80% germination at 10^{-11} M. In general, acetates of monohydroxy-SLs are approximately tenfold less active than the corresponding monohydroxy-SLs, while 7-oxoorobanchol is less active than its acetate, probably due to its instability. By contrast, rather lipophilic SL 5-deoxystrigol and sorgolactone are the least active SLs and induce 80% germination at 10^{-8} M. The synthetic SL GR24 (mixture of four stereoisomers) is 100-fold less active than the least active natural SLs. These results indicate that germination stimulation activity of natural SLs on *O. minor* seeds depends on the lipophilicity of SL molecules and their stability also affects activity. It is likely that seeds of different root parasitic plants have different sensitivities to each SL. Since plants produce and exude mixture of SLs, it is intriguing to understand which SL in the mixture contributes most to host-specific seed germination. For example, it has been known that only host plant root exudates can induce germination of host-specific root parasites such as *O. hederae* (ivy broomrape), whereas ivy root exudate was found to contain known SLs including orobanchol (Xie et al. unpublished data). Therefore, in addition to the germination tests with individual SL, various mixtures of SLs need to be examined for germination stimulation of host-specific root parasites.

Root parasitic weeds *Striga* and *Orobanche* (*Phelipanche*) spp. are causing devastating damages to agricultural production worldwide [21–23]. One of the most promising approaches to alleviate these damages is breeding of resistant genotypes. Although there are multiple layers of incompatibility to the parasitic weeds [71, 72], reduced production of SLs is a simple and straightforward characteristic for the resistance since genotypes with such a trait are expected to induce only a few parasite seeds located very closely to the host roots [73]. Indeed, root exudates from SL-deficient mutants of rice [11] and tomato [74] induced only low germination of root parasitic weeds.

In sorghum, *Striga*-resistant genotypes with reduced or no stimulant production were selected by comparing the germination stimulation activity of root exudates [73]. Therefore, quantitative and qualitative differences in SL production between *Striga*-resistant and *Striga*-susceptible sorghum cultivars were examined [5]. Unexpectedly, when they were grown hydroponically, root exudates from *Striga*-resistant sorghum cultivars induced *S. hermonthica* and *O. minor* seed germination at levels similar to those induced by *Striga*-susceptible cultivars. All of these sorghum cultivars produced sorgomol, sorgolactone, and 5-deoxystrigol as major SLs. The only difference was that *Striga*-resistant cultivars exuded lower amounts of more stable 5-deoxystrigol than did *Striga*-susceptible ones. Similar results were obtained with *Striga*-resistant and *Striga*-susceptible maize genotypes (Yoneyama et al. unpublished data). In the case of NERICA (new rice for Africa) cultivars, there was a positive relationship between the amount of SLs in the root exudate and *Striga* germination, attachment, and emergence rates [75]. Among the SLs detected in the root exudates, there was a strong correlation between *Striga* germination and amounts of more stable methoxy-5-deoxystrigol isomers and (*ent*-)2′-*epi*-5-deoxystrigol, whereas attachment, emergence, and dry biomass of *Striga* correlated best with orobanchol. These results indicate that more stable SLs are mainly involved in germination stimulation of *Striga* seeds and other less stable SLs also contribute to processes after germination including radicle elongation toward host root and subsequent attachment.

5.1.2 Signaling Molecules for Soilborne Microbes

AM symbiosis is the most widespread and common symbiosis on earth and evolved 460 Ma ago. AM fungi are soilborne microorganisms that form symbiotic relationship with more than 80% of land plants. They penetrate and colonize plant roots and develop branched structure called arbuscules, the sites of nutrient exchange. AM fungi uptake water and nutrients mainly phosphate and nitrogen through their network of fine hyphae and supply them to their hosts, and in turn the fungi receive photosynthates from their hosts. Like rhizobium–legume interaction, the interaction between plants and the AM fungi is initiated by mutual exchange of signaling molecules – branching factor (BF) and myc factor [76, 77]. 5-Deoxystrigol was the first BF identified in *L. japonicus* root exudate [7]. All natural SLs examined so far are active as BF at 1–100 pg/disc [48]. Synthetic SL GR24 is active at 100 pg/disc, but the karrikin KAR_1 (3-methyl-2*H*-furo[2,3-*c*]pyran-2-one), a germination stimulant in smoke [78], is totally inactive. Structure–activity relationships of natural and synthetic SLs have been reported [48]. SLs-deficient mutants of pea showed reduced AM colonization, which was rescued by SL application, suggesting SLs influence only presymbiotic phase of AM colonization [10]. On the other hand, AM colonization reduces SL production and/or exudation and thus renders the host plants less susceptible to root parasitic weeds [26, 79].

Several other soilborne pathogenic and nonpathogenic fungi have been examined for their response to the synthetic SL GR24. Steinkellner et al. reported that GR24 did not affect hyphal branching in *Rhizoctonia solani, Fusarium oxysporum*, and *Verticillium dahliae* [80]. In another report [81], GR24 inhibited growth of *F. oxysporum* f. sp. *melonis, F. solani* f. sp. *mango, Sclerotinina sclerotiorum, Macrophomia phaseolina*, *Alternaria alternata*, *Colletotrichum acutatum*, and *Botrytis cinerea*. Hyphal branching was promoted in *S. sclerotiorum, C. acutatum*, and *F. oxysporum* f. sp. *melonis* and also slightly in *Alternaria alternata*, *F. solani* f. sp. *mango*, and *B. cinerea*. GR24 was inhibitory on hyphal branching in *M. phaseolina*. These observations suggest that SLs would influence growth and development of broad range soilborne fungi. Since GR24 used in these studies consist of four (or two) stereoisomers, optically pure natural SLs may induce different responses.

SLs have been shown to regulate rhizobia nodulation. GR24 promoted nodulation in alfalfa (*Medicago sativa* L.) inoculated with *Sinorhizobium meliloti* without affecting bacterial growth or *nod* (nodulation) gene expression [17]. A similar promotive effect of GR24 on nodulation was observed in pea (*Pisum sativum* L.) inoculated with *Rhizobium leguminosarum*. Significantly reduced nodulation in the SL-deficient mutant *rms1* could be increased by GR24 application which also promoted nodulation in wild-type plants [18]. Grafting experiments with using wild type and *rms1* revealed that SLs are positive regulators of nodulation. SLs in roots appear to regulate nodule number but not nodule formation. Such an effect of SLs only on presymbiotic phase is similar to that observed in AM colonization where SLs play an important role in AM fungi infection but not in arbuscule formation. Recently, the GRAS-type transcription factors NODULATION SIGNALING PATHWAY1 (NSP1) and NSP2, which are functioning in Nod factor-induced signaling and thus essential for rhizobium symbiosis (NSP2, also for mycorrhizal symbiosis), have been shown to be indispensable for SL biosynthesis [45]. NSP1 and NSP2 are functionally conserved in nonlegume higher plants including non-mycotrophic *Arabidopsis*, and thus, the regulation of SL biosynthesis is considered an ancestral function.

It is difficult to simulate natural conditions in the soil as plant roots continuously release SLs into the rhizosphere, but artificially applied SLs would rapidly disappear due to degradation. It is likely, however, that SLs released from plant roots influence soil biota, and in turn some microbes utilize this rhizosphere signal for their growth and development.

5.2 Strigolactones as Plant Hormones

5.2.1 Inhibition of Shoot Branching

Inhibition of shoot branching or bud outgrowth by SLs has been reviewed extensively, and thus, we will not go into details here. Other plant hormones, in particular auxin and cytokinin, also influence shoot branching directly or indirectly through their effects on SL production and/or signaling [51, 82–84].

So far, all genes in the SL biosynthesis and signaling have been cloned from mutants with excessive branching (tillering in rice) [52]. The genes for SL biosynthesis are *RMS5*/*MAX3*/*D17(HTD1)* and *RMS1*/*MAX4*/*D10*/*DAD1* (RMS, MAX, D, and DAD are from pea, *Arabidopsis*, rice, and petunia, respectively) encoding carotenoid cleavage dioxygenase (CCD)7 and CCD8, respectively; *MAX1* in *Arabidopsis* encoding a cytochrome P450 (CYP711A1); and *D27* encoding an iron-containing isomerase [49]. *RMS4*/*MAX2*/*D3* encoding an F-box protein and *D14* encoding an α/β-hydrolase are required for perception and signaling of SLs [51, 52, 85].

Karrikins are smoke-derived germination stimulants which contain a butenolide structure similar to SLs [78]. Karrikins strongly elicit seed germination of fire-prone plant species but not those of root parasitic weeds [86]. They are active neither in hyphal branching of AM fungi nor inhibition of shoot branching [4, 48]. In *A. thaliana*, karrikins and SLs exhibit somewhat similar biological effects, for example, light-dependent inhibition on hypocotyl elongation, and MAX2 is required in the response to karrikins and SLs [87]. It has been demonstrated that *Arabidopsis D14* ortholog *AtD14* is necessary for SL response but *D14* paralog (*D14-like*) *KARRIKIN INSENSITIVE 2* (*KAI2*) is required for response to karrikins [88]. Both D14 and KAI2/D14-like homologs are distributed widely in the plant kingdom, and thus plants have two distinct signaling systems for karrikins and SLs.

5.2.2 Other Biological Functions

SLs have been shown to regulate not only shoot but also root architecture by controlling lateral root formation and root-hair elongation in a MAX2 dependent manner [12–14, 89]. As in the regulation of shoot architecture, SL–auxin cross talk seems to be involved in these root responses.

SLs appear to function in various processes of plant growth and development. For example, SLs induce seed germination of crops and weeds [90], promote photomorphogenesis [15], and restore germination in thermoinhibited seeds [91]. It is likely that additional functions and effects of SLs will be identified in the near future.

In addition to the effects of SLs on plants and microbes, they may influence other living organisms including human beings. Natural SLs were detected in fresh cucumber and coconut juice (Xie et al. unpublished data), and thus we would have a chance to receive SLs. SLs might protect us by inhibiting development of breast cancer [92]. Further study is needed to clarify effects of SLs on plant growth and development and also on other organisms including human beings.

6 Agronomical Uses

Root parasitic weeds are causing devastating damages to agriculture worldwide. They attach to host roots and spend most of their life cycle underground. The parasites become visible only after they emerge aboveground when growth of host plants had already been severely stunted. Single root parasitic weed can produce several hundreds of thousands of tiny seeds which remain viable for decades in soil. Therefore, the most important strategy in root parasite management is to decrease the number of parasite seeds (seed bank) in soil. As SLs can induce germination of parasite seeds in the absence of host plants and the germinated seeds will die unless they attach appropriate host roots within a few days, SLs and their synthetic analogs have been tested as inducers of seed germination in the absence of hosts – termed “suicidal germination” [93, 94]. Although natural SLs are not stable enough to induce acceptable levels of germination under field conditions, synthetic analogs like Nijmegen-1 provided promising results under field conditions [95–98]. Recently, novel types of SL mimics and nonmimics have been reported to be active on parasite seed germination, AM hyphal branching, and inhibition of shoot branching [99–101]. In general, these synthetic compounds are different from natural and synthetic SLs in that they have only one or two of the three biological activities – parasite seed germination, AM hyphal branching, and shoot branching. Therefore, it is likely to find useful compounds for the induction of suicidal germination of parasite seeds, for promotion of AM symbiosis, and for regulation of shoot and root architecture in plants.

7 Conclusion

Production of SLs by liverworts, the most basal land plants, and Charales, green algae closest to the land plants, suggests that SLs evolved before land colonization by plants and their primitive function may be regulation of rhizoid elongation because both liverworts and Charales have orthologs of D14-like which is involved in SL signaling [20]. Then, diffused or actively released SLs from tissue would be utilized by soilborne microbes including AM fungi as a cue of host presence nearby. Therefore, it is likely that SLs still have other functions in rhizosphere communications and in plant growth and development. In addition, it is intriguing to understand why and how plants produce and exude various SLs and their mixtures and how individual SL contributes to host recognition by root parasites and by AM fungi and to the regulation of shoot and root architectures in plants. Further studies are needed to answer to these questions.

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Biological Activity of Oleuropein and its Derivatives

119

Stefania Bulotta, Manuela Oliverio, Diego Russo, and Antonio Procopio

Contents

1 Introduction 3607
2 Chemistry and Analysis of Oleuropein and its Derivatives 3609
  2.1 Chemical Structure, Biosynthesis, and Enzymatic Degradation of Oleuropein ... 3609
  2.2 Analytical Methods for Oleuropein Derivative Determination 3611
3 Bioavailability 3616
4 Biological Activity and Pharmacological Properties 3618
  4.1 Antioxidant Activity 3618
  4.2 Anti-inflammatory Activity 3621
  4.3 Cardiovascular Protection 3622
  4.4 Antidiabetic Activity 3624
  4.5 Anticancer Activity 3626
  4.6 Protection Versus Infectious Diseases 3626
  4.7 Neuroprotective Activity 3627
  4.8 Skin Protection 3628
5 Conclusions 3628
References 3629

Abstract

Investigation of the biological effects of olive oil/leaf/fruit phenolic extracts and, more recently, of the isolated compounds oleuropein and hydroxytyrosol has revealed their health beneficial action opening the way for a potential pharmacological utilization as single drug or after enrichment of olive oil or other food component. After a brief description of the chemical structure, biosynthesis, and the enzymatic degradation of oleuropein, which generates other biological active derivatives (i.e., hydroxytyrosol), the present review will focus on the

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biological effects of oleuropein, oleuropein aglycone, and hydroxytyrosol, with particular attention on the molecular mechanism underlying their action, resulting from in vitro and in vivo experimentation performed using isolated compounds.

Keywords

Anti-inflammatory • antioxidant • cardiovascular diseases • hydroxytyrosol • oleuropein • oleuropein aglycones • phenols • virgin olive oil

Abbreviations

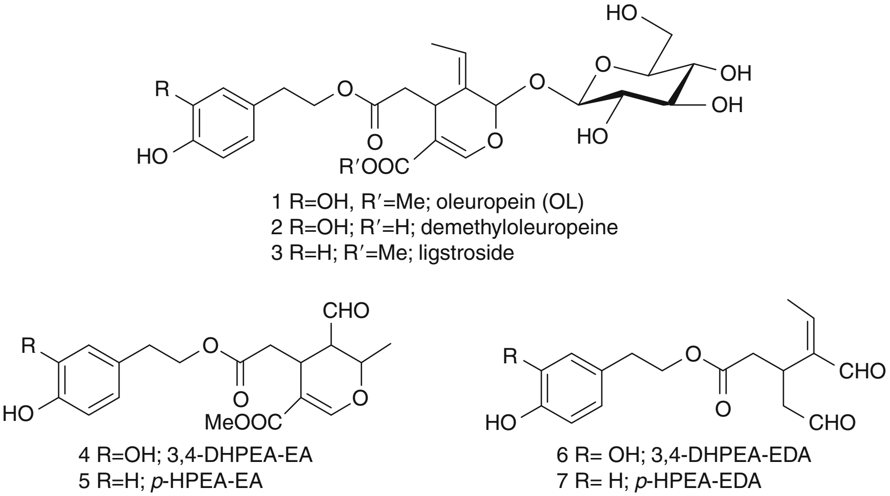
^{1}H-NMR	High-resolution proton nuclear magnetic resonance
3,4-DHPEA	3,4-Dihydroxyphenyl ethanol
3,4-DHPEA-EA	Oleuropein aglycone
3,4-DHPEA-EDA	3,4-Dihydroxyphenyl ethanol-decarboxymethyl elenolic acid dialdehydic form
AD	Alzheimer's disease
APCI	Atmospheric pressure chemical ionization
Aβ	Amyloid beta peptide
CE	Capillary electrophoresis
COX	Cyclooxygenase
DAD-UV	Diode array detector-ultraviolet
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EA	Elenolic acid
ESI	Electrospray ionization
FRAP	Ferric reducing antioxidant potential
GC	Gas chromatography
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HT	Hydroxytyrosol
ICAM-1	Intercellular adhesion molecule-1
iNOS	Inducible nitric oxide synthase
IT	Ion trap
LC	Liquid chromatography
LCN2	Lipocalin 2
LDL	Low-density lipoprotein
LLE	Liquid-liquid extraction
LPS	Lipopolysaccharide
MAE	Microwave-assisted extraction
MIR	Medium infrared spectroscopy
MMP	Matrix metalloproteinases
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
NF-kB	Nuclear factor kappa B
OL	Oleuropein
OLE	Olive leaf extracts

OMW	Olive mill wastewater
p-HPEA	*p*-Hydroxyphenyl ethanol
p-HPEA-EA	Ligstroside aglycone
p-HPEA-EDA	*p*-Hydroxyphenyl ethanol-decarboxymethyl elenolic acid dialdehydic form
PLE	Pressurized liquid extraction
QqQ	Triple quadrupole systems
ROS	Reactive oxygen species
SE	Soxhlet extraction
SHLE	Super Heated Liquid Extraction
SMC	Smooth muscle cell
SPE	Solid-phase extraction
TNF-α	Tumor necrosis factor alpha
TOF	Time of flight
UAE	Ultrasound-assisted extraction
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
VCAM-1	Vascular adhesion molecule-1
VOO	Virgin olive oil

1 Introduction

The Mediterranean diet has been associated with longevity and low incidence of chronic and degenerative diseases as shown in the Seven Countries Study [1–4] and several others studies, which attribute to the virgin olive oil (VOO) many of the healthy advantages of this diet [5–7].

The olive is an important crop in the Mediterranean basin, which produces 98% of the world total (approximately 11 million tons) [8]: it is estimated that seven million hectares is planted with around 600 million productive olive trees including more than 1275 autochthonous cultivars. Many customers identify olive oil as a food product that perfectly combines nutritional and sensory values, so that the production and consumption of olive oil are moving slowly but inexorably beyond the Mediterranean countries, and olive trees are being planted in countries as far from the Mediterranean basin as New Zealand and Argentina [9]. Historically, the healthful effects of VOO have been attributed to its high content of monounsaturated fatty acids (MUFAs), such as oleic acid, which represents 70–80% of fatty acids present in VOO [3]. However, several seed oils (including soybean, sunflower, and rapeseeds) rich in MUFA do not possess the same beneficial properties [10, 11]. Noteworthy, contrary to majority of edible oils, VOO can be consumed in crude form, conserving all the beneficial properties typical of its minor components. This unsaponifiable fraction of olive oil represents about 2% of the total weight



1 R=OH, R′=Me; oleuropein (OL)
2 R=OH; R′=H; demethyloleuropeine
3 R=H; R′=Me; ligstroside

4 R=OH; 3,4-DHPEA-EA
5 R=H; *p*-HPEA-EA

6 R= OH; 3,4-DHPEA-EDA
7 R= H; *p*-HPEA-EDA

Fig. 119.1 Chemical structures of OL derivatives

and includes a number of heterogeneous compounds (more than 230): some nonchemically related to fatty acids such as hydrocarbons, alcohols, sterols, volatile compounds, and antioxidants and fatty acid derivatives such as phospholipids, waxes, and sterol esters, as the most important [12, 13].

Virgin olive oil is a premium and highly priced product thanks to the nutraceutical properties of its antioxidant components. The most abundant antioxidants in VOO are lipophilic and hydrophilic phenols that affect the healthy and sensory characteristics of VOO and improve its resistance to oxidation [14]. Further, they are produced as a consequence of the reactivity to pathogen attacks and the response to insect injuries in the olive tree, thus playing an important role in the plant physiology [3, 15, 16]. Among the antioxidant compounds in VOO, the hydrophilic phenolic alcohols and their secondary metabolites are of paramount importance to keep a long oil shelf life and provide excellent organoleptic characteristics (taste and color) and beneficial effects on human health [14, 17, 18]. The main phenolic alcohols in VOO are 3,4-dihydroxyphenyl ethanol, also known as 3,4-DHPEA or hydroxytyrosol (HT), and *p*-hydroxyphenyl ethanol (*p*-HPEA or tyrosol) and their secoiridoid derivatives [3, 14].

The most abundant secoiridoids in intact olive fruit are oleuropein (OL), demethyloleuropein, ligstroside, and nüzhenide. During mechanical oil extraction, several hydrolysis reactions of OL, demethyloleuropein, and ligstroside take place due to the activity of endogenous β-glucosidases [19], and aglycone derivatives originate known as VOO secoiridoids. Thus, the most abundant in VOO are the dialdehydic form of decarboxymethyl EA (elenolic acid) linked to HT or tyrosol termed 3,4-DHPEA-EDA and *p*-HPEA-EDA, an isomer of OL aglycone (3,4-DHPEA-EA) and the ligstroside aglycone (*p*-HPEA-EA) (Fig. 119.1) [19].

The concentration of secoiridoids is largely affected by agronomic factors (cultivar, ripening stage, geographic origin of olive fruit, and olive trees irrigation) and by oil extraction conditions during crushing, malaxation, and VOO separation [20–22]. These compounds are present in olive fruit and are released to VOO during the mechanical extraction process. OL, discovered in 1908 by Bourquelot and Vintilesco, is a heterosidic ester of elenolic acid and HT [23]. HT is its principal degradation product [24]. OL is present in high amounts in unprocessed olive fruit and leaves, while HT is more abundant in the processed olive fruit and olive oil. The decrease in the concentration of OL and the increase in the concentration of HT occur due to chemical and enzymatic reactions that take place during maturation of the fruit or as a result of olive processing (e.g., oil production) [17, 19].

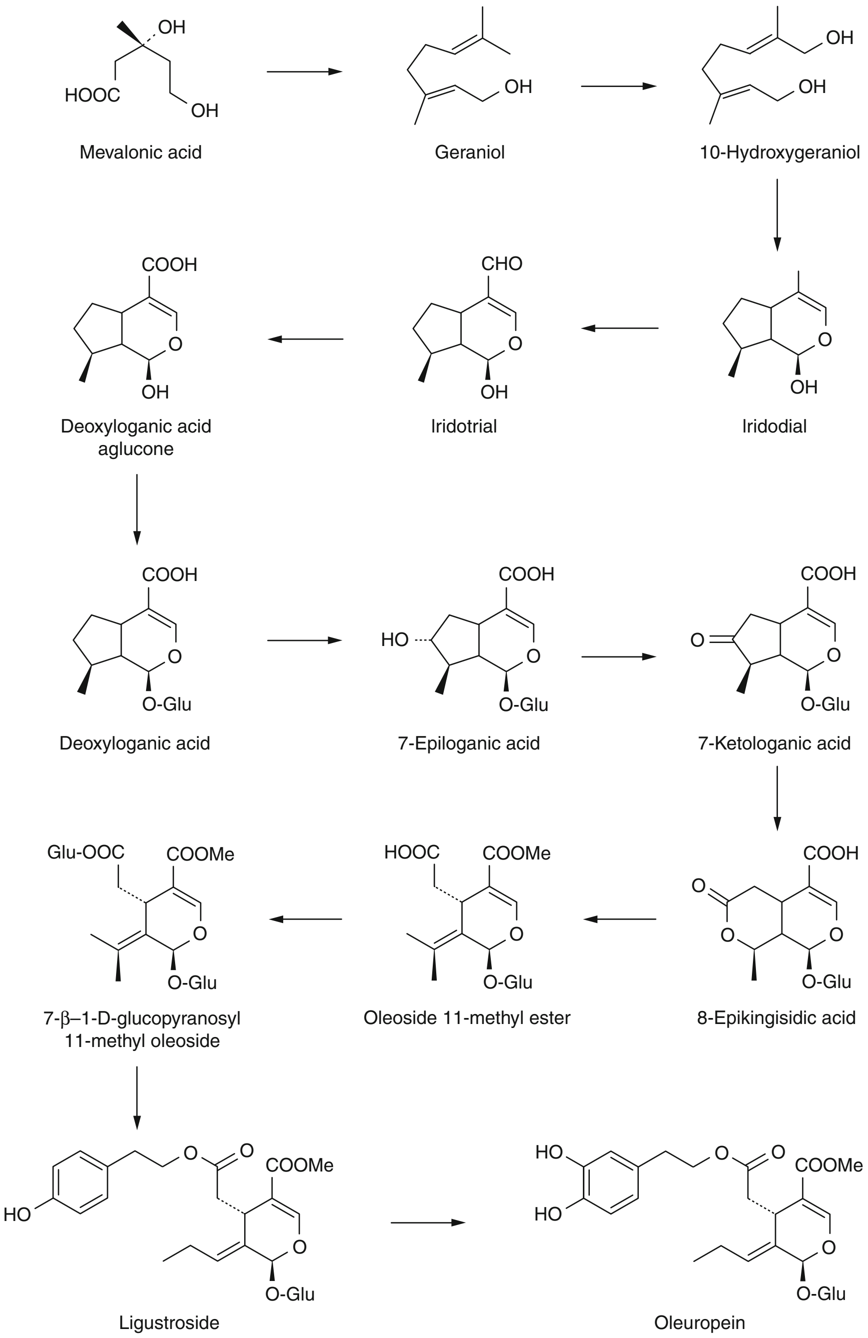
Among the all above-mentioned compounds, it is possible to consider the OL and its derivatives as the molecules of major interest for their biological and pharmacological properties and resulting among the most investigated antioxidant natural compounds [7, 14, 24].

2 Chemistry and Analysis of Oleuropein and its Derivatives

2.1 Chemical Structure, Biosynthesis, and Enzymatic Degradation of Oleuropein

Oleuropein (OL) and ligstroside are the major phenolic compounds in the olive fruit. OL belongs to the family of secoiridoids, which are abundant in *Oleaceae*, *Gentianaceae*, *Cornaceae*, as well as many other plants. Iridoids and secoiridoids are compounds that are usually glycosidically bound and are produced from the secondary metabolism of terpenes as precursors of various indole alkaloids. The secoiridoids in *Oleaceae* are usually derived from the oleoside type of glucosides (oleosides), which are characterized by an exocyclic 8,9-olefinic functionality, a combination of EA and a glucosidic residue. OL is an ester of HT and has the oleosidic skeleton that is common to the secoiridoid glucosides of *Oleaceae*, mainly in its aglycone form, which makes the sugar moiety insoluble in oil [24].

Probably, the biosynthesis of OL in *Oleaceae* proceeds via a branching in the mevalonic acid pathway in which terpene synthesis (oleoside moiety) and phenylpropanoid metabolism (phenolic moiety) merge [25–28], giving in the *Oleaceae* family the plausible biosynthetic route proposed, which, starting from deoxiloganin acid, produces ligstroside and then oleuropein oleosides [26–28]; thus, the biosynthesis of oleosides is similar to that of secologanin-derived secoiridoids in *Gentianales* and *Cornales*. In these compounds, the carbon skeleton is derived from mevalonic acid. Geraniol, 10-hydroxygeranoil as well as 10-hydroxynerol, and iridoidal are known precursors of loganin. Later, deoxyloganic acid, 7-epiloganic acid, and loganic acid are incorporated into ligustroside, a direct precursor of oleuropein, via a 7-ketologanic acid intermediate. The sequences of the steps between deoxyloganic acid and 7-ketologanin may differ between plant species and times of the year [28]. In *Olea europaea*, both


OH
HOOC
OH
Mevalonic acid
OH
Geraniol
OH
OH
10-Hydroxygeraniol
CHO
OH
Iridodial
CHO
O
OH
Iridotrial
COOH
O
OH
Deoxyloganic acid aglucone
COOH
O
O-Glu
Deoxyloganic acid
COOH
HO
O
O-Glu
7-Epiloganic acid
COOH
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7-Ketologanic acid
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HO
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O
COOMe
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O-Glu
Ligustroside
HO
HO
O
O
COOMe
O
O-Glu
Oleuropein

Scheme 119.1 Proposed biosynthetic pathway for OL in *Oleaceae* (From [7]) possible epoxides of secologanin and secoxyloganin can be precursors for OL [27], and a plausible biosynthetic route from deoxyloganic acid, 7-epiloganic acid, 7-ketologanic acid, 8-epikingisidic acid, oleoside 11-methyl ester, 7-β-1-D-glucopyranosyl-11-methyl oleoside, and ligustroside to OL was proposed by Damtoft et al. for *Oleaceae* (Scheme 119.1) [27, 29].

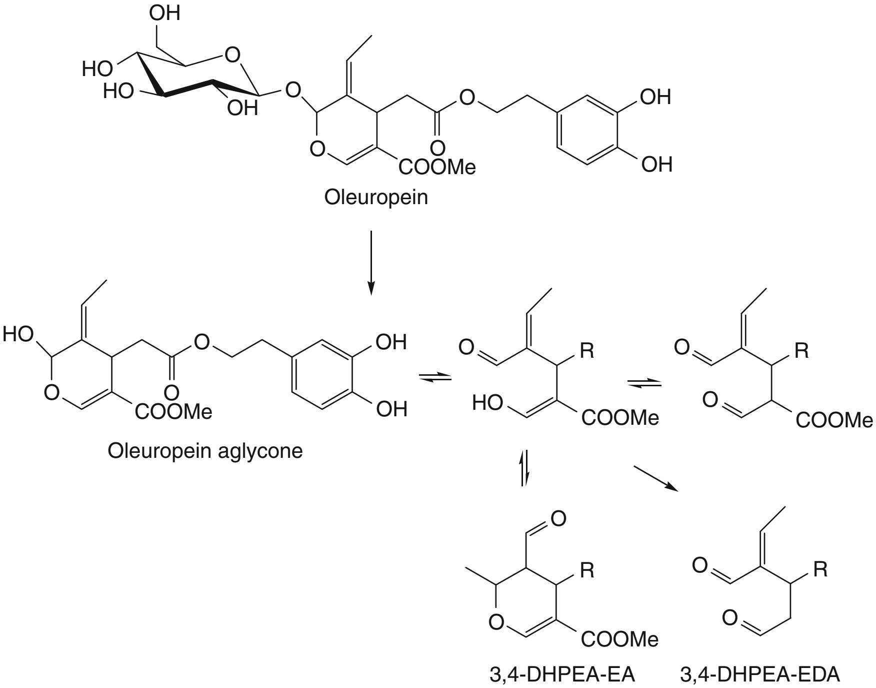
Phenols, particularly secoiridoids, generally decrease during VOO storage due to its hydrolysis in HT, tyrosol, and EA and formation of oxidized phenols. This reaction leads to a decrease in bitterness and pungent intensity, positive attributes that are characteristics of fresh VOO [30]. In the development of the olive fruit, three phases are usually distinguished: a growth phase, during which accumulation of OL occurs; a green maturation phase that coincides with a reduction in the levels of chlorophyll and OL; and a black maturation phase characterized by the appearance of anthocyanins and decrease of the OL levels [31]. Therefore, OL is very abundant in the early stages, and in young fruits, it can reach 14 % of dry matter [7]. Although lower, its levels are still very important at harvest for green picked cultivars [31]. In black cultivars, its levels decline rapidly during maturation [31, 32] in some varieties (*Olea europaea* var. *leccino*), falling to zero when the fruits are completely black [33]. Degradation of OL during maturation is accompanied by accumulation of demethyloleuropein and EA glucosides. The former replaces OL in about the same amount and constitutes the major components of black olive fruit [26, 31, 34–37].

So, EA glucoside and demethyloleuropein reach their maximum during black maturation, until demethyloleuropein becomes the major constituent of black olives [33]. It is possible that these two compounds are formed from OL by the action of esterases because esterase activity increases considerably during the first phase of maturation and reaches a maximum during the black maturation phase.

The degradation of OL occurs by two pathways: the first involves cleavage of OL to EA or demethyloleuropein by specific endogenous esterases, which are both found in mature olive fruit, which may produce the aglycone from the glycoside [38]; the second includes activation of β-glucosidases during crushing and malaxation of the fruits, which may produce the aglycone from the glycoside (Scheme 119.2) [39]. It is presumed that demethyloleuropein acts as a precursor for the formation of 3,4-DHPEA-EDA during crushing. However, high concentrations of the latter are found in cultivars characterized by low demethyloleuropein concentration. These results indicate that the concentration of 3,4-DHPEA-EDA is dependent not only on the concentration of demethyloleuropein but also on that of OL decomposed by methylesterase. During malaxation, the final composition of secoiridoid derivatives is mainly controlled by the activity of β-glucosidase.

2.2 Analytical Methods for Oleuropein Derivative Determination

Several different analytical approaches for the characterization of the secoiridoid profile, from both a qualitative and quantitative point of view, have been studied and applied in the last decade. Essentially two basic steps are required for analysis



Scheme 119.2 Degradation pathway of OL

of OL derivatives: extraction from the sample and separation/detection. The choice of the specific extraction method strongly depends on the sample characteristics, while the analytical technique to apply depends on the degree of information demanded [32, 40]. Besides the classical OL derivative sources (olive fruits and leaves, VOO), there are other cheaper sources coming from wastes of olive oil production, such as the olive mill wastewater (OMW) [41] and the alperujo, a semisolid residue obtained from the two-phase decantation used in olive oil industry [32]. Table 119.1 summarizes the analytical methods for quantification and identification of OL derivatives pointing out the average concentration of the most abundant derivatives in different matrix.

2.2.1 Extraction from the Matrix

With liquid samples as oils, OL isolation has been realized by two main techniques: liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [14, 73]. LLE is based on transfer of the phenolic fraction from VOO to a more hydrophilic phase such as pure methanol or methanol-water mixtures with different alcohol concentrations [42–44]. Ultrasounds can be used as auxiliary energy to accelerate and improve LLE. The main advantages of ultrasound-assisted LLE are shortened extraction time, reduced reagent and sample volume, and improved extraction efficiency [32, 48]. After extraction, cleanup and pre-concentration steps are

Table 119.1 Analytical methods for quantification and identification of OL derivatives

Entry	Matrix	Oleuropein derivative	Quantity range (g/Kg matrix)	Extraction technique	Separation technique	Identification technique
1.	VOO	3,4-DHPEA-EA	0.030–0.229	LLE [42–44]	GC [45–47]	UV [46]
						MS-EI [45]
		3,4-DHPEA-EDA	0.218–0.308	UAE [40, 48]	HPLC [44, 49]	MS-ESI [44, 49]
						APCI [50]
				SPE [45, 49, 51]	CE [52–55]	^{1}H-NMR [56, 57]
						Raman [58]
2.	Leaves	OL	6–133	SE [59–61]	HPLC [59, 60]	UV-AD [59, 62]
				SHLE [62, 63]		MS-ESI [64]
				PLE [65]		^{1}H-NMR [60]
				MAE [64]		MIR [61]
3.	Fruits	OL	0.17–3.25	SE [66]	HPLC [66, 67]	UV-AD [66, 67]
		DemethylOL	0.02–0.43	SPE [67]		MS-ESI [67, 68]
				UAE [69]		NMR [66]
4.	Alperujo	OL	0.64	UAE [70]	CE [70]	–
5.	OMW	OL	1.00–2.00[a]	LLE [41, 71]	–	MS-ESI [41]
		HT	12.0–16.0[a]			

[a] Expressed as g/L [72]

required to isolate OL derivatives [42]. SPE is based on the separation of the hydrophilic compounds of VOO from the lipophilic matter by fractionation between an unpolar sorbent packed in a cartridge and a polar solvent as methanol. SPE is a versatile technique thanks to the range of different sorbents and eluents [45, 49, 51]. The main advantage of SPE is its dual action of extraction and cleanup.

For solid samples, a time-consuming protocol is generally required. Olive leaves are normally extracted as dry powered matter, and the temperature in the drying step is strictly controlled, as OL is relatively thermolabile. The extraction methods used are maceration at room temperature or Soxhlet extraction (SE) using different solvents [59–61]. Super Heated Liquid Extraction (SHLE) [62, 63] and pressurized liquid extraction (PLE) using food-grade solvents [65] are some of the new techniques to valorize this cheap waste of oil production in a greener way. Moreover, olive leaves can be extracted using classical methanol/water mixture with a nonconventional microwave-assisted extraction (MAE) [64]. The whole olive fruits are normally blended and dried, but the OL derivative profile and the quantity of the extracted desired product strongly depend on the pretreatment and extraction procedure applied. The highest quantity of OL is obtained when the fruits are frozen before blending, to avoid the hydrolytic β-glucosidases action, and then extracted by maceration in a solvent [69]. When an acid or basic pretreatment is done to leach secoiridoids from the olive mesocarp, OL aglycone and hydroxytyrosol are the derivatives obtained in higher quantity [67]. Ultrasound-assisted extraction (UAE) applied on freeze-dried fruits was able to increase OL amount up to 33 % [66].

Semisolid matter, such as alperujo, has rarely been exploited for obtaining OL derivatives. The samples may be taken directly from the production line and stored at 20 °C until analysis. The extraction is realized using microwave or ultrasound assistance [70]. In particular, in the continuous UAE method, the sample is located in the leaching chamber, which is subjected to the action of an ultrasound probe; meanwhile, the leaching liquid is recirculated in a closed circuit for proper mass transfer to the liquid phase. No degradation of the target compounds was observed under the optimal working conditions for leaching [70].

Olive mill wastewater, generated by the oil extraction industry, represents a severe environmental problem due to its highly polluting organic load. However, with its more than 30 phenolic compounds, OMW is also regarded as a potent source of natural antioxidants [72]. Several techniques are used individually or in combination form to recover the phenolic compounds from olive OMW. Among them, solvent extraction is the most widely used technique for recovering OL and OL aglycones avoiding its hydrolysis to HT [72]. Emmons et al. [71] patented a process for collecting OL aglycone from OMW that involves addition of citric acid to the raw material, subsequent heating in order to precipitate the solids, and extraction of OL aglycone with a nonpolar organic solvent mixture. More recently, Cardoso et al. [41] proposed a method where the OMW was firstly freeze-dried, then defatted with n-hexane, and the residue was extracted with methanol.

2.2.2 Separation, Identification, and Quantification

The individual separation, identification, and quantification of OL derivatives from natural extracts have been realized basically by chromatographic separation or capillary electrophoresis methods. Both gas and liquid chromatographies have been exploited, combined with several detection methods such as UV, fluorescence, and mass spectrometry (MS) [14]. Soft spectroscopic techniques as midium infrared spectroscopy have been recently explored for rapid quantification of OL [61], while high-resolution spectroscopic techniques as nuclear magnetic resonance are considered interesting application in the analysis of the OL derivative structures [14].

Gas chromatography (GC) is a high-resolution separation technique based on the interaction of the sample components with a stationary phase and along a temperature gradient. GC assumes that the analytes are volatile at the temperature of the analysis and remain stable during analysis. Due to the low volatility and thermal stability of phenolic compounds, a suited derivatization step is mandatory in order to increase volatility and thermostability of the analytes. Derivatization of phenolic compounds is mostly based on silylation reactions: phenolic compounds are separated as more volatile trimethylsilyl esters, thus decreasing the temperature required for chromatographic separation. Ríos et al. proposed a simple and versatile method for silylation of phenolic compounds in VOO after an SPE diol cartridge extraction and cleanup procedure. Oxidation products coming from the aldehydic and dialdehydic forms of OL aglycones were detected [46]. In general, MS supported this technique as an excellent tool for identification [74]. Further improvement to GC-MS was obtained by the introduction of the ion trap (IT) and GC-tandem MS (GC-MS/MS) that provide excellent sensitivity and selectivity for target compound analysis [47, 75].

Liquid chromatography (LC) is nowadays the most popular technique for VOO phenols analysis because of the combination of high resolution and applicability to nonvolatile compounds. Among the different chromatographic modes, reverse-phase LC is the preferred option for phenols separation [76]. Over the past few years, various liquid chromatographic methods with fluorescence, UV-Vis (ultraviolet-visible) or DAD-UV (diode array detector-ultraviolet) absorption, and, more recently, with MS detection have been developed for the analysis of phenols [44, 45, 68, 76]. Using traditional approaches based on high-performance liquid chromatography (HPLC)-DAD or UV detection, spectra of phenolic compounds are very similar, and the possibility of unambiguous identification does not exist. Mass spectrometry coupled to HPLC or UPLC (ultra performance liquid chromatography) enables the collection of significant data on the structures of those compounds that show similar UV-Vis spectra [44, 76]. At present, in LC-MS analysis of phenolic compounds, atmospheric pressure chemical ionization (APCI) [50] and electrospray ionization (ESI) [49] are used almost exclusively, and both positive and negative ionizations are applied. The selection of the analyzer is determined by the required sensitivity and selectivity. IT or triple quadrupole systems (QqQ) provide the possibility of doing MS/MS or MS^n [77]. Di Donna et al. have recently applied a UPLC-ESI-MS/MS analysis to the separation and identification of the dialdehydes affording from the enzymatic hydrolysis of OL and of its demethylated analogue [78]. Finally, TOF (time of flight) MS, which is one of the most advanced MS analyzers, provides excellent mass accuracy and allows measurements of the correct isotopic pattern [79].

Capillary electrophoresis (CE) is a good alternative to GC and LC techniques for profile determination of OL derivatives. CE requires minimal sample preparation and represents a good compromise between analysis time and satisfactory characterization. The most efficient operative mode to separate phenolic compounds is the borate-based CE, which makes use of a borate run buffer at alkaline pH [52]. To date, the most widely used detector in CE is based on UV absorption [53, 54], although the coupling to MS analyzers such as QqQ, IT, TOF has revalorized the potential of this technique [55].

A new perspective for determination and quantification of OL in natural extracts is represented today by the medium infrared spectroscopy (MIR). It achieves high analysis speed and requires little or no sample preparation. Aouidi et al. have developed a direct and rapid tool to quantitative determination of OL in olive leaves by MIR associated to chemometric treatment [61].

Raman spectroscopy coupled to theoretical methods was also used to fully characterize OL derivative profile of olive oils. The Raman data, assisted by the theoretical simulations, allowed obtaining the main spectroscopic features of the olive oil constituents which determine their antioxidant and chemoprotective properties [58].

Finally, high-resolution proton nuclear magnetic resonance (^{1}H-NMR) has been a useful technique for identification and structural assignment of several OL derivatives. In the past, ^{1}H-NMR has permitted to elucidate the hydrolysis mechanism of OL that gives rise to aglycone contributing to recognize its five different isomeric forms in solution [66, 80]. Nevertheless, NMR identification of complex mixture of different derivatives in natural extracts is affected by several problems such as signal overlap or diversity of intensity due to various concentrations, finally leading to an incomplete assignment [56]. A useful approach to overcome this problem involves the combination of ^{1}H-NMR and chromatography. A good agreement of HPLC-DAD/MS and 1D and 2D NMR spectroscopy has been obtained by Valli et al. [57], by comparing the sum of monoaldehydic and dialdehydic forms of OL derivatives after microwave heating in VOO. The most promising application of ^{1}H-NMR is targeted to qualitative analysis by generation of characteristic fingerprinting registers to improve VOO quality [81].

3 Bioavailability

In addition to their effect on VOO stability and human health, phenolic and volatile compounds are the main responsible for the sensory attributes of VOO (e.g., bitter, astringent, pungent, throat catching), providing this oil with its delicate and unique flavor highly appreciated by costumers [6, 82, 83]. Recent studies showed that the sensory intensity of the bitterness attribute in VOO is associated mainly to secoiridoid derivatives of hydroxytyrosol, particularly 3,4-DHPEA-EDA and 3,4-DHPEA-EA [79]. Most importantly, VOO phenols are well adsorbed by the intestine and enter the blood circulation [84] to constitute one of the bases of nutritional and therapeutic effects of VOO. Recent papers have pointed out that HT, OL, and 3,4-DHPEA-EA reach human blood concentrations ranging from 1 to 18 μM [85, 86]. The structural similarity of 3,4-DHPEA-EA and 3,4-DHPEA-EDA with OL and their higher lipophilicity [log P(3,4-DHPEA-EDA) = 1.02; log P (OL) = 0.13] [87] suggest that these compounds may also be adsorbed into the bloodstream, according to the "Lipinski rule of 5" [88]. Moreover, both 3,4-DHPEA-EDA and 3,4-DHPEA-EA show good stability after 48 h at acidic pH values (pH 3.5 and 5.5) and do not change significantly in acidified water up to 4 h [89]. These studies suggest that these compounds may survive the acidic conditions of stomach and be available for absorption.

Vissers et al. found that absorption of administered HT and OL aglycone was 55–60 % in human subjects [90]. They also suggested that an important step in the metabolism of olive oil phenolics OL and ligstroside aglycones is their transformation into HT or tyrosol. This hypothesis was supported by finding that 15 % of OL supplement administered to healthy human subjects was excreted in urine as HT [90]. OL is rapidly absorbed after oral administration with a maximum plasma concentration occurring 2 h after administration. Both compounds (OL and HT) are rapidly distributed and excreted in urine mainly as glucuronides or in very low concentrations as free forms [91, 92]. Many other reports confirmed that HT is renally excreted: while part of HT is unchanged, an aliquot is also metabolized, and over 90 % of the urinary metabolites are conjugated [90, 93–96], mainly glucuronide metabolites, yet free phenols and methylconjugates, with or without glucuronidation, were also excreted in human urine. Sulfoconjugates of HT, tyrosol, or their metabolites (methyl or glucuronide conjugates) have been observed only in animal experiments [94, 97, 98].

Tuck et al. investigated the in vivo fate of HT after intravenous and oral dosing of either tritium labeled compound to rats [97]. The elimination of radioactivity in urine within 24 h for the intravenously and orally administered oil-based dosing was significantly greater than the oral, aqueous dosing method. HT is quantitatively transported into the small-intestinal epithelial cells by passive diffusion [99]. Data from in vivo experiments with animals and humans have confirmed that olive oil phenolics are well absorbed at the intestinal level [97]. Not the entire quantity of tyrosol and HT that was given was subsequently found in the urines; whether the remaining amount is not absorbed, excreted with the feces, destroyed in the gut, accumulated in organs or circulating cells such as red blood cells, or excreted after 24 h remains to be elucidated. Anyway, tyrosol and HT are absorbed by humans in a dose-dependent manner [93]. These observations indicate that the first-pass intestinal/hepatic metabolism of the ingested phenolics is extensive [100]. However, data regarding the metabolism of olive oil phenolics in the human body are very limited, and contrasting results have been obtained regarding the amounts and forms in which they are present in plasma and excreted in urine [92, 94–98, 101, 102]. HT appeared in plasma minutes after oral administration, with maximal concentrations of HT obtained in the 5–10-min period and minimal concentrations of HT observed after 60 min [103].

In situ intestinal perfusion on the absorption of OL showed that OL was absorbed under iso-osmotic conditions, with a permeability coefficient of 1.47×10^{-6} cm/s [104], and it was postulated that this increase was due to an enhancement in paracellular movement facilitated by opening of the paracellular junctions. OL could be poorly absorbed from the isolated perfused rat intestine [104].

Limited metabolism of olive oil phenols was observed using Caco-2/Tc7 cells as model of human intestinal epithelium: the methylated conjugates were the major metabolites detected [105]. Manna et al. investigated the kinetics of ^{14}C-labeled HT intestinal transport and metabolism in Caco-2 cells [99]. They observed that the rate of HT was linear, the intestinal transport system was not saturable, and transport occurred by a passive diffusion mechanism. It is likely that HT will be 100 % absorbed in humans. The only labeled metabolite found in this study was homovanillic alcohol (4-hydroxy-3-methoxy phenylethanol), a methylated derivative of HT which is a product of intestinal catechol-*O*-methyl-transferase activity [99]. A recent article by D'Angelo et al. investigated the fate of radiolabelled HT (^{14}C) in rats [106]. Pharmacokinetic analysis revealed that the HT is quickly absorbed. Over 90 % of the administered radioactivity is excreted in urine after 5 h indicating that renal excretion represents the preferential disposition, whereas about 5 % of radioactivity was observed in feces and gastrointestinal tract [106]. HT is converted to four oxidized and/or methylated derivatives. These metabolites were tentatively identified as homovanillic alcohol, homovanillic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylacetaldehyde, and sulfoconjugated derivatives.

To study the potential hepatic metabolism of olive oil phenols, human hepatoma HepG2 cells were incubated for 2 and 18 h with HT and HT acetate. Methylated and glucuronidated forms of HT were detected at 18 h of incubation, together with methylglucuronidated metabolites. HT acetate was largely converted into free HT and subsequently metabolized, yet small amounts of glucuronidated HT acetate were detected. The absence of the formation of sulfated metabolites in vitro is in agreement with data from human studies, in which methylated and glucuronidated metabolites were the only conjugates observed in plasma and urine samples [90, 93, 95, 96, 98, 99]. Sulfate metabolites were detected in urine from animals only after intravenous administration of HT [10, 94, 97], indicating that rat hepatocytes are capable of sulfation of this phenol. Nevertheless, when the in vitro sulfation of olive oil phenolics was attempted using microsomes obtained from rat liver, no metabolites could be detected after 2 h of incubation [107].

4 Biological Activity and Pharmacological Properties

In the last decades, many biological properties have been assigned to the olive tree phenols, investigated both as whole oil/leaf/fruit extracts and isolated compounds [7, 108–110]. The effects of the latter are of particular interest in view of a pharmacological use of the purified/neosynthesized compounds as single drug or after enrichment of olive oil or other food component. OL and its derivatives have shown antioxidant, anti-inflammatory, antiproliferative, antimicrobic, and antiviral activity, so representing promising pharmacological agents for the protection against/treatment of many human diseases, including cardiovascular diseases, cancer, and many others (Fig. 119.2). Some differences have been reported in the observed effects when single phenols were tested in comparison with “oil phenolic extracts” probably because some biological effects may derive from the mixture of various derivatives generated by enzymatic hydrolysis. In the following paragraphs, only the effects of OL, 3,4-DHPEA-EA, and HT will be examined, focusing on the molecular mechanism underlying their action, as resulting from in vitro and in vivo experimentation performed using isolated compounds.

4.1 Antioxidant Activity

Oxidation is a process naturally occurring during VOO storage by reaction with air. Initially, lipids are oxidized to hydroperoxides which decompose through homolytic cleavage of the hydroperoxide group, giving rise to volatile oxidation products responsible for typical unpleasant sensory characteristics [111, 112]. Moreover, oxidation is a degenerative process in biological systems due to the endogenous reactive oxygen species (ROS). ROS are the result of normal metabolic processes, and they can damage cellular macromolecules as proteins or DNA, possibly leading to the development of degenerative diseases. Phenolic compounds in general and OL derivatives in particular can act as natural antioxidant in various ways, thus becoming important molecules both for the food stability and the human health [3]. The antioxidant activity in food can be explained by the so-called polar paradox: in

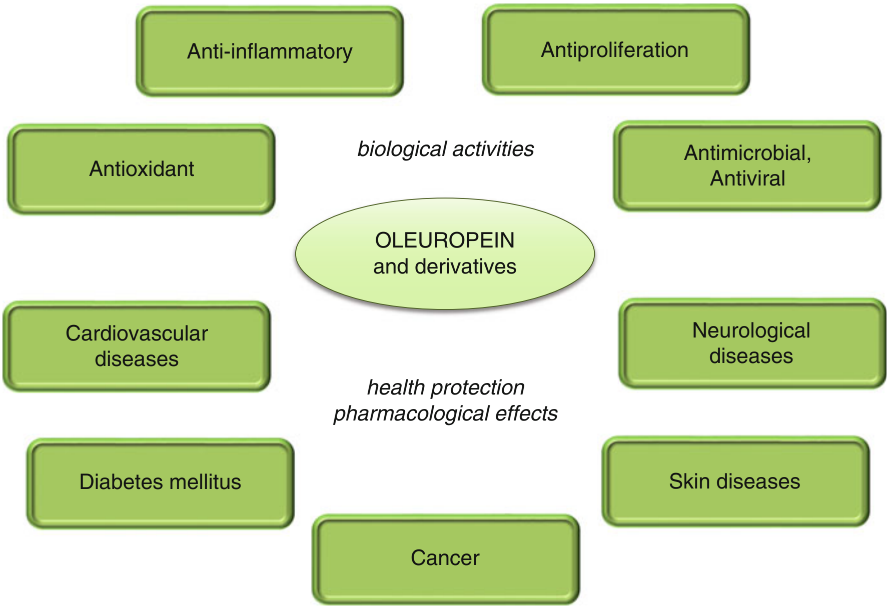
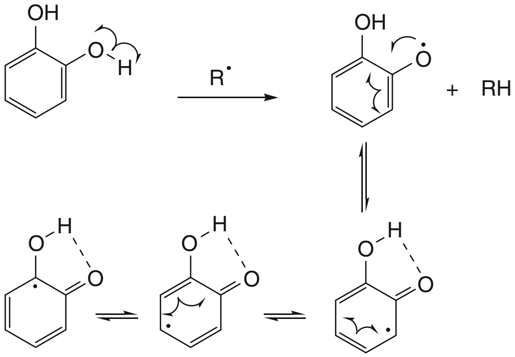

Anti-inflammatory
Antiproliferation
Antioxidant
biological activities
Antimicrobial, Antiviral
OLEUROPEIN and derivatives
Cardiovascular diseases
Neurological diseases
health protection pharmacological effects
Diabetes mellitus
Skin diseases
Cancer

Fig. 119.2 Biological and pharmacological effects of oleuropein and derivatives

a bulk oil system, the hydrophilic antioxidants such as OL derivatives are oriented in the air-oil interface, thus protecting lipophilic molecules against oxidation [113]. Endogenous defense against ROS includes the action of protective enzymes as superoxide dismutase, catalase, and glutathione peroxidase. When the defensive mechanism cannot balance the action of the free radicals, the subsequent cellular damages contribute to the pathogenesis of various diseases, as atherosclerosis and cardiovascular diseases, diabetes mellitus and metabolic syndrome, and skin and neurodegenerative diseases. In addition, the genotoxic effects of ROS have been correlated with the onset of cancer [114, 115]. Both natural and synthetic antioxidants are therefore of great interest for the disease prevention and treatment. The antioxidant activity of phenols in vivo is strictly correlated to their bioavailability, absorption, metabolism, and pharmacokinetics [116]. Depending on their action, the antioxidant can be classified as (a) free radical scavengers and radical chain breaking (primary antioxidants), (b) anti oxygen radicals (reducing and antioxidant molecules), and (c) metal chelators. OL derivatives can cover all of these three roles. They can both break the chain of reaction triggered by free radicals and scavenge the peroxyl radicals thanks to their catecholic structure: the ortho-diphenolic group in the HT moiety has the ability to form intramolecular hydrogen bonds between the hydroxyl group and the phenolic radical (Scheme 119.3). From the study of the resonance structures formed, the ortho-disubstituted radicals have higher stability than the meta-disubstituted and mono-substituted phenols [3].

The antioxidant properties of OL and its derivatives have been demonstrated in several in vitro and in vivo experimental models [7, 108, 109]. A strong chelating

Scheme 119.3 Resonance structures of phenolic radical



effect on metals as iron or cupper has been reported to diminish the appearance of ROS derived from reactions associated with these metals [117, 118]. In addition, the antioxidant activities of OL and HT have also been demonstrated using metal-independent oxidative systems [119] or measuring stable free radicals, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [120, 121]. In particular, the ability to scavenge or reduce the generation of ROS has been reported in intact leukocytes treated with phorbol 12-myristate 13-acetate as well as in a hypoxanthine/xanthine oxidase cell-free system using a chemiluminescence method to detect ROS generation [103, 122]. Finally, a scavenging effect of OL and HT was demonstrated in a model of hypochlorous acid (HOCl)-mediated inactivation of catalase [103].

Particular attention has been focused on the scavenger ability of inhibiting the process of low-density lipoprotein (LDL) cholesterol oxidation, since it represents a major prevention mechanism against atherosclerosis (see next paragraph). Various experimental evidences, using in vitro and in vivo preclinical models, showed a strong action of both OL and HT [109, 117]. Moreover, OL and HT were able to reduce 5-lipoxygenase-driven cellular recruitment of leukocytes and the damaging consequences of their ability to release ROS while leaving unimpaired the generation of prostaglandins, which promote microvascular blood flow and act as immunomodulators [122].

It has been suggested that protection by HT against oxidative stress is also conferred by the simultaneous activation of two critically important pathways, phase II detoxifying enzymes (a set of enzymes, such as glutathione S-transferase, heme oxygenase-1, NAD(P)H quinine oxidoreductase-1, and γ-glutamyl cysteine ligase, that lead to an increase in levels of endogenous antioxidants) and mitochondrial biogenesis [123]. Recently, Domitrovic et al. [124] have demonstrated that OL, in vivo, induces the antioxidant enzyme heme oxygenase in the liver. This enzyme catalyzes the degradation of heme into iron, carbon monoxide, and bilirubin, the latter of which provided with cytoprotective activity. Interestingly, 3,4-DHPEA-EA has revealed to be even stronger than oleuropein, as antioxidant as well as in other activities [125].

4.2 Anti-inflammatory Activity

The anti-inflammatory effects of olive oil phenolics, and particularly OL and HT, in humans have been described in many studies [7, 108–110]. When added to murine macrophages stimulated with a bacterial lipopolysaccharide (LPS), OL enhanced the functional activity of these immune-competent cells, as evaluated by a significant increase in the production of nitric oxide [126]. This increase was the consequence of a direct tonic effect of OL on the inducible form of the enzyme nitric oxide synthase (iNOS), as demonstrated by Western blot analysis of cell homogenates and by co-incubation of LPS-challenged cells with the iNOS inhibitor L-nitromethylarginine methylester. Also, OL elicited anti-inflammatory effects inhibiting leukocyte lipoxygenase activity and reducing the production of proinflammatory molecule leukotriene B4 [122]. Similarly, Maiuri et al. [127] reported that HT impeded the synthesis of prostaglandin E2 by indirectly blocking the enzymes iNOS and cyclooxygenase (COX)-2. This effect arose from the prevention of the activation of mouse macrophages subsequent to block of transcriptional activation of NF-kB, interferon regulatory factor-1, and transducer and activator of transcription 1. Suppression of COX-2 and iNOS expression was also described in human monocytes treated with HT [128]. To test the anti-inflammatory effect of OL and HT, De la Puerta et al. [129] studied a mouse model with inflammation induced in the ear by arachidonic acid and/or phorbol esters. These researchers found that the topical application of the two phenols inhibited the edematous tissue with a reduction of swelling related to the lower infiltration of neutrophils, as measured by the inhibition of myeloperoxidase. In another in vivo model of acute inflammation induced by intravenous injection of carrageenan, Gong et al. [130] demonstrated that a preparation containing HT (22 %) was able to inhibit both the acute inflammation as well as the pain: the analgesic action was not dose dependent, and levels of mRNA of the inflammatory cytokines interleukin-1β and tumor necrosis factor alpha (TNF-α) lowered without raising those of the anti-inflammatory cytokine interleukin-10 [130]. More recently, to gain insight into the mechanism of action and pharmacological value of OL on the inflammatory bowel disease, Giner et al. evaluated its efficacy in a dextran sulfate sodium-induced colitis in mice, associated with the inhibition of proinflammatory protein expression and release as well as block of NF-κB signaling [131].

Considering the functions of the prostaglandins and leukotrienes, the results of these studies have important implications not only for the genesis of the inflammatory response but also for the thrombotic processes, in particular platelet aggregation (see next paragraph). Thus, HT was found to inhibit in vitro platelet aggregation induced by collagen and thromboxane B2 production [132]. Moreover, González-Correa et al. [133] found a reduction in the synthesis of thromboxane A2 measured by the reduction of its metabolite, thromboxane B2, mainly due to the inhibition of activity of the COX. For these authors, the antithrombotic effects would be caused by decline in the production of vascular prostacyclin, effects similar to those determined by acetyl salicylic acid.

Recently, it has been shown that ligstroside aglycone (oleocanthal) inhibits COX-1 and COX-2 activity in the same way as the anti-inflammatory drug ibuprofen does [134]. Starting from these results, we speculated a similar activity for 3,4-DHPEA-EA, and for this reason, many efforts have been spent in our laboratory to design synthetic protocols that, starting from renewable sources of primary matter and adopting sustainable synthetic strategies, may give rise to biologically more active derivatives of OL [135]. By using a synthetic protocol to obtain 3,4-DHPEA-EA and HT from OL and their acetylated derivatives in good yields and very mild conditions, we demonstrated that peracetylation of OL and its derivatives may improve their capacity to permeate the molecular membrane. HT, peracetylated HT, and 3,4-DHPEA-EA were the strongest inhibitors of COX-1 and COX-2 activity as demonstrated by in vitro test and confirmed in vivo using the carrageenan-induced paw edema [5, 136]. In addition, 3,4-DHPEA-EA ameliorated development of arthritis caused by injection of collagen type II in mice, an effect associated with decreased iNOS and COX-2 expression and reduced levels of prostaglandin E-2, metabolite of COX-2, in the serum of treated mice. Amelioration of joint disease was associated with full inhibition of cytokines as well as of neutrophil infiltration [137]. The anti-inflammatory effect of 3,4-DHPEA-EA was recently demonstrated in the secondary injury associated with the mouse model of spinal cord trauma, in which this treatment significantly decreased histological damage and motor recovery [138]. Again, there was observed inhibition of proinflammatory enzymes COX-2, lipoxygenase, and iNOS and also of phosphoinositide 3-kinase.

Table 119.2 summarizes the molecular mechanisms involved in the anti-inflammatory action of OL, HT, and 3,4-DHPEA-EA.

4.3 Cardiovascular Protection

For decades, research on the health-promoting effects of Mediterranean diet has revealed that olive oil consumption is a key factor in the cardiovascular protection found in Mediterranean countries [139]. It is well established that the healthful properties of olive oil depend mostly on its high oleic acid content. However, many arguments demonstrate that in olive oil there are minor bioactive components, other than oleic acid, responsible for its cardiovascular protective properties: among them, the phenolic fraction of olive oil, and in particular OL and HT, has demonstrated antioxidant, anti-platelet aggregation, vasodilatatory, and anti-inflammatory effects, all involved in this health beneficial action [7, 108–110].

Oxidation of LDL cholesterol is one of the key steps in the initiation of atherosclerotic lesions by promoting injury to the arterial wall through several mechanisms, including growth factor and chemotactic protein expression, inflammation, and increased local macrophages [140]. Visioli et al. [117] have demonstrated that OL and HT strongly inhibit copper sulfate-induced oxidation of LDL, as result of measure of various indexes of lipid oxidation (vitamin E content, formation of thiobarbituric acid-reacting substances, lipid peroxides, levels of

Table 119.2 Molecular mechanisms of the anti-inflammatory action of OL and its derivatives

Phenolic compound	Molecular mechanisms	References
Oleuropein	↑, ↓ iNOS (↑,↓ NO)	[120, 131]
	↓COX 1/2	[64, 131, 136]
	↓Lipoxygenase	[122]
	↓Leukotriene B4	
	↓Myeloperoxidase	
	↓MMP-9	[131]
	↓NF-kB	
	↓TNF-1α	
	↓IL-1β	
Hydroxytyrosol	↓ iNOS (↓NO)	[127, 128]
	↓COX 1/2	[64, 128, 136]
	↓PGE-2	[137]
	↓Myeloperoxidase	[122]
	↓ IL-1β mRNA	[130]
	↓TNF-1α mRNA	
	↓Thromboxane A2, B2	[132, 133]
Oleuropein aglycone	↓ iNOS (↓NO)	[5, 136, 137]
	↓COX 1/2	
	↓PGE-2	
	↓Lipoxygenase	
	↓PI3-K	

polyunsaturated fatty acids, protein modification, conjugated diene formation). Production of chloramines via the myeloperoxidase-catalyzed formation of HOCl and subsequent chlorination of apoB-100 is an initiating agent in LDL lipid peroxidation [141]. As previously mentioned, OL and HT exert a scavenging effect toward HOCl, and this action, jointly to their protection against oxidation of LDL, plays a role in retarding the onset of the atherosclerotic damage. Postprandial lipemia is a well-known risk factor for atherosclerosis: OL, 3,4-DHPEA-EA, and HT promoted hypocholesterolemia, lowering LDL plasma levels and total cholesterol in rats fed a cholesterol-rich diet; also, they increased high-density lipoprotein levels and antioxidant enzyme activity reducing LDL oxidation [142, 143].

Although reduction of plasmatic cholesterol and LDL is the major mechanism underlying the antiatherogenic action of OL and HT, additional effects are also involved. It is well established that local leukocyte and monocyte recruitment into the vessel wall is an early step in atherogenesis; this event is correlated with the expression in the endothelial cells of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1). OL and HT showed capacity to reduce LPS-stimulated expression of VCAM-1 in human vascular endothelial cells inhibiting its mRNA levels. Simultaneously, they were able to decrease monocyte cell adhesion to endothelial cells [144]. Moreover, Dell'Agli et al. [145], investigating the action of OL in TNF-α-stimulated human monocyte cell line, have demonstrated that OL prevents the expression of matrix metalloproteinases (MMP), which contribute to atherosclerotic alteration of the vessels wall.

Platelet aggregation often accompanies and aggravates atherosclerosis development, since the damage to the vascular endothelium stimulates platelet activity and aggregation [146]. Recently, Dell'Agli et al. [147] have reported that OL inhibits platelet aggregation blocking cAMP and cGMP phosphodiesterases. Other anti-aggregate effects of OL and HT derive from their anti-inflammatory action, by reducing the production of eicosanoids derived from arachidonic acid, as result of inhibition of COX enzyme (see previous paragraph).

Analysis of the in vitro effects of OL on vascular smooth muscle cell (SMC) has revealed that OL inhibits proliferation of SMC acting through the extracellular-signal regulated kinase-1/2, an effect that participates in atherogenesis progression [148].

Furthermore, direct effects on myocardiocytes have also been reported. Manna et al. [149] analyzed OL effects in myocardial injury induced by ischemia, measuring in isolated rat heart perfused with OL before induction of ischemia the levels of creatine kinase, a biochemical marker of cellular damage, and oxidize glutathione, a marker of heart's exposure to OS and a key factor in the pathogenesis of atherosclerosis. OL significantly decreased levels of both markers suggesting a cardioprotective effect in the acute events that follow coronary occlusion. The impact of OL has been studied also in vivo in normal and hypercholesterolemic rabbits subjected to ischemia and reperfusion [150]. Treatment with OL for 3 or 6 weeks considerably reduced the infarct size in normal rabbits subjected to regional ischemia followed by reperfusion, while a higher dose reduced the infarct size. In this experimental model, OL protection of reperfused myocardium was associated with decreased total cholesterol and triglyceride levels. Finally, the cardioprotective effects of HT have been supported in a study conducted with cardiomyocytes extracted from rats treated with this phenol, in which administration of HT reduced the expression of proteins related to aging as well as the infarct size and cardiomyocyte apoptosis [151].

4.4 Antidiabetic Activity

A protective role for OL extracted from olive leaves against diabetic disease was first postulated by Gonzalez et al. in the early 1990s [152]. In that study, the hypoglycemic effect of OL in animals with alloxan-induced diabetes was attributed to a potentiation of glucose-induced insulin release and parallel increased peripheral uptake of glucose [152]. Subsequent studies evidenced a stronger link of the antidiabetic action with the antioxidant effects of OL. The role played by OS for diabetes complications such as retinopathy, nephropathy, and coronary heart disease is well established, so that the use of dietary antioxidant compounds was able to protect from the damages of oxidative stress and free radicals in diabetic patients [153].

In animal experimental models of alloxan-induced diabetes, both antioxidant and hypoglycemic effects of OL have been demonstrated. By treating alloxan-diabetic rabbits with 20 mg/kg body weight of OL for 16 weeks, Al-Azzawie and Alhamdani [154] found a significant decrease of blood glucose levels as compared with diabetic control rabbits. Such a hypoglycemic activity was related to its strong antioxidant potential: in fact, in parallel, the rabbits treated with OL showed also restoration of the levels of malondialdehyde and most of the enzymatic and nonenzymatic endogenous antioxidants [154]. A direct interaction of OL with free radicals was proposed as a possible explanation of this activity (see previous paragraph). Similar results were obtained in alloxan-diabetic rats: the administration, for 4 weeks, of OL- and HT-rich extracts exhibited significant hypoglycemic, hypolipidemic, and antioxidant effects in all of the tested diabetic rats [155]. In a similar experimental model, HT caused a decrease in glucose level in plasma and an increase in superoxide dismutase, catalase, and glutathione peroxidase activities in liver and kidney. Furthermore, a protective action against hepatic and renal toxicity in diabetic rats was also observed [156]. A close relationship between antioxidant and hypoglycemic activity of olive leaf extracts (OLE) was confirmed by Poudyal et al. [157] using a high-carbohydrate, high-fat diet-induced model of the metabolic syndrome in rats. Supplementation with OLE containing polyphenols such as OL and HT improved glucose tolerance and normalized abdominal fat deposition as well as plasma triglyceride and total cholesterol concentrations. Such effects were accompanied by reduced plasma uric acid and malondialdehyde concentrations, therefore suggesting a decrease in OS.

Recently, the effects of olive leaf polyphenols have been investigated also in insulin-secreting pancreatic β-cells, whose OS-induced alterations contribute to the pathogenesis of diabetes [158]. Cumaoglu et al. [159, 160] found that OL significantly suppressed cell death induced by cytokine- or H_2O_2-mediated apoptosis and/or necrosis and was able to inhibit ROS generation and to preserve insulin secretion in INS-1 cells. These effects were accompanied by the maintenance of intracellular glutathione content and the activity of antioxidant superoxide dismutase suggesting an involvement of the preservation of redox homeostasis in the protective action [159, 160].

An additional molecular mechanism of the protective action of OL against diabetes has been recently revealed by Rigacci et al. [161]. Using a rat insulinoma cell model, they demonstrated the ability of 3,4-DHPEA-EA to inhibit cytotoxic amyloid aggregation of amylin, a hallmark of type II diabetes. Moreover, by using gene expression profiling analysis in the liver of obese mice treated with OL, Kim et al. have found that the hepatic mRNA levels of lipocalin 2 (LCN2) (0.33-fold) were downregulated in the livers of the OSD-fed mice [162]. Since a recent study suggested that LCN2 deficiency protects mice from developing aging- and obesity-associated insulin resistance and hyperglycemia [163], the effect on this protein may represent an additional target of OL action.

4.5 Anticancer Activity

For a long time, the polyphenols of the olive oil of the diet have been considered to play a role for the prevention of certain types of cancer in the Mediterranean countries [164, 165]. Even more than in olive oil, constituents present in OLE have shown strong antioxidant potency and inhibition of cancer cell proliferation, thus suggesting the protection against the genotoxic action of the ROS as one of the mechanisms explaining the anticancer effects of these compounds. Indeed, either OLE or the isolated constituents OL, HT, and HT acetate exerted free radical scavenging activity and growth inhibition at low micromolar concentration on human breast cancer and urinary bladder carcinoma cells [166]. Such findings were further confirmed by other in vitro reports, testing the effects of OL or HT against human cancer cell lines [167–170]. Furthermore, Hamdi et al. demonstrated a regression of tumors caused by orally administrated OL in mice that developed spontaneous soft tissue sarcomas [171].

Several in vitro experiments support an action of OL or HT on diverse molecular pathways involved in human tumorigenesis; growth inhibition has in fact been associated with (a) induction of apoptosis [167, 172–177]; (b) upregulation of cyclin-dependent protein-kinase inhibitors $p21^{WAF/Cip1}$ and $p27^{Kip1}$ [178]; (c) blockage of messengers of pathways involved in cell proliferation, as inhibition of ERK1/2 activation [179], depletion of HER2 and reduction of its tyrosine autophosphorylation [180], and inhibition of fatty acid synthase enzyme [181]; and (d) inhibition of angiogenesis [182]. Interestingly, as for the antioxidant effect, 3,4-DHPEA-EA has shown a stronger activity than OL, when tested on cancer cells of various origins [168, 183, 184]. Moreover, semisynthetic peracetylated derivatives of OL and HT were also more effective to inhibit the growth of two breast cancer cell lines [185]. Finally, another mechanism active for cancer cell growth inhibition may derive from HT metabolism and interaction with hydrogen peroxide: production of catechol quinones, in fact, exerts anticarcinogenic properties, probably through the inhibition of NF-kB [186]. It must be remembered that, although very promising as anticancer agents, at present only one study [171] demonstrated the in vivo effectiveness of these compounds in blocking tumor proliferation.

4.6 Protection Versus Infectious Diseases

For thousands of years, folk medicine has used olive leaf for the treatment of infections. Such protective properties have been confirmed by several studies performed in the last decades using OLE [187] or isolated compounds [188–190]. Gram-positive and Gram-negative bacteria, as well as mycoplasma and also *Toxoplasma gondii*, resulted sensitive to the antiproliferative action of OL and its derivatives [188–192]. At present, the exact mechanism responsible for the antimicrobial activity of OL is still not completely clarified, although an interaction with the bacteria membrane determining a disruption of cell peptidoglycans involving the ortho-diphenolic system (catechol) of the molecule has been suggested [190].

Effective interference with the protein synthesis by altering the production of amino acids necessary for the growth of specific microorganisms has also been proposed [7]. Alternatively, nonspecific antimicrobial effects may derive from the direct stimulation of phagocytosis resulting from the activation of the immune system [7].

OL and its derivatives have demonstrated strong in vitro antimicrobial activity against several strains of bacteria causing intestinal and respiratory diseases. In fact, they were able to inhibit the development and production of enterotoxin B by *Staphylococcus aureus* [193, 194], the development of *Salmonella enteritidis* [195, 196], and the germination and consequent growth of spores of *Bacillus cereus* [197]. OL and other phenolic compounds completely inhibit the development of *Klebsiella pneumoniae, Escherichia coli*, and *Bacillus cereus* [189]. Interestingly, a synergistic growth inhibition effect of OL against *Salmonella enteritidis* has been reported when mixed with other phenolic compounds contained in olive leaves, as probably occurring in vivo [196].

Also, antiviral effects have been described for OL, and a US patent claims for a possible use of OL against herpes mononucleosis, hepatitis virus, rotavirus, bovine rhinovirus, canine parvovirus, and feline leukemia virus infections [198]. Antiviral activity of OL has been reported against respiratory syncytial virus and parainfluenza type 3 virus [199]: interestingly, in this study, the antiviral effects were not correlated with the antioxidative potency. Finally, OL and HT inhibition of cell-to-cell transmission of human immunodeficiency virus (HIV) as well as block of in vitro HIV replication has been reported [200, 201]. In the latter study, computational analysis complemented the corresponding experimental investigation proposing a direct interaction between OL and the HIV-1 surface glycoprotein subunit gp41, which is responsible for HIV entry into normal cells [201].

4.7 Neuroprotective Activity

As a consequence of their antioxidant and cardioprotective activity, including antiatherogenic and hypocholesterolemic effects, OL and its derivatives result effective also for protection against human neurodegenerative disease. In fact, a close link does exist between cardiovascular diseases caused by abnormal accumulation of metabolites like cholesterol and/or amyloid beta peptide (Aβ) and Alzheimer's disease (AD), so that modulation of such metabolites represents an outcoming therapeutic strategy currently explored even in this disorder [202, 203]. Furthermore, also inflammatory processes are clearly involved in the clinical manifestations of AD [204]. In this context, OL and HT have been proven to interact with Aβ preventing its aggregation [205, 206], thus protecting neural cells against Aβ-induced toxicity [207]. In addition, HT, OL, and 3,4-DHPEA-EA were able to prevent Tau aggregation into fibrillary tangles, an alteration proposed for playing a causative role in AD neurodegeneration [208]. Finally, neuroprotective effects of OL [209] and of 3,4-DHPEA-EA [138] were recently demonstrated in an experimental model of spinal cord injury. Further studies will clarify whether a neuroprotective action may be effective even against other neurodegenerative disorders (Parkinson's disease, vascular dementia, schizophrenia).

4.8 Skin Protection

The OL action as a free radical scavenger has opened the way to investigation of its protective effects also at the skin level. After the early report of Budiyanto [210] who demonstrated a protective effect of topically applied olive oil against photocarcinogenesis following UVB exposure of mice, a direct antioxidant action of OL on skin was first reported by Ancora et al. [211]. Subsequently, Perugini et al. [212] described the lenitive property of OL against UVB-induced erythema, suggesting a utilization in association with other active ingredients in cosmetics to repair UV damages. By using male hairless mice exposed to UVB irradiation, Kimura and Sumiyoshi [213] found that OL administered orally significantly inhibited increase of skin thickness and reduction of skin elasticity, as well as skin carcinogenesis and tumor growth, and that such an effect was associated with inhibition of the expression of VEGF, MMP-2, MMP-9, and MMP-13 through a reduction in COX-2 levels. The same authors confirmed such finding in another experimental model, proposing that the preventive effects on UVB-induced skin damage might be caused in part by inhibiting the degradation of extracellular matrixes in the corium and the proliferation of epidermal cells through reduction of MMP-13 and ROS levels induced by irradiation [214]. Interestingly, a set of HT conjugates with fatty acids at different molecular weights, synthesized under mild conditions, have shown optimal topical delivery features as a function of their permeation profiles through the human stratum corneum and viable epidermis membranes, appearing as novel promising agents in both anti-inflammatory and antioxidant topical therapies [215].

5 Conclusions

The beneficial health effects of olive oil phenolic compounds have also been proven by many randomized, crossover, controlled, human studies on biomarkers of health performed in the last years [165]. Several preclinical studies suggest that such beneficial effects may be mainly ascribed to the phenolic compounds OL and HT. However, at present, there are not final reports of studies using direct administration of OL or HT on humans; animal studies demonstrated an absolute absence of both acute and subchronic toxicity [106, 216], although it has been reported that OL may undergo CYP3A-mediated oxidation to reactive metabolite(s) capable of binding and inactivating CYP3A4, acting as inhibitor of androstenedione 6 beta-hydroxylase activity [217, 218].

With the demonstration of the intraluminal stability of OL in human gastric and small-intestinal contents [219] and considering their high bioavailability, attempts to enrich the olive oil with isolated/purified phenolic compounds are in progress [220].

In this regard, however, it should be taken into consideration, in addition to the evaluation of the phenolic content, also other important factors such as the feasibility of implementing the preparation process in the food industry. Noteworthy, recent results obtained with 3,4-DHPEA-EA or some semisynthetic derivatives [64, 185] suggest that attempts to improve the effects of OL by modifying the molecule to increase some advantageous pharmacological properties (stability, bioavailability) represent promising strategies in view of therapeutic use of these compounds. Further studies are necessary to better define the in vivo effects of individual OL derivatives used as single agents or in mixture, including their safety profile on humans. Moreover, a better characterization of their molecular mechanism of action may open the way to a larger utilization in human pharmacology.

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Bacosides and Neuroprotection 120

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Contents

1 Introduction 3641
2 Pharmacological Applications 3643
2.1 Memory-Enhancing Ability of Bacosides 3643
2.2 Protective Role of Bacosides in Chronic Cigarette Smoke 3643
2.3 Antioxidant Properties 3644
2.4 Antistress Effect of Bacosides 3644
2.5 Bacosides as Remedy for Epilepsy and Cognitive Dysfunctions 3644
2.6 Other Neuropharmacological Applications 3645
3 Mechanism of Action 3645
4 Toxicity 3647
5 Bioavailability and Metabolism 3647
6 Other Biological Activities 3652
6.1 Anti-inflammatory Role of Bacosides 3652
6.2 Protective Effect of Bacosides Against Heart and Kidney Damage 3652
6.3 Protective Role Against Gastric Ulcer 3652
6.4 Bacosides Stimulate Thyroid Hormone Secretion 3652
6.5 Potential Use as Antifertility Agent 3653
6.6 Anticancerous Property 3653
7 Clinical Trials 3653
7.1 On Antioxidant Properties 3653
7.2 On Cognitive Function 3654
7.3 On Memory-Enhancing Function 3655
7.4 On Anti-inflammatory Function 3655
7.5 On Gastrointestinal Disorders 3655

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7.6 On Diabetic Neuropathy 3656
7.7 On Anxiety and Depression 3656
7.8 Clinical Trials on Synthetic Drug-Bacosides Interaction 3656
8 Conclusion 3657
References 3657

Abstract

Bacosides are the putative bioactive component of the Indian medicinal plant *Bacopa monnieri* which was placed second in the most important medicinal plants' list by the Export-Import Bank of India. Among the bacoside components, bacoside A was found to be more pharmacologically active than bacoside B. Traditionally, *Bacopa* has been used in ayurvedic medicines as a cure for mental disorders and loss of memory. Later on, other pharmacological properties like antioxidant, antidepressant, antiulcer, hepatoprotective, anticancerous, vasodilator, smooth muscle relaxant, mast cell stabilizer, and various other functions are revealed. Increasing clinical trials indicate the potential role of bacosides even in Alzheimer's disease and in epilepsy. Bacosides attribute to the neuroprotective function mainly through modulating antioxidant enzymes, namely, SOD, catalase, etc. Bacosides also regulate the levels of different neurotransmitters in the brain. Interestingly, bacosides do not exert any side effects as proven both in animal models and in human volunteers. These features render *B. monnieri* as well as bacosides pharmacologically immensely important.

Keywords

Bacosides • *Bacopa monnieri* • neuroprotection • antioxidant • memory enhancer • antidepressant

Abbreviations

5-HT	Serotonin
ALP	Alkaline phosphatase
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid
AS	Acute stress
BME	*Bacopa monnieri* extract
CA1	Cornu ammonis 1
CAT	Catalase
CNS	Central nervous system
CUS	Chronic unpredictable stress
DA	Dopamine
EROD	7-ethoxyresorufin-o-deethylase
GABA	γ-aminobutyric acid
GPx	Glutathione peroxidase
GSH	Glutathione

GST	Glutathione S-transferase
Hsp70	Heat shock protein 70
IBS	Irritable bowel syndrome
IPP	Isopentenyl pyrophosphate
LPO	Lipid peroxidation
NA	Noradrenaline
NOS	Nitric oxide synthase
PMN cells	Polymorphonuclear cells
PROD	7-pentoxyresorufin-o-dealkylase
SOD	Super oxide dismutase

1 Introduction

Bacosides are the mixture of different triterpenoid saponins isolated from the plant *Bacopa monnieri* Linn., traditionally known for its immense therapeutic value. In ayurvedic medicine in India, it has been used for almost 3,000 years and is classified as medhya rasayana, a drug used to improve memory and intellect (medhya). On the basis of their medicinal importance, commercial value, and potential for further research and development, *B. monnieri* was placed second in a priority list of the most important medicinal plants by the Export-Import Bank of India. Bacoside A and bacoside B are the major bacosides found in *B. monnieri*. Bacoside A and bacoside B were elucidated as mixtures of triglycosidic and diglycosidic saponins, respectively. Bacoside A fraction was found to be more biologically active compounds.

B. monnieri is a rich source of many secondary metabolites, namely, alkaloids, glycosides, flavonoids, and triterpenoid saponins (Fig. 120.1). Although different triterpenoid compounds are well distributed in plant kingdom, *B. monnieri* (popularly known as Brahmi) is the only herbal source of bacosides. *B. monnieri* is a herbaceous plant, belongs to the family Scrophulariaceae, and grows naturally in the Indian subcontinent. Authentic ayurvedic treatise like Charaka Samhita and Sushruta Samhita, written in the first century AD, prescribed Brahmi as a cure for mental disorder leading to insanity and also beneficial in loss of intellect and memory [1]. Brahmi is recommended in formulations for the management of a range of mental conditions including anxiety, poor cognition, and lack of concentrations [2]. Besides this, it has the potential to act as antioxidant [3], antidepressant [4], antiulcer [5], hepatoprotective, anticancerous [6], Ca^{+2} antagonist, smooth muscle relaxant [7], vasodilator [8], and mast cell stabilizer [9].

Bacosides are most popular for their effect on nervous system mainly because of their neuroprotective activities. Neuroprotection signifies the mechanisms and strategies used to protect neuron from degeneration or malfunctioning. Various factors may involve in neuron malfunction (Fig. 120.2). Some of these factors are neuronal injury, exposure to chemical agents, adverse physiological conditions, and different disorders or diseases either decreasing efficiency of proper nervous

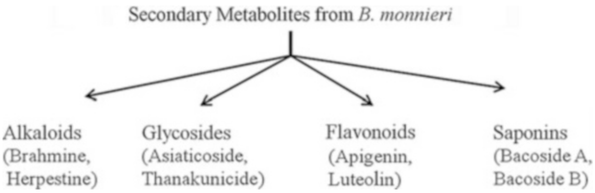
Secondary Metabolites from *B. monnieri*
Alkaloids (Brahmine, Herpestine)
Glycosides (Asiaticoside, Thanakunicide)
Flavonoids (Apigenin, Luteolin)
Saponins (Bacoside A, Bacoside B)

Fig. 120.1 Different types of secondary metabolites obtained from *B. monnieri*

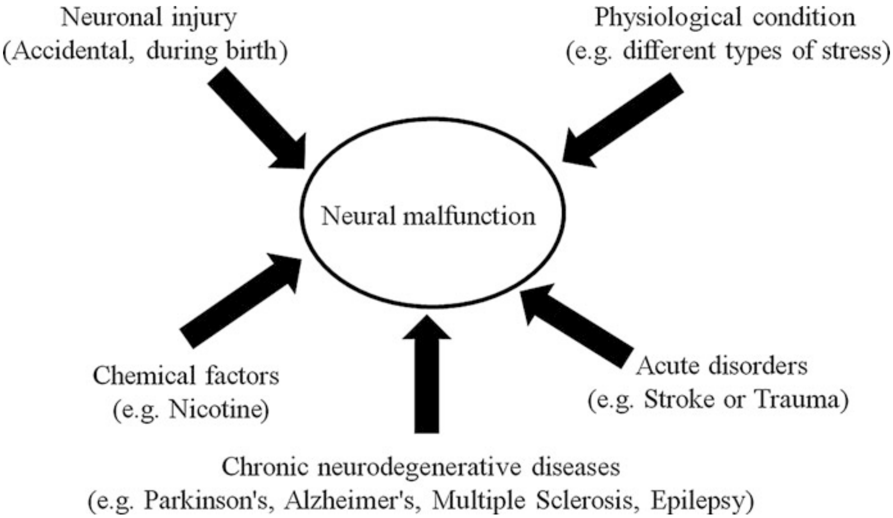
Neuronal injury (Accidental, during birth)
Physiological condition (e.g. different types of stress)
Neural malfunction
Chemical factors (e.g. Nicotine)
Acute disorders (e.g. Stroke or Trauma)
Chronic neurodegenerative diseases (e.g. Parkinson's, Alzheimer's, Multiple Sclerosis, Epilepsy)

Fig. 120.2 Factors involved in neuron malfunction

functions or causing degeneration of neuron. These factors are finally affecting the normal functions of the central nervous system (CNS) in various ways.

The goal of neuroprotection is to limit neuronal malfunction or to prevent neuronal death and to maintain the highest possible integrity of cellular interactions in the brain resulting in an undisturbed neural function. There is a wide range of chemical agents available or under investigation, which can potentially be used as neuroprotectant. An immunosuppressive calcineurin inhibitor, NOS inhibitor, σ-1 modulator, AMPA antagonist, and Ca^{2+} channel blocker have all demonstrated to show neuroprotective activity. An estrogen agonist and two glycoprotein IIb/IIIa antagonists also exhibit neuroprotective activity [10]. Effect of different neuroprotective agents can be achieved by different ways, as shown in the Fig. 120.3.

Bacosides have been experimentally shown to act as anti-inflammatory agents and free radical scavengers through modulating antioxidant enzymes. Recent studies revealed that bacosides also facilitate proper functioning of CNS through GABA receptor function regulation in the cerebellum, inhibition of acetylcholinesterase, and enhancement of kinase activity in damaged neurons. Unlike other synthetic

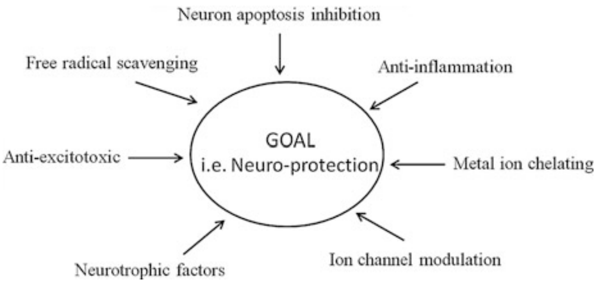

Fig. 120.3 Various strategies to achieve neuroprotection

neuroprotective drugs, bacosides show no apparent toxic effect in experimental animal models as well as in human volunteers. Bacosides are also shown to stimulate thyroxin hormone secretion, have protective function against gastric ulcer, and even have potential to be used as anticancerous agent.

2 Pharmacological Applications

Bacosides are well known for their neuropharmacological effects. Most of the studies on the pharmacological properties were made with the alcoholic extract of whole plant of *Bacopa monnieri* which chiefly constituted of bacosides A and B [1].

2.1 Memory-Enhancing Ability of Bacosides

B. monnieri extract (BME) has been traditionally used in ayurvedic medicine for the treatment of a number of memory-related disorders, particularly those involving intellect and poor memory [1]. Although bacosides A and B were reported to be active in facilitating effects on learning schedules [11], later on it was found that bacoside A alone was responsible for the facilitation of memory [12].

Bacosides promote the capacity for mental retention and were active in both positive and negative reinforcement experiments [13]. Bacosides are also reported to enhance retention of newly acquired information [14]. Kishore and Singh [15] described that bacosides facilitate anterograde memory and attenuate anterograde experimental amnesia induced by scopolamine and sodium nitrite possibly by improving acetylcholine level and hypoxic conditions, respectively.

2.2 Protective Role of Bacosides in Chronic Cigarette Smoke

Bacoside A was observed to exhibit protective role against chronic cigarette smoke. Cigarette smoke exposure disturbs the tissue defense system by enhancing oxidative stress, inducing mitochondrial dysfunction and membrane damage. Bacoside A works by exerting antioxidant role and maintaining level of trace elements like copper, zinc, and selenium [16]. Moreover, bacoside A maintains the structural and functional integrity of the mitochondrial membrane potential and impulse propagation which depends on membrane enzymes. Disturbances in the electrolyte balance due to cigarette smoke also contribute to membrane damage in brain. Anabarasi and coworkers [17] reported that bacoside A inhibits lipid peroxidation, improves the activities of ATPase, and maintains the ionic equilibrium.

2.3 Antioxidant Properties

The brain is extremely vulnerable to oxidative stress mainly because of abundance of nonheme iron in brain, which involves in the catalysis of oxygen free radical production. Besides, brain also possesses a relatively high amount of polyunsaturated fatty acids that are particularly good substrates for peroxidation reactions [18].

Antioxidant properties of bacoside A were studied by S. K. Bhattacharya [3], and he showed that it increases the activities of antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione peroxidase in the brain. The results also suggested that the increase in oxidative free radical scavenging activity of bacosides may be associated with cognition-facilitating action. Such cognition-enhancing effects of bacosides were revealed by Vohra et al. [19] and Stough et al. [20]. Both acquisition and retention of memory improvement was found to be associated with bacoside treatment, and the observed cognitive effects were noted to be independent of motor stimulation [19]. The later group of scientists suggested that the bacosides may improve higher-order cognitive processes that are critically dependent on the input of enforcement from the environment such as learning and memory.

2.4 Antistress Effect of Bacosides

Antistress effect of bacosides was studied by Shukia et al. [21] and Sheikh et al. [22]. The first group demonstrated that bacosides of *Bacopa monnieri* have been postulated to modulate the activities of Hsp70, cytochrome P450, and SOD allowing the brain to be prepared to act under adverse condition such as stress. The later group showed that treatment with bacosides attenuated the stress-induced changes in levels of serotonin and dopamine in cortex and hippocampus regions. The adaptogenic activity of bacosides might be due to the normalization of stress-induced changes in plasma corticosterone and levels of monoamines like noradrenaline, dopamine, and serotonin in cortex and hippocampus region of brain.

2.5 Bacosides as Remedy for Epilepsy and Cognitive Dysfunctions

BME has been indicated as a potential remedy for epilepsy in ayurvedic medicine. Bacoside A was reported to have beneficial effect on epilepsy-associated behavioral deficits [23]. High doses of BME were reported to exhibit anticonvulsive effect.

Hypobaric hypoxia-induced memory impairment has been attributed to several factors including increased oxidative stress, depleted mitochondrial bioenergetics, altered neurotransmission, and apoptosis. Hota et al. [24] expressed that administration of bacosides could be a useful therapeutic strategy in ameliorating hypobaric hypoxia-induced cognitive dysfunctions and other related neurological disorders.

2.6 Other Neuropharmacological Applications

Neuroprotective effect of bacosides was reported by Jyoti and group [25] against aluminium-induced changes in peroxidative products such as thiobarbituric acid-reactive substances and protein carbonyl contents and SOD activity. It was clearly observed that bacoside significantly prevented the aluminium-induced decrease in SOD activity as well as the increased oxidative damage to lipid and proteins. Neuroprotective effects of bacosides were shown to be comparable to those of l-deprenyl at both biochemical and microscopic levels.

It was found to have antidepressant activity and was comparable to that of standard antidepressant drug imipramine [4]. Bacoside was also known to possess potent adaptogenic property.

The role of bacosides on prevention of Alzheimer's disease has been exclusively studied by Holcomb et al. [26] and Singh et al. [27] concluding that these compounds have potential application in Alzheimer's disease. Anxiolytic activity of bacosides is also well established.

3 Mechanism of Action

Action of drugs is the biochemical and physiological mechanisms by which the chemical produces a response in living organisms. One major problem of pharmacology is that majority of the drugs produce single effect. The primary effect is the desired therapeutic outcome, and the secondary effects are all other consequences other than the desired effect which may be either beneficial or harmful (popularly known as side effects). The biological effects observed after a drug has been administered are the result of an interaction between that chemical and some part of the organism. Mechanisms of drug action can be viewed from different perspectives, namely, the site of action, the general nature of the drug-cell interactions, etc.

The bacosides aid in repair of damaged neurons by enhancing kinase activity, neuronal synthesis, and restoration of synaptic activity. Ultimately, nerve impulse transmission is relived, and a boost in the synthesis of new proteins in the brain is also observed [1].

Bacosides express antioxidant effect by increasing SOD, catalase (CAT), and GPx activities in all brain regions [3]. Bacoside A has a protective activity against nicotine-induced toxicity by reducing lipid peroxidation (LPO) and restoring SOD (superoxide dismutase), CAT, GSH, ALP, and GST levels [28], protecting the brain from damage by maintaining the structural and functional integrity of the

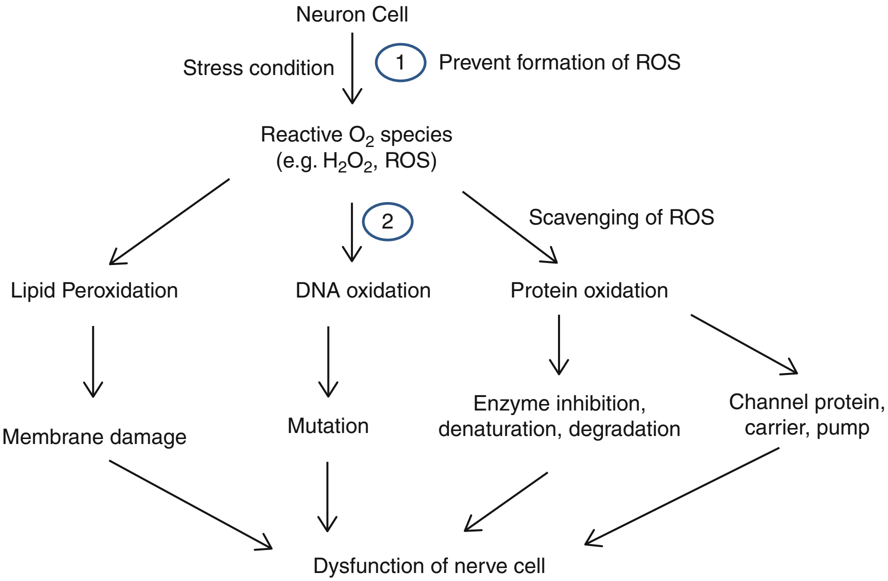
Neuron Cell
Stress condition
1
Prevent formation of ROS
Reactive O_2 species
(e.g. H_2O_2, ROS)
2
Scavenging of ROS
Lipid Peroxidation
DNA oxidation
Protein oxidation
Membrane damage
Mutation
Enzyme inhibition, denaturation, degradation
Channel protein, carrier, pump
Dysfunction of nerve cell

Fig. 120.4 Mechanism of oxidative damage on nerve cell. Damage can be prevented in two ways: (1) by preventing formation of ROS or (2) by scavenging ROS

mitochondrial membrane, inhibiting lipid peroxidation, improving the activities of ATPases, and maintaining the ionic equilibrium [17]. Figure 120.4 summarizes mechanism of oxidative damage on nerve cell. Neuroprotective activity of bacosides is mainly attributed to its ability to scavenge ROS.

Bacosides exhibit antistress effects modifying Hsp70 expression, superoxide dismutase, and cytochrome P450 activity in rat brain. During stressed condition, Hsp70 expression and cytochrome P450–dependent 7-pentoxyresorufin-o-dealkylase (PROD) and 7-ethoxyresorufin-o-deethylase (EROD) activity increase in all brain regions. As a result, the activity of SOD was found to decrease for lower dose of bacosides but increase for higher dose [29].

Bacosides have a positive effect in Alzheimer's disease. The levels of acrolein (one of the by-products of lipid peroxidation) and amyloid β peptide are much higher in vulnerable brain regions of the patients. This toxicity is due to oxidative stress generation in brain region. BME pretreatment significantly reduces intracellular reactive oxygen species (ROS) generation in the human neuroblastoma cell line SK-N-SH. Additionally, it preserves the mitochondrial membrane potential and activity of several redox-regulated proteins, i.e., NF-kappa β, Sirt1, ERK1/2, and p66Shc, to support cell survival in response to oxidative stress [27].

Shukia B. and coworkers [21] suggested that bacosides protect central nervous system from the nociceptive effect, electroshock seizures, and chemoconvulsions through GABAergic system. Additionally, Das et al. [30] showed that bacosides exert inhibitory effect on acetylcholinesterase activity and anti-dementia properties.

Bacosides have different effects on acute stress (AS)- and chronic unpredictable stress (CUS)-induced changes in plasma corticosterone and monoamines – noradrenaline (NA), dopamine (DA), and serotonin (5-HT) – in cortex and hippocampus regions of brain in rats. AS significantly elevates plasma corticosterone and 5-HT levels in both the brain regions, while DA content significantly increases only in cortex region. On the contrary, AS decreases NA content in both the brain regions and DA content in hippocampus regions. However, treatment with BME lowered the plasma corticosterone levels and increased the levels of 5-HT in both brain regions and DA in cortex. But it decreases DA in hippocampus regions and is also unsuccessful in normalizing the NA levels. CUS causes significant increase in plasma corticosterone levels and decrease in NA, DA, and 5-HT in cortex and hippocampus regions of rat brain. In summary, bacoside treatment weakens the stress-induced changes in levels of 5-HT and DA in cortex and hippocampus regions [22].

Bacosides were found to have a protective effect on morphine-challenged liver toxicity in rats. Also, it protects liver by controlling antioxidant enzyme levels [31].

4 Toxicity

In experimental studies, bacosides did not show any endocrine, metabolic, gastrointestinal, anabolic, or behavioral side effect; no lethality was observed on the oral administration also. Phase I clinical studies confirmed the safety of the bacosides in healthy male volunteers at both single and multiple doses administered over a period of 4 weeks [1]. In addition, bacosides showed no adverse effect on reproductive system in male mouse [32].

5 Bioavailability and Metabolism

Sivaramakrishna et al. [33] used column chromatography to obtain 12 pure saponin compounds from hydroalcohol extract of *B. monnieri*. Figure 120.5 represents all the saponins reported from *B. monnieri*. Table 120.1 compiles the chemical names of different saponins.

The neuroprotective actions of *B. monnieri* are mainly attributed by bacosides, which are dammarane type of triterpenoid saponins [34]. Bacosides were initially thought to be the mixture of bacoside A (melting point 250 °C) and B (melting point 203 °C). But later on, it was revealed that they are the stereoisomers of the same compound, where bacoside A was found levorotatory and bacoside B dextrorotatory [35]. The other active agents as extracted from this medicinal plant, namely, bacopasaponins A, B, C, D, E, and F [36] and also bacopasides I, II, III, IV, and V, were isolated and characterized [37, 38]. Figure 120.6 depicts chemical structures of some important saponins obtained from *B. monnieri*.

Bacoside A and B fractions were further enriched and elucidated as mixtures of triglycosidic and diglycosidic saponins, respectively. Bacoside A fraction

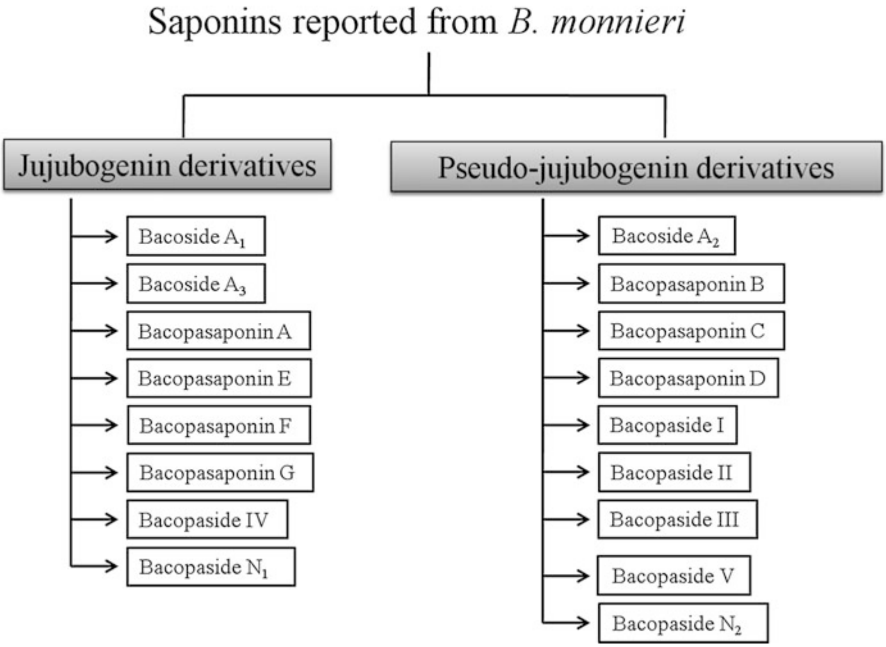
Saponins reported from *B. monnieri*
Jujubogenin derivatives
Bacoside A_1
Bacoside A_3
Bacopasaponin A
Bacopasaponin E
Bacopasaponin F
Bacopasaponin G
Bacopaside IV
Bacopaside N_1
Pseudo-jujubogenin derivatives
Bacoside A_2
Bacopasaponin B
Bacopasaponin C
Bacopasaponin D
Bacopaside I
Bacopaside II
Bacopaside III
Bacopaside V
Bacopaside N_2

Fig. 120.5 Different saponins reported from *B. monnieri*

comprises of bacoside A_3, bacopaside II, bacopaside X, and bacopasaponin C, whereas bacoside B was the mixture of bacopaside N_1, bacopaside N_2, bacopaside IV, and bacopaside V.

Saponins are a class of triterpene (C_{30}) glycosides, produced by many plant species as secondary metabolite. Like most triterpenoid compounds found in adaptogenic plants, saponins possess an aglycone (glycoside-free) portion, termed as sapogenins, along with various sugar molecules attached to the triterpene unit [39]. Glucose and arabinose are the glycone part, whereas jujubogenin and pseudojujubogenin are the aglycone part in the saponins obtained from *B. monnieri*.

Terpenoids are classified by the number of five-carbon units they contain like hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), and polyterpenes (more than 8 isoprene units). All terpenoids are derived by repetitive fusion of branched five-carbon units based on isopentane skeleton, i.e., isoprene units. At suitable chemical conditions, isoprene undergoes polymerization to generate numerous terpenoid skeletons.

At the turn of the twentieth century, structural investigations of many terpenoids led Otto Wallach to formulate the “isoprene rule,” which postulated that most terpenoids could be constructed hypothetically by repetitively joining isoprene units. This principle provided the first conceptual framework for a common structural relationship among terpenoid natural products. Wallach’s idea was refined in

Table 120.1 Chemical names of some important saponins obtained from *B. monnieri*

Saponin	Chemical name
Bacoside A_3	3-*O*-[β-D-glucopyranosyl-(1 → 3)-*O*-{α-L-arabinofuranosyl-(1 → 2)}-*O*-(β-D-glucopyranosyl)] jujubogenin
Bacopaside IV	3-*O*-[β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl] jujubogenin
Bacopaside X	3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)-}-α-L-arabinopyranosyl] jujubogenin
Bacopasaponin E	3-*O*-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-α-L-arabinopyranosyl]-20-*O*-(α-L-arabinopyranosyl) jujubogenin
Bacopasaponin F	3-*O*-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-β-D-glucopyranosyl]-20-*O*-(α-L-arabinopyranosyl) jujubogenin
Bacopaside N1	3-*O*-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl] jujubogenin
Bacopaside I	3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{6-*O*-sulphonyl-β-D-glucopyranosyl-(1 → 3)}-α-L-arabinopyranosyl] pseudojujubogenin
Bacopaside II	3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)}-β-D-glucopyranosyl] pseudojujubogenin
Bacopaside III	3-*O*-[{6-*O*-sulfonyl-β-D-glucopyranosyl-(1 → 3)}-α-L-arabinopyranosyl] pseudojujubogenin
Bacopaside V	3-O-[β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl] pseudojujubogenin
Bacopaside N2	3-*O*-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl] pseudojujubogenin
Bacopasaponin C	3-*O*-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-α-L-arabinopyranosyl] pseudojujubogenin

the 1930s when Leopold Ruzicka formulated the “biogenetic isoprene rule,” emphasizing mechanistic considerations of terpenoid synthesis in terms of electrophilic elongations, cyclizations, and rearrangements. This hypothesis ignores the precise character of the biological precursors and assumes only that they are “isoprenoid” in structure. As a working model for terpenoid biosynthesis, the biogenetic isoprene rule has been proved to be essentially correct [40].

Despite great diversity in form and function, the terpenoids are unified in their common biosynthetic origin. The biosynthesis of all terpenoids from simple, primary metabolites can be divided into four overall steps: (a) synthesis of the fundamental precursor IPP; (b) repetitive additions of IPP to form a series of prenyl diphosphate homologs, which serve as the immediate precursors of the different classes of terpenoids; (c) elaboration of these allylic prenyl diphosphates by specific terpenoid synthases to yield terpenoid skeletons; and (d) secondary enzymatic modifications to the skeletons (largely redox reactions) to give rise to the functional properties and great chemical diversity of this family of natural products. As bacosides are triterpenoid derivatives, they may probably follow the common biosynthetic pathway of terpenoid production [40].

Proper metabolic pathways that lead to the biosynthesis of bacosides are still not known. But most likely, as soon as the aglycone triterpenoid part is synthesized,

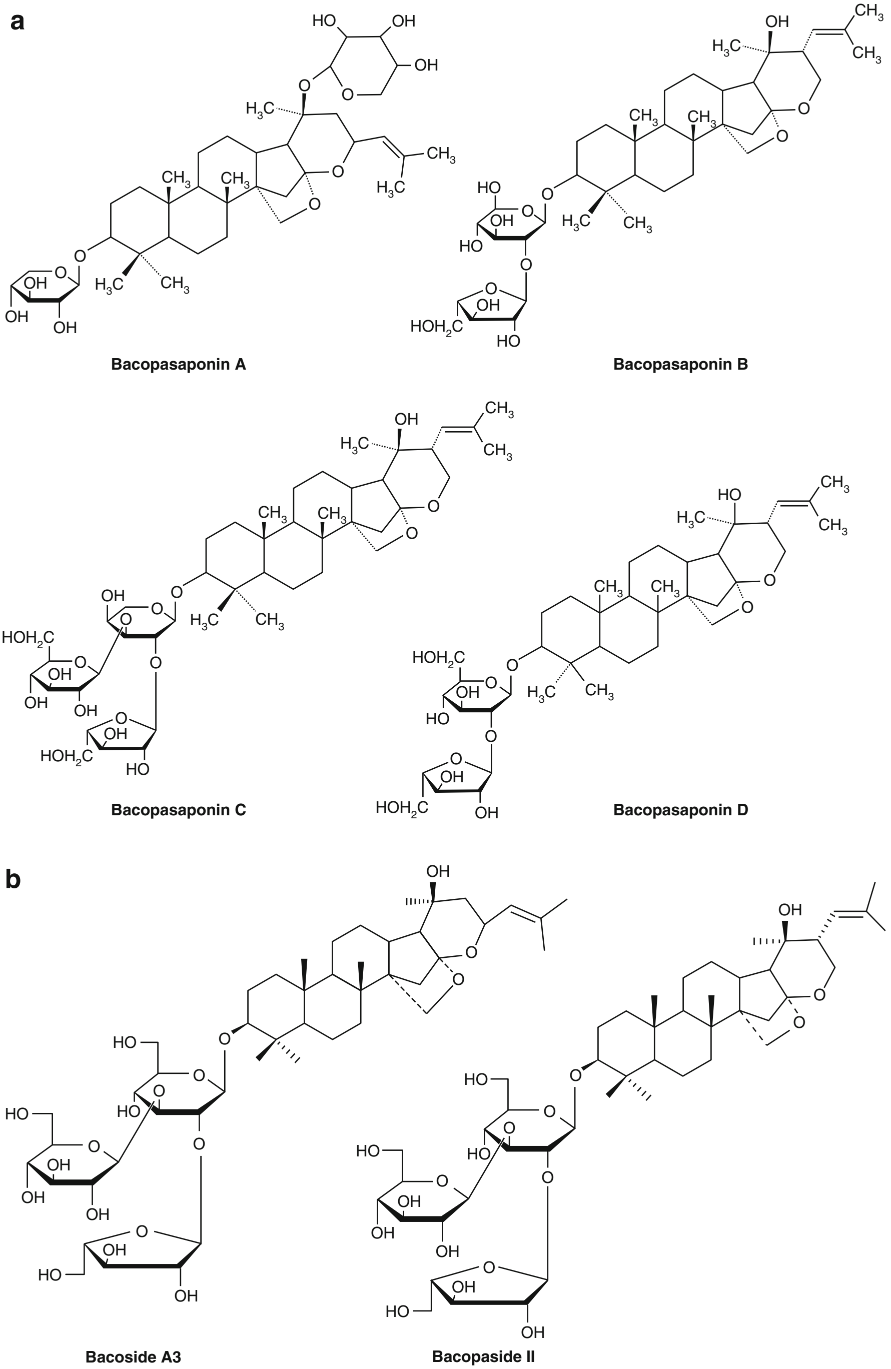
a
Bacopasaponin A
Bacopasaponin B
Bacopasaponin C
Bacopasaponin D
b
Bacoside A3
Bacopaside II

Fig. 120.6 (continued)

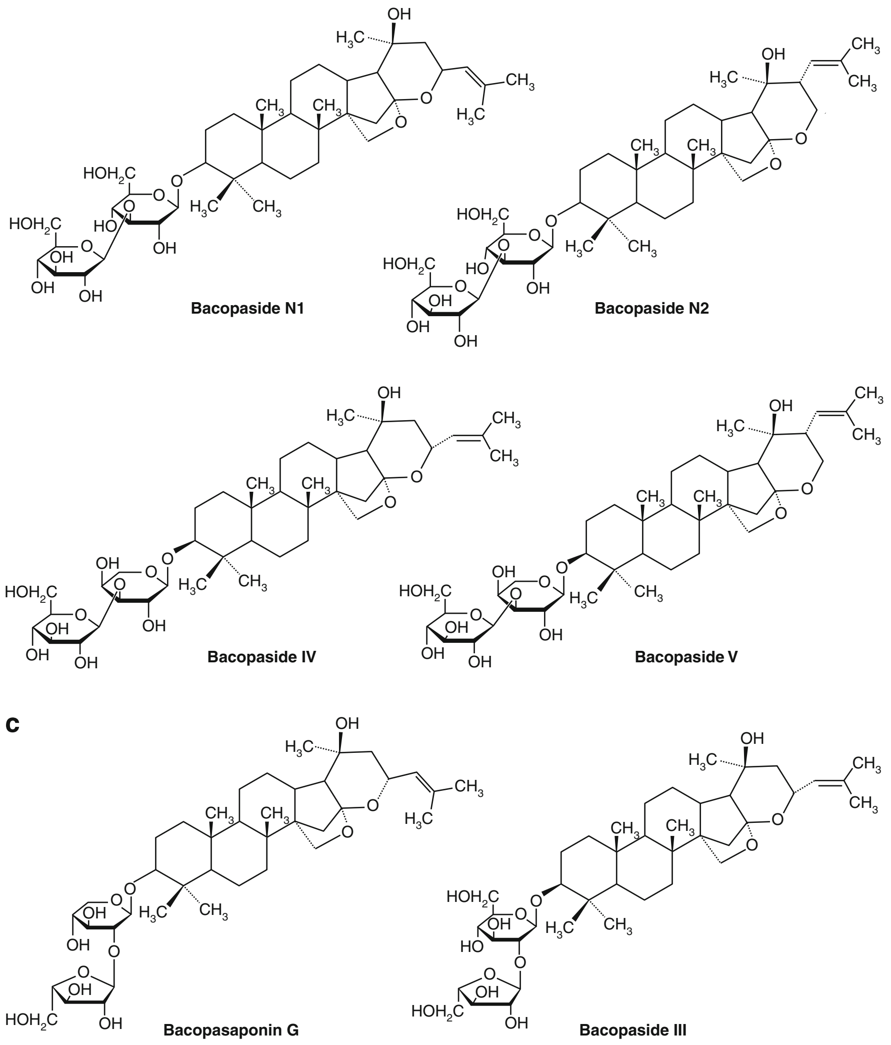
Bacopaside N1
Bacopaside N2
Bacopaside IV
Bacopaside V
c
Bacopasaponin G
Bacopaside III

Fig. 120.6 Structure of some important saponins obtained from *B. monnieri*: (**a**) bacopasaponins A, B, C, D; (**b**) bacoside A_3 and bacopasides II, N1, N2, IV, V; (**c**) bacopaside III G

the glycone part of the bacosides is then added to the aglycone part to yield different types of saponin molecules.

The following mechanism was proposed by James and Dubery [41] for the catabolism of bacosides. The sugar part of bacosides can easily be cleaved off in the gut by bacteria, allowing the aglycone (triterpene) to be absorbed. This allows them to be inserted into cell membranes and subsequently influence membrane fluidity, which can potentially affect many signaling pathways.

6 Other Biological Activities

6.1 Anti-inflammatory Role of Bacosides

Mast cells play a key role in the inflammatory process. A mast cell quickly releases its granules and various hormonal mediators into the interstitium when activated. Mast cell stabilizers are cromone medications (cromones prevent and relieve swelling of the airways and buildup of mucus. These are used to prevent asthmatic conditions) used to prevent or control certain allergic disorders. They block a calcium channel essential for mast cell degranulation, stabilizing the cell and thereby preventing the release of histamine and related mediators. Bacosides exhibit anti-inflammatory actions by stabilizing mast cells and inhibiting superoxide release from polymorphonuclear (PMN) cells [42].

6.2 Protective Effect of Bacosides Against Heart and Kidney Damage

B. monnieri along with four other herbs shows a considerable reduction of serum markers of heart and kidney damage. In addition, a decrease in lipid peroxidation with a concomitant increase in the enzymatic (SOD and CAT) and nonenzymatic antioxidants (reduced glutathione) suggested a protective effect against both damaged heart and kidneys [43]. Also, *B. monnieri* possesses broncho-vasodilatory activity, which is mainly characterized by interference with calcium ion movement [8].

6.3 Protective Role Against Gastric Ulcer

Bacoside A has a curative as well as protective effect in gastric ulcer. Sairam and coworkers [44] showed dose-dependent antiulcerogenic effect on various gastric ulcer models promoted by ethanol, acetic acid, aspirin, etc. This effect is due to increased mucin secretion and decreased cell shedding in stressed animals. But the extract does not have any effect on acid-pepsin secretion or cell proliferation. Bacosides were also shown to possess antimicrobial activity against *Helicobacter pylori*, a bacterium responsible for chronic gastric ulcers in vitro [45]. Accumulation of well-known protective agent for gastric mucosa, namely, prostaglandin E and prostacyclin, was also noted when *H. pylori*–infected human colonic mucosa cells were incubated with BME [45].

6.4 Bacosides Stimulate Thyroid Hormone Secretion

High doses of bacosides were found to increase thyroid hormone T4, but T3 remained unaffected. This data indicates that bacosides have the potential to induce thyroid stimulation, but probably they have no role in T4 to T3 conversion. Interestingly, this enhanced T4 secretion took place without increasing hepatic lipid peroxidation (LPO). This property of the extract suggests that it can be used as a thyroid-stimulating drug [46].

6.5 Potential Use as Antifertility Agent

Antifertility potential of bacosides has recently been demonstrated. In male mice, bacosides were shown to induce reversible suppression of spermatogenesis and fertility without causing toxic effect [32].

6.6 Anticancerous Property

Elangovan et al. [6] conducted in vitro studies on the anticancer activity of *Bacopa* extract. They saw that bacosides have cytotoxic activity for sarcoma-180 cells. This might be due to inhibition of DNA replication induced by bacosides in the cancerous cell line. Besides, in vitro studies demonstrated protective role of *B. monnieri* against DNA damage in astrocytes [47] and fibroblasts [48], which protects them from becoming cancerous.

7 Clinical Trials

Clinical trials are a set of procedures in medical research and drug development that are conducted to allow safety (or information about adverse drug reactions and adverse effects of other treatments) and efficacy data to be collected for health interventions (e.g., drugs, diagnostics, devices, therapy protocols). These trials can take place only after satisfactory information has been gathered on the quality of the nonclinical safety, and health authority/ethics committee approval is granted in the country where the trial is taking place. Clinical trials have different phases like phase I, phase II, phase III, and phase IV. After completion of different phases of clinical trial, a potential drug is available in markets for public use.

7.1 On Antioxidant Properties

It is reported that different salts of aluminium caused oxidative damages of lipids, proteins, and nucleic acids. Jyoti et al. [25] experimentally demonstrated that BME at a dose of 40 mg/kg/day shows similar antioxidant properties like l-deprenyl, a standard drug against $AlCl_3$ toxicity, in 8-month-old male Wistar rats. The extract significantly protects lipid and protein in cerebral cortex of rat brain from damage. It also restored the activity of endogenous antioxidant enzymes associated with aluminium administration. It extensively inhibits intraneuronal lipofuscin accumulation and necrotic alteration in the CA1 region of the hippocampus of rat brain [25].

Chronic cigarette smoke exposure also disturbs the tissue defense system by enhancing oxidative stress. Anbarasi and coworkers [16, 17] showed that when adult male albino rats and male Wistar albino rats were exposed to cigarette smoke for a period of 12 weeks, it induced mitochondrial dysfunction in rat brain by the production of ROS molecules. For assay of mitochondrial functional capacity, they used lipid peroxides, cholesterol, phospholipid levels, cholesterol/phospholipid (C/P) ratio, and the activities of isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, NADH dehydrogenase, and cytochrome c oxidase as a marker. Aqueous extract of *Bacopa monnieri* also protects from nicotine-induced toxicity by reducing lipid peroxidation (LPO) and restoring SOD (superoxide dismutase), catalase, GSH, ALP, and GST levels in mice liver [28].

When rats are administered with bacoside A (10 mg/kg), levels of glutathione, vitamin C, vitamin E, and vitamin A were reduced. The activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase were also assayed. Copper, iron, zinc, and selenium levels in brain and serum ceruloplasmin activity were also measured. Administration of bacoside A improved the antioxidant status and maintained the levels of trace elements [16].

7.2 On Cognitive Function

One of the significant effects of bacosides is to improve higher-order cognitive processes in human beings. Cognition helps people to function in day-to-day life. It includes concentrating, learning, strategizing, executing plans, comprehending language, etc. When it failed to work properly, people find difficulties in processing information quickly, remembering or recalling information, paying attention in work, solving problems, etc.

Stough and coworkers [20] studied the effect on a double-blind, placebo-controlled independent group. Subjects were indiscriminately assigned to one of two treatment conditions, ethanolic extract of *B. monnieri* (300 mg) or placebo. Neuropsychological status was monitored pre- and postdrug administration. Subjects were tested for speed of visual information processing, learning rate and memory consolidation, and state anxiety. Treated subjects were significantly improved above neurophysiological conditions compared to placebo.

Bacoside's capacity to improve cognitive effect has also been studied in Indian children. Tests were performed on 40 children (ages between 6 and 8). Children were grouped into control and treated. One teaspoon *Bacopa* syrup (350 mg *Bacopa* powder/teaspoonful) three times daily for 3 months was given to the children undergoing treatment. Visuomotor and perceptual abilities and memory span were measured before and after the treatment. Major improvements were noted in perceptual images of patterns, increased perceptual organization, and reasoning ability [49].

Another trial was done by another group of scientists to prove cognitive effect of bacoside. A double-blind, randomized, placebo-controlled trial was performed on 36 children with diagnosed attention deficit/hyperactivity disorder over a period of 16 weeks. Nineteen children were given *Bacopa* extract (containing 20 % bacosides) at a dosage of 50 mg twice daily for 12 weeks against a placebo. A significant improvement was observed in *Bacopa*-treated subjects at 12 weeks proven by an upgradation on sentence repetition and logical memory. Assessment showed that these improvements were maintained at 16 weeks [50].

7.3 On Memory-Enhancing Function

Roodenrys et al. [14] showed that bacosides also have chronic effect on human memory. In a double-blind, randomized, placebo-controlled study, they tested various memory functions and levels of anxiety in 76 adults (aged between 40 and 65 years). This study showed that bacosides help to retain new information, decrease the rate of forgetting newly gained information, increase verbal and short-term memory, and help in recovery of preexperimental knowledge.

7.4 On Anti-inflammatory Function

Carrageenan, a linear sulfated polysaccharide, induces paw edema in mice and rats which is an acute inflammatory model. The ethanolic extract of *B. monnieri* is injected intraperitoneally (100 mg/kg, 10 ml/kg) into right hind paw of mice. After 30 min, paw edema was induced by a single subplantar injection of 0.1 ml of arachidonic acid (0.5 %, w/v), bradykinin (20 μg/ml), histamine (1 mg/ml), prostaglandin E2 (0.01 μg/ml), or serotonin (1 mg/ml) into the right hind paw of rats. Three hours later, the animals were sacrificed, and paws were quickly amputated at the ankle-joint level and weighed. The edema in the right paw was determined by subtracting its weight from the weight of the left paw [51]. The extract (50 and 100 mg/kg) resulted in a considerable decrease in paw edema (33–95 %) which was 1.6 times more powerful than that caused by aspirin (28–60 %). Hence, *B. monnieri* has anti-inflammatory action toward paw edema.

7.5 On Gastrointestinal Disorders

Some in vitro animal and human studies have investigated the effect of bacosides on gastrointestinal tract. It has direct spasmolytic activity on intestinal smooth muscle, via inhibition of Ca^{2+} influx across cell membranes. Also, a similar effect was observed in rabbit's blood vessels and jejunum.

Bacosides have prophylactic and healing effects in five models of gastric ulcers. BME considerably healed penetrating ulcers induced by acetic acid at a dose of 20 mg/kg for 10 days, notably strengthened the mucosal barrier, and reduced mucosal exfoliation. The extract also reportedly reduces lipid peroxidation and balances SOD and catalase levels in rat gastric mucosa [44, 45].

In vitro studies on animal and human subjects regarding the effect of bacosides on gastrointestinal tract showed that they may have a positive effect on irritable bowel syndrome (IBS). Experiments showed that it has a direct spasmolytic effect on intestinal smooth muscle. A double-blind, randomized, placebo-controlled trial of 169 patients with IBS was done for both standard therapy (clidinium bromide, chlordiazepoxide, and psyllium) and Bacopa extract. Subjects were divided into five subgroups based on type of IBS and randomly assigned to standard drug treatment, BME therapy, or placebo for 6 weeks (5 g of each drug administered orally thrice daily). It was exposed that standard drug therapy is better than BME, except in IBS patients with diarrhea [52].

7.6 On Diabetic Neuropathy

A common complication of diabetes is diabetic neuropathy. It damages the nerves that allow feeling sensations such as pain. Recent treatments are insufficient to provide relief from pain and also cause side effects. Streptozotocin stimulates diabetes in 80 % of animals. Male Sprague Dawley rats were injected streptozotocin (65 mg/kg, i.p.) and subjected to thermal (cold and hot) and chemical (formalin) stimuli. Six weeks after streptozotocin administration, blood glucose levels increased and hyperalgesia developed. Hyperalgesia is an increased sensitivity to pain, which may be caused by damage to nociceptors or peripheral nerves. Ethanolic extract of *Bacopa monnieri* leaf (500 mg/kg, i.p.) containing bacoside A as main composition significantly set back the responses to thermal and chemical stimuli in diabetic rats [53].

7.7 On Anxiety and Depression

Traditionally, *Bacopa* was used as antianxiety remedy in ayurvedic medicine and is justified by both animal and clinical research. Lorazepam which is an antianxiety drug has amnesia as side effect. But BME having the same effect did not stimulate amnesia [54]. Thirty five patients diagnosed with anxiety neurosis were treated with Brahmi syrup with a dose of 30 mL twice daily. After this treatment the patients showed significant decrease in level of anxiety, disability, and mental fatigue whereas increase in immediate memory span. Some other positive changes that also occurred during treatment were increased body weight, decreased respiration rate, and decreased systolic blood pressure [55].

7.8 Clinical Trials on Synthetic Drug-Bacosides Interaction

Carbamylcholine chloride (carbachol) exerts bronchoconstrictor action. It is noticed that carbachol significantly increases inspiratory and expiratory pressures and decreases blood pressure and heart rate. Bronchodilation was monitored in anesthetized male albino rats, and ethanolic extract of *B. monnieri* (50 mg/kg) was administered before and after. The effects of carbachol were reversed within 2–5 min. At a dose of 25 mg/kg, the plant extract repressed the inspiratory pressure, while the doses 37 and 50 mg/kg inhibited both inspiratory and expiratory pressures, respectively. Increase in tracheal pressure due to carbachol reversed in the presence of the plant extract (50 mg/kg). Hence, the ethanolic extract antagonizes the bronchoconstrictor effects of carbachol [7].

Scopolamine is frequently used as amnesic agent. But being a synthetic drug, it has some undesirable effects. Oral dose (at 120 mg kg^{-1}) of ethanolic extract of *Bacopa monnieri* inverted scopolamine (0.5 mg kg^{-1} i.p.)-induced adverse effects. The ethanolic extract also stopped scopolamine (0.5 mg kg^{-1} i.p.)-induced retrograde amnesia [56].

8 Conclusion

With the increasing knowledge on pharmacological applications of bacosides, researchers are now also trying BIOTECHNOLOGICAL tools to obtain higher yields of bacosides from the plant as it is the exclusive source of these immensely useful compounds [57]. Diverse array of neuropharmacological functions of bacosides have the prospect to end the long quest for a neuroprotective drug which has no or minimal side effects. The therapeutic effects of BME were gradually established both in animal models and in human volunteers. But the detailed mechanism through which bacosides execute neuroprotective function or enhance memory is still not clear. Also, the exact functional component of BME as well as its metabolism is yet to be elucidated. Many studies indicated that there may exist interactions between herbal medicines and synthetic drugs. So, stringent clinical trials are necessary to negotiate these issues.

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Garlic and Cardiovascular Diseases 121

Syed Haris Omar

Contents

1 Introduction 3664
2 Bioactive Constituents and Preparations 3664
3 Garlic and Cardio Protection 3665
4 Hypolipidemic Effects 3668
4.1 Clinical Studies 3669
4.2 Proposed Mechanism 3670
5 Antioxidant Effects 3671
6 Clinical Studies 3674
7 Proposed Mechanism 3674
8 Antihypertensive Effects 3675
9 Clinical Studies 3676
10 Possible Mechanism 3677
11 Antithrombotic and Fibrinolytic Effect 3677
12 Clinical Studies 3679
13 Proposed Mechanism 3680
14 Antiatherosclerotic Effect 3681
15 Clinical Studies 3682
16 Proposed Mechanism 3683
17 Other Cardioprotective Activity 3684
18 Bioavailability and Metabolism 3685
19 Side Effects and Toxicity 3686
20 Conclusion 3686
References 3687

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Abstract

Globally, there is an increasing prevalence of cardiovascular diseases (CVD) and is still maintaining its apex position. It includes elevation in low density lipoproteins cholesterol (LDL-C), increase in LDL-C oxidation, irregular clumping of blood platelets, and increase in C-reactive proteins and homocysteine. These abnormalities are also associated with atherosclerosis, high blood pressure, and hypercholesterolemia. Garlic (*Allium sativum* L.) is among the world's oldest cultivated plants, popular in food and for medicinal purposes. Commercially available garlic preparations in the form of garlic oil, powder, and pills are widely used for therapeutic purposes. The water-soluble and insoluble sulfur compounds in garlic showed beneficial effects in all these cardiac abnormalities, especially for the treatment of hypercholesterolemia and prevention of arteriosclerosis through antioxidant ability involving induction and inhibition of various metabolic enzymes and via chelating activity. There is continuous investigation being carried out to find out the other potent bioactive constituent in garlic. Likewise, phenolic compounds have emerged as minor but potent compounds, which are responsible for its antioxidant activity. Most of the studies are in the preliminary stage and need to be clarified in clinical trials.

Keywords

Atherosclerosis • cardiovascular disease • garlic • hypercholesterolemia • sulfur compounds

Abbreviations

AA	Arachidonic acid
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
AGE	Age garlic extract
AMS	Allyl methyl sulfide
BC	Before Christ
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BP	British Pharmacopeia
Ca^{2+}	Calcium ion
CAC	Coronary arterial calcification
CETP	Cholesteryl ester transfer protein
cNOS	Endothelial nitric oxide synthase
CVD	Cardiovascular disease
DADS	Diallyl disulfide
DAS	Diallyl sulfide
DASO	Diallyl sulfoxide
$DASO_2$	Diallyl sulfone

DBP	Diastolic blood pressure
DNA	Deoxyribose nucleic acid
DOX	Doxorubicin
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DTS	Diallyl trisulfide
EH	Essential hypertension
FDA	Food and Drug Administration
GC–MS	Gas chromatography–Mass spectrometry
GSH	Glutathione
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
Hcy	Homocysteine
HDL-C	High density lipoprotein cholesterol
$HepG_2$	Liver hepatocellular carcinoma cell
HETE	Hydroxyeicosatetraenoic acid
HMG-CoA	3 Hydroxy-3-methylglutaryl coenzyme A
HOCl	Hypochlorous acid
iNOS	Inducible nitric oxide synthase
IP_3	Inositol phosphate
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
L-NAME	L-Arginine methyl ester
MDA	Malondialdehyde
NO	Nitric oxide
OxLDL	Oxidized low density lipoprotein
PGG2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
Ph Eur	European Pharmacopeia
ROS	Reactive oxygen species
SAC	*S*-Allylcysteine
SACS	*S*-Allylcysteine sulfoxide
SBP	Systolic blood pressure
SBS	*S*-Benzylcysteine
SPC	*S*-Propylcysteine
TAS	Total antioxidant status
TBARS	Thiobarbituric acid reactive species
TC	Total cholesterol
TG	Triglycerides
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
USP	United States Pharmacopeia
VF	Ventricular fibrillation
VT	Ventricular tachycardia

1 Introduction

Garlic, scientifically known as *Allium sativum*, is a close relative of onions, leeks, and chives. The word *Allium* is derived from the Celtic word *al*, meaning pungent, burning, or stinging and *sativum* meaning planted, cultivated, or sown. The English name "garlic" comes from the Anglo-Saxon *gar-leac* or spear plant, which refers to its flowering stalk. It is one of the oldest cultivated plants with its origin in central Asia. It has also been found in Egyptian pyramids, ancient Greek temples, and on Sumerian clay tablets dating from 2600 to 2100 BC [1, 2]. Garlic was used as medicine by the ancient Egyptians especially for the working class involved in heavy labor and is listed in the Egyptian medical papyrus *Codex Elsers* (1500 BC). The ancient medical manuscript of India, *Charaka-Samhita*, recommends garlic for the treatment of heart disease and arthritis, and another ancient Indian medical textbook, *Bower Manuscript* recommends garlic for fatigue, parasitic disease, digestive disorder, and leprosy [2]. During World Wars I and II, the injured soldier wounds were dressed with garlic, and it was used as an antiseptic in the prevention of gangrene.

Garlic and its preparations are prescribed in many pharmacopeias around the world, including Ph Eur 6 [3], USP 31 [4], and BP 2007 [5]. Garlic is also incorporated in the list of German Commission E, which is a therapeutic guide in herbal medicine, complied by a special expert commission of the German Federal Institute of Medicines and Medical Inventions. German Commission E recommends usage of an average dose of 4 g of fresh garlic or equivalent preparations of garlic as a dietary supplement to hyperlipidemic patients and in prevention of vascular alterations caused by aging. Garlic is popularly used as one of the major spices, and its medicinal use is both widespread and growing due to proven potential health benefits, which have been published in more than 3,000 research articles. Studies suggests that garlic and its components not only prevent cardiovascular disease (including lowering of serum cholesterol level, inhibition of platelet aggregation, and increased fibrinolysis) but also other chronic diseases associated with aging, stimulation of immune function through activation of macrophages, induction of T cell proliferation, reduction of blood glucose level, radioprotection, improvement of memory and learning deficit, and protection against microbial (viral and fungal infections) and anticancer effects.

2 Bioactive Constituents and Preparations

Garlic bulbs develop and grow entirely underground and are composed of several bulbils structure called cloves. Each clove is enclosed in a white or pink skin of the parent bulb, mainly consists of active secondary plant metabolites, which are responsible for taste, flavor, and health benefits. There are over 600 cultivated subvarieties of garlic available in the world, and scientifically, all the true garlic comes under the species *Allium sativum* with two most common subspecies, *ophioscorodon* or hard-necked garlic (ophios for short) and *Sativum* or soft-necked garlics.

Fresh raw garlic bulbs contain ~65% of water, ~28% carbohydrate, ~2% proteins, ~1.2% amino acids, ~1.5% fibers, fatty acids, phenols, and trace elements, as well as more than 33 (~2.3%) sulfur (Fig. 121.1)-containing compounds [6, 7]. Hundred grams of garlic provides ~149 kcal energy, 33.07 g of carbohydrate, 6.93 g of protein, and 0.5 g of fat. It has been estimated that ~97% of chemical constituents in garlic are water soluble, and very small amounts of oil-soluble constituents which vary from 0.15–0.7% (Table 121.1). The major trace elements found in the fresh garlic cloves are shown in Table 121.2. Further compounds present in a small amount are flavonoids, steroids, and triterpene saponins from the β-sitosterol or F-gitogenin.

The sulfur-containing compounds are generally classified into nonvolatile precursor and organosulfur compound. δ-glutamyl-*S*-allyl-L-cysteines and *S*-allyl-L-cysteine sulfoxides (alliin) are abundant in intact garlic and serve as a precursor of allicin, methiin, (+)-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide, and cycloalliin. When there is any mechanical injury to the garlic bulb then, there is formation of thiosulfinate compound called allicin through enzymatic reaction of sulfur-substituted cysteine sulfoxides, which are present in the cytoplasm with alliinase in the vacuole, via sulfur-substituted sulfenic acids. Key studies by Cavallito and Bailey [9] and Stoll and Seebeck [10] identified the compound allicin. Allicin further decomposes into diallyl disulfide (DADS), diallyl sulfide (DAS), diallyl trisulfide (DTS), and sulfur dioxide. In addition, there are other thiosulfinates present in garlic homogenate, including allyl methyl, methyl allyl, and *trans*-1-propenyl thiosulfinate, that are also unstable like allicin.

There are various available brands of garlic products in stores/on shelves that provide a convenient way to obtain the health benefits of garlic. The most commonly used garlic preparations are raw garlic per se, aged garlic, garlic oil, allicin extract powder (Table 121.3) and commercially prepared lyophilized garlic powder, garlic oil, garlic oil macerate, and aged garlic extract (AGE) [11, 12]. Among the popular commercial garlic preparations investigated in trials are KWAI® (Lichtwer Pharma, Berlin, Germany), Garlicin® (Nature's Way, Springville, UT, USA), and Kyolic-100® (Wakunaga of America, Mission Viejo, CA, USA).

3 Garlic and Cardio Protection

Globally, CVD remain the biggest cause of death and disability. According to WHO report 2011, 17.3 million people died from CVD in 2008, which accounts for over 80 % of deaths in low- and middle-income countries. It is estimated that by 2030, almost 23.6 million people will die from CVD [13]. CVD refers to a group of disorders of the heart and vascular system and includes coronary heart disease, congestive heart failure, stroke, congenital heart defects, myocardial infarction, and high blood pressure. Imbalance between free radicals production and scavenging leads to oxidative damage of membrane lipids, proteins, carbohydrates, and finally

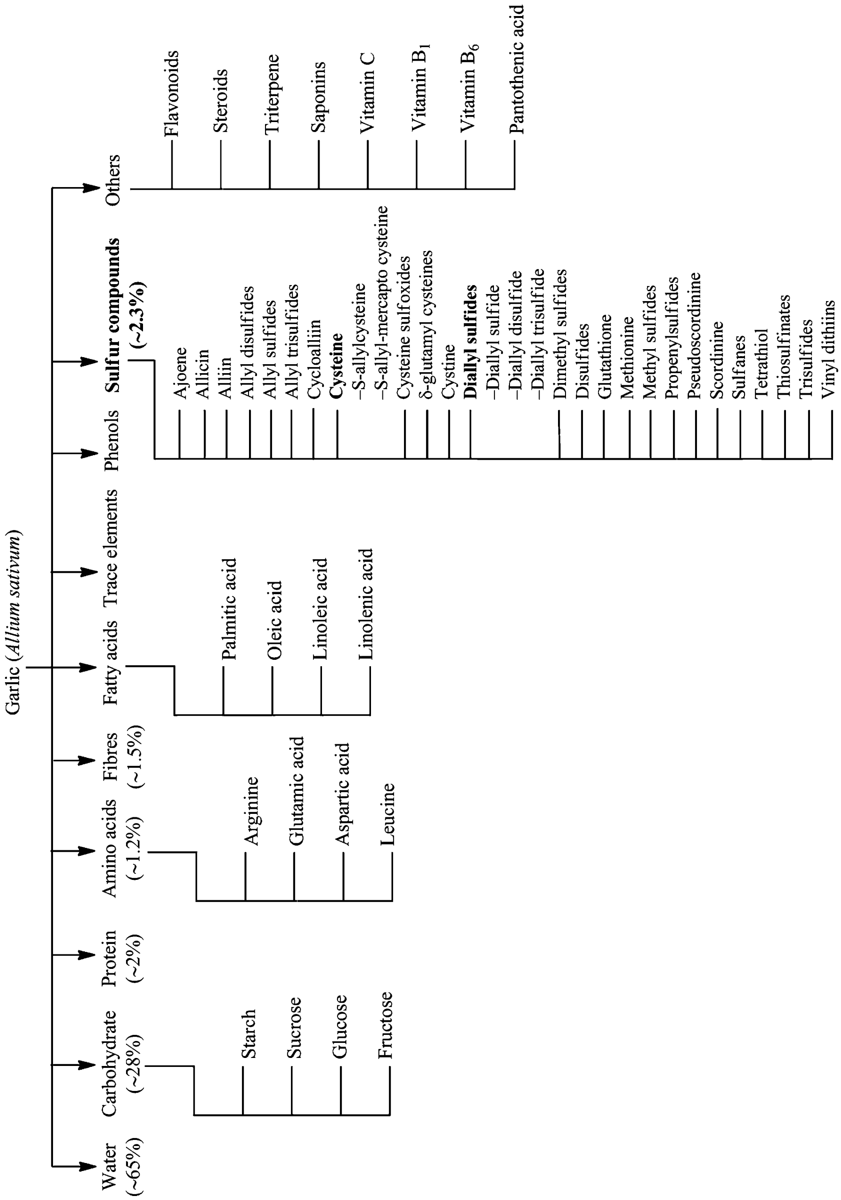
Garlic (*Allium sativum*)
Water (~65%)
Carbohydrate (~28%)
Starch
Sucrose
Glucose
Fructose
Protein (~2%)
Amino acids (~1.2%)
Arginine
Glutamic acid
Aspartic acid
Leucine
Fibres (~1.5%)
Fatty acids
Palmitic acid
Oleic acid
Linoleic acid
Linolenic acid
Trace elements
Phenols
Sulfur compounds (~2.3%)
Ajoene
Allicin
Alliin
Allyl disulfides
Allyl sulfides
Allyl trisulfides
Cycloallin
Cysteine
–S-allylcysteine
–S-allyl-mercapto cysteine
Cysteine sulfoxides
δ-glutamyl cysteines
Cystine
Diallyl sulfides
–Diallyl sulfide
–Diallyl disulfide
–Diallyl trisulfide
Dimethyl sulfides
Disulfides
Glutathione
Methionine
Methyl sulfides
Propenylsulfides
Pseudoscordinine
Scordinine
Sulfanes
Tetrathiol
Thiosulfinates
Trisulfides
Vinyl dithiins
Others
Flavonoids
Steroids
Triterpene
Saponins
Vitamin C
Vitamin B_1
Vitamin B_6
Pantothenic acid

Fig. 121.1 Major classification of the bioactive constituents in garlic

Table 121.1 Water-soluble and oil-soluble constituent in garlic [8]

Water soluble	Oil soluble
S-allylcysteine	Diallyl disulfide
Alliin	Diallyl trisulfide
S-propylcysteine	Methyl allyl sulfide
S-ethylcysteine	Dipropyl disulfide
S-methylcysteine	Dipropyl sulfide
Se-(methyl) selenocysteine	Allixin
Selenomethionine	Allyl mercaptan
Selenocysteine	Allyl methyl sulfide

Table 121.2 Important trace elements in fresh garlic bulb

Constituents	Quantity per 100 g
Aluminum	0.5–1 mg
Barium	0.2–1 mg
Boron	0.3–0.6 mg
Calcium	50–90 μg
Chromium	0.3–0.5 mg
Copper	0.02–0.03 μg
Germanium	14 μg
Iron	2.8–3.9 μg
Manganese	0.2–0.6 mg
Magnesium	43–77 μg
Nicotinic acid	0.5 mg
Phosphorus	390–460 mg
Potassium	100–120 μg
Retinal	15 μg
Riboflavin	0.08 mg
Selenium	15–35 μg
Sodium	10–22 mg
Thiamine	0.25 mg
Vitamin C	5 mg
Zinc	1.8–3.1 mg

to DNA which brings changes in the structural, mechanical, electrical, and biochemical properties of the heart. Nowadays, natural herbal drugs are gaining greater acceptance from the researchers and public due to advances in understanding the mechanism of action, fewer side effects, and lesser cost effective therapy. Extensive in vitro, in vivo, and clinical studies showed that garlic with its sulfur and nonsulfur bioactive compounds is involved in the prevention and treatment of CVD.

Table 121.3 Commonly available and used garlic preparations

Preparation	Major sulfur constituents	Preparation process	Properties
Raw garlic per se	δ-glutamyl-*S*-allyl-L-cysteines, *S*-allyl-L-cysteine sulfoxides, allicin, adenosine	Intact clove with the white or pink skin by simple pull off	Precursor of allicin, methiin, (+)-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide, and cycloalliin
Garlic essential oil	Diallyl disulfide (DADS), diallyl sulfide (DAS), diallyl trisulfide (DTS), methyl allyl disulfide, methyl allyl trisulfide, vinyldithiins, and ajoenes	Steam distillation or ether extracted	Most potent source of garlic odor and causes body odor, used in perfumery and cosmetic industry
Garlic oil macerate oil	DAS, allyl methyl dimethyl, mono-to hexasulfides, allyl 1-propenyl and methyl 1-propenyl di-, tri-, and tetrasulfides	Cold extraction method (maceration) and steam distillation	Manufactured as capsule in diluted form for therapeutic use
Garlic powder	Alliin accumulates naturally during storage of the bulbs at cool temperature	Simply dehydrated, pulverized garlic clove with (<60 °C) temperature control	Commercially available for household use
Allicin powder extract	Allicin	Specialized patented extraction process produces allicin liquid that is spray dried	Stable at cool temperatures but at high temperature allicin degraded
Aged garlic extract (AGE)	Stable and water-soluble organosulfur compounds, such as *S*-allylcysteine, *S*-allylmercaptocysteine, with allixin, selenium, and *N*-alpha-(1-deoxy-D-fructos-1-yl)-L-arginine	Sliced raw garlic is stored in 15–20 % ethanol, fermented bulb of age ~2 years	Highly bioavailable and shown to be superior to raw garlic in terms of its antioxidant properties

4 Hypolipidemic Effects

Hyperlipidemia, which is characterized by an increase in the level of cholesterol or lipids (LDL, triglycerides) in the blood, serves as a major risk factor of atherosclerosis. The growing interest in complementary and alternative medicine has led to an increasing number of nonpharmacological therapies for lipid management and treatment of hyperlipidemia/hypercholesterolemia with dietary intervention. These include garlic, which reduces total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), and triglyceride (TG) levels; furthermore, it increases high density lipoprotein cholesterol (HDL-C), which has been confirmed in several research studies.

Preparations of garlic including garlic paste, garlic oil, allicin, and ajoene have been found to significantly reduce cholesterol biosynthesis in rat hepatocytes via inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and

14-α-demethylase [14, 15] and human HepG$_2$ cells [16]. After measurement of the enzyme activity, it has been indicated that garlic and its constituents inhibit human squalene monooxygenase along with HMG-CoA reductase, the enzymes involved in cholesterol biosynthesis [8, 17]. In addition, garlic supplementation significantly decreased the cholesterol 7-α-hydroxylase activity [18]. Augusti et al. [19] in 2005 confirmed the inhibition of HMG-CoA reductase by garlic. Some authors postulate that garlic's trace minerals, such as tellurium (Te), are involved in the inhibition of hepatic cholesterol synthesis [20]. It was found that the more water-soluble compounds like *S*-allylcysteine (SAC) present in aged garlic extract are less cytotoxic but more efficient in inhibiting cholesterol biosynthesis; in contrast the lipid-soluble sulfur compounds such as diallyl sulfide (DAS) are less efficient [16]. In rabbits that were fed with a high-cholesterol diet and supplemented with garlic or allicin, it was found that hypercholesterolemia was significantly inhibited by 50% and showed a decrease in tissue cholesterol and LDL-C concentrations and raised HDL-C concentrations along with reduced atheromatous changes [21]. Koch [22] indicated that the cholesterol-lowering effect of garlic was probably due to the nonsulfur component, saponin. In addition, another study [23] also supported the saponin fraction from methanolic raw garlic extracts, which mainly contains spirostanol saponins produced by the conversion of furostanol saponins via β-glucosidase. It lowered TC and LDL-C cholesterol without changing HDL-C levels in hypercholesterolemic animal models. A recently published animal study results showed that garlic significantly reduced TC, TG, LDL-C, very low density lipoprotein (VLDL-C), liver triglyceride, plasma malondialdehyde (MDA), and elevated plasma antioxidant in garlic-treated rats along with decrease in liver phosphatidate phosphohydrolase (PAP) activity [24].

4.1 Clinical Studies

Several clinical trials on garlic preparation have investigated, but only a few of them have showed significant hypolipidemic effects. In a meta-analysis on 28 clinical trials, Warshafsky et al. [25] found only five randomized, placebo-controlled studies that met their criteria for inclusion in the meta-analysis. The analysis result showed that treatment with garlic or garlic preparations caused decrease an approximately 9% in TC level. In addition, another meta-analysis of 16 published studies [26] in 1994, revealed that treatment with garlic preparations resulted in nearly a 12% decrease in TC and a similar decrease in LDL-C. Out of 16 studies, only 8 studies included the data on TG; when analyzed together, they revealed a 13% decrease in TG levels. A 12-week, randomized, placebo-controlled study [27] using Kwai garlic powder (900 mg/day) showed a reduction in TC and LDL-C levels by 6% and 11%, respectively, in the garlic supplement group, while TC and LDL-C levels decreased by 1% and 3%, respectively, in the placebo control group. In a long-term (10 months) randomized, double-blind, crossover study [28] in moderately hypercholesterolemic men, using AGE versus placebo, the measurements were made six times during the first intervention (AGE or placebo) and five times over the 120 days of crossover to the alternate treatment. AGE resulted in a maximum TC reduction of 6% for all study subjects compared with placebo and 7% compared with their baseline values. While the LDL-C reduction was 4.5% and 4% compared against placebo and baseline, respectively. A comparative study [29] was reported in 1997, in which garlic (900 mg powder/day) and fish oil (12 g fish oil/day) were used as dietary supplements. This was a randomized, placebo-controlled (partially double-blind) study with four arms: garlic with fish oil placebo, fish oil with garlic placebo, garlic and fish oil, and both placebos. Potential subjects began a 3-week run-in period for dietary stabilization. During this run-in period, TC level had to exceed 5.2 mmol/L (200 mg/dL). They reported that garlic significantly reduced both TC and LDL-C levels, whereas fish oil also significantly decreased TG and increased LDL-C levels as expected. An interesting outcome by Zhang et al. [30] showed that gender might be affect the action of garlic on plasma cholesterol and glucose levels of normal subjects. Alder et al. [31] in 2003 published a systematic review of the effectiveness of garlic as an antihyperlipidemic agent. They included ten studies and found that in six studies garlic was effective in reducing serum cholesterol levels. The average drop in total cholesterol was 9.9%, LDL-C 11.4%, and triglycerides 9.9%. In a study, consumption of enteric-coated garlic supplements, standardized to produce 9.6 mg allicin, significantly decreased TC (4%) and LDL-C (7%) in mild to moderated hypercholesterolemic patients when combined with a low-fat diet [32]. A clinical study confirmed the hypolipidemic effect of raw garlic in hyperlipidemic subjects and reported significantly reduction of TC and TG along with significantly increase in HDL-C [33]. Recently published meta-analysis studies, in which 13 trials including 1,056 subjects, do not produce any statistically significant reduction in serum total cholesterol level from garlic [34]. In addition, another recently published meta-analysis of 29 trials suggested intake of garlic causes significant reduction in TC and TG but does not exhibit any significant effect on LDL-C or HDL-C [35].

4.2 Proposed Mechanism

There are four possible mechanisms through which garlic inhibit the cholesterol synthesis and enhance the cholesterol excretion from the body (Fig. 121.2). The first one is that garlic decreases cholesterol absorption in the intestine, as shown in hypercholesterolemic rat models [23]. Second, experiments using cultures of rat hepatocytes have shown that garlic inhibits the enzymes involved in cholesterol synthesis [16, 36, 37]. Third, Borek [38] has suggested that the cholesterol-lowering effect of garlic is caused by deactivation of HMG-CoA reductase, involved in the synthesis of cholesterol. Fourth, garlic also increased the excretion of cholesterol, as manifested by enhanced excretion of acidic and neutral steroids after garlic feeding [39].

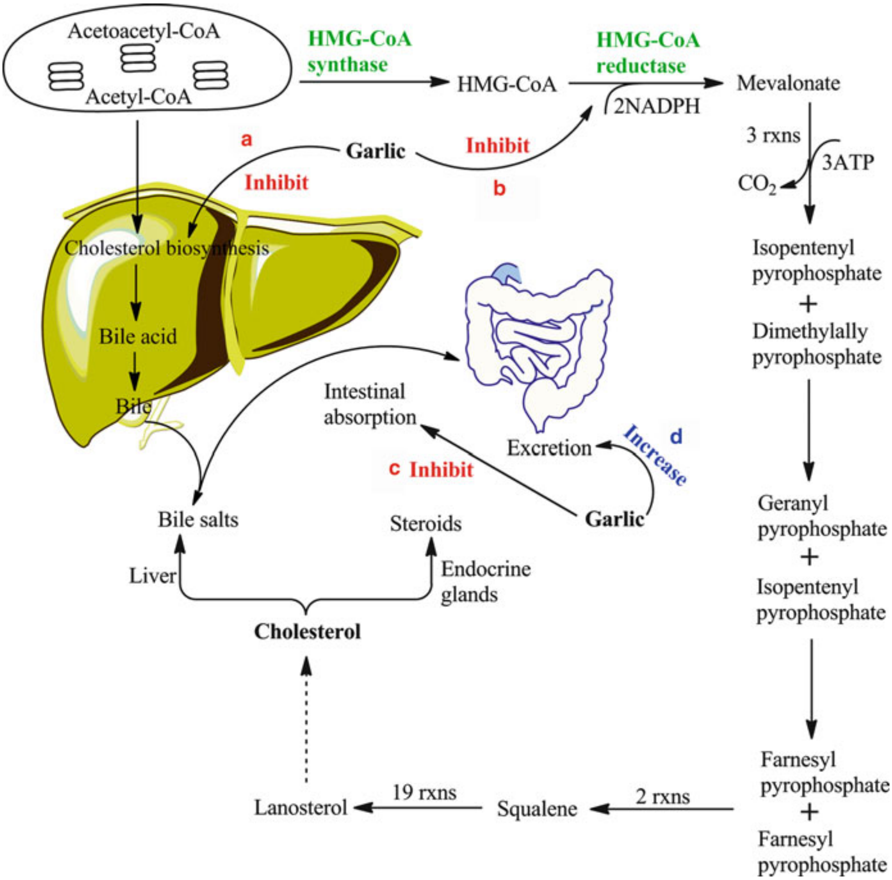
Acetoacetyl-CoA
Acetyl-CoA
HMG-CoA synthase
HMG-CoA
HMG-CoA reductase
2NADPH
Mevalonate
a
Garlic
Inhibit
Inhibit
b
3 rxns
3ATP
CO_2
Cholesterol biosynthesis
Bile acid
Bile
Isopentenyl pyrophosphate
+
Dimethylally pyrophosphate
Intestinal absorption
Excretion
Increase
d
c Inhibit
Garlic
Bile salts
Steroids
Liver
Endocrine glands
Cholesterol
Geranyl pyrophosphate
+
Isopentenyl pyrophosphate
Lanosterol
19 rxns
Squalene
2 rxns
Farnesyl pyrophosphate
+
Farnesyl pyrophosphate

Fig. 121.2 Hypolipidemic mechanism of garlic. (*a*) Inhibit the cholesterol biosynthesis in liver; (*b*) Inhibit the rate limiting enzyme HMG-CoA reductase; (*c*) Inhibit intestinal absorption; (*d*) Induces the rate of cholesterol excretion

5 Antioxidant Effects

There is a continuous production of free radicals from the body's metabolic process that use oxygen, such as respiration and some cell-mediated immune functions. The LDL oxidation in the artery wall gives rise to thrombosis, atherosclerosis, and CVD. Besides reactive oxygen species (ROS) and reactive nitrogen species (RNS), hypochlorous acid (HOCl) is also a strong endogenous oxidant and is excessively produced in inflammatory, degenerative, and neoplastic disorders; thus, effective therapies and/or prophylaxes using exogenous scavengers of high specificity are required. Homeostasis and self-defense system of the body maintain a balance between the amount of generated free radicals in the body and body internal antioxidants glutathione (GSH) to quench and/or scavenge or even detoxify them

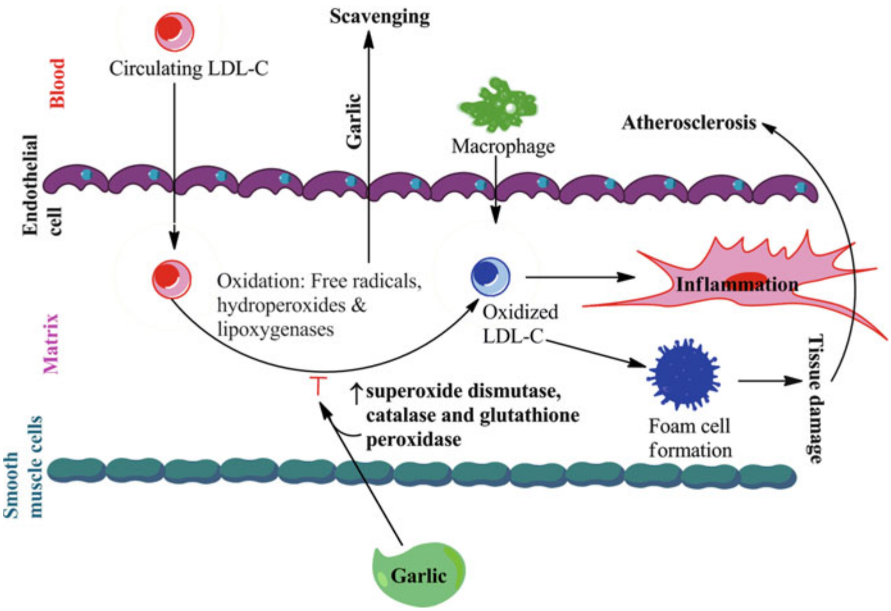
Scavenging
Circulating LDL-C
Blood
Garlic
Macrophage
Atherosclerosis
Endothelial cell
Oxidation: Free radicals, hydroperoxides & lipoxygenases
Oxidized LDL-C
Inflammation
Matrix
Tissue damage
↑superoxide dismutase, catalase and glutathione peroxidase
Foam cell formation
Smooth muscle cells
Garlic

Fig. 121.3 Antioxidant mechanism: garlic inhibiting oxidative modification of LDL-C, scavenging ROS, enhancing the cellular antioxidant enzymes superoxide dismutase, catalase and glutothione peroxidase and glutothione in the cells, thus protecting endothelial cells from the injury by the oxidized molecules

and finally protect the body against their harmful effects. Nowadays, commercially available synthetic antioxidants such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) are being replaced with natural origin antioxidants because of their toxicity and carcinogenicity. Garlic and its preparations show antioxidant action by scavenging ROS, enhancing the cellular antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase, and increasing glutathione in the cells (Fig. 121.3). It has been observed that aqueous extracts from raw garlic and unpeeled cloves treated with distinct thermal processes were able to differentially scavenge HOCl and that SAC was the only effective garlic compound [40].

Alliin scavenges superoxide, while allyl cysteine and allyl disulfide do not react with superoxide. Allicin suppress the formation of superoxide by the xanthine/xanthine oxidase system, probably via a thiol exchange mechanism. It is now concluded that alliin, allyl cysteine, and allyl disulfide all scavenges hydroxyl radicals ($OH^{\bullet}$). Allyl disulfide, alliin, allicin, and allyl cysteine exhibit different patterns of antioxidant activities as protective compounds against free radical damage [41–43] and appear in fresh garlic approximately 1,000 times more potent as antioxidants than those found in aged garlic extract, while whole garlic and aqueous garlic extract exhibit direct antioxidant effects and enhance the serum levels of two antioxidant enzymes: catalase and glutathione peroxidase [44]. Several studies have been performed to test the antioxidant activity of raw and boiled garlic by using different assays: β-carotene linoleate model system (β-carotene), radical scavenging activity by 1, 1-diphenyl-2-picrylhydrazyl (DPPH), scavenging activity against nitric oxide (NO) with 2, 29-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^+$), ferric-reducing/antioxidant power (FRAP), and Cu^{2+}-induced LDL-C oxidation [45–49]. It has been investigated that an aqueous extract obtained from 1 mg of a garlic preparation was as effective as an antioxidant as 30 nmol ascorbic acid and/or 36 nmol α-tocopherol [50]. In rat liver microsomes, garlic extract prevented formation of thiobarbituric-acid-reactive substances in cell membranes during lipid peroxidation in a dose-dependent manner [51]. In an in vitro and animal study, there was a significant improvement in the plasma lipid levels in rats fed cholesterol-containing diets and an increase in the plasma antioxidant activity in groups of rats fed cholesterol-free diets supplemented with raw and boiled garlic at 100°C for 20 min [52, 53]. A concentration-dependent inhibition of LDL-C oxidation was observed with the oil-soluble garlic compound, allixin [54]. In another in vitro study, AGE significantly reduced Cu^{2+} and 15-lipoxygenase-mediated lipid peroxidation of isolated human LDL-C by 81% and 37%, respectively [55]. Aqueous garlic extracts have the ability to scavenge superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) in the following aqueous preparations: (a) extracts of boiled garlic cloves (BG), (b) extracts of microwave-treated garlic cloves (MG), and (c) extracts of pickled garlic (PG) and heated extracts of (a) garlic powder (HGP) and (b) raw garlic (HRG). The data were compared with the unheated raw garlic (RG) or with the unheated garlic powder (GP). Extracts of GP and RG scavenged $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$ in a concentration-dependent way. The ROS scavenging capacity was not decreased in the aqueous garlic extracts except in MG and HRG (for $O_2^{\bullet-}$) and in HGP and PG (for H_2O_2), while the heating before or after garlic cutting was unable to eliminate the capacity of the extracts to scavenge H_2O_2, $O_2^{\bullet-}$, and $OH^{\bullet}$ [56]. Moreover, it was also found that fresh garlic, subjected to a cooking regimen of 100 °C during 20 min, preserves its bioactivity: the decrease in the contents of the studied compounds and the decrease in the total antioxidant potential were statistically not significant [53]. Recently, Lei et al. [57] suggested that DADS and DATS protect eNOS activity against ox-LDL-C insult. This protection can be attributed partly to their mediation of phosphatidylinositol 3-kinase/protein kinase B signaling and prevention of eNOS degradation. Subsequently, another recent study [58] suggested that SAC, *S*-benzylcysteine (SBC), and *S*-propylcysteine (SPC) to be excellent hydroxyl radical (•OH) scavengers, while SAC only as a modest peroxyl radical (HOO•) scavenger. As described, the sulfur content in garlic responsible for antioxidant activity, furthermore some recent studies reported the abundance of phenolic compound in garlic leaves including gallic acid and quercetin, contributing the antioxidant activity [7, 59, 60].

6 Clinical Studies

In a randomized, double-blind, placebo-controlled study with three parallel arms [61], 17 participants consumed garlic oil (4 mg), 18 participants consumed garlic powder (0.5 g), and 17 participants consumed placebo for 11 weeks. Garlic oil caused a relatively rapid (4 weeks) rise in total antioxidant capacity compared with placebo or garlic powder. However, at 6 weeks, a significant rise also could be seen with garlic powder; by 11 weeks, it reached the same level as that obtained with garlic oil. Moreover, in a randomized, double-blind, placebo-controlled crossover trial [62], in which 10 normolipidemic subjects (5 males, 5 females) took six capsules each day containing either 100 mg of garlic powder/tablet or placebo followed by a 1-week washout period and then another 2 weeks on the alternate substance. The study data indicated that, for individuals taking garlic, there was increased antioxidant status as evidenced by an increase in the resistance of LDL-C to oxidative stress as compared with participants taking placebo [62]. However, the TBARS assay used in this study did not use purified LDL-C, and it is now known that the use of purified LDL-C yields more reliable data. In a small-scale preliminary double-blind, placebo-controlled, crossover study [54] involving eight subjects (4 men and 4 women), four subjects took 1.2 g AGE three times a day for 2 week, then 2 week of no garlic (washout period), followed by 2 week of placebo. The use of the garlic supplement was found to significantly increase the resistance of LDL-C to oxidation [54]. A study [63] showed that after short-term garlic supplementation in essential hypertensive patients (EH) regarding indices of oxidative stress, there is a significant reduction in ox-LDL-C and 8-iso-PGF2α levels. In a study [64] on six organosulfur compounds, derived from garlic showed marked antioxidative and antiglycative effects in partially oxidized (or glycated) LDL-C and plasma against further deterioration in 36 diabetic patients. Durak et al. [65] showed that consumption of 10 g of garlic/day for 4 months causes significant increase in blood antioxidant capacity and improved blood lipid profile in hypertensive patient. Dhawan and Jain [66] in 2005 demonstrated in a clinical trial of hypertensive patients, supplementation with garlic leads to significant reduction in 8-Hydroxy-2′-deoxyguanosine (8-OHdG), nitric oxide (NO) levels and lipid peroxidation along with an increase in vitamin levels (A, E, and C) and total antioxidant status (TAS).

7 Proposed Mechanism

Many authors suggest various possible garlic antioxidant mechanism of action, in which scavenging of ROS is most common mechanisms whereby garlic derivatives confer its important health care benefits. Vaidya et al. [67] suggested that the peroxyl-radical-trapping activity of garlic is primarily due to 2-propenesulfenic acid formed by the decomposition of allicin. Thus, sulfenic acids are very probably the most potent of all peroxyl-radical-trapping antioxidants. New insights on the antioxidant mechanism of garlic derivatives *S*-allylcysteine and its corresponding sulfoxide (alliin) showed the highest and lowest HOCl-scavenging capacities [68]. This scavenging activity is enhanced by increasing the number of S atoms or by the alanyl group ($-CH_2CH–NH_2–COOH$) and decreased in the absence of the C=C bond or in the presence of a sulfoxide group in the thioallyl group [68]. Recently, Miron et al. [69] showed that the allicin diffuses through cell membranes and exerts its biological effects by rapidly reacting with intracellular free thiols, such as reduced glutathione (GSH), cysteine, and sulfhydryl groups of proteins. The reaction of the allylthio group with those cellular components constitutes the major beneficial effects of allicin. The first product is most likely that of the *S*-allylthio-mixed disulfide (AS-SX) with GSH. Another recent study [58] suggested the mechanism by which SAC, SBC, and *S*-propylcysteine (SPC) scavenge •OH and ROO• due to amelioration when the allyl group was replaced by benzyl or propyl groups.

8 Antihypertensive Effects

Hypertension is a collective risk factor for cerebrovascular disease, ischemic heart disease, peripheral vascular disease, and renal disease characterized by systolic blood pressure of 140 mmHg or greater, and/or a diastolic blood pressure of 90 mmHg or greater, in people who are not taking antihypertensive medication. A number of studies have documented the hypotensive effect of garlic and their bioactive preparations. In 1973, Chanderkar and Jain [70] reported the hypotensive (10–50 mmHg) effect of alcoholic garlic extract (2.5–25 mg/kg) after oral administration in experimentally induced hypertension. Some studies [71, 72] showed a slight decrease in both systolic and diastolic pressures after intravenous injection of garlic extracts in experimental animals. Gastric administration of encapsuled garlic powder to anesthetized dogs induced a dose-dependent (2.5–15 mg/kg) prolonged decrease in arterial blood pressure [73]. Single or multiple doses of 0.5 mL of aqueous extract of garlic were given orally to two-kidney-one-clip (2K–1C) model rats that showed a maximum antihypertensive effect at 2–6 h after administration [74]. Experimental rats who were fed a 2% high-cholesterol diet exhibited a 23.50% increase in systolic blood pressure which was significantly reduced when the rats were fed an aqueous extract of garlic powder containing allicin on a daily basis [75]. In another study [76], chronic feeding of diets containing either AGE or raw garlic (RG) powder for 10 weeks resulted in a reduction of the increase of systolic blood pressure compared with the control group from 4 weeks after beginning the experimental diets. The effect of AGE was accompanied by a decrease of pulse pressure (PP), suggesting an improvement of the pliability of the artery, although RG did not affect PP. In a recent animal study [77], combination of fresh garlic homogenate compound *S*-allyl cysteine sulfoxide and captopril exerted super-additive (synergistic) interaction with respect to fall in blood pressure and angiotensin converting enzyme (ACE) inhibition and suggested the combination of garlic with captopril should be avoided. Continuous researches have been carried out on animals and have repeatedly documented the significant hypotensive activity of garlic. Another recent study [78] demonstrated that garlic homogenate in moderate dose (250 mg/kg) with added hydrochlorothiazide possesses synergistic cardioprotective and antihypertensive properties against fructose- and isoproterenol-induced toxicities in albino rats.

9 Clinical Studies

Evidence has been found that people's belief in continuing garlic use for the management of hypertension is justified and well documented. A very early research in 1921 showed the hypotensive effect of garlic tincture [79]. Allimin tablets containing 4.75 g of garlic concentrate (0.31 g of desiccated garlic and 2.375 g of desiccated parsley) administered to 26 hypertensive patients three times daily for 3 days resulted in reduction of systolic and diastolic blood pressure (12.3 mmHg and 6.5 mmHg) in 85% of the patients [80]. Intake of about 900 mg/day garlic powder in hypercholesterolemic [81], mild hypertension patients [82], and normotensive subjects [83] resulted in reduction of diastolic blood pressures as compared to the nongarlic consuming groups. In another study [28], there was a 5.5% decrease in systolic blood pressure and a modest reduction of diastolic blood pressure in response to 900 mg/day aged garlic consumption. Short-term consumption of garlic supplementation (250 mg/day for 2 months) in essential hypertensive patients resulted significant decline in both systolic and diastolic blood pressures [63]. Garlic extract consumption for 4 months caused significant reductions in systolic and diastolic blood pressures in 13 hypertensive patient [65]. A study [84] showed that the undamaged garlic (swallowed) had no lowering effect on lipid level of serum, while crushed garlic (chewed) reduces cholesterol, triglyceride, MDA, and systolic and diastolic blood pressures. In a double-blind parallel randomized placebo-controlled trial [85] involving 50 patients, receiving four capsules of aged garlic extract (960 mg containing 2.4 mg SAC) daily for 12 weeks resulted in lowering of systolic blood pressure similar to current first-line medications in patients with treated but uncontrolled hypertension. Recently, a randomized, placebo-controlled parallel feeding trial [86] showed a significant reduction in both systolic and diastolic blood pressures in hypertensive subjects after taking two 500 mg capsules of processed garlic for 8 weeks. There was one meta-analysis performed by Silagy and Neil in 1994 [87] which included eight trials using the same dried garlic powder preparation (Kwai). The results from the data of 415 subjects after analysis showed only three of the trials were specifically conducted in hypertensive subjects, and out of the seven trials that compared the effect of garlic with that of placebo, three showed a significant reduction in systolic blood pressure (SBP) and four in diastolic blood pressure (DBP). In 2008, a study [88] searched the databases for studies published between 1955 and October 2007 in which 11 of 25 studies included in the systematic review were suitable for meta-analysis. The author suggested that garlic preparations are superior to placebo in reducing blood pressure in individuals with hypertension. Another meta-analysis study [89] was published in the same year 2008 which included ten trials in the analysis. The result showed that garlic reduced SBP by 16.3 mmHg and DBP by 9.3 mmHg compared with placebo in patients with elevated SBP in the three trials. However, the use of garlic did not reduce SBP or DBP in patients without elevated SBP. A very recently published meta-analysis [90] containing the data from January 1994 to December 2010 including 13 studies included 659 subjects. The result of all studies showed a mean decrease of 4.2 ± 2.4 mmHg for SBP in the garlic group compared to placebo, while the mean decrease in the hypertensive subgroup was 7.3 ± 2.2 mmHg for SBP and 6.7 ± 1.4 mmHg for DBP. There were no statistically significant effects of garlic (compared to placebo) observed for DBP of all subjects and the nonhypertension subgroup.

10 Possible Mechanism

Many authors proposed the possible mechanism by which garlic influences antihypertensive effects. Early investigation [72] proposed that prostaglandin-like activity responsible for antihypertensive action of garlic decreases peripheral vascular resistance. Whenever, the body increases production of angiotensin II converting enzyme (ACE), blood pressure increases. Garlic showed blood pressure reducing properties which have been linked to its hydrogen sulfide (H_2S) production [91]. It has been demonstrated that garlic and garlic-derived organic polysulfides, such as diallyl trisulfide (DATS) and diallyl disulfide (DADS), induce H_2S production in a thiol-dependent manner and allicin content liberated from alliin and the enzyme alliinase which has angiotensin II inhibiting and vasodilating effects in in vitro [92], animal [93] studies. Nitric oxide (NO) is a well-recognized vasodilator and vasorelaxant for endothelial and smooth muscle cells. Garlic extract and *S*-allyl-γ-cysteine significantly increase NO production in endothelial cells [94, 95] which results in lowering blood pressure [96]. In addition, in in vitro [97] and animal studies [98], garlic shows nitric-oxide-dependent relaxation in pulmonary arteries. Amino acid analysis of garlic powder demonstrated that it is a rich source of arginine, the precursor of NO. This was explained by the fact that NG-nitro-L-arginine methyl ester (L-NAME, a NOS inhibitor) abolished the vasodilatory effect of garlic. In a single in vitro study [99], garlic opens K^+ channels, which can reduce calcium influx and cause vasodilation which was ultimately responsible for the antihypertensive activity.

11 Antithrombotic and Fibrinolytic Effect

Blood platelets are mainly responsible for maintaining the hemostatic integrity of blood vessels and to stop bleeding after injury. Due to coronary artery disease and rupture of atherosclerotic plaque, there is increase in the platelet count and activation of the coagulation cascade with platelet thrombus formation, and that finally leads to vessel embolism. Garlic inhibits the platelet aggregation and prevents the thrombosis in in vitro animal models, and clinical studies. During the 1970s, there were three animal studies which reported the antiplatelet activity of garlic. The essential oil was more effective than clofibrate in the usual clinical dose of 33 mg/kg/day prevent lipid accumulation in the rabbit aorta [100], and essential oils of garlic protect against experimental atherosclerosis by preventing the fall in the alpha lipoprotein fraction and by enhancing fibrinolytic activity [101]. Sainani et al. [102] in 1979 showed enhanced fibrinolytic activity in albino rabbits when administered with garlic juice (0.25–25 g/day) in 10 mL distilled water. Aqueous garlic extracts inhibit platelet aggregation in vivo when added to plasma rich platelet at a concentration of 10 μM, suggesting allicin as principle inhibitor [103]. ADP-, epinephrine-, collagen-, and arachidonate-induced platelet aggregation is inhibited in vitro by garlic extract in a dose-dependent manner by inhibition of the prostacyclin biosynthesis in rat aorta [104]. Diallyl disulfide and diallyl trisulfide are mainly responsible for the prevention of acute platelet thrombus formation in stenosed canine coronary arteries model [105]. Several in vitro and in vivo studies were continuously performed and have proven the previous work with proposed mechanism hypothesis. In an ex vivo study [106], infusion of different garlic extract (10, 2, 5 and 100 mg/kg) in the ear vein of the rabbit significantly inhibits serum TXB_2 production in a distinct dose and time-dependent pattern. Chloroform/acetone extracts of fresh garlic have been shown to inhibit cyclooxygenase activity directly in in vitro, with the acetone extract being more effective [107]. In 1992, Lawson et al. [108] suggested that the antiaggregatory activity of garlic clove homogenates (*S*-allyl cysteine sulfoxide) in platelet rich plasma was due to adenosine; however, in whole blood neither adenosine nor the polar fraction had any effect, and all of the antiaggregatory activity was due to allicin and other thiosulfinates compound. Ajoene is a well-established antiplatelet agent in garlic, and its inhibitory effect on platelet aggregation has been extensively studied and documented both by in vivo and in vitro experiments [109, 110] including inhibition of baboon platelet aggregation in vitro (75 μg/mL) and in vivo (25 mg/kg) induced by adenosine diphosphate (ADP) or collagen [111]. Diallyl trisulfide inhibited platelet aggregation and Ca^{2+} mobilization induced by thrombin without affecting the production of IP_3 [112]. Another study suggested that garlic component sodium 2-propenyl thiosulfate modulated cyclooxygenase activity in canine platelets in a dose-dependent manner, thus preventing their aggregation [113]. Administration of the garlic in a rat in situ loop model,suggested that odorless garlic not only activates fibrinolytic activity by accelerating tPA-mediated plasminogen activation but also suppresses the coagulation system by downregulating thrombin formation [114]. Recently, an in vitro study [115] suggested the alcoholic wild garlic extract is more potent, while *Allium sativum* and *Allium ursinum* exert similar antiaggregatory effects in a dose dependant manner and inhibit platelet aggregation induced via the ADP pathway.

12 Clinical Studies

Several clinical studies done by Bordia and colleagues [116, 117] in the late 1970s and early 1980s reported that a garlic oil preparation rich in vinyldithiins, sulfides, and ajoene could inhibit platelet aggregation and result in increased fibrinolytic activity. In 1977, study on 10 healthy individuals, 10 patients with old myocardial infarction, and 20 patients with acute myocardial infarction showed garlic (1 g/kg b.w.) significantly increased fibrinolytic activity in all subjects especially in healthy group [116]. Later on, Bordia et al. [118] showed that garlic oil preparation inhibits platelet function in both healthy subjects and in patients with coronary heart disease. A randomized, double-blind, placebo-controlled crossover study [119] of 12 healthy subjects reported that garlic powder (Kwai, 900 mg/day) inhibited platelet aggregation induced by ADP and collagen. Administration of garlic in a daily dose of 2 × 2 capsules (each capsule containing ethyl acetate extract from 1 g peeled and crushed raw garlic) showed antiplatelet activity and also inhibited platelet thromboxane formation [120]. In another study [121] of garlic powder, it was found that feeding 7.2 g of AGE powder/day (~25 mL/day of liquid AGE) to hypercholesterolemic men resulted in inhibition of epinephrine (another platelet aggregating agent) and collagen-induced aggregation, but not ADP-induced aggregation. A 13-week study [122] involving normolipidemic subjects who ingested 5 mL of aged garlic extract per day significantly inhibited both the total percentage and initial rate of platelet aggregation at concentrations of ADP up to 10 μmol/L. Besides sulfur containing compound, other nonsulfur compounds, such as β-chlorogenin and quercetin, have also been shown to inhibit platelet aggregation [123]. A meta-analytical survey based on 11 electronic databases study [124], which included 1,798 pertinent records, 45 randomized trials, and 73 additional studies, reported cardiovascular-related effects were limited to randomized controlled trials lasting at least 4 weeks, and it has been found that in comparison with placebo, garlic preparations lead to a significant reduction in platelet aggregation [124]. Cavagnaro et al. [125] describe the effect of cooked garlic on antiplatelet activity on the blood sample from two healthy subjects, who had abstained from eating *Alliums* or other known platelet inhibitory foods for at least 1 week. The result showed oven heating at 200 °C or immersing in boiling water for 3 min or less did not affect the ability of garlic to inhibit platelet aggregation (as compared to raw garlic), whereas heating for 6 min completely suppressed in vitro antiaggregatory activity in uncrushed, but not in previously crushed, samples [125]. Prolonged incubation (more than 10 min) at these temperatures completely suppressed in vitro antiaggregatory activity, while microwaved garlic had no effect on platelet aggregation. A randomized, double-blind, placebo-controlled, crossover study involving 14 healthy subjects showed one large dose of garlic oil (~9.9 g garlic) slightly but significantly affected adrenaline but not ADP or collagen-induced platelet aggregation [126]. In a recently published report, it was shown that there was reduction in adenosine-induced platelet aggregation by garlic diallyl sulfide (2.2 μg) compound in women participants with type 2 diabetes mellitus [127].

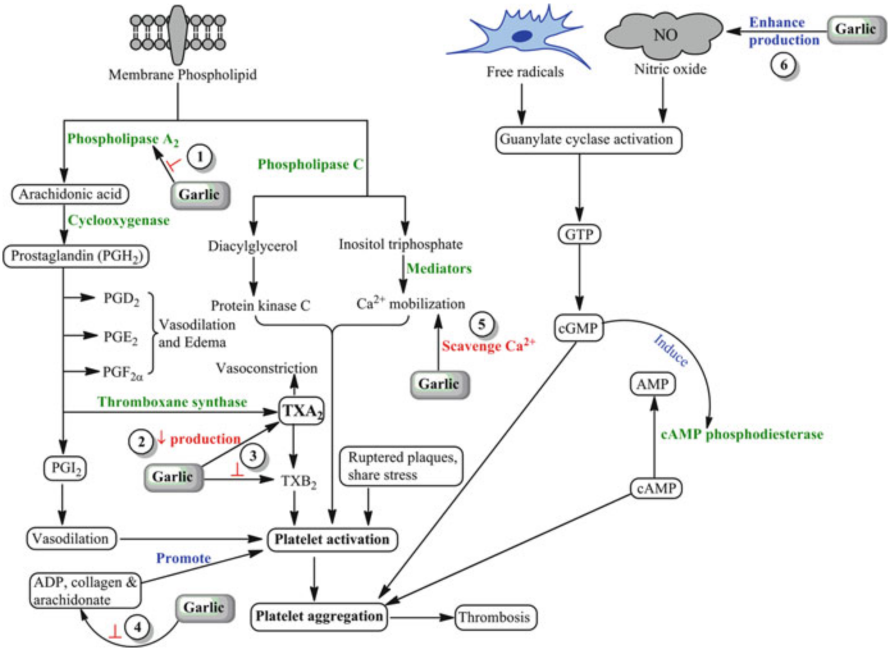
Membrane Phospholipid
Free radicals
NO
Nitric oxide
Enhance production
Garlic
6
Phospholipase A$_2$
1
Garlic
Phospholipase C
Guanylate cyclase activation
Arachidonic acid
Cyclooxygenase
Prostaglandin (PGH$_2$)
Diacylglycerol
Inositol triphosphate
Mediators
GTP
PGD$_2$
PGE$_2$
PGF$_{2\alpha}$
Vasodilation and Edema
Protein kinase C
Ca^{2+} mobilization
5
Scavenge Ca^{2+}
Garlic
cGMP
Induce
Vasoconstriction
Thromboxane synthase
TXA$_2$
AMP
2
↓ production
3
Garlic
TXB$_2$
Ruptered plaques, share stress
cAMP phosphodiesterase
PGI$_2$
cAMP
Vasodilation
Platelet activation
Promote
ADP, collagen & arachidonate
Garlic
4
Platelet aggregation
Thrombosis

Fig. 121.4 Proposed antiplatelet mechanism of garlic: *1*-directly inhibit the phospholipase A2; *2*-modulate the TXA_2 and decrease the production; *3*-directly inhibit the TXB_2 function; *4*-inhibit the ADP, collagen and arachidonate induced platelet aggregation; *5*-effect on Ca^{2+} mobilization via scavenging the available Ca^{2+}; *6*-directly enhance the nitric oxide production

13 Proposed Mechanism

Arachidonic acid (AA) is an essential fatty acid precursor in the biosynthesis of leukotrienes, prostaglandins, and thromboxanes (Fig. 121.4). Various platelet agonists mobilize calcium through G-protein-coupled receptors. Calcium activates phospholipase A_2, which liberates arachidonic acid from phosphatidylcholine and phosphatidylethanolamine. Calcium also activates myosin light-chain kinase. AA is liberated from phospholipids and, in the presence of the enzyme cyclooxygenase, incorporates oxygen to form the endoperoxide prostaglandin G_2 (PGG_2). PGG_2 is then quickly transformed to prostaglandin H_2 (PGH_2). PGH_2, in the presence of thromboxane synthase, produces thromboxane A_2 (TXA_2) which further mobilizes calcium from intracellular storage sites. TXA_2 and activated myosin light-chain kinase together lead to platelet coagulant activation by stimulating secretion of products of platelet granules, allowing tenase and prothrombinase formation [128]. TXA_2 is a vasoconstrictor and platelet aggregating compound which serves as precursor for inactive thromboxane-B_2 (TXB_2). Ajoene strongly inhibits the metabolism of arachidonic acid by both cyclooxygenase and lipoxygenase pathways [129, 130], thus inhibiting the synthesis of TXA_2, AA metabolite, and

12-hydroxy-eicosatetraenoic acid (12-HETE). AGE has been shown to reduce thromboxane formation [106]. It has also been reported that *N*-ethylmaleimide causes the disaggregation of both ADP and thrombin-induced platelet aggregation and that this disaggregation is a result of the removal of calcium ions (Ca^{2+}) from the platelet cytosol. Therefore, the effects of AGE on calcium mobilization were investigated in both A23187 and ADP-stimulated platelets [131]. In the presence of AGE, the initial concentration of calcium ions was significantly less than when the experiments were performed in the absence of AGE. This could be due to the metal chelation properties of AGE, as reported earlier [55]. In support of this, garlic extract has been shown to strongly inhibit calcium binding, suppressing the influx of calcium ions by chelating calcium within platelet cytosol and arteriosclerotic nanoplaque formation [132, 133]. AGE may inhibit phospholipase A_2, thus reducing levels of lysophosphatidic acid, which causes platelet aggregation and increases intracellular calcium ions [131]. Antiaggregatory effect of ajoene may also be causally related to its direct interaction with the putative fibrinogen receptor (GPIIb/IIIa) in a dose-dependent manner [134]. The GPIIb–IIIa receptor has a high content of –SH groups, and binding of fibrinogen is inhibited by the organosulfur compound ajoene [135]. AGE has been reported to enhance NO production by activating cNOS, but not iNOS [136], which may increase the GTP concentration and ultimately induces platelet aggregation.

14 Antiatherosclerotic Effect

Atherosclerosis is a result of an interaction between fat and cholesterol within the cellular components of the arterial wall and buildup in the walls of arteries to form hard structures called plaques, the pathogenic substratum of many cardiovascular diseases. Various in vitro and clinical studies have shown and confirm the effect of garlic in the prevention and treatment of atherosclerosis. Early studies on experimental animals showed the reduction in aortic lipid content of garlic fed animals by 72%, while in the control group there was no significant reduction. The data suggests that cholesterol is depleted from experimentally induced atherosclerosis by garlic administration [137, 138]. Treatment with aged garlic extract reduces fatty streak development, vessel wall cholesterol accumulation, and the development of fibro fatty plaques in neointimas of cholesterol-fed rabbits, thus providing protection against the onset of atherosclerosis [139]. In cell cultures, aqueous solutions of dried garlic powder containing allicin and ajoene significantly inhibit the proliferative activity of smooth muscle cells from atherosclerotic aortic plaques [140, 141]. In hypercholesterolemic rabbits, garlic supplements significantly reduced the aortic lesions and lipid content of existing fatty plaques [142]. AGE exerts antiatherogenic effects through inhibition of smooth muscle phenotypic change and proliferation and by another (unclarified) effect on lipid accumulation in the artery wall [143]. Garlic exerts hypocholesterolemic and antiatherogenic activity by inhibition of plasma cholesteryl ester transfer protein (CETP) activity, which may delay the progression of atherosclerosis, thereby supporting the atherogenicity of CETP and the inhibitory activity of garlic supplementation against CETP [21]. An in vitro study's results show the formation of the ternary proteoheparan sulfate HS-PG/LDL/Ca^{2+} complex, which is initially responsible for the "nanoplaque" composition and ultimately for the arteriosclerotic plaque generation, where the garlic extract strongly inhibits Ca^{2+} binding to HS-PG [132]. A notable restoration of arterial blood pressure; significantly enhanced vasorelaxant response to adenosine, acetylcholine, and isoproterenol; and reduction in atherogenic properties of cholesterol were seen in animals on garlic-supplemented diet [144]. Daily dietary supplement of allicin, 9 mg/kg body weight, reduced the atherosclerotic plaque area by 68.9% and 56.8% in apolipoprotein E-deficient and low density lipoprotein (LDL) receptor knockout mice, respectively, as compared with control mice and also by using pure allicin preparation; an in vitro study results showed that allicin may affect atherosclerosis not only by acting as an antioxidant but also by other mechanisms, such as lipoprotein modification and inhibition of LDL uptake and degradation by macrophages [145]. A recent in vitro study suggested that AGE inhibit monocyte differentiation into macrophages, CD36 expression, and oxidized LDL-C (oxLDL-C) uptake into macrophages induced by the cardiovascular risk factor homocysteine (Hcy) [146].

15 Clinical Studies

In a randomized, placebo-controlled trial in ten healthy adults, there was a significant improvement in plasma viscosity and capillary blood flow within 5 h after taking 900 mg of standardized garlic powder [147]. Another randomized placebo-controlled double-blind crossover study in healthy volunteers showed increased erythrocyte velocity results from vasodilation of precapillary arterioles which increased diameter of erythrocyte column by an average of 8.6 % along with simultaneous inflow of interstitial fluidity accompanied by a significant decrease in hematocrit and plasma viscosity (rheoregulation) [148]. In a placebo-controlled trial of patients with stage II peripheral arterial occlusive disease, garlic powder supplements (800 mg/day) were associated with a significant increase in walking distance by 46 m; the improvement started after the fifth week of treatment mainly by simultaneous decrease in spontaneous thrombocyte aggregation [149]. A cohort study including 101 healthy adults who took at least 300 mg daily of dried garlic powder for at least 2 years were compared with 101 age and gender matched controls who were not taking supplements; pulse wave velocity and elastic vascular resistance (two measures of arterial elasticity) were significantly lower in the garlic group than in the control group, even after controlling for age and systolic blood pressure, that is, chronic garlic powder intake was associated with an attenuation in age-related increases in aortic stiffness [150]. In a prospective, 4-year clinical trial of patients treated with 900 mg daily of standardized garlic powder, there was a 9–18 % reduction in plaque volume, a 4 % decrease in LDL-C levels, an 8 % increase in HDL-C concentrations, and a 7 % decrease in blood pressure [151]. Similar results were reported in a 4-year German trial in 152 older adults; those who took high-dose garlic for 4 years, demonstrated reduced atherosclerotic plaque in both carotid and femoral arteries by 5–18 % [152]. A study of 11 atherosclerotic patients with oxidative stress showed prevention of oxidation reaction by eliminating this oxidative stress and significantly lowered plasma and erythrocyte malondialdehyde (MDA) levels after the ingestion of garlic extract [153]. A placebo-controlled, double-blind, randomized pilot study indicates the potential ability of AGE to inhibit the rate of progression of coronary calcification in 19 patients, as compared to placebo over 1 year [154]. AGE consumption increases plasma nitric oxide synthase (NOS) activity in 11 atherosclerotic patients and suggested that AGE may arise from its NOS-inducing and nitric-oxide (NO)-producing activities [155].

16 Proposed Mechanism

The exact molecular mechanism of garlic by which it shows antiatherosclerotic effect is not fully understood. The development of atherosclerotic plaque or lesions is a result of endothelial dysfunction induced by elevated and modified LDL and ox-LDL, free radicals, toxins, homocysteine, hypertension, and other unknown risk factors. It has also seen that disturbance in immune system supports the formation of atherosclerotic plaques. Diets supplemented with garlic are able to restore endothelial function in experimental laboratory animals [144] and investigations of humans [156]. It has been shown that raw garlic possibly works via its active metabolite allicin action on coronary endothelial function and vasoreactivity [157]. The electrophysiological correlation to vasodilatation in human coronary arteries under the influence of garlic extract showed decrease in the isometric wall tension. Allicin and ajoene hyperpolarized the cell membrane and relaxed the vascular strips in a concentration-dependent manner and suggested that garlic extract and its compounds can be classified as phytopharmacological K^+ channel openers [99]. OxLDL-C, but not native LDL-C, contributed to atherogenesis and promoting vascular dysfunction by exerting direct cytotoxicity toward endothelial cells, by increasing chemotactic properties for monocytes, by transforming macrophages to foam cells via scavenger receptors, and by enhancing the proliferation of endothelial cells, monocytes, and smooth muscle cells. Garlic compounds can effectively suppress LDL-C oxidation in vitro and that short-term supplementation of garlic to humans increases resistance of LDL-C to oxidation [54, 158]. Hcy, a metabolite from methionine, serves as an independent cardiovascular disease risk factor, which causes thrombosis and oxidative-stress damage and is often associated with atherosclerosis and a higher risk of coronary heart disease, stroke, and peripheral vascular disease by damaging the inner lining of arteries and promoting blood clotting. Hcy has an inverse relationship with folate deficiency and decreased NO production. AGE may at least partly prevent a decrease in bioavailable NO and endothelium-derived hyperpolarizing factor during acute hyperhomocysteinemia [159] and also decrease plasma total hcy concentration by 30 % without changing the protein-bound/free hcy ratio [160]. Coronary arterial calcification (CAC), a marker of plaque formation in human coronary arteries and atherosclerosis, has been linked to an increased risk for cardiovascular events such as myocardial infarction, fatal arrhythmia, and congestive heart failure. AGE inhibits the rate of progression of coronary calcification as compared to placebo over 1 year [154]. C-reactive protein (CRP) is one of the strongest predictors for the risk of atherosclerosis and cardiovascular events in subjects with and without cardiovascular disease. But there was only one study which showed that 12 weeks of treatment with a high-dose, chemically well-characterized, production-controlled garlic powder had no significant effect on CRP protein in normolipidemic subjects with risk factors for CVD [161].

17 Other Cardioprotective Activity

Cardiac arrhythmias are any abnormality or perturbation in the normal activation sequence of the myocardium. Some earlier studies showed beneficial effects of garlic in cardiac arrhythmias. Garlic powder (1 % corresponding to Kwai/Sapec added to a standard chow for a 10-week period) significantly reduces the incidence of ventricular tachycardia (VT) and fibrillation (VF) in isolated perfused rat heart [162]. Another study in the same manner using garlic powder (1 % added to a standard chow for an 8-week period) also showed significantly reduced ischemia reperfusion-induced ventricular fibrillation (VF) in isolated perfused rat heart and suggested that an intact alliin–alliinase system is important for this activity of garlic [163]. Garlic dialysate decreased the positive inotropic and chronotropic effects of isoproterenol in a concentration-dependent manner and suggesting via β-adrenoceptor blocking action produced by the garlic dialysate [164]. Another study by same author suggested that garlic dialysate has a significant antiarrhythmic effect in both ventricular and supraventricular arrhythmias [165]. Aqueous garlic extract increased the amplitudes of atrial complex "p" wave and the ventricular complex "QRS" of the rat ECG. This is suggestive of increase in voltage output of the atria and ventricles probably in accordance with positive inotropism [166]. A recent animal study suggested that garlic cannot alter the ventricular fibrillation threshold (VFT), but it significantly decreases the upper limit of vulnerability (ULV) in a dose-dependent pattern, indicating that it can reduce the range of the stimulation strength between the VFT and ULV (vulnerability window) during the vulnerable period of a cardiac cycle [167].

A few studies have shown the beneficial effect of garlic and its active constituents in conjugation with some drugs. Allylmercaptocaptopril is an example of a conjugate of the ACE inhibitor drug captopril with garlic allicin. Allylmercaptocaptopril prevented progressive weight gain, without a detectable effect on food intake, lowered blood pressure, and improved cardiac hypertrophy, as indicated by heart weight and ventricular-wall thickness [168]. Garlic therapy in animals with myocardial infection showed improved survival and cardiac function by add-on captopril [169, 170] and propranolol [171]. Garlic juice inhibited the contractions of rabbit and guinea pig aortic rings induced by norepinephrine in Ca^{2+}-free and Ca^{2+}-containing Krebs–Henseleit solutions and indicated that it produces concentration-dependent synergistic effect by its calcium-blocking property [172]. Beneficial effects of combined therapy of garlic and hydrochlorothiazide were also demonstrated and confirmed in the recent past [173, 174]. The same author also reported that combination of *S*-allyl cysteine sulfoxide (SACS) from fresh garlic homogenate and captopril exerted super-additive (synergistic) interaction with respect to fall in blood pressure and ACE inhibition [77]. In one preliminary study, in which pretreatment with aged garlic extract for 27 days ameliorated the effect of an active anticancer agent doxorubicin (DOX) administration on cardiac tissue; cardiomyocytes looked more or less similar to those of control and suggested that aged garlic extract is potentially protective against doxorubicin-induced cardiotoxicity [175].

18 Bioavailability and Metabolism

Very few studies have been conducted on the bioavailability of garlic compounds. An animal study [176] showed that in a very short time (10 min) after orally administering alliin (10 mg/mouse), it was observed in the stomach (7.2%), intestine (22.4%), and liver (2.5%) without the production of allicin and its degradation compounds such as DADS, vinyldithiins, and allyl-SS conjugated compounds, suggesting alliin is not metabolized to respective organosulfur compounds without an appropriate enzyme (allinase). In a pharmacokinetic study using synthesized 35S-labeled alliin, 60–70% was absorbed in rats [177]. Alliin along with DADS has been detected in the perfusate after the isolated rat liver passage, while there was absence of allicin [178] even in human serum or urine from 1 to 24 h after ingesting 25 g of raw garlic containing a significant amount of allicin [108]. These findings indicate that alliin itself is never converted to allicin in the body and metabolized into various organosulfur compounds such as DADS by liver enzymes. When allicin is added to fresh blood, it is quickly transformed into allyl mercaptan, but this compound was not found in blood or urine of people who consume garlic, also demonstrating that the level of allyl methyl sulfide (AMS) in the exhaled air depended on amount of the ingested allicin or its derivatives [179]. Vinyldithiins, 2-vinyl-4H-1,3-dithiin, and 3-vinyl-4H-1,2-dithiin, have been detected in the serum, kidney, and fat tissue >24 h after oral ingestion, while only 1,3-vinyldithiin was found in the rat liver [180]. The metabolic fate of [^{35}S]-labeled DADS in rats after intraperitoneal injection with the maximum concentration of [^{35}S]-labeled DADS by mice livers occurred 90 min after treatment, and about 70% of the radioactivity was distributed in the liver cytosol, of which 80% was metabolized to sulfate [181]. The pharmacokinetics of SAC is well established in animal studies, which is detected in the blood, and its pharmacokinetic parameters are well associated with oral dose administration. Significant concentration of *N*-acetyl-*S*-allyl-L-cysteine is also identified as a metabolite of SAC in the urine. This indicates that SAC could be transformed into *N*-acetylated metabolite by *N*-acetyltransferase in the body. The bioavailability of SAC is 103.0 % in mice, 98.2% in rats, and 87.2% in dogs [182]. SAC was found also in human blood after ingestion of AGE, the main component of which is SAC [183]. DAS could be metabolized by one of the cytochrome P_{450} isoenzymes to form diallyl sulfoxide (DASO) and then diallyl sulfone ($DASO_2$) [184]. After GC–MS analysis, two major peaks, which were identical to allyl mercaptan and DADS after ingesting grated garlic, could be detected in human breath without other organosulfur volatiles [185]. There is a need of major clinical trial which confirm the bioavailability of sulfur and nonsulfur compounds in garlic.

19 Side Effects and Toxicity

According to the suggested doses from the studies and optimum pharmacological responses, the following doses are recommended: 2–5 g of fresh raw garlic, 0.4–1.2 g of dried garlic powder, 2–5 mg garlic oil, and 300–1,000 mg of garlic extract (as solid material). Other preparations should correspond to 4–12 mg of alliin or approximately 2–5 mg of allicin, an active constituent of garlic. Various studies have been investigated in search of toxicity from garlic, but till now there is no known toxic constituents in garlic and its preparations. Garlic is considered to have very low toxicity and is listed as Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (FDA). The most common side effects produced by intake of small amounts of garlic are bad breath and body odor. When garlic is taken in a high dose by a sensitive individual, it is known to cause gastric irritation. A clinical trial showed side effects which include heartburn, nausea, vomiting, diarrhea, flatulence, bloating, mild orthostatic hypotension, flushing, tachycardia, headache, insomnia, sweating, and dizziness as well as offensive body odor [186]. In some cases, people are allergic to sulfur-based compounds and reported allergic reactions to garlic; namely, contact dermatitis, asthma, rhinitis, conjunctivitis, urticaria, anaphylaxis, and angioedema [187–189]. Garlic may be used safely in pregnant and breast-feeding mothers. Consumption of garlic enhances the pharmacological effects of anticoagulants (warfarin, fluindione) but reduces the efficacy of anti-AIDS drug saquinavir [190]. A clinical trial reported that coadministration of garlic did not significantly alter warfarin pharmacokinetics or pharmacodynamics [191].

20 Conclusion

Evidence from in vitro, in vivo, and clinical studies support the beneficial effects of garlic consumption in various preparations in the prevention of cardiovascular disease. The raw garlic and AGE are the most important among the available preparation and showed maximum pharmacological effect in low dose. The present report suggests that garlic has the ability to prevent excess free radical production, maintain the oxidative balance via increase in antioxidant status and increase in bioavailability of nitric oxide, prevent vascular inflammation, reduce cholesterol content and plaque formation, and inhibit platelet aggregation.

Evidence suggests that garlic may produce modest but not clinically significant effects in the treatment of hyperlipidemia and hypertension via reduction in DBP. Indeed, the results from clinical trials are very few and inconsistent, probably due to differences in garlic preparations, unknown active constituents and their bioavailability, and small sample size. Systematic reviews are available for the possible antilipidemic, antihypertensive, antithrombotic, and chemopreventive effects. However, the clinical evidence is far from compelling. Garlic appears to be generally safe, although some allergic reactions may occur [192]; therefore, it would be a safe tool for the treatment and prevention of CVD. In conclusion, the proposed in vitro, in vivo, and animal models should be further verified in human studies in order to establish a causative link between molecular properties and the role of garlic active constituents in the prevention and treatment of CVD.

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Ginkgolides and Neuroprotective Effects 122

Syed Haris Omar

Contents

1 Introduction 3700
2 Major Bioactive Constituents and Preparations 3700
3 Ginkgolides 3701
4 Ginkgolides Pharmacology 3701
5 Neuroprotective Activities of Ginkgolides 3701
6 CNS Effects of *Ginkgo Biloba* 3703
6.1 Cerebral Blood Flow 3703
6.2 Cognition 3707
6.3 Neurotransmitter System 3709
6.4 Free Radical Scavenging 3711
6.5 Apoptosis 3713
7 *Ginkgo biloba* in CNS Disorders 3713
7.1 Alzheimer's Disease 3713
7.2 Parkinson's Disease 3718
7.3 Anxiety 3719
7.4 Cerebral Insufficiency/Ischemia and PAF 3720
7.5 Retinal Degeneration and Glaucoma 3723
7.6 Vestibular Dysfunction 3724
7.7 Other Neuroprotective Activities 3726
8 Ginkgolides Pharmacokinetics 3726
9 Side Effect 3727
10 Conclusion 3727
References 3728

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Abstract

Ginkgo biloba is commonly known as maidenhair tree, available as a popular herbal supplementary in Asian, European, and American countries. Extensive in vitro, in vivo, and clinical studies demonstrated and confirmed the neuroprotective effects of a commercial standard ginkgo extract formulation known as EGb-761. Terpene trilactone comprises 5–7 % in EGb-761, collectively called as ginkgolides (G-A, G-B, G-C, G-J, G-K, G-L and G-M) and bilobides. Its clinical application gained popularity in herbal medicine due to treatment of early-stage Alzheimer's disease, cerebrovascular disorders, PAF antagonism, and vestibular disorders. In addition, ginkgolides showed potent antioxidant activities via scavenging of reactive oxygen and nitrogen species. The physiological dosage of ginkgo extracts ranges between 120 and 240 mg/day in humans, and it is readily available as an over the counter product/supplementary product. According to the recent clinical findings, EGb-761 did not confirm its effect on long-term cognitive functioning, while it showed effectiveness and enhancement in shor-term cognitive and related activities. Ginkgo is generally well tolerated, but in a high dose, it can cause gastric upset, skin allergy, and increase the risk of bleeding in patients with risk factors (anticoagulant or antiplatelet treatment, surgery, etc.). Neuropharmacology, clinical issues of safety and usage are addressed in this book chapter.

Keywords

Ginkgo • ginkgolides • neuroprotection • PAF antagonism • terpenoids

Abbreviations

5-HT	5-Hydroxytryptamine
8-OHdG	Hydroxyl-deoxyguanosine
ACh	Acetylcholine
AD	Alzheimer's disease
ADDLs	Amyloid beta-derived diffusible soluble ligands
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BALF	Bronchoalveolar lavage fluid
BBB	Blood–brain barrier
BN-52021	Ginkgolide B
cAMP	Cyclic adenosine monophosphate
D_2	Dopaminergic receptor 2
DA	Dopamine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSC-MRI	Dynamic susceptibility contrast-enhanced magnetic resonance imaging
EGb	*Ginkgo biloba* extract

EGb-761	Standard ginkgo extract
FFA	Free fatty acid
G. biloba	*Ginkgo biloba*
G-A	Ginkgolide A
GABA_A	Gamma-amino butyric acid
G-B	Ginkgolide B
G-C	Ginkgolide C
G-J	Ginkgolide J
G-K	Ginkgolide K
G-L	Ginkgolide L
G-M	Ginkgolide M
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase-1
i.p.	Intraperitoneally
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-jun N-terminal kinase
KYNA	Kynurenic acid
LI-1370	Standard *Gingko* extract
MAO	Monoamine oxidase
MDA	Malondialdeide
MGluR	Metabotropic glutamate receptor
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribose nucleic acid
MS	Multiple sclerosis
NCE	Noncontact erection
NE	Norepinephrine
NF-kappa B	Nuclear factor kappa B
NFT	Neurofibrillary tangles
NIDDM	Non-insulin dependent diabetes mellitus
NMDA	*N*-methyl D-aspartate receptor
NO	Nitric oxide
P8A	Standard *Gingko* extract
PAF	Platelet activating factor
PBR	Peripheral-type benzodiazepine receptor
PD	Parkinson's disease
PKA	Protein kinase-A
PVN	Paraventricular nucleus
RAGE	Receptors for advanced glycation end products
RNA	Ribose nucleic acid
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species

USP	United States Pharmacopeia
VF	Ventricular fibrillation
VSMCs	Vascular smooth muscle cells
VT	Ventricular tachycardia
WHO	World Health Organization

1 Introduction

Ginkgo biloba, maidenhair tree, is one of the ancient tree classified in its own division called, Ginkgophyta which belongs to family Ginkgocea, the only species still surviving and found in Asian countries (China, Japan, and Korea) [1]. There are unequivocal representatives of the genus Ginkgo which have been described as a new species, *Ginkgo yimaensis* and another extinct "species," *Ginkgo adiantoides* [2]. Genus, *Ginkgo* has been derived from the Japanese name Yin-Kwo, meaning silver fruit and the species *biloba*, describes the bilobed shape of the leaves. *G. biloba* is also known as a living fossil because of the finding of the fossil plants quite similar to *G. biloba* which date back to 180 million years ago [3]. In the ancient period, ginkgo seeds were used against cough, asthma, enuresis, alcohol misuse, pyogenic skin infections, and worm infestations in the intestinal tract. This is first mentioned in the great herbal Pen Ts'ao Kang Mu of 1596 by Li Shih-chen [4]. The leaf extracts were used for the improvement of the blood circulation, both peripherally and centrally. This started in the 1960s in Germany [5]. The draft monographs on ginkgo folium and extract mentioned in the USP [6, 7] and ginkgo folium and standardized ginkgo extract are in the European Pharmacopeia [6]. The positive monographs on *G. biloba* were published in the German Commission E, which are available in an English translation as well [8]. In addition to the Commission E, WHO also published a positive monograph on *Ginkgo* leaf extracts, which is in principle comparable [9]. This chapter will summarize the beneficial effects and provide an update on gingkolides, mostly in the form of EGb-761 preparation in the various neurological conditions and diseases.

2 Major Bioactive Constituents and Preparations

The commonly used parts of *G. biloba* are the seeds and leaves, which contains most of their pharmacologically important constituents and makes them medicinally important. The seeds contain mainly polysaccharide and protein (ginkbilobin), which stimulates apoptosis of human hepatoma SMMC-7721 cells and exhibit antifungal activity. However, seed also contains 4-*O*-methylpyridoxine, a ginkgotoxin, a poisonous compound whose primary mode of action is to antagonize the activity of vitamin B_6 [10]. The leaf extract is generally categorized into two types: full extracts and standardized extracts. The full leaf extracts are usually prepared with alcohol and contain all alcohol soluble constituents. The standardized extracts are EGb-761 and LI-1370, but EGb-761 is more common in use and contains a variety of active constituents. The major bioactive compounds in EGb-761 are classified as flavonol glycosides (24 %), terpene trilactones (5–7 %), proanthocyanidins (7 %), carboxylic acids (13 %), catechins (2 %), non-flavonol glycosides (20 %), alkylphenols (5 ppm), and other (unknown 28 %) shown in Fig. 122.1. Other preparation like ginkgo extract (P8A) that is approximately 10-fold enriched in terpene trilactones and contains bilobalide and the four ginkgolides (G-A, G-B, G-C, G-J) extracted from the leaves of the plant [11]. While the preparation BN-52021 is specific for ginkgolide B and used as ginkgolides activity.

3 Ginkgolides

Ginkgolides were first isolated by Furukawa in 1932 from the root bark of *G. biloba* [12]. Maruyama et al. [13–17] and Nakanishi [18] isolated and elucidated the structure of ginkgolides G-A, G-B, G-C, and G-M from the root bark. Meanwhile, Okabe and Sakabe independently determined the structures of G-A, G-B and G-C from leaves of *G. Biloba* by means of X-ray crystallography [19, 20]. Later, ginkgolide G-J [21] and two new traces ginkgolide G-K and G-L have been identified [22]. From the total of 5–7 % terpene trilactones, 2.8–3.4 % are ginkgolides A, B, and C, and 2.6–3.2 % are bilobalides, ginkgolides contributes 50–60 %. Chemically, ginkgolides (Fig. 122.2) are diterpene trilactones consist of C-20 terpenes, 6 five-membered rings, that is, a spiro[4, 4]-nonane carbocylic ring, three lactones, and a tetrahydrofuran ring. The presence of a tertiary butyl group makes ginkgolides unique among natural products. All are pharmacologically active; however, ginkgolide B is by far the most potent and has received far more attention from researchers due to their distinguishing structure, specific occurrence, and broad pharmacology.

4 Ginkgolides Pharmacology

A number of studies have been conducted on the pharmacological activities of *G. biloba* crude extract, commercial available extract (EGb-761), and leaf extract. G-B and ginkgolide extract (EGb-761) have received more attention due to their higher potency, broad action, and versatile constituents [23]. The in vitro, in vivo, and clinical studies demonstrated the anti-inflammatory, cardioprotective, anticancer, antidiabetic, antioxidant, and gastroprotective activities as shown in Table 122.1. Out of the various beneficial studies, few of them have not been confirm by further researcher, in animal models, and which are confirmed in animals did not further conducted in human (clinical trial).

5 Neuroprotective Activities of Ginkgolides

Neuroprotection refers to maintaining the intactness of cellular interactions/intercellular communication in the brain resulting in an overall undisturbed

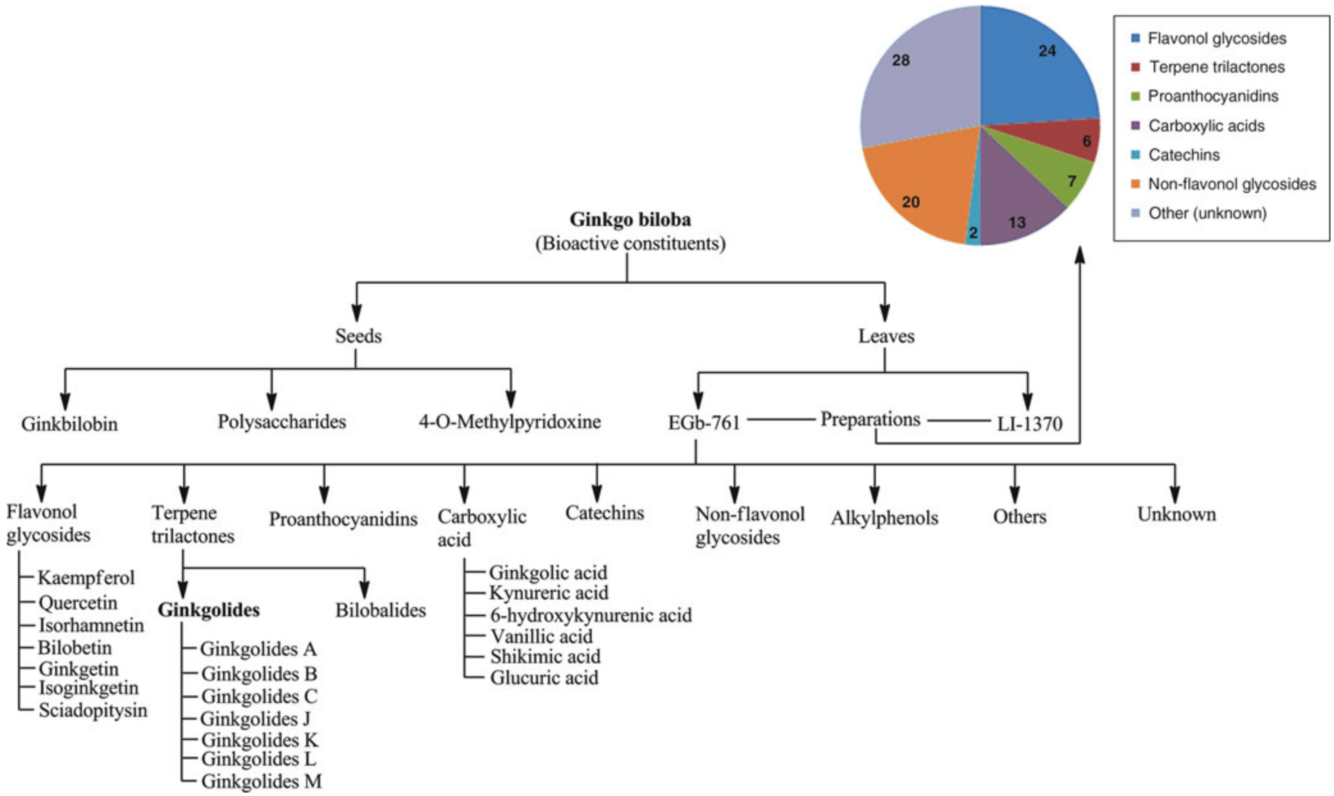
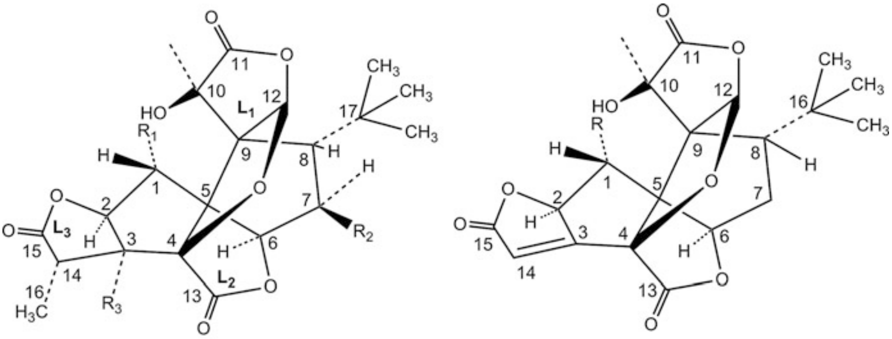

Ginkgo biloba
(Bioactive constituents)
Seeds
Leaves
Ginkbilobin
Polysaccharides
4-O-Methylpyridoxine
EGb-761
Preparations
LI-1370
Flavonol glycosides
Kaempferol
Quercetin
Isorhamnetin
Bilobetin
Ginkgetin
Isoginkgetin
Sciadopitysin
Terpene trilactones
Ginkgolides
Ginkgolides A
Ginkgolides B
Ginkgolides C
Ginkgolides J
Ginkgolides K
Ginkgolides L
Ginkgolides M
Bilobalides
Proanthocyanidins
Carboxylic acid
Ginkgolic acid
Kynureric acid
6-hydroxykynurenic acid
Vanillic acid
Shikimic acid
Glucuric acid
Catechins
Non-flavonol glycosides
Alkylphenols
Others
Unknown
24
6
7
13
2
20
28
Flavonol glycosides
Terpene trilactones
Proanthocyanidins
Carboxylic acids
Catechins
Non-flavonol glycosides
Other (unknown)

Fig. 122.1 Major bioactive components and their broad classification in *Ginkgo biloba* extract (EGb-761)



	R1	R2	R3
Ginkgolide G-A	H	H	OH
Ginkgolide G-B	OH	H	OH
Ginkgolide G-C	OH	OH	OH
Ginkgolide G-J	H	OH	OH
Ginkgolide G-M	OH	OH	H

	R
Ginkgolide G-K	OH
Ginkgolide G-L	H
	L
Lactone ring	1,2,3

Fig. 122.2 Chemical structures of ginkgolides

function [45]. Both prevention and delayed onset of neurological disorders would have a large impact in terms of reducing both suffering and costs. Nowadays, researcher interest in herbal medicine has grown in several countries. Moreover, efforts have been made to find new therapeutic agents from these natural products for the prevention or treatment of memory disorders, such as the gradual impairment of memory in aging or in neurodegenerative pathology and even lifestyle factors. Ginkgolides from *G. biloba*, especially G-B and preparation EGb-761, have emerged as natural/herbal source of neuroprotective agents. Various in vitro/in vivo and preclinical/clinical studies confer their neuroprotective activities (Fig. 122.3).

6 CNS Effects of *Ginkgo Biloba*

6.1 Cerebral Blood Flow

Interruption or inclusion in cerebral blood flow leads to a decrease in supply of oxygen and nutrient to the brain, which ultimately leads to several neurodegenerative diseases. Table 122.2 showed the protective effect of EGb-761 in cerebral blood flow disorder animal models.

There are various mechanisms involved in decrease in cerebral blood flow. Research studies suggested three major events including increase uptake of glucose or decrease tissue glucose content, leads to increase glucose utilization and cerebral embolism which have cumulative effects on the cerebral blood flow (Fig. 122.4).

Table 122.1 Common pharmacological activities of *G. biloba* (Ginkgolides)

Pharmacological activities	Preparations	Effects	References
Anti-inflammatory	Ginkgolide B, A, C and J	Inhibit the increase of T-helper 2 cytokines, such as interleukin IL-5 and IL-13, in bronchoalveolar lavage fluid (BALF), suppression of extracellular regulating kinase/MAPK (mitogen activated protein kinase) pathway; ginkgolide B, A, C, and J inhibited PAF-mediated aggregation of human platelets at concentrations of 2.5, 15.8, 29.8, and 43.5 μg/mL,	[24, 25]
Antiarrhythmic	EGb-761	Dose-dependently reduction in ventricular fibrillation (VF) and ventricular tachycardia (VT) in the hearts of rats	[26]
Anticancer	EGb-761	Dose-dependent decreases in xenograft growth of both MDA-MB-231 breast cancer and U-87 glioma cell lines in nude mice; suppress proliferation and increase cytotoxicity in HepG2 and Hep3B cells	[27, 28]
Antidiabetic	EGb-761 and G-B	Ingestion of *G. biloba* extract by an NIDDM subject may increase the hepatic metabolic clearance rate of not only insulin but also the hypoglycemic agents; protect beta cells	[29–31]
Anti-ischemic reperfusion	EGb-761	preventive effect on ischemia-reperfusion injury in rat urinary bladder; reduced hydroxyl-deoxyguanosine (8-OHdG) formation in the DNA from liver undergoing ischemia-reperfusion	[32, 33]
Anti-atherosclerotic	EGb-761	Significantly suppressed the proliferation and migration of VSMCs, promoted apoptosis and reduced inflammatory processes	[34]
Antimicrobial	G-A, G-B, and the standardized Ginkgo leaf extracts	Exhibited antimicrobial activity against *Streptococcus pyogenes*; crude extracts of Ginkgo leaves (7.8 μg/mL) possess inhibitory activity against Gram-positive and Gram-negative bacteria	[35, 36]
Antioxidant	*G. biloba* extract; G-A & G-B	Inhibit NF-κB activation induced by H_2O_2; antagonize iNOS-mediated NO production in macrophages stimulatory effect via antioxidation and attenuation of NF-κB activation	[37–39]
Antithrombotic	EGb-761	In a rat model of thrombosis, the antithrombotic effects of EGb-761 combination therapy were more effective than with ticlopidine alone; combinative therapy of G-B and cilostazol enhanced antithrombotic efficacies without increasing side effects	[40, 41]

(*continued*)

Table 122.1 (continued)

Pharmacological activities	Preparations	Effects	References
Gastroprotective	*G. biloba* extract	EGb (25, 50, and 100 mg/kg, ig) inhibit the increase of MDA both in gastric mucosa and in serum; significantly inhibit the ethanol-induced gastric lesions in rats	[42, 43]
Vasodilation	*G. biloba* extract injectable solution	Treatment in healthy elderly adults leads to the increase of LAD (left anterior descending) blood flow and improved endothelium-dependent vasodilatory capacity	[44]

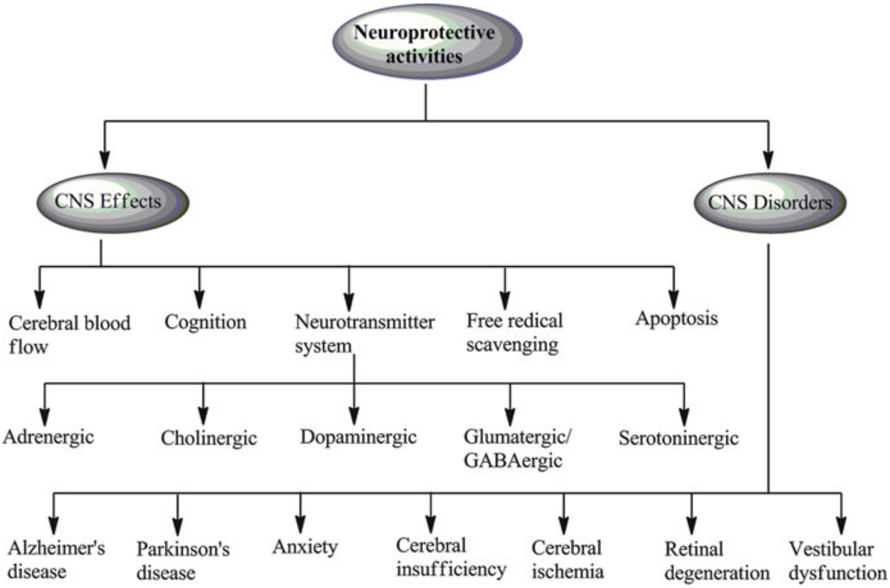

Neuroprotective activities
CNS Effects
CNS Disorders
Cerebral blood flow
Cognition
Neurotransmitter system
Free redical scavenging
Apoptosis
Adrenergic
Cholinergic
Dopaminergic
Glumatergic/ GABAergic
Serotoninergic
Alzheimer's disease
Parkinson's disease
Anxiety
Cerebral insufficiency
Cerebral ischemia
Retinal degeneration
Vestibular dysfunction

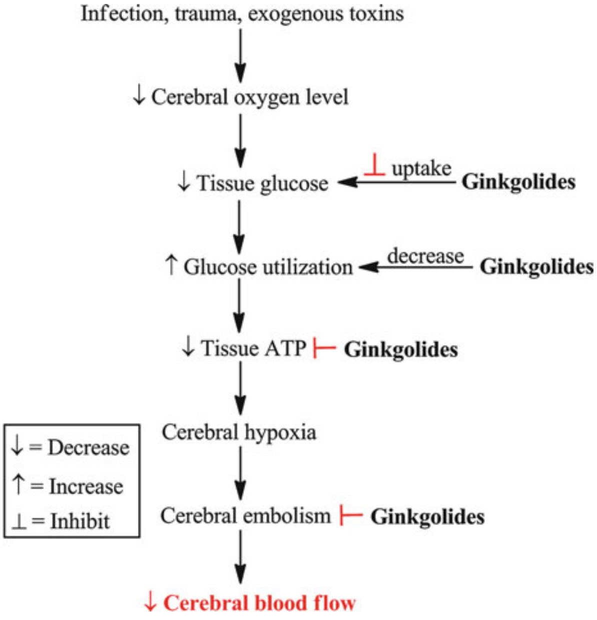
Fig. 122.3 Ginkgolides involved in multiple neuroprotective activities

6.1.1 Clinical Trials

In a double-blind randomized placebo-controlled trial of 72 outpatients with cerebral insufficiency of at least 24 weeks duration, EGb-761 improved mental/mnestic performance [53]. A meta-analysis was conducted to evaluate *G. biloba* (120–240 mg/day) effectiveness in treating cerebrovascular insufficiency [54]. All of the seven studies included in the analysis were double-blind, placebo-controlled

Table 122.2 Protective effect of ginkgolides in animal models of cerebral blood flow disorders

Compound	Study	Action	Reference
EGb-761	Rats	Partially suppressed the effects of the brain embolization	[46]
EGb-761	Rats	(100 mg/kg, i.p.) survived hypobaric hypoxia for a much longer period than controls	[47]
EGb-761	Rats	Dose (10 mg/mL) dependently inhibition of glucose uptake and decrease in the cortical glucose concentration	[48]
EGb-761	In vitro	0.5–1 μg/mL inhibit the ATP decrease induced by hypoxia	[49]
EGb-761	Rats	50 mg/kg/day decreases in glucose utilization in the frontoparietal somatosensory cortex, nucleus accumbens, cerebellar cortex, and pons	[50]
EGB	Dogs	Injection of an extract of *G. biloba*; the cerebral blood flow was increased and decreased in cerebrovascular resistance	[51]
EGb-761	Rats	Reduction in cranial perfusion pressure and regional cerebral blood flow	[52]



Infection, trauma, exogenous toxins
↓ Cerebral oxygen level
↓ Tissue glucose
⊥ uptake
Ginkgolides
↑ Glucose utilization
decrease
Ginkgolides
↓ Tissue ATP
Ginkgolides
Cerebral hypoxia
Cerebral embolism
Ginkgolides
↓ Cerebral blood flow
↓ = Decrease
↑ = Increase
⊥ = Inhibit

Fig. 122.4 Ginkgolides in cerebral blood flow: inhibits the glucose uptake, decrease glucose utilization, inhibit decrease in ATP contents and inhibits cerebral embolism

trials that showed significant improvements compared to placebo on all of the individual symptoms that were analyzed (one study was inconclusive) [54]. Recently, in a pilot study [55] of 9 healthy subjects, the dynamic susceptibility contrast-enhanced magnetic resonance imaging (DSC-MRI) showed a significant increase of non-normalized cerebral blood flow after *G. biloba* extract capsule (15 % in white and 13 % in gray matter).

Table 122.3 Beneficial effects of ginkgolides in animals models of cognitive disorders

Compound	Study	Action	References
EGb-761	Mice	Oral administration (100 mg/kg) facilitate memory processes, acquisition, and improved the performance	[56]
EGb-761	Mice	100 mg/kg dose per day showed improvement in short-term memory but only in the aged group mice	[57]
EGb-761	Mice	a daily i.p. injection (40 mg/kg) cause enhancing effect on the performance of a learning task as observed in both group of mice (adult and aged)	[58]
EGb-761	Rats	200 mg/kg dose showed significant improve in continuous learning and delayed nonmatching to position tasks in aged rats	[59]
EGb-761	Rats	Chronic (60 mg/kg/day i.p.) and acute (60 or 120 mg/kg i.p.) injections enhanced olfactory recognition in young rats and facilitative effects in aged rats that received a single 60 mg/kg i.p. injection	[60]
EGb-761	Chicks	(3 mg/mL) dose facilitate memory in chicks with poor long-term retention	[61]
EGb-761	Rats	high doses (150 mg/kg/b.w./day) significantly improved considerable memory, cognitive performance, and exploratory behavior	[62]
Leaf extract (gingkoselect)	Rats	(100 mg/kg/day, orally, 21 days) normalized cognitive deficits in rats chronically treated with corticosterone and improved memory in the chronically stressed rats	[63]
EGb-761	Rats	50 or 100 mg/kg doses per day protect against intermittent hypoxia-induced memory impairment, oxidative stress, and neuronal DNA damage	[64]

6.2 Cognition

Cognitive abilities include perception, memory, judgment, perceptual speed, spatial manipulation, and reasoning. These declines as part of normal aging. Dementia is a loss of cognitive abilities in multiple domains that results in impairment in normal activities of daily living and loss of independence. AD is the most common cause of dementia, responsible for 60–80 % of all dementia. *Gingko* extract has been involved in improvement of cognitive disorder in various animal models (Table 122.3).

6.2.1 Clinical Trials

Numerous clinical studies have been conducted that showed the *G. biloba* has a beneficial effect in age-related decrease in cognition.

A double-blind, crossover comparative trial [65] with healthy female volunteers documented the ability of EGB ability to improve short-term memory. There was no any significant effect observed at lower doses (120 and 240 mg). However, when the women were given 600 mg of EGB and tested 1 h later, the results showed a very significant improvement in short-term memory compared to women taking a placebo [65]. In a double-blind, randomized placebo-controlled long-term study (24 weeks) involving 72 outpatients with cerebral insufficiency using EGb-761, it showed statistically significant improvement in the short-term memory after 6 weeks and of the learning rate after 24 weeks were observed in the ginkgo group, but not in the placebo group (longitudinal analysis) [53]. In a randomized, double-blind, placebo-controlled study [66] on aged patients receiving either *G. biloba* alcohol/water extract in a high dose (RD), a low dose (LD) or a placebo (PL) for 24 weeks resulted increase in short-term visual memory by 18 %, 26 %, and 11 % in the RD, LD, and PL groves respectively and indicating that the use of ginkgo extracts in elderly individuals with cognitive impairment might be promising. In contrast, another double-blind, placebo-controlled study [67], in 30 healthy male subjects who were receiving 260-mg tablets of Bio Ginkgo (LI-1370) daily for 5 days did not showed any significant results, furthermore another 24-week, randomized, double-blind, placebo-controlled, parallel-group, multicenter trial [68] did not show any statistically significant differences in mean change of scores between ginkgo (either 240 mg/day or 160 mg/day) or placebo. In a 6-week, double-blind, fixed-dose, placebo-controlled trial on intact persons over the age of 55 years who received EGb-761 (180 mg/day) [69], results showed significantly more improvement on a task, assessing speed of processing abilities (i.e., Stroop Color and Word Test color-naming task) and improved overall abilities to remember by the end of treatment as compared to participants who received placebo [69]. A placebo-controlled, multi-dose, double-blind, balanced, crossover trial of 20 healthy subjects who received 120, 240, and 360 mg of a standardized extract of ginkgo (GK501, Pharmaton, SA) or a matching placebo [70] was conducted. The results showed a number of significant changes on the performance measures after administration of ginkgo. The most striking of these was a dose-dependent improvement of the "speed of attention" factor following both 240 mg and 360 mg of the extract, which was evident at 2.5 h and was still present at 6 h. Additionally, there were a number of time and dose-specific changes (both positive and negative) in performance of the other factors [70]. In a very short-term study [71] on healthy postmenopausal, women aged 53–65 years, of one week of ginkgo treatment, showed improved performance in three of the cognitive tasks – the tests of short-term nonverbal recognition memory, mental flexibility, and sustained attention. In another placebo-controlled double-blind design study [72] on young healthy volunteers showed an acute dose of ginkgo significantly improved performance on the sustained attention task and pattern recognition memory task. In a prospective community-based cohort study involved 3,534 subjects aged 65 years and older [73], the results showed that initial consumption of EGb-761 did not modify the risk of dementia, whereas the consumption of other treatments for memory impairment was associated with a higher risk of dementia. Subjects who took *G. biloba* had a significantly lower risk of mortality in the long-term, even after adjustment for potentially confounding factors [73]. In a placebo-controlled, multi-dose, double-blind, balanced-crossover study [74] where combination treatment of 120 mg EGB complexed with phosphatidylserine resulted both in improved secondary memory performance and significantly increased speed of memory task performance across all of the post-dose testing sessions. In a small randomized, double-blind, placebo-controlled trial [75] of GB, 120 mg significantly improves the cognitive performance of subjects with multiple sclerosis (MS) via the Stroop test. In a meta-analysis study [76], which included 6 randomized placebo-controlled trial. Considering baseline risk in the assessment of treatment effect, EGb was found to be effective for cognitive functions in dementia with the treatment of 6 months [76]. Recently, Kaschel [77] showed that the EGb-761 (240 mg once daily) improves free recall of appointments in middle-aged healthy volunteers, which requires high demands on self-initiated retrieval of learned material.

6.3 Neurotransmitter System

Neurotransmitters are the chemical messengers in the nervous system, which relay information across synapses via excitation or inhibition of the next neuron or effector tissue. Neurotransmitters can be classified into two broad categories, small-molecule neurotransmitters and neuropeptides. Small-molecule neurotransmitters, such as acetylcholine and the monoamines, are synthesized in the axon terminal of the neuron and larger neuropeptides, such as somatostatin and vasopressin, are synthesized in the neuron's cell body. It has been shown that *G. biloba* and its gingkolides produces effect on a number of neurotransmitter systems, including serotonergic, adrenergic, dopaminergic, and cholinergic systems.

6.3.1 Adrenergic Transmission

A neurotransmitter formed in sympathetic postganglionic synapses, known as noradrenaline. Very few studies have been conducted to show the effect of *G. biloba* on adrenergic system. Chronic treatment with *G. biloba* extract on rat cerebral cortex resulted in increase in noradrenaline release along with decrease in the density of cerebral β-adrenoceptors and suggested as an adaptive mechanism (after 27 days or 2 months) [78]. The age-related decrease of α-2-binding-sites in rat cerebral cortex was prevented by EGb-761 treatment, indicating a relative increase of noradrenergic neurotransmission in aged rats, while the reduction in binding affinity was unaffected [79]. Oral administration of *G. biloba* extract at a dose of 90 mg/kg for seven consecutive days on rat brain modulate the β-adrenoceptors and implicated in the favorable effects of *G. biloba* extracts on learning and memory [80]. After 14 days of daily oral treatment with 100 mg/kg of EGb-761, which resulted significantly only in decrease of NE uptake in mice [81].

6.3.2 Cholinergic Transmission

Choline is a precursor for biosynthesis of the neurotransmitter acetylcholine; any modulation in the cholinergic system is known to influence cognitive processes, learning processes, and working memory [82]. Indeed, increases in cholinergic transmission are known to enhance working memory performance [83] and vice versa [84]. *G. biloba* extracts have been shown to enhance cholinergic processes in various cortical regions. In vitro studies indicate that EGb-761 (100 μg/mL) increases acetylcholine (ACh) release in hippocampal synaptosomes [85]. In vivo studies showed that EGB attenuate the amnesia induced by scopolamine [86], and chronic oral treatment with an extract of *G. biloba* increases the apparent muscarinic receptor population in the hippocampus of the aged rat [87].

6.3.3 Dopaminergic Transmission

Dopamine (DA) is a monoamine neurotransmitter which has a number of important physiological roles and influences on brain function, including playing a role in regulating attention, cognition, movement, pleasure, and hormonal processes. Studies have shown the benefits of *Gingko* extract in the improvement of DA neuron or its physiological function under the influences of neurotoxicity. Administration of EGb-761 (20, 50, 100 mg/kg/day i.p.) for 7 days before or after MPTP treatment effectively protects against MPTP-induced nigrostriatal dopaminergic neurotoxicity and suggesting that the inhibitory effect of EGb-761 on brain MAO may be involved in its neuroprotective effect [88]. Another study showed the significant recovery of rats observed after EGb (50, 100, and 150 mg/kg) treatment for 3 weeks in 6-OHDA induced decrease in the level of DA and its metabolites and an increase in the number of dopaminergic D_2 receptors in striatum [89]. The neuroprotective effect of EGb-761 against MPTP neurotoxicity in mice, receiving EGb-761, had significantly attenuated MPTP-induced loss of striatal dopamine levels and tyrosine hydroxylase immune staining in the striatum and substantia nigra pars compacta. Moreover, the author suggested that the neuroprotection was associated with blockade of lipid peroxidation and reduction of superoxide radical production (indicated by a downregulation of Mn-superoxide dismutase activity) [90]. Chronic (100 mg/kg/14 days/once daily) treatment with EGb-761 showed dose-dependent increases in frontocortical dopamine levels and, to a lesser extent, in the striatum [91]. A recent study results suggest that administration of EGb-761 increases dopaminergic activity in the paraventricular nucleus (PVN) and the mesolimbic system to facilitate noncontact erection (NCE) in male rats [92].

6.3.4 Glutamateric and GABAergic Transmission

Glutamate is the major excitatory neurotransmitter of the cortex and hippocampus, released from vesicles in presynaptic terminals by a Ca^{2+}, and is involved in many aspects of higher mental function. In particular, loss and dysfunction (hyperactivity) of both the pre- and postsynaptic glutamatergic system have been linked to neurodegenerative disorders. *Gingko* extract treatments have shown the positive effect in the glutamate transmission either from loss or excitotoxitcity. Ginkgolide B reduced excitotoxic damage in cultured chick embryo telencephalic neurons overexposed to glutamate [93]. Furthermore, BN-52021 (100 mM) showed protection against glutamate toxicity when it was added to rat neuronal cultures 24 h after glutamate exposure [94]. EGb (2.5 mg/L) and its constituent G-B (2 mg/L) protected the neuronal viability against glutamate-induced injury and prevented the glutamate-induced elevation in the intracellular free calcium (Ca^{2+}) concentration. EGb (3–10 mg/kg) attenuated the decrease of nucleus areas in arcuate nuclei induced by glutamate (1 g/kg, s.c.) [95]. In contrast, another study showed an increase in protein kinase A (PKA) activation by G-B, which subsequently enhances the Ca^{2+} entry through voltage-dependent N and P/Q-type Ca^{2+} channels to cause increase in evoked glutamate release from rat hippocampal nerve terminals [96]. Recently, EGb-761 showed neuroprotection at a concentration 200 μg/mL to the spinal cord neurons from glutamate excitotoxicity and oxidative stress-induced cell death [97]. Kynurenic acid (KYNA) belongs to the group of low-affinity metabotropic glutamate receptor (mGluR) antagonists and interferes with the glycine B site of the NMDA receptor. An earlier study showed that EGb-761 modulating the glutamatergic systems are KYNA and 6-hydroxykynurenic acid (6-HKA) [98]. Ginkgolides are selective and potent antagonize at a concentration 10 μM the glycine receptor action and at IC_{50} 73 μM inhibit gamma-aminobutyric acid ($GABA_A$) receptors activity [99]. Ginkgolides A, B, and C noncompetitive inhibit the direct action of α, β, and γ $GABA_A$ receptor [100]. In vivo treatment of rats with EGb and its bioactive components G-A and G-B reduces the ligand-binding capacity, protein, and messenger RNA expression of the adrenocortical mitochondrial peripheral-type benzodiazepine receptor (PBR) [101].

6.3.5 Serotoninergic Transmission

Serotonergic neurotransmission plays a pivotal role in the etiology and expression of stress and anxiety disorders. In vitro EGb-761 (4–16 μg/mL) significantly increase the 5-HT uptake (23 %) and also similar effects have been found in ex vivo synaptosomes preparation from the cortex of mice treated orally (100 mg/kg/day) with EGb-761 [102]. It was found that an age-related decrease in $5\text{-}HT_{1A}$-receptor binding density in human cerebral cortex. Intraperitoneally administration of EGb-761 (5 mg/kg) resulting significantly (33 %) increase the binding density in aged rats [103]. These findings indicate that changes in $5\text{-}HT_{1A}$ receptors may reflect changes in the brain that are responsible for the impaired cognition that occurs with aging. *G. biloba* extract (14 mg/kg p.o.) restored restraint stress-induced elevation in whole brain levels of catecholamines (NE, DA), 5-HT, and plasma corticosterone to near normal levels [104]. The administration of EGb-761 (50 mg/kg per o.s./14 days) antistress property via enhancing the stimulation of $5\text{-}HT_{1A}$ receptors and preventing their desensitization after subchronic cold stress [105]. In addition another study showed the anti-aggressive effect of EGb-761 may be mediated by $5\text{-}HT_{2A}$ receptors in the MAO-A deficient mice [106]. EGb-761 also induces a stimulus control similar to that of $5\text{-}HT_{1A}$ receptor agonists, and indeed, changes in behavior induced by EGb-761 (10 mg/kg i.p.) were antagonized by the selective $5\text{-}HT_{1A}$ receptor antagonist WAY 100635 [107].

6.4 Free Radical Scavenging

Oxidation and reduction process is one of the cellular activity occurred in each and every kind of cells, whereas reactive oxygen and nitrogen species (RONS) are the major by product formed. Whenever the rate of formation of RONS is more than the

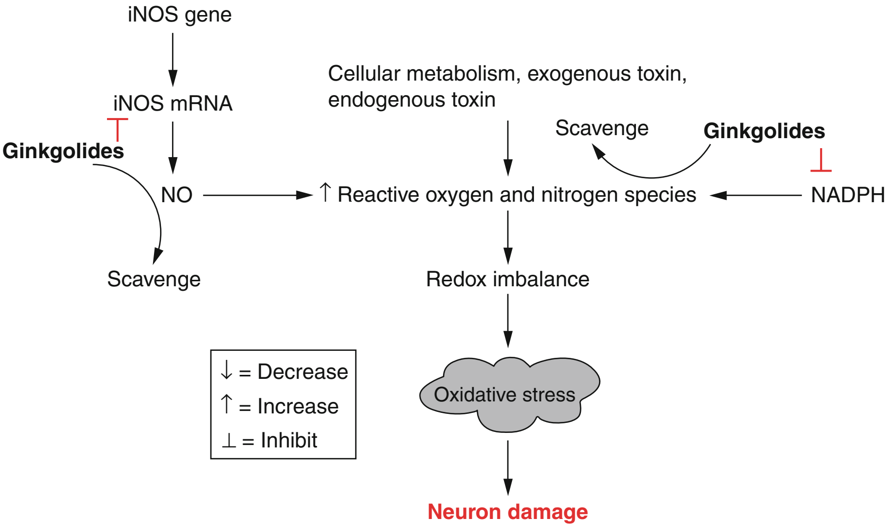

iNOS gene
iNOS mRNA
Ginkgolides
NO
Scavenge
Cellular metabolism, exogenous toxin, endogenous toxin
Scavenge
Ginkgolides
↑ Reactive oxygen and nitrogen species
NADPH
Redox imbalance
↓ = Decrease
↑ = Increase
⊥ = Inhibit
Oxidative stress
Neuron damage

Fig. 122.5 Antioxidant activity of ginkgolides: inhibits NADPH and scavenge RONS activities, inhibits iNOS and scavenge NO

rate of clearance from the cells then the oxidative stress environment created, which cause a number of neurodegenerative disorders including cerebral ischemia, neuronal hypoxia, and AD. Extensive research have been conducted and shown the antioxidant activity of *G. biloba* constituent (Fig. 122.5). In vitro, EGb-761 is a potent free radical scavenger via inhibition of NADPH-oxidase, decreased in the concentration of superoxide anion (O^{-2}) and hydrogen peroxide (H_2O_2) along with the reduction of hydroxyl radical generation ($OH^{\cdot}$) at concentrations as low as 15.6 μg EGb/mL [108, 109]. EGb-761 showed the dose-dependently inhibition of nitric oxide (NO) production in lipopolysaccharide/gamma interferon (LPS/IFNγ)-activated macrophages by concomitantly scavenging NO and inhibiting inducible nitric oxide synthase (iNOS) mRNA and enzyme activity [110, 111]. Excessive iron deposition and mitochondrial insufficiency are responsible for aging and degenerating nervous system. Heme oxygenase-1 (HO-1) is the rate-limiting enzyme that degrades heme to biliverdin, free iron, and carbon monoxide; its immunoreactivity is enhanced greatly in neurons and astrocytes of the hippocampus and cerebral cortex of Alzheimer subjects and co-localizes to senile plaques and neurofibrillary tangles (NFT) [112]. A study showed that EGb-761 induces HO-1 in a dose-dependent manner (0, 10, 50, 100 and 500 μg/mL) and suggested the protective activity in ischemia [113]. In an in vitro study, EGb-761 also displayed protective effects against toxicity produced by either H_2O_2 or nitric oxide which possibly mediate Aβ toxicity and completely blocked Aβ-induced events, such as reactive oxygen species accumulation and apoptosis [114]. Recently, EGb-761 pretreatment (100 mg/kg/o.s.) significantly increased the protein expression levels of Nuclear factor E_2 (Nrf2), HO-1, GAPDH, β-actin,

$CRMP_2$, and histone H_3 during t-BuOOH-induced oxidative stress and showed antioxidant as well as neuritogenic potential and suggesting the beneficial effect of extract in stroke and ischemic brain injury [115]. EGb-761 has been shown to increase the protein level and activity of antioxidant enzymes such as superoxide dismutase (60 %) and catalase (22 %) in rat hippocampus [116] and rat ileum [117] as well as of glutathione (GSH) reductase in mouse liver specifically from G-A [118]. Similarly, the activity of γ-glutamylcysteinyl synthetase, the rate-limiting enzyme of GSH synthesis, was enhanced by EGb-761 (200 μg/mL) in a dose-dependent manner [119]. In vitro study on human neuroblastoma cell line (SK-N-SH), where G-A, and G-B inhibit the NO-induced cytotoxicity in SK-N-SH cells via scavenging [120].

6.5 Apoptosis

EGb-761 (100 mg/L), G-J (100 mM/L), and G-B (10 mM/L) reduces apoptosis from staurosporine-induced apoptotic chick embryonic neurons to 24 %, 62 %, and 31 %, respectively [121]. It is well established that ROS may trigger apoptosis in various types of cells including T cells and neuronal cells. Mice were treated daily with 100 mg/kg EGb-761 per o.s. over a period of 2 weeks showed significantly reduction in ROS-induced apoptosis and protects spleen T-lymphocyte [122]. In vitro study showed that EGb-761 (100 mg/mL) prevented the hydroxyl radical-induced thymocyte apoptosis [123]. In contrast, in vivo study using EGb-761 (250 μg/mL) shown to effectively decrease oral cavity tumors by inducing apoptosis via caspase-3 activation [124]. In addition, another study also support and showed that treatment of mouse blastocysts with 5–10 μM G-A and G-B dose-dependently induced five- to eightfold increases in apoptosis and suggesting their pro-apoptotic activity [125]. Moreover, a recent animal study revealed after administration of 10 and 20 mg/kg G-B significantly suppress gene expressions of TLR-4 and NF-κB, lessen concentrations of TNF-α, IL-1β and IL-6 as well as reduce number of apoptotic neuronal cells in haemorrhagic rat brain tissues [126].

7 *Ginkgo biloba* in CNS Disorders

7.1 Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, pathologically characterized by deposition of amyloid beta (Aβ) plaques and neurofibril tangles ultimately leads to decline cognitive function and memory. A number of researches have been done on anti-Alzheimer's activity of ginkgo extract, and most of them have shown significant beneficial effect (Fig. 122.6). But also there were some studies which do not show any beneficial activity of ginkgo extract. Even though the results of nonsignificant, we cannot neglect the plenty of significant outcomes from the ginkgo extract. EGb-761 (100 μg/mL) was even able to protect (up to 8 h)

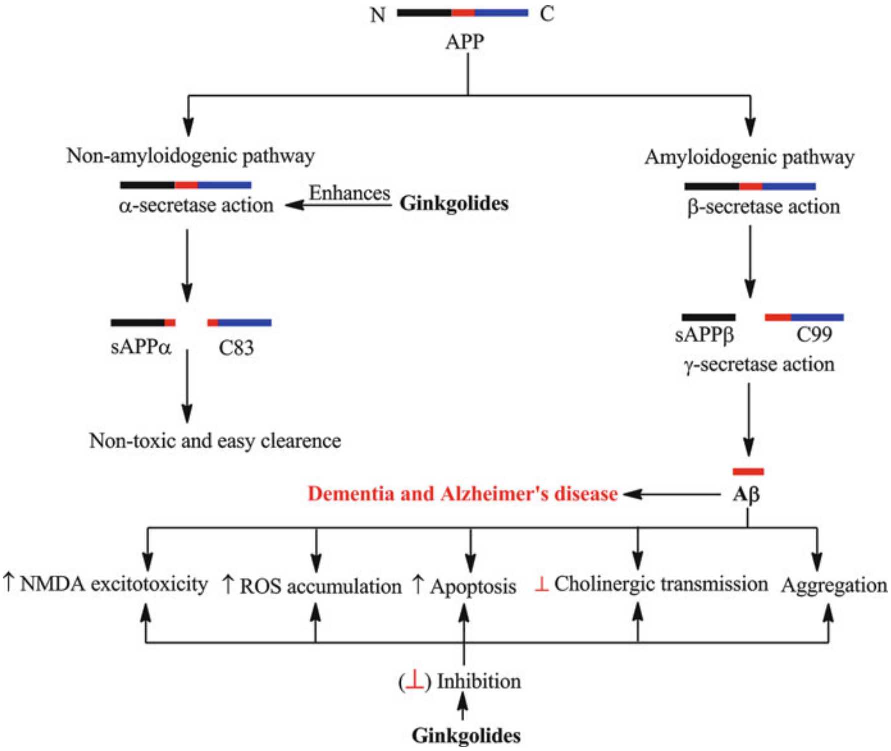

N
C
APP
Non-amyloidogenic pathway
α-secretase action
Enhances
Ginkgolides
Amyloidogenic pathway
β-secretase action
sAPPα
C83
Non-toxic and easy clearence
sAPPβ
C99
γ-secretase action
Dementia and Alzheimer's disease
Aβ
↑ NMDA excitotoxicity
↑ ROS accumulation
↑ Apoptosis
⊥ Cholinergic transmission
Aggregation
(⊥) Inhibition
Ginkgolides

Fig. 122.6 Alzheimer's disease pathogenesis and ginkgolides effect: amyloid precursor protein found in the cytosol and upon action of various enzymes forms soluble nontoxic amyloid and toxic amyloid beta via nonamyloidogenic and amyloidogenic pathway. Secretase involved in non-toxic amyloid formation and ginkgolides enhances this enzyme activity and protect the brain. While amyloid beta from the amyloidogenic pathway induces toxicity by increasing NMDA excitotoxicity, increase in ROS accumulation, increase in apoptosis, inhibition of cholinergic transmission and aggregation. These all events are inhibited ginkgolides and showed neuroproecion against demetia and AD

hippocampal cells against toxicity induced by $A\beta_{25–35}$ and $A\beta_{1–40}$ [127]. Diffusible, nonfibrillar ligands derived from $A\beta_{1–42}$ are potent central nervous system neurotoxins, and these ADDLs soluble oligomers of Aβ have been found in AD brains. An in vitro study [128] showed that EGb-761 inhibits the formation of amyloid beta-derived diffusible neurotoxic soluble ligands (ADDLs) in a dose-dependent manner. Alpha-secretase (α-secretase), the enzyme regulating the non-amyloidogenic processing of APP (cuts within the Aβ segment) and the release of α-APPs, EGb-761, enhance the effect on the α-secretase pathway observed at low concentrations (5 and 25 μg/mL) in hippocampal slices could be counterbalanced by an α-secretase PKC-dependent pathway at higher concentration (100 and 200 μg/mL) [129]. Free cholesterol may be involved in the production of APP and Aβ peptide, key events in the development of AD. EGb-761 (50 mg/kg) lowered circulating free cholesterol and inhibited the production of brain APP and Aβ after 28 weeks of treatment, as compared with controls rats [130]. As Aβ elicit its neurodegenerating effects by interfering with the central cholinergic system, therefore an in vitro study [131] results showed that G-B (0.01–10 μM) caused a concentration-related reversion of the inhibitory effect elicited by the effective concentration of Aβ (1 μM) and suggesting its anti-amnesic effect by minimizing the inhibitory effect of Aβ peptides on cholinergic transmission. Chronic *G. biloba* extract (similar to EGb-761) treatment (70 mg/kg/day) block an age-dependent decline in spatial cognition without altering Aβ levels and without suppressing protein oxidation in a transgenic mouse model of AD [132]. EGb-761 (100 μg/mL) directly inhibits amyloid fibril formation in solution in vitro and in the cell culture medium, moreover G-J also inhibit (72 %) the Aβ aggregation [133]. In a (SH-SY5Y) neuroblastoma cell line study [134], ginkgolides (A and B) inhibit PAF and that platelet-activating factor antagonists block the toxicity of amyloid-$\beta_{1–42}$ or sPrP106. The results suggested that PAF antagonists such as the ginkgolides may be relevant treatments for prion or AD [134]. Furthermore, pretreatment with ginkgolides A or B protects neurons against A$\beta_{1–42}$-induced synapse damage, reduced the effects of PAF, and suggested that the ginkgolides are active components of *G. biloba* preparations and may protect against the synapse damage and the cognitive loss seen during the early stages of AD [135]. There is also evidence which showed suppression of Aβ-related pathological behaviors. Among six single components of EGb-761, only G-A and G-J (100 μg/mL) exhibited a statistically significant delay of Aβ-induced paralysis in transgenic worms [136]. Transthyretin plays an important role in hormone transport in the brain and possibly a neuroprotective role by Aβ sequestration. The only gene on the array whose expression was upregulated more than threefold that encodes transthyretin in the hippocampus by dietary supplementation with EGb-761 in a dose of 36 mg/kg [137]. Aβ_{1-42} induces cell apoptosis, reactive oxygen species (ROS) accumulation, mitochondrial dysfunction and activation of c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2), and Akt signaling pathways. In a double-transgenic mouse model (TgAPP/PS1) study [138], EGb-761 (100 mg/kg) significantly increases cell proliferation in the hippocampus of both young (6 months) and old (22 months) TgAPP/PS1 mice, and the total number of neuronal precursor cells in vitro in a (0–120 μg/mL) dose-dependent manner. Furthermore, Aβ oligomers inhibit phosphorylation of cAMP response element-binding protein (CREB) and cell proliferation in the hippocampus of TgAPP/PS1 mice. Administration of EGb-761 (100 mg/kg) reduces Aβ oligomers and restores CREB phosphorylation in the hippocampus of these mice and suggesting therapeutic potential for the prevention and improved treatment of AD [138]. In other transgenic mice for human APP (Tg2576) model study [139], long-term treatment (16 months) with EGb-761 (300 mg/kg diet) significantly lowered human APP protein levels by approximately 50 % as compared to controls in the cortex but not in the hippocampus. However, APP levels were not affected by EGb-761 in young mice, suggesting the potential neuroprotective properties of EGb-761 may be, at least partly, related to its APP lowering activity [139]. An in vitro study [140] revealed that EGb-761 prevent the activation of NF-κB, ERK1/2, and JNK pathways induced by Aβ in neuroblastoma cell line N2a. Another human neuroblastoma SH-SY5Y cell line study [141] showed that EGb-761 (50–200 μg/mL) constituents G-B (5–20 μg/mL) along with quercetin (1.5–6 μg/mL) involved in the inhibitory effects on Aβ_{1-42} induces cell apoptosis, reactive oxygen species (ROS) accumulation, JNK, ERK1/2, and Akt signaling pathways. While only G-B helped to improve mitochondrial functions. Decreased clearance of Aβ from brain is the main root cause of their deposition in sporadic AD. However, the mechanisms underlying ischemia-mediated AD pathogenesis remain unclear. The receptors for advanced glycation end products (RAGE) and low-density lipoprotein receptor-related protein-1 (LRP-1) expressed at blood–brain barrier (BBB) are actively involved in Aβ clearance. In vitro study [142] suggested that EGb-761 favor clearance of Aβ via regulating the expression of RAGE and LRP-1 during brain ischemia. Synaptic dysfunction is likely to occur at early stages of AD. Low levels of oligomeric Aβ alter mechanisms underlying the excitatory response at single synapses producing synaptic dysfunction before synapse loss, cell death, and a complex series of events including inflammation, deposition of Aβ in senile plaques and within the walls of the cerebral microvasculature and appearance of neurofibrillary tangles. A new *G. biloba* extract P8A, 70 % enriched with terpene trilactones, prevents Aβ_{1-42} induced inhibition of long-term potentiation in the region I of hippocampus proper (CA1) in mouse hippocampal slices and also capable of inhibiting cell death of rodent hippocampal neurons caused by Aβ_{1-42}, which is attributed in large part to G-J (1–5 μM) that completely replicates the effect of the extract [143]. EGb LI-1370 (100 μg/mL) significantly improved oxidative phosphorylation system performance and was able to restore Aβ-induced mitochondria failure [144]. An in vitro study [145] showed that G-B (40 μg/mL) significantly dampens Aβ_{25-35}-induced apoptosis, and the neuroprotective effects may be intimately associated with brain-derived neurotrophic factor upregulation caused by G-B. The *N*-methyl D-aspartate receptor (NMDA) plays a pivotal role in the process of glutamate-induced excitotoxicity associated with many neurological disorders including AD. Studies in isolated rat hippocampal neurons indicated that the modulatory effects of EGB on NMDA-activated currents may contribute to the neuroprotective effects of two solvent preparations that is mEGB (0.1 mg/mL, dissolved in DMSO) and nEGB (0.1 mg/mL) either dissolved in DMSO or dissolved in standard extracellular solution where the modulatory effect of nEGB on NMDA-activated current was greater than that of mEGB [146].

7.1.1 Clinical Trials

In a randomized, double-blind, placebo-controlled, multicenter study (24 weeks) on 156 (222 patients at entry) outpatients with presenile and senile primary degenerative dementia of the Alzheimer type (DAT) and multi-infarct dementia (MID) [147], where patients received a daily oral dose of 240 mg of EGb-761 or placebo. There was a significant difference in the number of responders at the end of the treatment (28 % for EGb-761 compared with 10 % for placebo), suggesting that EGb-761 is of clinical efficacy in the treatment of outpatients with dementia. Furthermore a placebo-controlled, randomized, double-blind clinical trial [148] of 40 patients with moderate dementia received intravenous infusions of either

EGb-761 or placebo 4 days per week for 4 weeks. The result showed in an improvement of psychopathology and cognitive performance, which is reflected in an increased ability to cope with the demands of daily living. In addition, placebo-controlled, double-blind, randomized trials [149], 137 patients (327 patients at entry) were treated for 52 weeks with 120 mg of EGb-761. The patients showed significant improvement in learning, memory, visual and spatial orientation, and social behavior. A meta-analysis of effect size on the results of the 4 studies of more than 50 studies considered that met the author's inclusions criteria [150]. This analysis revealed a highly significant overall effect of ginkgo compared with placebo. The results showed that there is a small but significant effect of 3- to 6-month treatment with 120–240 mg of *G. biloba* extract on objective measures of cognitive function in AD [150]. In a long-term (26 weeks) study [151] on mild to severe AD patient, EGb-761 treatment with a 120-mg dose (40 mg t.i.d.) and the placebo group showed a statistically significant worsening in all domains of assessment, while the group receiving EGb-761 was considered slightly improved on the cognitive assessment and the daily living and social behavior. A retrospective analysis [152] explored whether the therapeutic effect of EGb-761 (120 mg) in AD depends on baseline severity. Treatment effect favorable for EGb-761 could be observed with respect to cognitive performance and social functioning, regardless of the stage of dementia. However, improvement was observed only in the group of patients with very mild cognitive impairment, while in more severe dementia, the mean effect of EGb-761 should be considered in terms of stabilization or slowing down of worsening [152]. An interesting result of a 6-week study [153] on healthy volunteers (203 completed out of 230) indicates that ginkgo (40 mg, t.i.d.) did not facilitate performance on standard neuropsychological tests of learning, memory, attention, and concentration or naming and verbal fluency in elderly adults without cognitive impairment. The ginkgo group also did not differ from the control group in terms of self-reported memory function or global rating by spouses, friends, and relatives. These data suggest that ginkgo provides no measurable benefit in memory or related cognitive function to adults with healthy cognitive function [153]. In a randomized controlled trial [154] of 359 dementic patient aged 50 years or above, treated with EGb-761 (240 mg/day) or placebo for 22 weeks. Their short syndrome test (SKT) score improved by −3.0+/−2.3 and −3.4+/−2.3 points in patients with AD and VaD, respectively, whereas the patients on placebo deteriorated by +1.2+/−2.5 and +1.5+/−2.2 points [154]. Another, randomized, double-blind exploratory trial [155] of 96 outpatients, aged 50 years or above, where EGb-761 (240 mg/day), donepezil (initially 5 mg, after 4 weeks 10 mg/day) or EGb- 761 and donepezil combined (same doses) were administered for 22 weeks. The results showed no significant difference in the efficiency between EGb-761 and donepezil, but a combination therapy will be superior to a mono-therapy with one of both substances with fewer side effects under a combination therapy than under monotherapy with donepezil [155]. In a German study [156], it was reported that the efficacy of EGb-761 has its place in the treatment of dementia. EGb-761 in the treatment of dementia (AD and VD) had been studied in 10 randomized controlled, double-blind clinical trials. In 3 of the

4 large trials conducted in accordance with recent recommendations, EGb-761 was significantly superior to placebo with respect to cognitive performance and one or more further (global, functional, or behavioral) outcomes demonstrating the clinical relevance of the findings. The findings from the 6 smaller trials were in line with those of the large trials [156]. Recently, published systematic review and meta-analysis [157] using 9 relevant trials were found statistically significant advantage of *G. biloba* extract compared to placebo in improving cognition for the whole group of patients with AD, vascular, or mixed dementia. While regarding activities of daily living, there was no significant difference for the whole group. However, in the subgroup of patients with AD, there was a statistically significant advantage of *G. biloba* extract compared to placebo [157]. Moreover, a recent multicenter trial [158] of 410 outpatients with mild to moderate dementia (AD, VD, or mixed form) with neuropsychiatric features, where the patients were treatment with 240 mg of EGb-761 or placebo once daily for 24 weeks. The results showed significantly superior to placebo in the treatment of patients with dementia with neuropsychiatric symptoms [158]. More recently, in a multicenter, double-blind, randomized, placebo-controlled, 24-week trial [159] with 410 outpatients, treatment with EGb-761 at a once daily dose of 240 mg was safe, confers the previous study findings, which is resulted in a significant clinically relevant improvement in cognition, psychopathology, functional measures, and quality of life of patients and caregivers.

7.2 Parkinson's Disease

Parkinson's disease (PD) is characterized by the degeneration of the dopaminergic nigrostriatal pathway, as indicated by the severe loss of substantia nigra neurons and by the decrease in striatal dopamine (DA) concentration. N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) selectively causes degeneration of the nigrostriatal dopaminergic neuronal pathway and used as lesion model in several studies to investigate the possible protective effect of EGb-761. Semi-chronic ingestion of EGb-761 for 17 days (100 mg/kg/day) prevents MPTP-induced reduction (approximately 25 %) in the striatal dopaminergic nerve endings in mice [160]. The 2 mono-amino oxidase (MAO) isoforms (A and B) regulate levels in the brain of most biogenic amines (e.g., dopamine, serotonin, and norepinephrine), and it is also well established that MAO-B activity increased with age and that some MAO-B inhibitors may improve quality of life in the elderly. Interestingly, 2 *G. biloba* extracts different from EGb (dried leaved extract) have been reported to induce reversible inhibition of both MAO-A and MAO-B activity in rat brain mitochondrial extracts [161]. Moreover, EGb-761 administered before (20, 50, 100 mg/kg/d i.p.) or after (50 mg/kg/d i.p.) MPTP treatment effectively protects against MPTP-induced nigrostriatal dopaminergic neurotoxicity and that the inhibitory effect of EGb-761 on brain MAO may be involved in its neuroprotective effect in mice [88]. EGb (100 mg/kg/d) decreased the duration and frequency of the rotation of rats ($P < 0.05$, $n = 10$) while EGb (50 or 100 mg/L) inhibited the decreases of dopamine (DA) and superoxide dismutase (SOD) and the increase of malondialdehyde (MDA) induced by MPTP [162]. 6-OHDA-induced rat models of PD study suggested that levodopa (50 mg/kg/day for 3 days, 5 days, 7 days, L-dopa group) had neurotoxic effect, and EGb (100 mg/kg/day) decreases the toxicity of levodopa [163]. EGb-761 (100 mg/kg/day) was investigated on 6-OHDA-induced neurotoxicity in the nigrostriatal dopaminergic system of the rat brain and reduces the behavioral deficit in 6-OHDA lesions in rat and also indicates a possible role for the extract in the treatment of PD [164]. Rats were treated with 50, 100, and 150 mg/kg EGb for 3 weeks showed dose-dependent protection against 6-OHDA-induced Parkinsonism in rats [89]. EGb-761 attenuates MPTP-induced neurodegeneration of the nigrostriatal pathway and suggesting that an inhibitory effect against oxidative stress possibly partly responsible for its observed neuroprotective effects [90]. Recently, chronic treatment with EGb-761 (100 mg/kg/14 days/once daily) showed dose-dependent increases in frontocortical dopamine levels [91].

7.2.1 Clinical Trials

The in vitro and animal data suggesting the beneficial effect of EGb-761 in PD, but there is no any clinical trial conduct to investigate the protective effect in human. Therefore, there is a need of conduction of such trials.

7.3 Anxiety

Anxiety is a condition which describes a normal feeling person experience when faced with threat, danger, or when stressed and the anxious condition makes a person upset feeling, uncomfortable, and tense. EGb-761 (100 mg/kg in 5 % ethanol) treatment group inhibited the development of polydipsia in rats due to the stress of daily handling and intubation [165]. In a 20 days of oral treatment with an extract EGb-761 (50 or 100 mg/kg/day) showed that auditory perturbation (stress) during the discriminative phase of learning decreased the percentage of correct responses and increased the number of errors in young as well as old rats [166]. Chronic administration of EGb-761 (50 or 100 mg/kg p.o. daily for 14 days) inhibits stress-induced corticosterone hypersecretion through a reduction in the number of adrenal peripheral benzodiazepine receptors [101]. Another, long-term EGb-761 (50 or 100 mg/kg p.o. daily for 14 days) administration study [167] on rats resulted in a decreased basal corticosterone secretion and an attenuation of the related increase in corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) gene expression, while chronic i.p. injection of G-B reduced basal corticosterone secretion without alteration in the subsequent CRH and AVP increase. Under intense surgical stress CRH, ACTH, and corticosterone plasma concentrations were markedly elevated in control animals but significantly less so in EGb-761 treated animals. These findings confirm that the administration of EGb-761 and G-B reduces corticosterone secretion suggest that EGb-761 interferes with the regulation of the activity of the hypothalamic-pituitary-adrenocortical (HPA) axis [167]. There were few studies conducted and showed the anxiolytic effect of EGb-761 in combinational therapy. Hasenohrl et al., in the year 1996 and 1998 [168, 169], showed the anxiolytic effect of a combination preparation of standardized extracts of *G. biloba* and *Zingiber officinale*. Ginkgolic acid conjugates (GAC), isolated from the *G. biloba* leaf, having similar profile with EGb-761, GAC (0.6 mg/kg) significantly increased ambulation and reduced the immobility time, suggesting the anxiolytic activity [170]. EGb-761 (100 mg/kg/day) serve as an antistress buffer, showed attenuating the increase in anxiety in mice after cold water exposure [171]. EGB (0.125 g/kg, p.o.) produces a significant anxiolytic-like effect following repeated administration and that G-A (1 or 2 mg/kg, p.o.) is most likely responsible for this effect [172]. It was found that *G. biloba* extract (14 mg/kg p.o.) restored restraint stress-induced elevation in whole brain levels of catecholamines (NE, DA), 5-HT, and plasma corticosterone to near normal level [104]. In prevention and treatment of the post-stress memory dysfunctions study [173], repeated administration of EGb-761 (100 mg/kg) prevents stress and corticosterone-induced impairments of information retrieval in rats.

7.3.1 Clinical Trials

A few clinical studies have conducted to evaluate the anxiolytic activity of ginkgolides. An early German studies, patients enrolled with cerebrovascular disease induced cognitive impairment, in which anxiety was one of the noncognitive symptoms relieved significantly by EGb-761 treatment [174]. In a pilot study [175], EGb (240 mg/day) significantly improved sleep pattern by an increase of sleep efficiency and a reduction of awakenings especially in the treatment of the depressive syndrome with sleep disturbance. In a trial on healthy volunteers [176], single treatment with EGb-761 (120 mg) reduced stress-induced rise in blood pressure without affecting the heart rate and influence salivary cortisol release in response to some stress stimuli. A randomized, double-blind, placebo-controlled trial [177] suggested that EGb-761 (240 and 480 mg/day) has a specific anxiolytic effect that is dose-dependent and significantly exceeds the placebo effect commonly seen in trials of psychoactive drugs.

7.4 Cerebral Insufficiency/Ischemia and PAF

Cerebral insufficiency refers to describe the people with age-related decline in mental function and decrease blood flow to the brain cause by clogged arteries. Platelet activating factor (PAF) is an inflammatory mediator, plays an important role in allergy, inflammatory processes, coronary, and cerebral vasoconstriction [178]. The production and release of PAF in the brain under various pathological conditions, including oxidant stress-induced ischemic injury [179]. The efficiency of EGb-761on cerebral circulation and metabolism has been demonstrated in various models of cerebrovascular insufficiency and showed beneficial effect like cerebral edema in rats intoxicated with triethyltin chloride (TET) [180]. Moreover, oral or intravenous administrations EGb decrease cerebral edema development in gerbils [181]. In an animal study [182], EGb-761 and an extract of local ginkgo leaf (LGb) improved spatial memory function with chronic cerebral insufficiency (produced by bilateral common carotid artery ligation) from the second week after operation, but only EGb-761 delayed deterioration of motor functions from the fifth week after operation.

EGb enhanced the local cerebral blood flow as well as the blood glucose level dose-dependently but decreased the cortical glucose concentration without other substrate levels being changed in rat [180]. EGb-761 (110 mg/kg/day) could prevent the ischemia-induced impairment of the Na,K-ATPase activity [183]. Pretreatment (15 days) with oral administration of *G. biloba* extract (Ph-GB; 37.5–150 mg/kg) significantly and in a dose-dependent way reduced post-ischemic brain MDA (malondialdeide) levels and post-ischemic brain edema in gerbils [184]. Oral administration of EGb-761 at a dose of 25, 50, and 100 mg/kg/day protects against ischemia-induced neuron death and reductions in mitochondrial gene expression in gebrils [185]. In addition, EGb-761 (100–200 mg/kg) protects against transient and permanent focal cerebral ischemia and was effective after a prolonged reperfusion period even when therapy is delayed up to 2 h in rats [186]. EGb-761 (100 mg/kg) also showed protection of neuronal cells against ischemic brain injury by preventing injury-induced decreases in p70S6 kinase and S6 phosphorylation in rats [187]. In contrast, a recent study showed that after p.o. administration of G-B (6 mg/kg) once daily for 7 days does not cause acute increase in cerebral blood flow after reperfusion, especially in hyperglycemia condition but effective in reactive oxygen species or MDA control in hyperglycemia ischemic rats [188]. G-B protects against cerebral ischemic injury by inhibiting excitotoxicity by modulating the imbalance of excitatory amino acids versus inhibitory amino acids, which may support the traditional use of *G. biloba* leaves for the treatment of stroke [189]. Pretreatment with G-K (2, 4, and 8 mg/kg (i.v.) once a day for 5 days) significantly diminished the volume of infarction and brain water content and improved neurological deficit score [190]. Moreover, G-K markedly reversed the level of MDA, NO, NOS, and SOD to their normal state in serum or cerebral ischemic section. Another recent in vitro (brain slices) and in vivo study [191] revealed that administration of EGb-761 (300 mg/kg) strongly reduces cellular edema formation and neurodegeneration under conditions of ischemia possibly via reduction of excitotoxicity because ischemia-induced release of glutamate was strongly suppressed.

Administration of ginkgolide B (BN-52021) in dose of 10 mg/kg/day via i.p. or oral-improved stroke index scores (determined by symptoms ranging from piloerection to seizures) and protect brain against hypoxic damage with their PAF antagonistic properties following bilateral carotid artery occlusion in the gerbil [192]. Free fatty acids, diacylglycerols, and polyphosphoinositides were accumulated in ischemic condition. BN-52021 (10 mg/kg, i.p.) inhibited the maturation of ischemic injury; increased cerebral blood flow and increased free fatty acid levels were reduced likely by inhibition of phospholipase A [193]. Moreover pretreatment with BN52021 (10 mg/kg, i.p.) reduces the injury-induced activation of phospholipase A2 and lysophospholipase, which mediate the accumulation of FFA in mice brain [194]. A temperature-controlled model of transient forebrain ischemia in the rat receiving BN-52021 (25 mg/kg, s.c.), 1 h before and 1 h after the induction of transient forebrain ischemia, exhibited a significant reduction in hippocampal and neocortical damage and proposed that PAF plays an important role in the pathophysiology of ischemic/excitotoxic neuronal injury via a direct action on neurons [93]. A comparative study [25] confirmed that induction of aggregation of human platelets by PAF requires at least 200 times higher concentration when compared to rabbit cells. Under the chosen experimental conditions, PAF-mediated aggregation of human platelets was half-maximally inhibited by ginkgolide B, A, C, and J at concentrations of 2.5, 15.8, 29.8, and 43.5 μg/mL, respectively [25]. In an animal study [195], both pre- and posthypoxic treatment with BN 52021 (25 mg/kg/dose, two serial doses) decreased the incidence of cerebral infarction from 90 % to about 30 %. The result suggested either prophylactic or rescue administration of PAF antagonists decreases the incidence and severity of brain injury associated with an episode of perinatal cerebral hypoxia-ischemia [195]. In a photochemically induced thrombotic cerebral ischemia in tree shrews model, G-B (5 mg/kg, i.v.) 6 h after photochemical reaction, cortical NA, DA, and 5-HT contents recovered to control levels and water, and calcium contents decreased significantly [196]. The results suggested that PAF may play an important role in inducing calcium overload, brain edema, and secondary brain damage in penumbra and that G-B produces its neuroprotective effects by inhibiting the pathological manifestation of PAF [196]. A recent animal study [197] showed that administration of G-B (10 or 20 mg/kg) before ischemia reduced the ischemia-induced elevation of levels of glutamate, aspartic acid, and glycine, increased the elevation of extracellular GABA, decreased the excitotoxic index, and diminished the volume of cerebral infarction. The results suggested the protection against cerebral ischemic injury by G-B-induced inhibition excitotoxicity by modulating the imbalance of excitatory amino acids versus inhibitory amino acids [197].

7.4.1 Clinical Trails

There were numerous clinical trial conducted between 1980s and 1990s, and most of them were shown a significant result. In an open one year German trial [198], *G. biloba* extract at a dose of 120 mg/day showed a statistically significant regression of the major symptoms of vertigo, headache, tinnitus, short-term memory, vigilance, and mood disturbance in 112 outpatient with chronic cerebral insufficiency. In a multicentric, double-blind, EGb versus placebo French trial [199] involving 166 patients confirmed that *G. biloba* extract is effective in 3 months against cerebral disorders due to aging. Moreover, in double-blind, randomized placebo-controlled study [53] of 24 weeks duration in outpatients with cerebral insufficiency showed statistically significant improvement in the short-term memory after 6 weeks and of the learning rate after 24 weeks in the test substance group. A critical review identified 40 such trials included small patient numbers [200]. Nevertheless, 8 out of 40 trials were found to be well performed, and the qualities of trials were sufficient enough to make credible conclusions. In these 8 trials, patients were typically given 120- to 160-mg *G. biloba* extract daily for at least 4–6 weeks. All 8 trials reported positive results and supported the conclusion that *G. biloba* extracts reduce the symptoms of cerebral insufficiency to the extent that is clinically relevant [200]. A meta-analysis including 11 clinical trials revealed the usefulness of the *G. biloba* extract, Kaveri (LI-1370), in cerebral insufficiency [54]. Three studies were excluded due to methodological inadequacies. In one study, the findings were inconclusive, but the pooled data from the remaining trials confirmed the effectiveness of the extract compared to placebo controls. Patients received Kaveri (150 mg per day, oral) for a period of 12 weeks, and the results support the clinical use of *G. biloba* extracts for cerebral insufficiency [54]. The results suggested the further need of clinical evaluation investigation in healthy as well as cerebral insufficiency/ischemic patient.

7.5 Retinal Degeneration and Glaucoma

The lens of the eye focuses an image of an object on a portion of the retina called the *macula*, the area of finest visual perception. *Gingko* extract have shown beneficial effect in macular degeneration, reduced intraocular pressure, and reduced ocular blood flow and glaucoma. Early studies showed that EGb reduces ischemia-reperfusion injury in rat retina [201, 202] as well as inhibits the preretinal proliferation in experimental tractional retinal detachment [203]. In an isolated rat retina model study suggested the existence of PAF-acether-specific receptors inside the retina. Simultaneous administration of ginkgolide B (BN 52021; 2×10^{-5} M) inhibited an irreversible decrease of the electroretinogram β-wave amplitude [204]. EGb-761 (50 mg/kg, per o.s.) was administered in a daily dose for 10 days showed significantly reduction in the maldistribution of ions induced by ischemia and reperfusion in rat retina obtained from normotensive and spontaneously hypertensive rats [205]. It was reported that EGb-761 (40 mg/kg) protects against susceptibility of rabbit retinal cells from proteolytic enzymes [206]. EGb have a protective effect against the progression of diabetic retinopathy and neuropathy [207]. Pretreatment and early posttreatment with EGb-761 protect and effective against neurotoxicity of retinal ganglion cells of rats with chronic moderately elevated intraocular pressure (IOP) [208]. Intraperitoneal injection of EGb-761 enhances the antioxidation ability of retina and partially inhibits the apoptosis of photoreceptors and exerts a protective effect on photoreceptors [209]. Intragastral administration of a *G. biloba* extract applied after an experimental and standardized optic nerve crush in rats were associated with a higher survival rate of retinal ganglion cells in a dosage-dependent manner [210]. Intraperitoneal injections of a *G. biloba* extract given prior to and daily after an experimental and standardized optic nerve crush in rats were shown associated with a higher survival rate of retinal ganglion cells [211]. In a recent study [212], pretreatment with EGb-761 prevented the focal cerebral ischemic injury-induced decrease in PEA-15 (phosphoprotein enriched in astrocytes 15) expression in rats.

7.5.1 Clinical Trials

In a 6-month, double-blind, placebo-controlled study of 10 people with macular degeneration, use of ginkgo at a dose of 160 mg daily resulted in a statistically significant improvement in long-distance visual acuity [213]. In a French double-blind trial, in 29 diabetic subjects with an early diabetic retinopathy (6 months period) showed an improvement tendency was evidenced in EGb treated subjects [214]. Phase I crossover trial [215] of with either EGb 40 mg or placebo 3 times daily orally in 11 healthy volunteers was treated for 2 days showed significantly increased end diastolic velocity (EDV) in the ophthalmic artery (OA). EGb-761 was investigated in a controlled, double-blind trial involving 99 patients with impaired vision due to senile, dry macular degeneration for 6 months [216]. Both the dosages (240 mg/day or 60 mg/day) of EGb-761 results in increase therapeutic efficacy of EGb-761 in patients with senile, dry macular degeneration, with obvious benefits in everyday life [216]. A small double-blind, placebo-controlled trial found that use of ginkgo extract at a dose of 120 mg daily for 8 weeks significantly improved vision in people with glaucoma [217]. In a randomized, double-masked, placebo-controlled, two-way crossover study [218] included 15 healthy male volunteers, before and up to 3 h after oral intake of 240 mg EGb-761 cause significantly decreased retinal venous diameters, but there was no significant difference between the two groups. The optic nerve head blood flow was significantly increased in response to *G. biloba*, but this effect was not significant compared with that of placebo. However, the results suggesting the drug may influence ocular blood flow in patients with ocular vascular disease after long-term treatment [218]. In a case study [219], 11 months after commencing *G. biloba* (120 mg/day) treatment, visual acuity improved to 20/80 OD and 20/40 OS, and subsequently at 30 months follow-up, his visual acuity improved further to 20/40 OD and 20/30 OS.

7.6 Vestibular Dysfunction

Vertigo is a type of dizziness characterized by “spinning” sensation in the head and is usually brought on by sudden changes in position. Ginkgo extract have shown significant beneficial influence on the vestibular system, particularly on compensation after vestibular lesions in experimental animals. In a chemical-induced labyrinthectomy rats, EGb-761 (50 mg/kg per day, i.p.) administration for 73 days post-surgery significantly accelerated compensation of static postural symptoms and spontaneous nystagmus compared with non-treated controls [220]. But there was a limitation of all of these studies that control animals have not received vehicle injections. Due to missing in conduction of a dose–response analysis, there was no evident whether 50 mg/kg/day i.p. was the optimal dose [220]. In an animal study, postoperative administration of EGb-761 (50 mg/kg/day, i.p.) for 30 days following UVD in cats has been shown to accelerate the compensation of postural, locomotor dysequilibrium, and oculomotor symptoms [221]. EGb-761 was administered over 30 days at daily doses of 50 mg/kg i.p. in cat strongly accelerated postural and locomotor balance recovery and demonstrated that EGb-761 acts on recovery mechanisms considered as key processes in vestibular compensation [222]. Guinea pig vestibular nuclei perfused with EGb-761 has a direct excitatory effect on the lateral vestibular nuclei (LVN) neurons and also i.p. administration of EGb-761 led to a reversible, dose-dependent decrease of the horizontal vestibule-ocular reflex (HVOR) gain without affecting the phase of the reflex [223]. In a comparative study [224] of EGb-761 components (terpenes vs. flavonoids) contained extract, examining on equilibrium function recovery in the unilateral vestibular neurectomized cat. Administration of EGb-761 orally (p.o./2 groups; 40 mg and 80 mg/kg) or intraperitoneally (i.p/2 groups; 50 mg and 25 mg/kg), whereas the 2 others received only a special extract that did not contain the terpenes (i.p. administration: 25 mg and 10 mg/kg) significantly improved the locomotor balance recovery in all the experimental groups as compared to the control groups [224]. There was also significant pharmacological activity of the extract when given i.p. as compared to the p.o. route of administration, and dose-dependent effects were evidenced with the i.p. administration of the special extract without the terpenes, with a lower efficacy for the lowest dose (10 mg/kg) [224]. In an animal study [225], G-B was investigating for the behavioral recovery process (vestibular compensation) which occurs following surgical removal of the vestibular receptor cells in one labyrinth (unilateral labyrinthectomy, UL). Guinea pigs received a single i.p. injection of G-B at the time of the UL (25, 50, or 100 mg/kg), and the effects were evaluated on the compensation of the UL symptoms, spontaneous ocular nystagmus (SN), yaw head tilt (YHT), and roll head tilt (RHT). A single i.p. injection of G-B (25 mg/kg) at the time of the UL produced an acceleration of SN compensation [225].

7.6.1 Clinical Trials

In a randomized, placebo-controlled, double-blind trial in patients (out of 50, 33 completed) with vertigo and ataxia symptoms using EGb-761 (120 mg/day) for 12 weeks showed lateral sway amplitude in the cranio-corpography (CCG) and proportion of subjective improvement [226]. In another randomized, placebo-control, double-blind trial on 35 patients with peripheral vestibular vertigo treated with EGb-761 (160 mg/day) for 12 weeks resulted in sway amplitude in posturography and suggesting combinational therapy with *G. biloba* [227]. In a double-blind trial extending over a 3-month period, the patient (out of 70, 67 completed) with vestibular vertigo were given either EGb-761 (160 mg/day) or a placebo. At the end of trial 47 % of the patients treated were rid of their symptoms as against 18 % of those who received the placebo [228]. In a non-vestibular group of trial, 80 patient receiving EGb-761 (160 mg/day) for 12 weeks showed proportion of the patients free from the symptoms and greatly improved [229]. An open, randomized study of 45 patients suffering from vertigo induced by peripheral vestibular lesions is interesting [230]. All patients participated in a physical training program, 23 patients received EGb-761 in addition. In these patients, posturographic investigations showed a more rapid reduction in sway amplitude [230]. A systematic review published in 2007 showed the beneficial effect of EGb-761 on vestibular compensation in various preclinical and clinical studies [231]. The author suggested the presence of efficacy of EGb-761 for the treatment of vertiginous syndromes in the available studies [231].

7.7 Other Neuroprotective Activities

Neuroinflammation is characterized by activation of local glial cells and production of various pro-inflammatory mediators which lead to the abnormalities in neurons and astrocytes. Cytokine IL-1β has been implicated in the extensive inflammation and progressive neurodegeneration that occurs after ischemia. Brain ischemia induces production of both TNF-α and IL-1β which may disrupt phosphatidylcholine homeostasis by increasing its hydrolysis and inhibiting the synthesis. In a trial of 79 patients suffering from chronic, age-related neurological disorders, treatment with 9.6 mg of EGB (ginkgo extract) twice daily for 8 weeks shown a statistically significant decline IL-6 level to near normal values, but there were no significant changes observed in serum levels of IL-1β and TNF-α [232]. In a pilot study [233] of 10 multiple sclerosis patients in acute relapse were treated with a 5-day course of intravenous G-B. 8 patients had improvement of their neurological score, beginning 2–6 days after the initiation of therapy of G-B [233]. In contrast, a randomized double-blind placebo-controlled trial [234] on 104 multiple sclerosis patients, 43 received placebo, 29 received 240 mg/day G-B, and 32 received 360 mg/day ginkgolide B for 7 days. The result does not showed any significant result, and suggesting G-B is not an effective treatment of exacerbations of multiple sclerosis [234]. Treatment with BN-52021 (10 mg/kg) attenuates the development of early posttraumatic cerebral edema in rats subjected to a mild traumatic insult [235]. The author suggested that PAF may be involved in the pathogenesis of posttraumatic cerebral edema [235]. Moreover, administration of BN-52021 (1 or 10 mg/kg i.v.) 15 min prior to, and 120 min after, fluid percussion-induced traumatic brain injury resulted in the reduction of neurological deterioration due to traumatic brain injury [236].

8 Ginkgolides Pharmacokinetics

Ginkgolide B showed about 50 % of metabolism in vivo and suggesting its hydroxyl metabolites its principal metabolites [237]. A dosage of 40-mg G-B twice daily (every 12 h) is accompanied by a significantly longer half-life ($t_{1/2}$) and mean residence time (MRT) than a single 80-mg dose, even though the latter causes a higher concentration peak (C_{max}). The maximum concentration time (T_{max}) is 2.3 h after administration in both treatments [238]. After each single dose of G-A, G-B and bilobalide ranging from 0.90 mg to 3.36 mg, blood and urine samples were collected for up to 36 h and 48 h, while fasting, the extents of bioavailability are high, as shown by bioavailability coefficients (FAUC) mean (+/− SD) values equal to 0.80 (+/− 0.09) and 0.88 (+/− 0.21) for G-A and G-B respectively [239]. In short, G-A and G- B are nearly completely bioavailable [240].

9 Side Effect

As *G. biloba* is one of the very popular herbal supplementary in dose range of 120–240 mg/day due to its versatility in human health benefits and safer efficacy. But, there were few cases have been reported the side effect of *G. biloba* including minor (stomach upset, skin reaction and headache or dizziness) and severe (haemorrhage). At a higher dose of ginkgo extract in initial supplementary or therapy dose leads to stomach upset. It is recommended to start with a lower dose and titrate as tolerated to minimize or ovoid the gastrointestinal side effects. In some cases, allergic skin reactions is caused by the herbal remedy *G. biloba* extract and that resolve after discontinuing ginkgo therapy [241]. There is also a case of Stevens-Johnson syndrome reported in a patient after their second dose of a combination herbal product containing EGB [242]. Ginkgo seeds contain ginkgo toxin, which can cause seizures, difficulty breathing, loss of consciousness, and shock when consuming more than 10 roasted seeds daily. It is also found that the availability of ginkgo toxin is much higher concentrations in ginkgo seeds than in ginkgo leaf extract. There is an evidence of about 20 detailed reports of hemorrhage (usually cerebral, ocular, or postsurgical) in patients using *G. biloba* extracts have been published [243]. One third of these patients were also taking drugs that increase the risk of bleeding (anticoagulants or antiplatelet drugs). Therefore there is a cautionary recommendation established in practice, patients with risk factors for bleeding (anticoagulant or antiplatelet treatment, surgery, etc.) should avoid using *G. biloba* extracts [243].

10 Conclusion

Aging, which is a fate of all creatures, is a challenge to every living being. There is a cellular, structural, and functional changes occur in the brain during aging. Nerve cells may respond to these changes adaptively, or they may succumb to neurodegenerative cascades that result in disorders such as AD and PD. Neurodegenerative diseases have drawn a lot of attention due to their irreversibility, lack of effective treatment, and accompanied social and economic burdens. *G. biloba* showed various pharmacological effects, among them multiple researches have been demonstrated their neuroprotective effect in the prevention and recovery of cognition, ischemic, antioxidant, anti-amyloidogenic, and PAF inhibitory abilities. It maintains blood vessel health, reduces blood viscosity, and enhances blood supply to the brain, which has recently been implicated as a causative factor for stroke.

At the present time, there are no definitive prevention and treatments for dementias or age-related cognitive decline. The significance of these neurological disorders mandates that every therapeutic option should be investigated with rigorous scientific methodology. There is a major portion (28 %) of unknown or unidentified compounds in ginkgo extract which are need to be investigating because it may be possible that they exhibit the same effects like ginkgolides or other active constituents. Moreover the basic pharmacology and therapeutic potential of *G. biloba* extracts (EGB-761) in neuroprotection have understood and also the activity of ginkgolide has also been evaluated separately in neurological disorders. But there is a lack of clinical research in PD which is one of the major neurodegenerative disorders. The question is EGb-761 does not contains only ginkgolides at a major constituents, it contributes about 6–7 % of the total components. Moreover, it may be possible that the other bioactive constituents also participating in neuroprotection activities. Even though the gingkolides were used combination and fewer in the total content, we cannot underestimate their role in neuroprotection. Therefore, the complete neuropharmacological potential of ginkgolides will be understood through coordinated in vitro/in vivo animal investigations and should be confirmed with human placebo-controlled, double-blinded research designed to objectively measure relevant functional parameters including their safety parameters.

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Cardiac Glycosides and Anticancer Activity

123

Yuan Lin, Dapeng Chen, Li Wang, and Dongmei Ye

Contents

1 Introduction 3745
1.1 Sources of Cardiac Glycosides 3745
1.2 Structure Activity Relationship 3745
1.3 Mechanism of Action of CGs 3746
1.4 Major Clinical Application of CGs 3746
2 Anticancer Activity of CGs 3746
2.1 In Vivo Efficacy of Ouabain in a Xenograft Model of Retinoblastoma 3747
2.2 Anticancer Selectivity of CGs 3747
2.3 Inhibitors of Human Tissue Kallikrein Expression 3749
2.4 Anoikis Sensitizers to Decrease Tumor Metastasis 3749
2.5 Inhibitory Effects on Topoisomerase 3750
2.6 Anticancer Activity via Blocking NF-kB Activation 3750
2.7 Anticancer Activity by Inhibiting General Protein Synthesis 3751
3 Estimation of Clinical Anticancer Property of CGs 3751
3.1 Beneficial Effect of CGs in the Treatment of Breast Tumors 3751
3.2 Negative Effect of CG Reported in the Treatment of Breast Tumors 3752
3.3 Digoxin and Estrogen-Sensitive Cancer 3752
3.4 Prospect of Clinical Implication 3752
4 Conclusion 3753
References 3753

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Abstract

Cardiac glycosides (CGs) which are composed of aglycone moiety and glycone moiety occur mainly in plants. CGs increase cardiac contractility by inhibiting the sodium-potassium-adenosine triphosphatase (Na^+/K^+ ATPase) of plasma membrane and are widely used in the treatment of chronic heart failure. New findings within recent years have revealed that CGs are involved in selective control of tumor proliferation. Inhibition of Na^+/K^+ ATPase by CGs induces antiproliferative downstream effects which are related to cell growth and apoptosis. As anticancer effects of CGs occur also below their cardiotoxic concentration, Na^+/K^+ ATPase independent pathways are also proposed. Some CGs are almost completely nontoxic to rodent-derived tumor cell lines but potently inhibit proliferation human tumor cell lines. Some of the CGs, in nontoxic concentrations, are able to induce apoptosis in human promyelocytic leukemia cells (HL60) but not in normal leukocytes. The anticancer effects of CGs are found to be related to the inhibition of tissue kallikrein expression, anoikis sensitizers, inhibition on topoisomerase, blockade of NF-kB activation, and suppression of general protein synthesis. However, the viewpoint and evidence of CGs as anticancer agents in clinical application are still controversial.

Keywords

Anticancer activity • cardiac glycosides • clinical implication • mechanism

Abbreviations

bcl-2	B cell lymphoma/leukemia-2
Ca^{2+}	Calcium ion
c-FOS	FBJ osteosarcoma oncogene
CGs	Cardiac glycosides
c-myc	v-myc myelocytomatosis viral oncogene homolog (avian)
DNA	Deoxyribonucleic acid
EDLC	Endogenous digitalis-like compounds
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
HL60	Human promyelocytic leukemia cells
IkBa	I kappa B alpha
JAK2	Janus kinase 2
JNK	c-Jun NH_2-terminal kinase
KLK	Kallikreins
MAPK	Mitogen-activated protein kinase
MCF-7	Michigan Cancer Foundation – 7

MEK	MAPK/ERK kinase
mRNA	Messenger ribose nucleic acid
Na^+/Ca^{2+} exchanger	Sodium-calcium exchanger
Na^+/K^+ ATPase	Sodium-potassium-adenosine triphosphatase
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
p21	Protein 21
p53	Protein 53 or tumor protein 53
PI3K	Phosphoinositide-3 kinase
PPC-1	Primary prostatic carcinoma cell line
PSA	Prostate-specific antigen
PUMA	p53 upregulated modulator of apoptosis
Rac1	Ras-related C3 botulinum toxin substrate l
SCID	Severe combined immune deficiency
Src	Sarcoma
$t_{1/2}$	Half-life
TNFR	Tumor necrosis factor receptor

1 Introduction

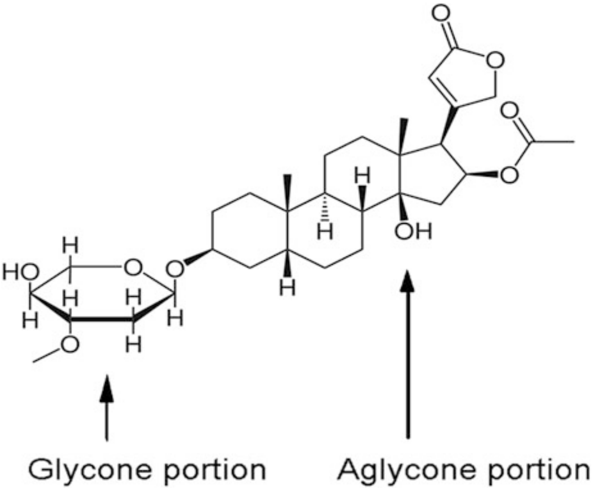
1.1 Sources of Cardiac Glycosides

Cardiac glycosides (CGs, digitalis-like compounds, cardenolides) occur mainly in plants, such as *Digitalis purpurea, Digitalis lanata, Strophanthus gratus*, and *Strophanthus kombe*. Plants containing CGs have been used as poisons and heart drugs at least since 1500 B.C. CGs are found in amphibian, e.g., toad venom, such as bufadienolides, and in mammals, e.g., endogenous digitalis-like compounds (EDLCs). EDLCs are identified in mammalian tissues, e.g., ouabain and digoxin, which are purified from human plasma and hypothalamus.

1.2 Structure Activity Relationship

CGs are composed of two structural features. Aglycone (steroid, nonsugar portion) moiety is responsible for biological activities, e.g., positive inotropic action on cardiac muscle. Glycone moiety (sugar portion, 1–4 sugars present in most CGs) possesses no biological activity; however, the polarity differences caused by the number of sugars differ markedly in their degree of absorption, half-life ($t_{1/2}$), and the time to maximal effect. For instance, ouabain which contains one sugar molecule has a $t_{1/2}$ of 21 h, and digoxin, which possesses a three-sugar side chain, has $t_{1/2}$ of 36 h (Fig. 123.1).

Fig. 123.1 Chemical structure of cardiac glycosides



1.3 Mechanism of Action of CGs

The most widely accepted mechanism of action of CGs involves the ability to inhibit the activity of the membrane-bound sodium-potassium-adenosine triphosphatase (Na^+/K^+ ATPase) pump (Fig. 123.2).

CGs inhibit the cardiac myocyte membrane Na^+/K^+ ATPase causing intracellular sodium concentration to increase. The increased intracellular Na^+ reduces the concentration gradient driving Na + into the cell across the Na^+/Ca^{2+} exchanger, thereby decreasing the activity of the exchanger, which lowers the movement of Ca^{2+} out of the cell. The increased intracellular Ca^{2+} results in more Ca^{2+} to be released by the sarcoplasmic reticulum, thereby making more Ca^{2+} available to bind to troponin C, increasing contractility.

1.4 Major Clinical Application of CGs

CGs are widely used in the treatment of chronic heart failure owing to their cardiotonic effects and are also used for patients with atrial fibrillation, especially if they are diagnosed with congestive heart failure via vagal activation.

2 Anticancer Activity of CGs

It is known that the inhibition of Na^+/K^+ ATPase activates tyrosine kinase sarcoma (Src), phosphoinositide-3 kinase (PI3K), and phospholipase C signalosome complex, which can induce many antiproliferative downstream effects related to cell growth and apoptosis [1–3]. Since some anticancer effects of cardiac glycosides

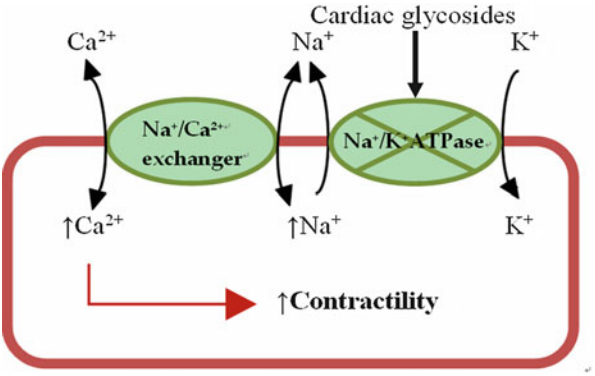


Fig. 123.2 Inhibition of plasma membrane Na^+/K^+ ATPase by cardiac glycosides

occur below their cardiotoxic concentration, many Na^+/K^+ ATPase independent pathways are also proposed [1, 4].

New findings within recent years have revealed that CGs are involved in complex cell-signal transduction mechanisms, resulting in selective control of tumor rather than normal cellular proliferation. As such, CGs are promising in cancer chemotherapy.

2.1 In Vivo Efficacy of Ouabain in a Xenograft Model of Retinoblastoma

Anticancer activity of CGs ouabain is found on a xenograft model of retinoblastoma. Three groups of two 8-week-old ICR/SCID male mice bearing Y79 tumors implanted in the flank are treated with vehicle only, 1.5 and 15 mg/kg ouabain, respectively [5]. The results show that the average tumor size for the control group reached 1,000 mm^3 at day 14 and in animals treated with 15 mg/kg ouabain, the tumors are nearly eradicated by day 14 (18 mm^3 in average). At a lower dose of 1.5 mg/kg, ouabain also reduces the tumor burden compared with that in the control. However, the average body weight of treated and control animals does not differ significantly, implicating that even at the high dose of 15 mg/kg, ouabain does not induce any significant toxicity.

2.2 Anticancer Selectivity of CGs

2.2.1 Characteristics of the Species-Related Sensitivity

Some CGs, such as oleandrin, bufalin, and digitoxin, are almost completely nontoxic to rodent (mouse and rat)-derived tumor cell lines but potently inhibit proliferation of monkey and human tumor cell lines at nanomolar concentration [6, 7]. The species-dependent disparity in tumor cell sensitivity to antitumor agents is unusual. The difference in response of murine as compared to human tumor cell lines implicates that a fundamental difference in drug targeting exists and, thus, serves as a probe or model for reexamination of the pharmacologic role of this class of compounds in treatment of human cancers [7].

A difference in the subunit composition of Na^+/K^+ ATPase might explain the differential species-dependent sensitivity and selectivity to human tumor cell to CGs [8]. Human tumor cells commonly express both $\alpha1$ and $\alpha3$ subunits; however, all rodent tumor cell lines now known only express the $\alpha1$ subunit. Recent studies indicate a clear selective binding of CGs to the $\alpha3$ form over that of the $\alpha1$ or $\alpha2$ isoforms [9]. For instance, a 1,000-fold difference in binding of ouabain to the $\alpha3$ isoform over that of $\alpha1$ is found in the study [9]. The increased expression of $\alpha3$ over $\alpha1$ subunits is also noted in human colon or colorectal cancer [7], whereas no significant expression of the $\alpha3$ isoform protein is noted in the normal kidney [10]. Human tumor cell lines with a low ratio of $\alpha3{:}\alpha1$ are relatively resistant to growth inhibition with CGs, and the tumor cell lines with high $\alpha3{:}\alpha1$ ratios are very sensitive to CGs.

2.2.2 Cardiac Glycosides Selectively Inhibiting Tumor Cells

Selectively Inducing Apoptotic Effects on Tumor Cells

The different effects of CGs on human tumor compared to normal cells are observed. For instance, oleandrin inhibits the activation of certain transcription factors and potentiates ceramide-induced apoptosis in human tumor cells rather than in normal, primary human cells [11]. Researchers also [12] indicate that CGs sensitize human tumor but not normal cells to subsequent radiation treatment. These data suggest that it may be possible to exploit differences in the Na^+/K^+ ATPase pumps of normal as opposed to tumor cells to improve the therapeutic index of radiation therapy.

CGs have been shown to induce apoptosis in different malignant cell lines [13, 14]. In light of the pivotal role of apoptosis in cancer development and progression and this new experimental finding concerning CGs, it seems probable that the apoptosis-inducing capability is explained by mechanisms other than just Na^+/K^+ ATPase inhibition. The role of sustained $[Ca^{2+}]$ increases in the cells, persistent activation of the mitogen-activated protein kinase (MAPK) pathway, altered expression of the c-myc and B cell lymphoma/leukemia-2(bcl-2) genes involved in apoptosis, and activation of ras-related C3 botulinum toxin substrate 1 (Rac1), protein 21 (p21)-activated kinase, and c-Jun NH_2-terminal kinase (JNK) pathway have all been considered as potentially involved.

Malignant cells in general are more susceptible to CGs than normal cells. It may be due to the fact that in many cases, Na^+/K^+ ATPase activity is different in tumor or transformed cells compared to their normal counterparts. For instance, one of the CGs, bufalin, in nontoxic concentrations, is able to induce apoptosis in human promyelocytic leukemia cells (HL60) but not in normal leukocytes. Therefore, bufalin seems to act as a potent differentiation and apoptosis-inducing agent in cancer cells. Also, oleandrin [15] is found to increase the expression of Fas and tumor necrosis factor receptor 1 (TNFR1), resulting in potentiation of apoptosis in tumor cells but not in normal primary cells, such as peripheral blood mononuclear cells or neutrophils. Oleandrin also initiates apoptosis in non-small-cell lung cancer cells by increasing the expression of death receptors 4 and 5 [16] with little if any toxicity to normal cells.

Inhibiting Mutant-Protein 53 Containing Sensitive Cancer Cells

Protein 53 (p53) can prevent tumor formation through transcriptional-dependent mechanism, which is mainly mediated by p53 upregulation of its downstream targets. Upon activation by a variety of stimuli, p53 induces the expression of proarrest genes such as p21, to induce growth arrest or pro-apoptotic genes, and p53 upregulated modulator of apoptosis (PUMA), to induce apoptosis [17]. p53 acts as a guardian of the genome by inducing growth arrest to allow cells to repair the damage or apoptosis, if the damage is too severe and irreparable.

As p53 plays a pivotal role in controlling abnormal cell growth and is inactivated by point mutations in more than 50 % human cancers, p53 has been a central target for mechanism-driven cancer drug discovery [17, 18]. CGs such as digoxin and ouabain selectively kill mutant p53 containing sensitive cancer cells via a synthetic lethal mechanism [19]. CGs sensitivity to p53 reduction is cancer cell line dependent, but independent of p53 status of a wild type or mutants. CGs are completely inactive in reducing wt p53 in normal "immortalized" cells. CGs-induced p53 decrease occurs not at the messenger ribose nucleic acid (mRNA) levels but at the protein levels, as a result of reduced synthesis rather than enhanced degradation. The drug-induced p53 reduction can be rescued by the inhibitors of Src and MAPK/ERK kinase (MEK), suggesting an involvement of Src/MAPK signaling pathways, initiate upon the drug binding to Na^+/K^+ ATPase.

2.3 Inhibitors of Human Tissue Kallikrein Expression

Human tissue kallikrein (KLK) comprises a subgroup of 15 homologous-secreted serine proteases. KLK is used as cancer biomarkers (e.g., prostate-specific antigen (PSA)) and is directly implicated in cancer-related invasion, angiogenesis, and tumor growth [20]. Acting at low concentrations (10–50 nmol/L), CGs are found to inhibit KLK expression and induce marked decrease in c-myc and c-FBJ osteosarcoma oncogene (c-FOS) expression, in a dose-dependent manner that is correlated the KLK inhibition, suggesting a transcriptional mechanism of regulation of KLK expression.

2.4 Anoikis Sensitizers to Decrease Tumor Metastasis

Normal epithelial cells undergo apoptosis upon detachment from the extracellular matrix, a process termed "anoikis" [21]. However, malignant epithelial cells with metastatic potential resist anoikis and can survive in an anchorage-independent manner. One study is carried out to identify novel anoikis sensitizers in anoikis-resistant PPC-1 prostate adenocarcinoma cells. CGs ouabain is found as anoikis sensitizers. The underlying mechanisms of ouabain to sensitize cells include initiation of mitochondrial pathway of caspase activation, inhibition of Na^+/K^+ ATPase pump, and induction of hypoosmotic stress. Ouabain is found to inhibit tumor metastases but rather than alter the growth of subcutaneous tumors. Since the resistance to anoikis permits cancer cells to metastasize to distant organs, to sensitize resistant cells to anoikis using ouabain can be considered as a novel mechanism to decrease tumor metastasis. The clinical evidence also implicates the reduction in metastasis and relapse in breast cancer patients who have undergone treatments with CGs. CGs peruvoside, digoxin, digitoxin, and strophanthidin are also anoikis sensitizers.

2.5 Inhibitory Effects on Topoisomerase

Topoisomerases are key cellular enzymes that adjust DNA's topological structure, transcription, replication, and chromosome structure. Apoptosis of certain type of cancer cell is induced when topoisomerases are inhibited. Topoisomerase inhibitors are among the most active anticancer agents.

Topoisomerase II enzymes are important for cancer chemotherapy, especially since overexpression of these proteins has been demonstrated in many human tumor types, such as breast cancer [22, 23]. Digoxin, ouabain, and proscillaridin exert significant inhibitory effects on the proliferation of the tested MCF-7 breast cancer cells. The degree to which these compounds inhibited cell growth in breast cancer cells is correlated to topoisomerase II-inhibiting activity. Ouabain and digoxin inhibited topoisomerase II catalytic activity at nanomolar concentrations (100 nM), but neither agent inhibited topoisomerase I catalytic activity even at concentrations as high as 100 mM. On the other hand, proscillaridin A was a potent inhibitor of both topoisomerase I and II activities at nanomolar drug concentrations (30, 100 nM, respectively), suggesting that this agent may produce its cytotoxic activity by targeting both enzymes simultaneously [13].

2.6 Anticancer Activity via Blocking NF-kB Activation

Nuclear factor kappa-light-chain enhancer of activated B (NF-κB) cells were first discovered in the lab of Nobel Prize laureate David Baltimore via its interaction with an 11-base pair sequence in the immunoglobulin light-chain enhancer in B cells [24]. NF-κB is a protein complex that controls the transcription of DNA. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli. Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis. NF-kB is activated in response to various inflammatory stimuli including cytokines, mitogens, bacterial products, viral proteins, and apoptosis-inducing agents [25]. Incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development.

NF-kB is an ideal target for anticancer drug development for several reasons [26] described as follows. (a) In tumor cells, NF-κB is active either due to mutations in genes encoding the NF-κB transcription factors themselves or in genes that control NF-κB activity (such as IκB genes); some tumor cells secrete factors that cause NF-κB to become active. (b) Activation of NF-kB has been shown to block apoptosis and promote proliferation, and blockade of NF-κB causes tumor cells to stop proliferating or to become more sensitive to the action of antitumor agents. (c) Constitutive expression of NF-kB in tumor cells induces proliferation, and NF-kB activation induces resistance to chemotherapeutic agents. Thus, NF-κB is the subject of much active research among pharmaceutical companies as a target for anticancer therapy.

The CG oleandrin is found to block tumor necrosis factor (TNF)-induced activation of NF-kB in a concentration- and time-dependent manner [25]. Oleandrin blocked NF-kB activation induced by phorbol ester and lipopolysaccharide. The inhibitory effect of oleandrin on NF-kB is mediated via suppressing phosphorylation and degradation of I kappa B alpha (IkBa), an inhibitor of NF-kB; however, these effects of oleandrin were not cell-type specific, because it blocked TNF-induced NF-kB activation in a variety of cells.

2.7 Anticancer Activity by Inhibiting General Protein Synthesis

Studies proposed that inhibition of the Na^+/K^+ ATPase pump was responsible for the inhibitory effects of CGs on protein synthesis. They expressed the naturally cardiac glycoside-resistant alpha1 chain of the murine Na^+/K^+ ATPase pump, which is about 1,000 times less sensitive for CGs than the human alpha1 subunit, in human cells [27]. Expression of the murine but not the human alpha chain largely rescued the inhibitory effects of digitoxin on intracellular protein expression. The other study also further indicates that inhibition of the Na^+/K^+ ATPase pump is responsible for the inhibition of CGs on protein synthesis. For instance, Na^+/K^+ ATPase pump activity is inhibited by digitoxin in incubating cells for 4–8 h in a sodium-free buffer. The Na^+-free buffer inhibited both murine and human Na^+/K^+ ATPase pump activity as determined by intracellular K^+ levels and did not affect cell viability in the timeframe of the experiment, resulting in a decreased expression of relatively short-lived proteins such as p53 and Janus kinase 2 (JAK2).

3 Estimation of Clinical Anticancer Property of CGs

3.1 Beneficial Effect of CGs in the Treatment of Breast Tumors

An unusual finding was reported in 1979 that breast cancer of women ($n = 142$) [28, 29] who were taking CGs (mostly digoxin) at the time of their breast cancer diagnosis had tumors with less aggressive phenotypes than breast tumors of women not taking CGs [30]. They later reported a higher recurrence rate among the women not taking CGs after 5 years and approximately 22 years of follow-up [28, 31]. These observations suggested a beneficial effect of CGs for women with breast tumors.

A 5-year follow-up study indicates that the recurrence rate of breast cancer in patients not on digitalis was 9.6 times higher than in patients treated with digitalis. In the subsequent 20-year follow-up, the death rate from breast carcinoma (excluding other causes of death and confounding factors) was 6 % (2 of 32) among patients on digitalis compared with 34 % (48 of 143) among patients not on digitalis ($p = 0.002$); the study confirmed these results by conducting a retrospective study of 127 cancer patients in their records. Of a total of 21 deaths, they found only one cancer death among those who had taken digitalis. Additionally, a study with 9,271 patients showed a relationship between high plasma concentrations of digitoxin and a lower risk for leukemia lymphoma [30].

3.2 Negative Effect of CG Reported in the Treatment of Breast Tumors

After comparison of site-specific cancer incidence rates among digitalis-treated Norwegian patients with expected rates in the general population [32], the results showed that several cancers, including female breast cancer, occurred at higher rates among those treated with digitalis compared with the general population [32], showing the association between digoxin treatment and breast tumor incidence rate in a population-based prospective case-control study of postmenopausal Danish women.

3.3 Digoxin and Estrogen-Sensitive Cancer

Among women given digoxin, breast and uterus cancer incidences are significantly increased (risk ratios is about 1.3–1.5); both cancers are estrogen sensitive [33]. In contrast, ovary and cervix cancers are relatively estrogen insensitive, and incidence is unaffected by digoxin administration. These phenomena parallel those of estrogen, suggesting that digoxin works via estrogen receptor (ER)-stimulated proliferation, accelerating the growth of nascent cancers. Also consistent with an estrogenic effect, men using digoxin have a small but significant reduction in prostate cancer (risk ratio, 0.76).

3.4 Prospect of Clinical Implication

Whether CGs may sound as potential anticancer agents is still controversy [27–29].

3.4.1 CGs not Expected as Potential Anticancer Agents

As described in the previous sections, reports about CGs are controversial in clinical observations, and successful randomized trials have thus far not been reported. Since it is found that normal diploid fibroblasts, non-tumorigenic breast epithelial cells MCF10A, and peripheral blood mononuclear cells are equally sensitive to CGs as neoplastic cells, and also the mechanism of cytotoxicity is correlated to general protein synthesis inhibition, CGs should not be considered to possess specific anticancer activities.

3.4.2 CGs Expected as Potential Anticancer Agents

The changes occur in Na^+/K^+ ATPase activity in premalignant mucosa, months before gross tumors develop, and these changes may partially explain the altered levels of Na^+ and K^+ in the cytoplasm of premalignant and malignant colonocytes; the changes in the transmembrane transport of cations during the course of malignant cell transformation are due to increases in Na^+/K^+ ATPase activity [29, 34].

Studies indicate that digitoxin may be one of the most promising anticancer CGs since it shows significant anticancer effect against several types of cancer including lung cancer, pancreatic cancer, leukemia, and breast cancer, all at therapeutic concentrations.

4 Conclusion

The major characterization of the anticancer activities of CGs is as follows. (a) Some of the CGs are found almost completely nontoxic to rodent-derived tumor cell lines but potently inhibit proliferation of human tumor cell lines at nanomolar concentration, some of the CGs induce apoptosis in human tumor cells rather than in normal, and some of the CGs can selectively kill mutant p53 containing sensitive cancer cells. (b) KLK is directly implicated in cancer-related invasion, angiogenesis, and tumor growth; CGs are found to inhibit KLK expression. (c) Some of the CGs are found to inhibit tumor metastases by acting as anoikis sensitizers because some malignant epithelial cells have metastatic potential resist anoikis. (d) Topoisomerase inhibitors are among the most active anticancer agents, and some of the CGs inhibit topoisomerase II catalytic activity at nanomolar concentrations. (e) Constitutive expression of NF-kB in tumor cells induces proliferation, and NF-kB activation induces resistance to chemotherapeutic agents; some of the CGs are found to block tumor necrosis factor (TNF)-induced activation of NF-kB in a concentration- and time-dependent manner. (f) Clinical evidence for whether CGs may sound as potential anticancer agents is still controversy. Comparing with those women with breast cancer not taking CGs, both a higher and a lower recurrence rate among the women taking CGs are reported by follow-up study. (g) Further study is needed for whether and in what condition CGs can be used as anticancer agents in clinics.

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Terpenes and Improvement of Transdermal Drug Delivery

124

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Contents

1 Introduction 3758
2 Structure of the Human Skin 3759
3 Human Skin Permeation and Transdermal Drug Delivery 3759
4 Terpenes 3760
4.1 Source, Nomenclature, Classification, and Uses of Terpenes 3760
4.2 Terpenes as Chemical Penetration Enhancers 3762
4.3 Mechanisms of Skin Permeation of Terpenes 3764
4.4 Reversible Effects of Terpenes 3770
4.5 Terpenes in Transdermal Formulations 3770
5 Conclusions 3771
References 3772

Abstract

The human skin itself hinders the widespread use of transdermal drug delivery (TDD) for administration of medications. Despite the different strategies devised and employed to reversibly overcome the skin barrier, this noninvasive delivery mode is restricted to potent, low molar mass therapeutic agents. As most drugs would not be able to penetrate the skin in a sufficient quantity to reach the desired therapeutic level, chemical penetration enhancers (CPE) are commonly used to breach the skin barrier and increase drug permeation. Over the years, extensive screening and testing have identified different classes of chemicals as potential

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adjuvants. Among these, terpenes, which are constituents of plant essential oils, have been widely investigated as skin penetration enhancers for both hydrophilic and hydrophobic drugs. Their enhancing effects on human skin and interactions with skin lipids have been extensively studied. The mechanisms of action of terpenes on excised human skin as determined by several analytical techniques were found to be the extraction and phase separation of stratum corneum intercellular lipids. The enhancing efficacies of terpenes with various physicochemical properties for lipophilic and hydrophilic drugs could be compared and ranked, while their enhancing effects on the skin were found to be reversible and the in vitro permeability of skin recovered once they were removed from the excised skin. Terpenes have been incorporated as adjuvants in the form of penetration enhancers or sorption promoters for improved drug delivery from various dosage forms including solutions, gels, and transdermal therapeutic systems.

Keywords

Transdermal • human skin • drug delivery • chemical penetration enhancers • terpenes

Abbreviations

CPE	Chemical penetration enhancers
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared
GP1	Dibutyllauroylglutamide
ITC	Isothermal titration
LPP	Lipid-protein-partitioning
PG	Propylene glycol
SC	Stratum corneum
SMGA	Small molecule gelling agents
TDD	Transdermal drug delivery
TTS	Transdermal therapeutic system

1 Introduction

The human skin barrier hinders the widespread use of transdermal drug delivery (TDD) for drug administration. Despite the range of strategies devised and employed to reversibly overcome the skin barrier, TDD is restricted to potent and low molar mass therapeutic agents. Nonetheless, this noninvasive delivery mode offers advantages such as sustained and controlled drug release, reduced side effects, improved bioavailability, better patient acceptance and compliance, and easy termination of drug therapy. The use of chemical penetration enhancers (CPE) is the conventional approach to modify the skin structure and lower its resistance, so as to allow sufficient drug to reach desired therapeutic levels for systemic effects. Over the years, extensive screening and testing have identified different classes of chemicals as potential adjuvants. Among these, terpenes, the natural volatile oils extracted from plant sources, have been widely used as CPE by permeating into the human skin and reversibly decreasing its barrier resistance.

2 Structure of the Human Skin

The skin, with a surface area of ca. 1.72 m^2, is the most accessible organ of the human body and continues to be the preferred site for the application of topical dosage forms [1]. It consists of three main histological layers: the epidermis, dermis, and subcutis as show in Fig. 124.1 [2]. The stratum corneum (SC) of the epidermis, the outermost part of the skin [3, 4] is a remarkable transport barrier which effectively retards the diffusion of exogenous and endogenous moieties into and out of the host and may be regarded as the rate-limiting layer [5]. Superficial SC is formed in the final stage of differentiation from several layers of dead cells embedded in a lipid matrix, and its morphology resembles a “brick and mortar” array [6].

The protective quality of the skin is firmly attributed to the unique composition and structural configuration of the intercellular SC lipids. Lipid bilayers of ceramides, fatty acids, cholesterol, and cholesterol esters are arranged into a continuous semicrystalline and crystalline interconnecting domain [7–9]. As sweat glands and hair follicles occupy a fractional skin area of 0.1% [3], absorption across the SC layer can be geometrically subdivided into transcellular and intercellular routes. Tortuous intercellular pathway is supposedly the principal route [10–12].

3 Human Skin Permeation and Transdermal Drug Delivery

The continuous SC has been the usual target in TDD. In recent years, researchers have employed physical and chemical methods to temporarily disrupt its elegant molecular structure. Physical methods such as low-frequency ultrasound (sonophoresis) and electrical current (iontophoresis and electroporation) may facilitate absorption of drug molecules by physically altering the skin morphology [13]. Ultrasound causes cavitation, and the shock waves from the collapsing vacuum bubbles increase the free volume space within the lipid lamellae [14, 15]. Iontophoresis electrically drives or repels ionized drug molecules and peptides through current-induced defects [16, 17]. Skin electroporation creates transient aqueous pores in the lipid bilayers.

The conventional strategy of disrupting the skin barrier and improving the skin permeation of poorly absorbed drugs is to incorporate CPE into drug preparations [10, 18]. Lipid-protein-partitioning (LPP) model [19] theorizes that CPE intensifies TDD by increasing drug solubility in the donor formulation, increasing drug partitioning between the formulation and the skin, or disrupting the intercellular SC lipids [13, 20]. At clinically acceptable concentrations, most CPE interact with

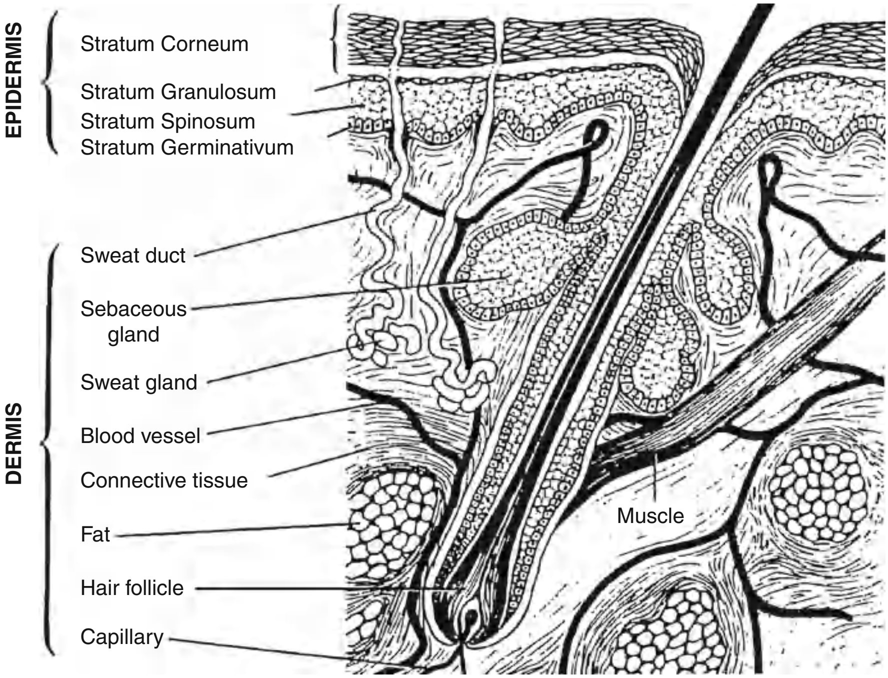
EPIDERMIS
Stratum Corneum
Stratum Granulosum
Stratum Spinosum
Stratum Germinativum
DERMIS
Sweat duct
Sebaceous gland
Sweat gland
Blood vessel
Connective tissue
Fat
Hair follicle
Capillary
Muscle

Fig. 124.1 Cross-sectional view of the uppermost layers of human skin (stratum corneum, viable epidermis, and dermis) (Adapted from Venkatraman and Gale [2])

the lipoidal domains. These interactions include lipid fluidization, polarity alteration, phase separation, and lipid extraction as illustrated in Fig. 124.2 [10, 20]. Compounds such as sulfoxides, pyrrolidones, fatty acids, terpenes, and alcohols are potential enhancers. The ideal CPE would be pharmacologically inert, nontoxic, nonirritating, nonallergenic, and compatible with other components of the transdermal formulation and device as well as upon removal, the skin barrier properties would be restored rapidly and fully. In addition, the onset and duration of the action of a CPE should be predictable and reproducible [21].

4 Terpenes

4.1 Source, Nomenclature, Classification, and Uses of Terpenes

Terpenes are constituents of essential oils which are the volatile and fragrant substances present in flowers, fruits, and leaves of plants. They are named “terpenes” after “turpentine” as turpentine oil is a mixture of these compounds [22, 23]. They are usually named after the plants from which they were first isolated. Some terpenes share the same composition by percentage, and some have even the same molecular

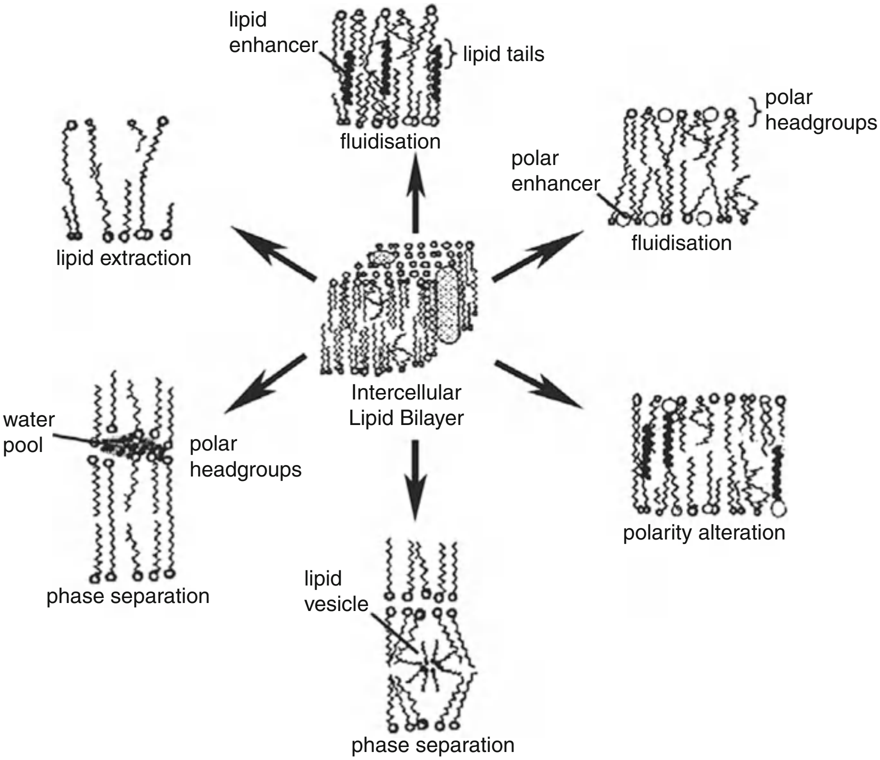
lipid
enhancer
lipid tails
fluidisation
polar
headgroups
polar
enhancer
fluidisation
lipid extraction
Intercellular
Lipid Bilayer
water
pool
polar
headgroups
phase separation
polarity alteration
lipid
vesicle
phase separation

Fig. 124.2 Action of chemical penetration enhancers within the intercellular lipid domain (Adapted from Williams and Barry [20])

weights and similar boiling points. However, they smell different, have different optical properties, and behave differently in chemical reactions, they are not identical.

The term "terpene" is used to describe a compound, which is a constituent of an essential oil containing carbon and hydrogen or carbon atoms, hydrogen, and oxygen atoms, and is not aromatic in character [24, 25]. This definition is usually extended to include other compounds called terpenoids, which are not of natural occurrence but are very closely related to the natural terpenes. Most terpenes, which include terpenoids, are invariably hydrocarbons, alcohols, aldehydes, ketones, or oxides, and they may be solids or liquids. Terpene hydrocarbons are usually liquids, while terpenes of higher molecular weights, mostly obtained from the natural gums and resins of plants and trees, are not steam volatile.

Terpenes are defined and classified by the so-called isoprene rule, introduced by Wallach in 1887 [22]. Two isoprene units make one "terpene unit." They are grouped according to the number of isoprene units, for example, monoterpenes [C_{10}], sesquiterpenes [C_{15}], diterpenes [C_{20}], triterpenes [C_{30}], and tetraterpenes [C_{40}] contain 2, 3, 4, 6, and 8 isoprene units, respectively. A subsidiary classification is based on the number of carbon rings present in the terpene;

monoterpenes, for example, may be acyclic, monocyclic, or bicyclic. Each group is further broken down into chemical divisions of hydrocarbons, alcohols, oxides (ethers), and ketones.

Terpenes have been utilized for a number of therapeutic purposes, such as in antispasmodics, carminatives, and perfumery. They have been found to be useful when incorporated into topical and transdermal pharmaceutical formulations as they act as chemical penetration enhancers facilitating the permeation of drugs through the skin barrier, both healthy and diseased in comprehensive reviews [26, 27].

4.2 Terpenes as Chemical Penetration Enhancers

Terpenes, in particular monoterpenes (Fig. 124.3), are generally recognized as safe (GRAS) adjuvants as indicated by high percutaneous enhancement [28–30], reversible effect on skin lipids, and mild cutaneous irritancy at low concentrations (1–5%w/v) [31, 32]. No skin irritation or sensitization was noted in humans for formulations containing hydrophobic limonene [31] and oxygen-containing anethole, linalool, carvacrol, thymol, and menthol [33, 34]. Sesquiterpenes (Fig. 124.4), probably due to a more bulky molecular structure, tend to be less effective and favorable. They are less active [21] and have a longer duration of action, implying poor reversibility, that is, they do wash out of the skin readily [29]. However, in a recent work by Nokhodchi et al. [35], sesquiterpenes (farnesol and nerolidol) were found to be more potent enhancers than monoterpenes (carvone, limonene oxide) for diclofenac sodium.

The activity of terpenes is dependent on terpene concentration, but there is no clear activity-concentration relationship. Kunta et al. [32] examined the influence of terpene concentration on propranolol permeation. The terpenes investigated were carvacrol, linalool, menthol, and limonene. In general, propranolol transport initially increased and subsequently remained constant with terpene concentration. This phenomenon was also documented by Krishnaiah et al. [34], for menthol in the delivery of nicardipine hydrochloride. Probable reasons are limited terpene solubility in the vehicle and terpene saturation in the skin. In a study by Kararli et al. [33], the permeation flux of zidovudine dropped significantly as thymol and carvacrol concentrations increased from 5% to 10% w/w. Similarly, Babu and Pandit [36] noted a decrease in bupranolol permeation as menthol concentration increased from 2% to 5%w/v. No clear explanations were given for the reduced activities with terpene concentration. Many researchers have chosen a terpene concentration of around 5%w/v in their preparations, and at this level, significant improvements in drug permeation were obtained [28, 37–40]. The bottom line is that the enhancer concentration should be high enough to produce an adequate skin penetration but not high enough to elicit an irritating cutaneous response.

Oxygen-containing terpenes were more potent for hydrophilic drugs (Table 124.1) such as 5-fluorouracil [41], propranolol [32, 49], and zidovudine [33, 45]. Hydrocarbon terpenes apparently gave a better enhancement for lipophilic drugs (Table 124.1) such as ketoprofen [1] and indomethacin [20]. These collectively give rise to the

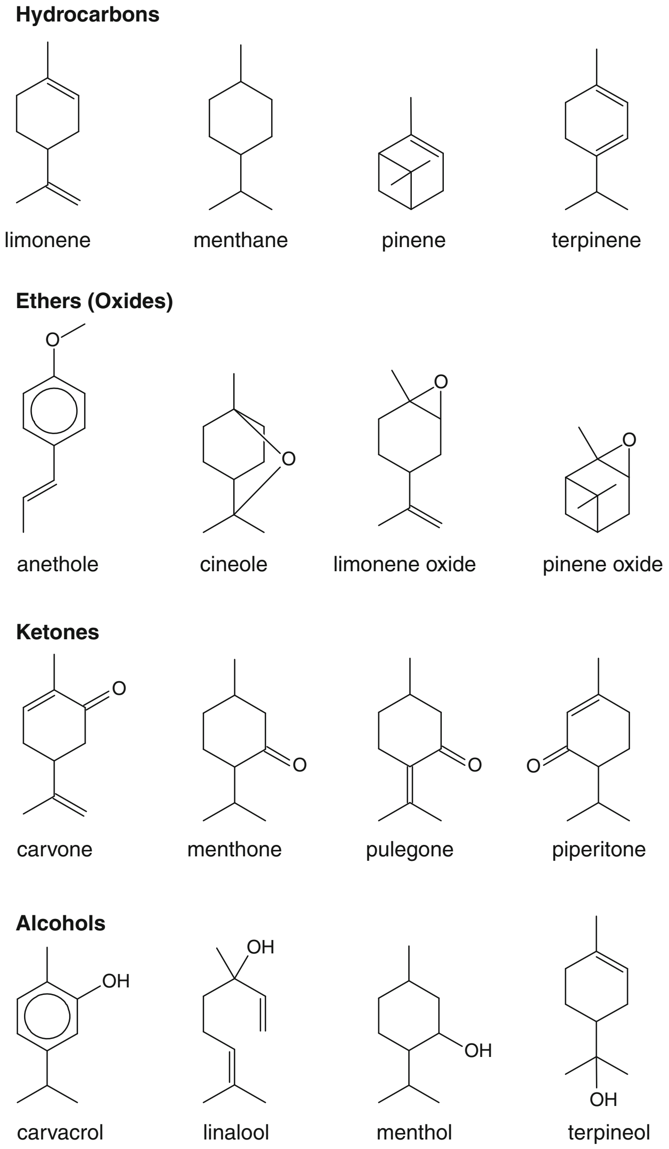
Hydrocarbons
limonene
menthane
pinene
terpinene
Ethers (Oxides)
O
anethole
O
cineole
O
limonene oxide
O
pinene oxide
Ketones
O
carvone
O
menthone
O
pulegone
O
piperitone
Alcohols
OH
carvacrol
OH
linalool
OH
menthol
OH
terpineol

Fig. 124.3 Molecular structures of the different chemical divisions of monoterpenes tested as skin penetration enhancers. Monoterpenes (C_{10}) are made up of two isoprene units

general rule of thumb – the use of hydrophilic terpenes for polar drugs and the use of hydrocarbon terpenes for nonpolar drugs. Evidently, there are exceptions to the rule. Font et al. [37] and Ueda et al. [50] showed that hydrophobic limonene improved the deliveries of hydrophilic sumatriptan succinate (Table 124.1) and aminopyrine

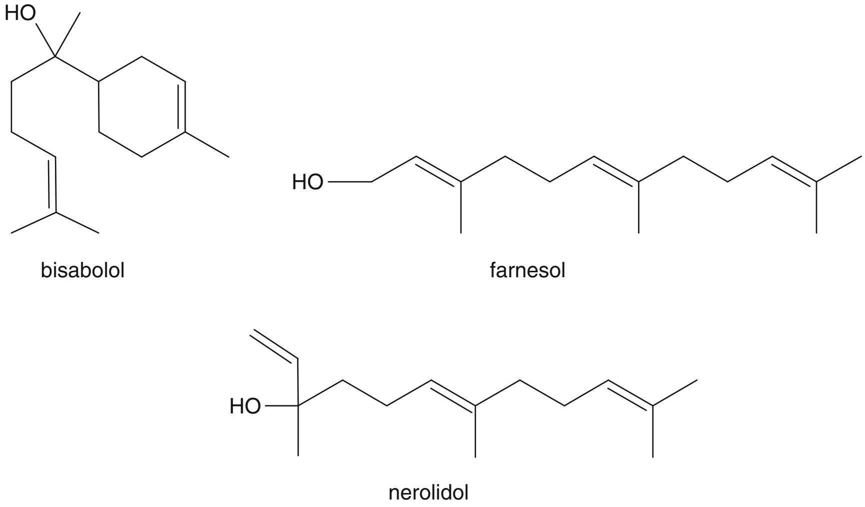

Fig. 124.4 Molecular structures of sesquiterpenes tested as skin penetration enhancers. Sesquiterpenes (C_{15}) are made up of three isoprene units

through excised animal skins the most as compared to oxygen-containing terpenes. The generic trend acts as a guideline for the screening of terpenes, but it does not provide any information on the enhancement level.

The use of terpenes in conjunction with physical enhancers aims to further enhance drug permeation. Ueda and coworkers explored the coupled effect of ultrasound and several chemical enhancers on the percutaneous delivery of aminopyrine [50]. A greater enhancement in aminopyrine flux was obtained when ultrasound was used with monoterpenes (menthol, carvone, and limonene); the coupled effect was much greater than the sum of its individual effects. Rastogi and Singh [51] demonstrated a similar synergistic effect when iontophoresis was combined with limonene pretreatment in the percutaneous absorption of insulin through porcine epidermis.

4.3 Mechanisms of Skin Permeation of Terpenes

Penetration enhancers are applied onto the skin in an amount that is many times more than the skin lipid molecules beneath the area of administration. This means that there are possibly multiple mechanisms taking place at the same time, making it difficult to determine the relative importance and contribution of each mechanism [52, 53].

4.3.1 Techniques to Investigate the Mechanisms of Skin Permeation of Terpenes

Analytical techniques are used in elucidating changes in lipid conformation and composition caused by skin enhancers [54]. Differential scanning calorimetry (DSC),

Table 124.1 Terpenes as skin penetration enhancers for hydrophilic and hydrophobic drugs

Drug	Log *P* of drug	Terpene	Log *P* of terpene	Class and type of terpene	Ref.
Diclofenac sodium	0.70^a	Nerolidol	5.68^c	Alcohol; sesquiterpene	[35]
		Farnesol	5.77^c	Alcohol; sesquiterpene	[35]
5-Fluorouracil	−0.89^a	Cineole	2.50^a	Oxide; monoterpene	[19, 41]
		Menthone	2.87^c	Ketone; monoterpene	[30]
		Pulegone	3.20^c	Ketone; monoterpene	[19]
Haloperidol	3.36^a	Farnesol	5.77^c	Alcohol; sesquiterpene	[38]
		Limonene	4.83^a	Hydrocarbon; monoterpene	[42]
		Limonene oxide	3.43^c	Oxide; monoterpene	[43]
		Linalool	2.97^a	Alcohol; monoterpene	[44]
Indomethacin	4.27^a	Limonene	4.83^a	Hydrocarbon; monoterpene	[20]
Ketoprofen	3.12^a	Limonene	4.83^a	Hydrocarbon; monoterpene	[1]
Nimodipine	3.15^b	Limonene	4.83^a	Hydrocarbon; monoterpene	[31]
Sumatriptan succinate	0.97^b	Limonene	4.83^a	Hydrocarbon; monoterpene	[37]
Zidovudine	0.05^b	Anethole	3.39^a	Oxide; monoterpene	[38]
		Cineole	2.50^a	Oxide; monoterpene	[45]
		Menthol	3.30^a	Alcohol; monoterpene	[45]
		Thymol	3.30^a	Alcohol; monoterpene	[33]

[a] Log*P* was obtained from Howard and Meylan [46]
[b] Log*P* was obtained from DrugBank website [47]
[c] Log*P* was obtained from Syracuse Research Corporation website [48]

Fourier transform infrared (FTIR), and isothermal calorimetry (ITC) are techniques which have been utilized to examine macroscopic and molecular transitions of the skin.

DSC is sensitive to the thermal effects accompanying phase changes or transitions of the components of the SC layer. DSC thermograms of human SC, extracted lipids, and protein residue showed three endotherms at ca. 65 °C, 75 °C, and 95 °C [55]. The first is due to the melting of lipids. The second is believed to be the melting of lipid-protein complexes and the last represents protein denaturation. A decrease in lipid melting transition of the enhancer-treated skin denotes a plausible phase separation [39, 56, 57]. Phase separation involves the formation of interfacial defects in the SC lamellae, resulting in decreased diffusional path length or resistance. Based on the lowered lipid endotherms, Vaddi and our fellow researchers suggested that alcoholic terpenes in ethanol-water could exist as pools of fluid within the intercellular lipids [39]. The presence of fluid terpenes at physiological temperatures presumably aids molecular transport. Moghimi et al. reported some degree of phase transformation, the replacement of the lamellae by a reversed viscous isotropic phase, in limonene-treated matrices [57]. These macroscopic defects within the lipid structure may lead to enhanced drug permeation.

To better understand the effects of the terpenes on the permeability of a drug through the skin, the interactions of the terpenes with SC intercellular lipids were studied using the isothermal titration (ITC) method by our research group [58]. Cholesterol, palmitic acid, and stearic acid were found to be the most soluble among all the lipids in propylene glycol, and they were further significantly solubilized upon the addition of farnesol. The interactions between farnesol and four representative lipids, that is, cholesterol, behenic acid, ceramide 3, and ceramide 9 were studied using the ITC method. The binding ratios of farnesol to cholesterol, behenic acid, ceramide 3, and ceramide 9 were found to be 1, 2, 2, and 2, respectively. All were endothermic and entropy driven except for that between farnesol and behenic acid, which was exothermic and enthalpy driven. Hydrogen bonding may be the driving force of these interactions. The results suggested that the skin permeation enhancement mechanism of farnesol, the terpene of interest, could be due to lipid extraction and/or triggering lipid phase transition of the SC lamella. This finding was also consistent with the permeation study results, which showed the permeability coefficients of the drug increased as the lipophilicities of monoterpene and sesquiterpenes increased. It is perceivable that terpenes with high lipophilicities will have more interactions with skin lipids.

Infrared spectra bands of the SC can be attributed to the lipid or protein molecular vibrations. The hydrocarbon chains of SC lipids give rise to asymmetric and symmetric CH_2 stretching vibrations at 2,920 and 2,850 cm^{-1}, respectively [39]. The shift of these bands to a higher frequency occurs when the methylene groups change from a trans to a more energetic gauche conformation, and this shift is associated with lipid fluidization. The absorbance of the CH_2 stretching bands is proportional to the amount of SC lipids, and thus, the extraction of lipids by a skin enhancer results in a decrease in absorbance [5, 39, 53, 59]. SC proteins give rise to CN stretching and NH bending vibrations at 1,550 cm^{-1}, and $C = O$ stretching vibration at 1,650 cm^{-1}. The shift of these bands to either a higher or lower frequency signifies a change in the protein conformation [45, 53]. FTIR findings of several research groups identified partial lipid extraction as a dominant mechanism for alcoholic, oxide, and hydrocarbon terpenes [39, 40, 60, 61] and showed that delipidization was consistent with enhanced drug permeation.

After the permeation behaviors of the model drug and the enhancers have been investigated by studying the interactions between skin lipids and terpene enhancers, the modeling of drug permeation process through excised skin using both Franz cell and flow-through cell was used to compare the effects of terpenes on drug permeation [62]. A mathematical solution based on finite outflow volume was derived from Fick's law. It can serve as a statistical model to estimate the permeability coefficient from in vitro skin permeation study with the accumulation of penetrants in the receptor compartment of the static diffusion cells. The model is suitable to describe the in vitro drug or chemical permeation studies using Franz cells. However, the flow-though cells have infinite outflow volume, so a different model that could enable the parameter estimation without impairing the integrity and quality of the original permeation data was proposed. The nonlinear regression model derived from Fick's law is appropriate.

Bootstrap sampling is useful for checking the precision of parameter inference based on the large-sample theory. For the in vitro permeation study that we conducted with flow-through cells, the method proved to be robust. The estimates of permeated drug/chemical are important in that, unlike in vivo environment where stratum corneum is replenished by the adjacent live stratum granulosum through keratinization, the excised stratum corneum, though composed of dead corneocytes, will deteriorate after days in contact with solvents, which will cause overhydration of stratum corneum that can destroy the lamella and decomposition that will leave highly permeable passages in the stratum corneum. The predictions are relevant for transdermal drug delivery, the cosmetic industry, and regulatory risk assessment on dermal exposure to toxic substances.

4.3.2 Comparison of the Skin Permeation Effects of Terpenes

The effects of a terpene as a CPE are very much dependent on its physicochemical properties and molecular structure. Increases in the skin transports of propranolol [32, 45], haloperidol [39], and zidovudine [33] were partially attributed to the hydrogen-bonding ability of oxygen-containing monoterpenes. The ether and hydroxyl groups of terpenes interact with the polar head groups of skin ceramides and fatty acids, thereby disrupting the lateral/traverse hydrogen bonding of the lipid bilayers [45, 49]. On the contrary, hydrocarbon terpenes would reside preferentially and cause structural perturbation in the alkyl tail regions of the lipid bilayers [61].

Cornwell and Barry [29] obtained a positive, linear relation between skin conductivity ratio and 5-fluorouracil enhancement ratio for eight terpenes (limonene, terpineol, carvone, pulegone, nerolidol, menthone, ascaridole, and cineole) with oxygen-containing cineole and hydrocarbon limonene positioned at the higher and lower ends of the curve, respectively. Increases in both skin conductivity and permeability after treatment with terpenes suggest the creation of new polar channels through which both ions and polar molecules traverse. These polar routes would be confined in the aqueous regions near the head groups of SC lipids [45], and their formation is possibly linked to the hydrogen-bonding potential of hydrophilic terpenes.

The enhancing effects of 49 terpenes were compared by in vitro drug permeation studies of haloperidol through excised human epidermis by our research group [62]. The derived multiple linear regression models which provided estimations of the permeability coefficients of a drug or chemical through the human skin were found to be useful for the preliminary screening of CPE. The authors reported that for monoterpenes and sesquiterpenes, the permeability coefficients of haloperidol increased as the lipophilicities of terpenes increased. For all terpenes studied, their enhancing abilities decreased as their molecular weights increased. Melting points and boiling points of terpenes were negatively correlated with the permeability coefficients of haloperidol. Sesquiterpenes were better than monoterpenes when only their enhancing effects were considered. The effects of the skin permeation enhancement by terpenes were ranked as follows: ester > aldehyde > oxide > hydrocarbon > alcohol > ketone > phenol > acid (Table 124.2).

Table 124.2 The second column is the name of each terpene, its CAS entry, and purity. The third column T indicates the terpene category (key: 1 monoterpene, 2 sesquiterpene, 3 diterpene, 4 triterpene, 5 tetraterpene). The fourth to seventh columns are molecular weight, melting point, boiling point, and logP of each terpene. The boiling point of (−)-isolongifolol is not available and is estimated at 300 °C to be similar to the boiling points of other sesquiterpenes. The eighth column, Sol, is the solubility of HP in PG at 37 °C with or without 5 % (w/v) enhancer. The last column *LogKp* is the in vitro permeability coefficient of HP though human stratum corneum. Data are given as mean ± SD. For columns 8 and 9, the data were determined experimentally in our lab. The other data were obtained from SciFinder Scholar® and original product information

No	Terpene name, [CAS] and purity/%	T	MW	mp/°C	bp/°C	*LogP*	Sol/mg.ml^{-1}	*LogKp*/cm.h^{-1}
0	Haloperidol	–	–	–	–	3.36	3.08 ± 0.28	−9.04 ± 0.06
1	(−)-Guaiol [489-86-1] 99	2	222.37	90	288	4.75	4.73 ± 0.31	−8.88 ± 0.61
2	(−)-Carveol [99-48-9] 97	1	152.23	Liquid	232	2.68	6.32 ± 0.59	−6.45 ± 0.15
3	(−)-Caryophyllene oxide [1139-30-6] 99	2	220.35	63	280	4.57	4.49 ± 0.38	−6.78 ± 0.68
4	(−)-Dihydrocarveol [20549-47-7] 97	1	154.25	Liquid	220	2.92	5.89 ± 0.45	−8.87 ± 0.10
5	(−)-Epiglobulol [88728-58-9] 95	2	222.37	Liquid	294	4.81	4.95 ± 0.38	−4.41 ± 0.01
6	(−)-Isolongifolol [1139-17-9] 99	2	222.37	112	–	4.05	4.63 ± 0.19	−8.55 ± 0.06
7	(−)-Isopulegol [89-79-2] 99	1	154.25	Liquid	197	2.93	6.60 ± 0.49	−8.35 ± 0.21
8	(−)-Trans-caryophyllene [87-44-5] 99	2	204.35	Liquid	268	6.78	5.09 ± 0.02	−7.28 ± 0.02
9	(−)-α-Cedrene [469-61-4] 99	2	204.35	Liquid	263	6.38	4.62 ± 0.10	−6.89 ± 0.03
10	(−)-α-Santonin [481-06-1] 98	2	246.30	171	423	1.80	5.71 ± 0.38	−7.58 ± 0.30
11	(−)-α-Thujone [76231-76-0] 96	1	152.23	Liquid	206	1.90	6.83 ± 0.08	−8.52 ± 0.14
12	(+)-Aromadendrene [489-39-4] 97	2	204.35	Liquid	258	6.41	4.77 ± 0.15	−7.40 ± 0.08
13	(+)-Cedrol [77-53-2] 99	2	222.37	84	277	4.77	4.35 ± 0.12	−7.96 ± 0.00
14	(+)-Cedryl acetate [77-54-3] 95	2	264.40	45	292	5.67	5.76 ± 0.35	−5.52 ± 0.33
15	(+)-Dihydrocarveol [22567-21-1] 97	1	154.25	Liquid	220	2.92	6.28 ± 0.53	−8.71 ± 0.08
16	(+)-Dihydrocarvone [7764-50-3] 98	1	152.23	Liquid	222	2.47	6.92 ± 0.18	−7.17 ± 0.05
17	(+)-Longifolene [475-20-7] 99	2	204.35	Liquid	252	6.39	4.55 ± 0.18	−7.42 ± 0.01
18	(+)-β -Cedrene [546-28-1] 97	2	204.35	Liquid	263	6.37	4.72 ± 0.13	−7.01 ± 0.06
19	(±)-Linalool [78-70-6] 96	1	154.25	Liquid	199	3.28	5.05 ± 0.13	−8.97 ± 0.29
20	(±)-Nerolidol [7212-44-4] 97	2	222.37	Liquid	276	5.31	5.10 ± 0.20	−4.59 ± 0.05

(*continued*)

Table 124.2 (continued)

No	Terpene name, [CAS] and purity/%	T	MW	mp/°C	bp/°C	*LogP*	Sol/mg.ml^{-1}	*LogKp*/cm.h^{-1}
21	(±)-α-Bisabolol [515-69-5] 99	2	222.37	Liquid	315	5.01	6.26 ± 0.48	−5.25 ± 0.30
22	(1R)-(−)-myrtenal [564-94-3] 98	1	150.22	Liquid	216	2.52	7.27 ± 0.16	−5.29 ± 0.06
23	(1R)-(−)-myrtenol [515-00-4] 95	1	152.23	Liquid	225	2.64	5.51 ± 0.05	−828 ± 0.04
24	(R)-(−)-carvone [6485-40-1] 98	1	150.22	Liquid	231	2.27	2.43 ± 0.19	−7.56 ± 0.11
25	(R)-(+)-pulegone [89-82-7] 98	1	152.23	Liquid	229	2.56	3.53 ± 0.07	−6.63 ± 0.25
26	(S)-(−)-citronellal [5949-05-3] 96	1	154.25	Liquid	208	3.48	9.43 ± 0.61	−4.83 ± 0.03
27	(S)-(−)-perillaldehyde [18031-40-8]	1	150.22	Liquid	238	2.81	6.34 ± 0.06	−6.59 ± 0.09
28	Carvacrol [499-75-2] 98	1	150.22	3.5	237	3.28	5.84 ± 0.26	−8.44 ± 032
29	Citral [5392-40-5] 96	1	152.23	Liquid	229	3.17	6.33 ± 0.62	−5.08 ± 0.03
30	Cyclohexanemethanol [565-50-4] 99	1	172.76	117	265	1.07	5.16 ± 0.14	−8.08 ± 0.55
31	Eucarvone [503-93-5]	1	150.22	Liquid	227	2.21	5.47 ± 0.02	−7.60 ± 0.04
32	Farnesol [4602-84-0] 97	2	222.37	Liquid	283	5.31	5.65 ± 0.26	−6.72 ± 0.36
33	Geraniol [106-24-1] 98	1	154.25	Liquid	230	3.28	6.11 ± 0.69	−7.43 ± 024
34	L-(−)-menthol [2216-51-5] 98	1	156.27	43	215	3.20	5.11 ± 0.51	−7.34 ± 0.05
35	Menthone [14073-97-3] 90	1	154.25	Liquid	209	2.63	7.53 ± 0.08	−8.72 ± 0.05
36	Myrcene [123-35-3] 95	1	136.23	Liquid	167	4.58	6.03 ± 0.66	−5.43 ± 020
37	Nerol [106-25-2] 97	1	154.25	Liquid	230	3.28	5.54 ± 0.20	−7.80 ± 0.01
38	Ocimene [3338-55-4] 70	1	136.23	Liquid	175	4.70	7.74 ± 0.70	−5.41 ± 0.01
39	Octisalate [118-60-5] 99	2	250.33	Liquid	332	5.77	3.14 ± 0.34	−5.19 ± 014
40	Phytol [7541-49-3] 97	3	296.53	Liquid	336	8.66	4.77 ± 0.31	−5.13 ± 0.02
41	Retinoic acid [302-79-4] 98	3	300.44	146	463	6.83	8.79 ± 1.46	−12.13 ± .90
42	Retinol [68-26-8] 97	3	286.45	63	421	6.84	7.11 ± 0.23	−6.71 ± 0.06
43	Squalene [111-02-4] 97	4	410.72	Liquid	429	13.09	3.91 ± 0.16	−8.56 ± 0.07
44	Terpinolene [586-62-9] 97	1	136.23	Liquid	182	4.67	2.30 ± 0.10	−7.01 ± 0.48
45	Thymol [89-83-8] 98	1	150.22	51	233	3.28	6.69 ± 0.55	−8.29 ± 0.10
46	α-Humulene [6753-98-6] 99	2	204.35	Liquid	276	7.03	5.28 ± 0.43	−6.23 ± 0.03
47	α-Phellandrene [99-83-2] 92	1	136.23	Liquid	171	4.43	4.83 ± 0.21	−4.96 ± 0.00
48	β-Carotene [7235-40-7] 102.8	5	536.87	181	655	15.51	18.6 ± 1.60	−11.15 ± .19
49	β-Citronellol [106-22-9] 95	1	156.27	Liquid	225	3.38	5.29 ± 0.20	−7.66 ± 0.26

4.4 Reversible Effects of Terpenes

In addition to the enhancing effects, the reversibility of the effects of terpenes on the skin is also an important characteristic of an ideal CPE. The permeability of the pretreated epidermis was comparable to that of the control, so the insult to the barrier function of the skin caused by the enhancers was restored as reported by our research group [43]. As an in vitro study was performed, the recovery of the epidermal barrier function could not be due to the physical barrier being restored via cellular regeneration of the horny layer. The mechanism for this reversible enhancement would be attributed to the insertion of these enhancers within the SC intercellular lipid lamella. The disruptions in the lipid lamella eased the permeation of the lipophilic drug through the tortuous pathway, hence resulting in enhancement of drug permeation. Likewise, once the enhancers were removed, bonds between the lipids could start to re-form, and the depletion of the enhancers could allow the packing of the lipids to revert back to its original alignment. Of the terpenes studied, (R) – (−) carvone had a much faster elution profile out of the epidermis than eucarvone. The results also showed that (R) – (−) carvone, rather than eucarvone, retained more drug, haloperidol, within the epidermis, which suggests that (R) – (−) carvone could be useful as an enhancer for depot HP therapy. Both (R) – (−) carvone and eucarvone were shown to be effective and reversible enhancers for the in vitro permeation of haloperidol through the human epidermis.

4.5 Terpenes in Transdermal Formulations

The transdermal drug formulations could be in the form of a suspension, solution, gel, ointment, or multilayer transdermal patch. A controlled drug release from the patch is usually achieved by changing the properties of either the rate-controlling membrane or the drug matrix.

Lim et al. [42] demonstrated a controlled drug release by varying the gelator content of an organogel vehicle which presented different degrees of resistance to drug diffusion. He also went on to show the effect of CPE, such as terpenes, on the physical, rheological, and chemical characteristics of a model pharmaceutical formulation for topical and transdermal drug delivery [63] by examining the effects of three terpenes (linalool, cineole, limonene) on the rheology and chemical stability of an organogel composed of dibutyllauroylglutamide (GP1) and propylene glycol (PG). At a given GP1 concentration, oxygen-containing linalool and cineole decreased gel moduli (elastic and viscous) and brittleness, and the reverse was obtained for hydrocarbon limonene. Probably, linalool and cineole interfered with hydrogen bonding between GP1 molecules, while limonene could have initiated a phase separation-mediated gelation, changing the gel morphology. Microcalorimetry detected minute heat endotherms for gels (with and without terpenes) subjected to accelerated heat testing. These heat changes could arise from a small degree of structural disruption of the gel network. Heat endotherms normalized with respect to GP1 content were used to assess gel chemical stability. Although the terpenes altered rheology, they did not significantly affect the chemical stability of the gels. This is the first literature report on the effect of penetration enhancers, such as terpenes, on the physical, rheological, and chemical characteristics of a model pharmaceutical formulation for topical and transdermal drug delivery.

The enhancing effect of a selected enhancer, farnesol, incorporated into gels containing small molecule gelling agents (SMGA), was reported by our research group [38]. The SMGA gels developed for application on the skin retained their characteristic aesthetic and rheological properties with the incorporation of the drug and enhancer. These in vitro human skin permeation studies showed that the gels possessed desirable properties for both topical and transdermal delivery. The translucent lipophilic gels with ISA were stable and the permeation of the drug reached the pseudo steady state in less time compared to the PG-based gel. The latter, opaque white in color, delivered the drug at a faster rate with the addition of the enhancer. The gelator, GP1, did not influence the drug permeation rate but increased its permeation lag-time.

Experimental transdermal patches have also been fabricated by flanking a solution or gel containing the test drug and other excipients between an impermeable backing laminate and a rate-controlling membrane. A pressure-sensitive adhesive coated on the membrane ensures an intimate patch-skin contact. The patch is kept in a sealed aluminum pouch to minimize solvent loss [31, 62, 64].

Krishnaiah et al. [31] designed a transdermal therapeutic system (TTS) for nimodipine. The drug reservoir was an ethanolic gel with 4%w/w limonene as the penetration enhancer. A copolymer film (rate-controlling membrane) was coated with a pressure-sensitive adhesive. In vivo study performed on human volunteers showed that the TTS provided a steady-state nimodipine plasma level with minimal fluctuations for around 20 h and a much-improved bioavailability relative to a tablet dosage form of nimodipine. The intersubject variation in the drug plasma level was observed to be significantly lower for the transdermal route than for the oral route. Hepatic first-pass metabolism, differences in gastric emptying, and gastrointestinal absorption among the subjects are the key causes of a low bioavailability and a less reproducible pharmacokinetics, commonly associated with oral administration. Also, the absence of any local irritation at the application sites of the volunteers demonstrated that the components of the TTS patch (drug, terpene, vehicle, and adhesive) were well tolerated by the skin. Similar findings and conclusions were obtained for the TTS for nicardipine hydrochloride and nicorandil with menthol and nerolidol as the penetration enhancer, respectively [34, 65, 66].

5 Conclusions

Terpenes, as constituents present in plant essential oils, have been investigated as chemical enhancers in transdermal drug delivery. In particular, monoterpenes were generally more efficacious probably due to their small molecular sizes. The main mechanisms of action of terpenes on the skin as determined by several analytical techniques were lipid extraction and phase separation. Terpenes such as limonene and menthol in experimental patch formulations were demonstrated to be safe on the skin and effective in improving drug delivery across the skin.

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Quassinoids: Anticancer and Antimalarial Activities

125

Emeline Houël, Didier Stien, Geneviève Bourdy, and Eric Deharo

Contents

1 Introduction 3776
1.1 Structural Diversity and Natural Occurrence of the Quassinoids 3776
1.2 From Kwasi to Quassin, or How a Traditional Pharmacological Application Led to a New Promising Family of Molecules 3781
1.3 Pharmacological Activities: A Focus on Cancer and Malaria 3783
2 Pharmacological Applications 3784
2.1 Historical Perspective and In Vitro Cytotoxic Activities 3784
2.2 In Vivo Anticancer Assays with Quassinoids, Clinical Trials 3785
2.3 Historical Perspective and In Vitro Antiplasmodial Activities 3786
2.4 In Vivo Antimalarial Activity of Quassinoids in Murine Models 3787
3 Mechanisms of Action 3789
4 Other Biological Activities and Ethnopharmacological Relevance 3791
5 Conclusion 3796
References 3796

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Abstract

Quassinoids were initially isolated as bitter principles of plants of the Simaroubaceae family. These natural products are formed by oxidative degradation of triterpene derivatives. Since the 1970s, these molecules have attracted attention because of their promising biological activities, especially in the context of research regarding active anticancer and antimalarial principles. In this chapter, the structural diversity of quassinoids and their botanical and geographical occurrence are described, combining a historical perspective from the literature references regarding these two major biological activities and focusing on the results obtained in vivo with the most promising compounds; in vitro studies are less relevant and have already been extensively reviewed in the literature. The biological activities with respect to the uses of the corresponding Simaroubaceae in traditional medicine are also analyzed.

Species names have been transcribed according to the nomenclature system used by The Plant List (http://www.theplantlist.org). Full names of species with determinant are given when cited for the first time only.

Keywords

Anticancer • Antimalarial • Ethnopharmacology • Simaroubaceae • Quassinoids

Abbreviations

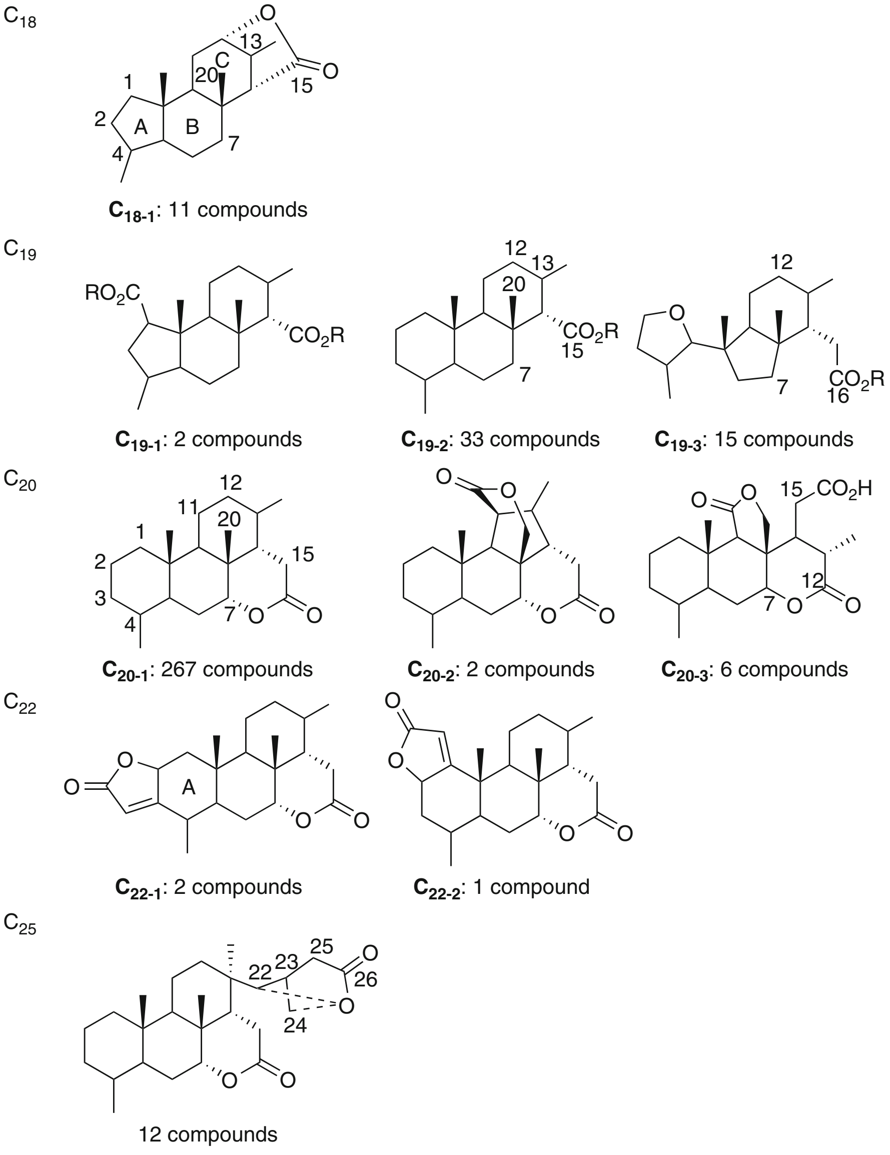
ED_{50}	Drug dose inducing 50% response
SkD	Simalikalactone D
SkE	Simalikalactone E

1 Introduction

1.1 Structural Diversity and Natural Occurrence of the Quassinoids

Quassinoids are natural products formed by oxidative degradation of triterpene derivatives; their biosynthetic precursors are similar to those of limonoids but the biosynthetic pathways of quassinoids have not been established so far [1]. At least 351 different natural quassinoids have been described in the literature (Fig. 125.1), and a large number of semisynthetic and synthetic analogues have been prepared for synthesis or medicinal chemistry purposes, mostly in last 30 years [see, for example, 2–4]. Several base skeletons have been described in the literature, and these can be classified into five distinct groups according to the number of atoms of the main chain (Fig. 125.1).

The C_{18} quassinoids comprise laurycolactones A and B, eurycolactone B-D, and samaderin A and derivatives. Here, the lactone linkage between carbon atoms C-15


C18
O
13
C
O
1
20
15
2
A
B
4
7
C19
12
13
12
RO2C
20
O
CO2R
CO2R
15
7
7
CO2R
16
C20
12
O
O
O
O
15 CO2H
11
1
20
2
15
3
12
7 O
O
O
7 O
O
4
C22
O
O
O
A
O
O
O
O
C25
25
O
22 23
26
O
24
O
O

C18-1: 11 compounds

C19-1: 2 compounds

C19-2: 33 compounds

C19-3: 15 compounds

C20-1: 267 compounds

C20-2: 2 compounds

C20-3: 6 compounds

C22-1: 2 compounds

C22-2: 1 compound

12 compounds

Fig. 125.1 Natural quassinoids: base skeletons and occurrence

and C-12 is always present, and rings A and B are oxidized with carbonyl groups at positions 1 and 7. In samaderin A, carbon atoms C-20 and C-13 are interconnected with an ether moiety.

The C_{19} quassinoids can be subdivided into three structural groups (Fig. 125.2). The first includes eurycolactone A and samaderolactone A. The second is more diverse, with 33 compounds, including eurycomalactone, eurycomalide, and

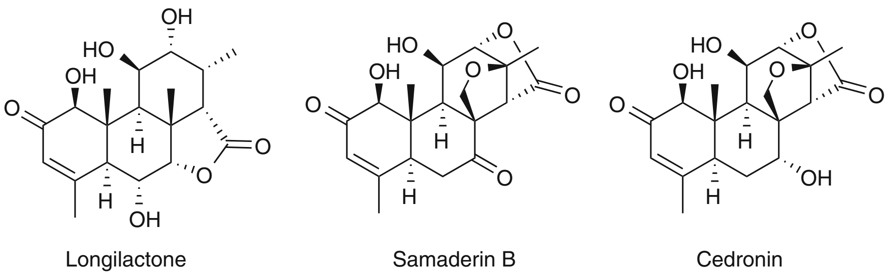


Fig. 125.2 Examples of C_{19} quassinoids

longilactone derivatives, more samaderin derivatives and eurycolactones E and F, indaquassin A, and cedronin. The first representatives of this series were the samaderins B and C, isolated and characterized in 1962 from *Quassia indica* (Gaertn.) Noot. by Polonsky [5].

In this series, longilactones have a lactone linkage between C-15 and C-7, while all others have a lactone ring closure between carbon atoms C-15 and C-12. The only exceptions are eurycolactone F and eurycomaoside, which should have been named after longilactone. In addition, cedronin and samaderins have an ether linkage between C-20 and C-13. The third group of C_{19} quassinoids is smaller. It is composed of 15 different compounds with a contracted B ring. In general, C-16 is linked to C-7, forming a six-membered lactone ring. The A ring is always a γ-butyrolactone and is almost always unsaturated.

More than 75 % of all natural quassinoids described in the literature have a C_{20} skeleton. In the first type of C_{20} quassinoids, a lactone is usually formed between C-16 and C-7 (as represented here), although some members of this group are lactonized between C-16 and C-12. All positions can be oxidized with double bonds or oxygenated functional groups, and again, an ether moiety linking C-20 to C-13 and sometimes C-11 can be encountered. When the C-15 atom is hydroxylated, the hydroxyl group is often esterified with small lipophilic side chains. Quassin, the first isolated quassinoid, belongs to this C_{20} group, and most in vivo studies and clinical trials with quassinoids were conducted on compounds of that group. Quassin is used as natural insecticide and bitter food flavoring. The C_{20} quassinoids that have attracted most attention in the literature for their pharmacological interest are ailanthone and its analogues (ailanthinone, glaucarubinone, chaparrinone, 15-desacetylundulatone, and peninsularinone), bruceins, brusatol, bruceolide, bruceantin, and simalikalactones D and E (Fig. 125.3).

The second group of C_{20} quassinoids with a contracted C ring is represented by shinjudilactone and ailantinol D, and the third group with a cleaved C ring is composed of vilmorinines A-F isolated from *Ailanthus vilmoriniana* (Dode) [6].

The three C_{22} natural quassinoids known in the literature have a butenolide moiety attached to the A ring, presumably originating from the aldol cyclization of a α-acetoxycarbonyl moiety from the normal C_{20} skeleton. In this series, sergeolide has attracted much attention due to its very good antimalarial potential (Fig. 125.4) [7].

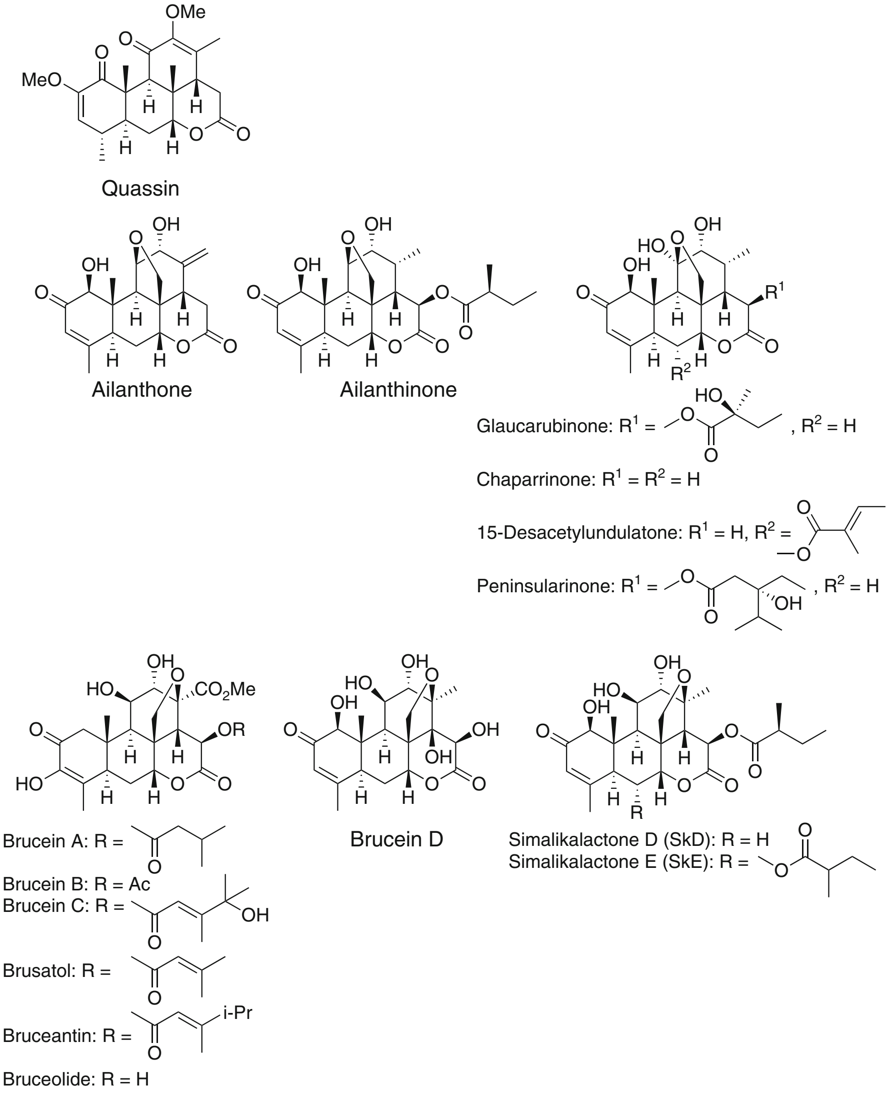

Quassin
Ailanthone
Ailanthinone
Glaucarubinone: R1 = , R2 = H
Chaparrinone: R1 = R2 = H
15-Desacetylundulatone: R1 = H, R2 =
Peninsularinone: R1 = , R2 = H
Brucein A: R =
Brucein B: R = Ac
Brucein C: R =
Brusatol: R =
Bruceantin: R =
Bruceolide: R = H
Brucein D
Simalikalactone D (SkD): R = H
Simalikalactone E (SkE): R =

Fig. 125.3 Examples of naturally occurring C_{20} quassinoids

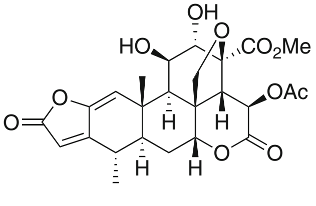


Fig. 125.4 Structure of sergeolide

C_{25} quassinoids are rather rare. Simarolide was the first member of this group to be isolated and characterized by Polonsky in the early 1960s [8, 9]. The side chain consisting of carbon atoms C-22 to C-26 is always lactonized, either forming an O–C-22 or a O–C-24 bond. In this series, the C-15 position is never hydroxylated so the side chain that seems to impact on the biological activity of the C_{20} quassinoids is absent. Also, C-20 is always a methyl group, whereas, the A ring of soulameolide and indaquassin F does bear the α,β-unsaturated ketone with the carbonyl functional group on C-2.

Quassinoids are natural products occurring in the Simaroubaceae family and are known as the bitter principles of these plants. The Simaroubaceae belong to the Sapindales order and are considered as emerging from a protorutaceous stock because of the presence of tryptophan-derived alkaloids (canthinones and β-carbolines) common to the Rutaceae and the Simaroubaceae. The major metabolic difference with Rutaceae species originates from the presence of limonoids in Rutaceae, whereas the Simaroubaceae generate quassinoids [10–12]. The correlation between the presence of alkaloids and quassinoids and the geographical distribution of the species has also been studied to clarify the phylogenetic relationships within the Simaroubaceae family [11].

Nevertheless, some exceptions to this rule must be highlighted, with the examples of the genera *Samadera* and *Harrisonia*. In 1997, the first example of joint occurrence of quassinoids and limonoids was uncovered in a new Australian Simaroubaceae species SAC-2825, tentatively assigned as aff. *Samadera bidwillii* Oliv. [12]. The genus *Harrisonia* was also shown to contain both families of molecules [13]. However, with the evolution of botanical nomenclature, *Samadera bidwillii* is considered unresolved and some *Samadera* species have been placed in synonymy with *Quassia* species, whereas the genus *Harrisonia* is now included in the Rutaceae family. These examples of species containing simultaneously limonoids and quassinoids could illustrate the chemistry of primitive Rutales, before the metabolic separation between the two families, and quassinoids therefore appear to be of chemotaxonomic relevance.

Currently, the Simaroubaceae family includes 16 genera divided into 102 species exclusively distributed in tropical and subtropical areas, with the exception of the *Ailanthus* and *Picrasma* genera, the distribution area of which extends to temperate Asia. The most species-rich genera are *Quassia* (37 species), followed by *Castela* (17), *Simaba* (12), and *Brucea* (10). *Armoria*, *Gymnostemon*, *Iridosma*, *Leitneria*, and *Simarouba* are monospecific. Some genera have distribution areas restricted to Asia (*Eurycoma*) or southwest Africa (*Odyendyea* and *Hannoa*). *Brucea* is present in East Africa and Asia, *Picrasma* and *Quassia* are considered cosmopolitan, and *Castela* is essentially neo-tropical [14]. A biogeographic study of the Simaroubaceae family suggested that this family may originate from North America with a migration through the Bering Strait by ancestral taxa [15].

Morphologically, species of this family are trees of medium to small size, or branched and bushy shrubs, sometimes spiny. The leaves are alternate, compound, rarely simple, without stipules. The bitter taste of all parts of the plants of this family is also a criterion for botanic identification. The scientific names and even

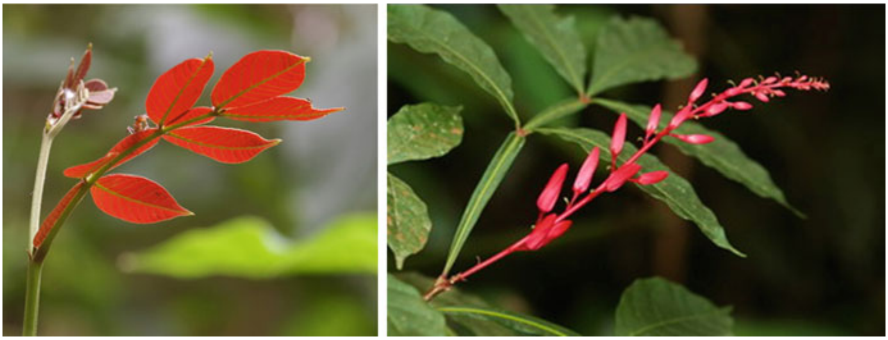


Fig. 125.5 Young leaf (*left*) and mature leaf and flower (*right*) of *Quassia amara* L. (Picture: G. Bouchon)

more the vernacular names of these species bear witness to this feature. For example, *Quassia africana* (Baill.) Baill., an African Simaroubaceae, is known in Congo under the name of "simalikali" which means "bitter than everything else" [16].

1.2 From Kwasi to Quassin, or How a Traditional Pharmacological Application Led to a New Promising Family of Molecules

The history of quassinoids began in the mid-eighteenth century, after the discovery in 1760 of the febrifuge properties of a Simaroubaceae, *Quassia amara* L. (Fig. 125.5). The medicinal property of the roots of this species was revealed to Carl G. Dahlberg, a Dutch army officer, by a Suriname slave and famous healer named Kwasi. This recipe was subsequently made public by Daniel Rolander, a Swedish naturalist. Linnaeus, excited by the discovery of this plant and its uses, named it in honor of the healer. The botanist, however, committed an error in its description, corrected in 1763 by his disciple C.M. Blom [17–20].

Under the name of "quassia" or "quassia wood" (*Quassiae lignum*), two indiscriminate species were then sold in Europe: *Quassia amara* (mainly root, wood and stems) and *Picrasma excelsa* (Sw.) Planch. (formerly *Picraena excelsa* (Sw.) Lindley) or Jamaican quassia (trunk wood) [17, 18, 20]. Quassia wood was initially used as an antiseptic, for meat preservation and as antipyretic. But because of its bitter principles its main recommendation was as a digestive and tonic [17, 21]. *Q. amara* was rapidly registered in various European pharmacopoeias, alone or with other Simaroubaceae species with the same reputation, such as *Picrasma excelsa* or *Simarouba amara* Aubl. [22–25]. The reputation of quassia wood then spread to the United States, where the medicinal use of cups mostly made of *Q. amara* wood became popular [26]. Meanwhile, a few Simaroubaceae were registered in North American official pharmaceutical documents, such as the King's American Dispensatory [27] or the United States Dispensatory [28].

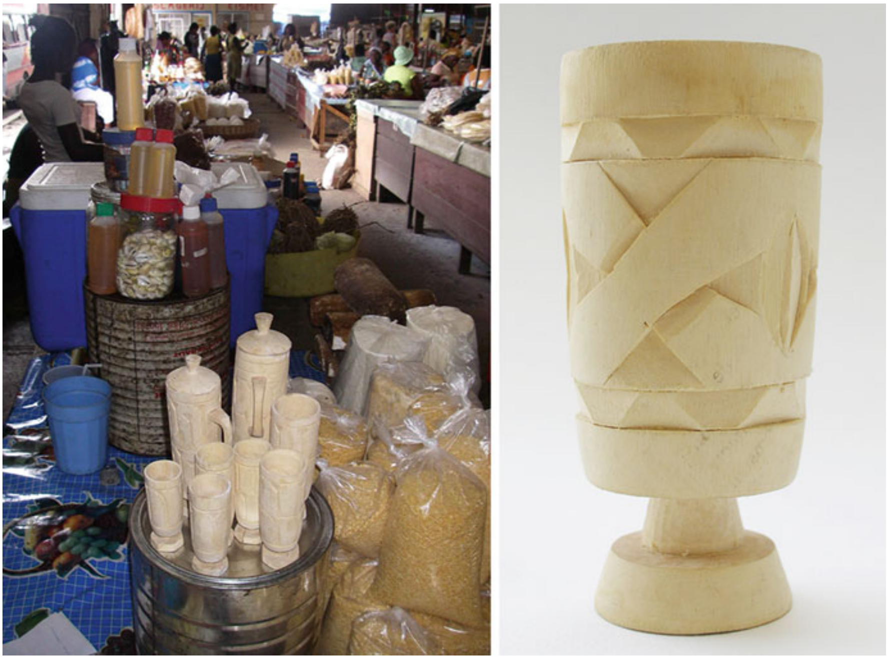


Fig. 125.6 *Left*: *Middags Markt* stall, Paramaribo, Suriname, with a choice of bitter-cups (Picture: G. Odonne) – *Righ*t: close-up view of a bitter-cup (Picture: E. Deharo)

These cups, called tonic- or bitter-cups, are still in use for their tonic properties in Suriname today (Fig. 125.6), where they are also known under the name "Kwasi bita beker" [29].

The bitter substances of *Quassia amara* were first named quassin by Thompson in the beginning of the nineteenth century [17 ,21] and were obtained in crystalline form by Winckler in 1835 [30]. It was more than a century later that a method for preparation and purification of quassin and neoquassin was described, highlighting that the crude extract was, in fact, a mixture of these two components [31, 32]. Studies on quassinoids from *Quassia amara* and *Picrasma excelsa* – mainly quassin, neoquassin, and isoquassin, the latter initially named picrasmine – then continued until the 1950s [33–36]. In particular, their structures were partially determined by Robertson et al. based on physical and chemical observations. The authors also synthesized norquassine, isolated thereafter and named simalikalactone B or picrasine B [37]. The complete structure of these molecules (quassin and neoquassin) and their stereochemistry, however, were only fully established in the early 1960s, when nuclear magnetic resonance techniques could be applied to them [38, 39]. This step marked the beginning of many studies leading to the isolation of quassinoids from natural sources.

Technical advances in structural analysis also initiated subsequent advances in synthesis of quassinoids, leading in particular to the first total synthesis of quassin

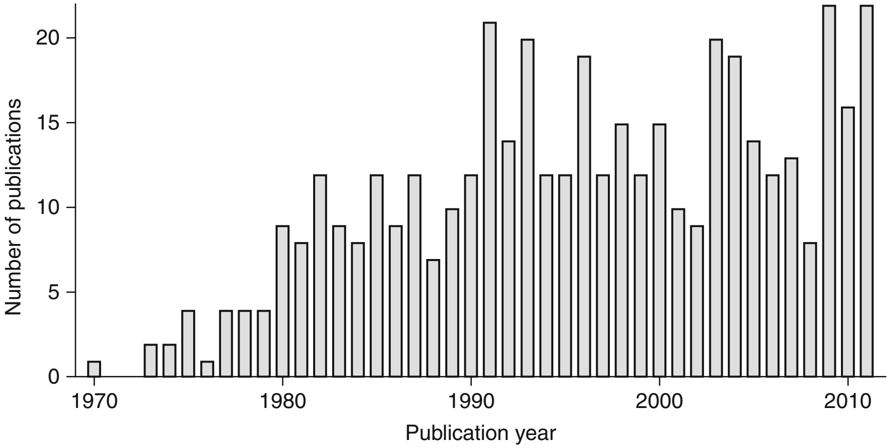


Fig. 125.7 Yearly number of publications related to quassinoids between 1970 and 2011 (Source Scopus + Science Direct)

by Grieco et al. in 1980 [40]. This milestone contribution to the study of quassinoids and the marked antileukemic activity of some of them – especially quassinoids with the C_{20} skeleton – initiated a considerable increase in publications related to quassinoids in the 1980s [41].

A literature search conducted for the terms "quassinoid" and "simaroubolide" (a term used in the 1970s to designate these molecules isolated from plants of the Simaroubaceae family) on the search engines Scopus and Science Direct provided a total of 453 references from 1970 to 2011; Fig. 125.7 illustrates the yearly profile.

This graph highlights the growing interest in quassinoids between the 1970s and 1980s. This trend could be explained simply by the increasing number of scientific publications at the time, but a study of the literature shows clearly that it is also correlated with the discovery of the anticancer properties of these molecules. Since 1990, the number of articles on this family of molecules has remained stable.

1.3 Pharmacological Activities: A Focus on Cancer and Malaria

The quassinoids are renowned for two major pharmacological activities: their anticancer and their antiplasmodial potential. Looking more closely at the evolution of publications on these two major classes of biological activities, we obtained the curves shown in Fig. 125.8. The linear regressions represented here demonstrate an early interest in their anticancer activities (1970–1985) and the increasing interest in their antimalarial potential (since 1990). Subsequent sections will therefore focus on these two pathologies.

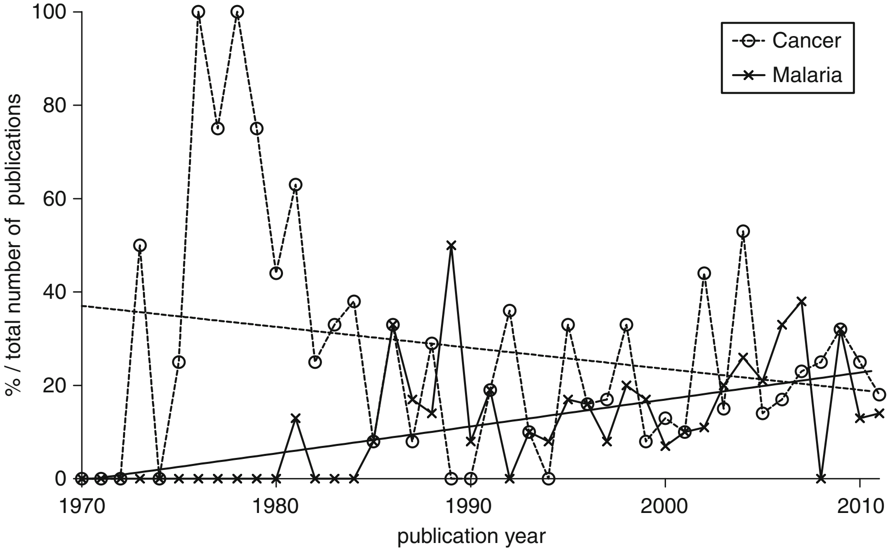


Fig. 125.8 Trends in numbers of publications on anticancer and antimalarial activities of quassinoids from 1970 to 2011

2 Pharmacological Applications

2.1 Historical Perspective and In Vitro Cytotoxic Activities

The decade that followed the discovery of the antileukemic activity of bruceantin is marked with a strong interest of the scientific community for the study of the anticancer properties of quassinoids. Bruceatin was isolated in 1973 by Kupchan from the stem bark of *Brucea antidysenterica* Mill. and identified as the active ingredient of this tree used against cancer in Ethiopia [41]. Following this discovery, many plants of the Simaroubaceae family were studied, and more than twenty molecules with high in vitro cytotoxic activity were isolated between 1973 and 1985, including dehydroailanthinone from *Pierreodendron kerstingii* (Engl.) Little [42], quassimarin extracted from *Quassia amara* [43], bruceoside A from *Brucea javanica* (L.) Merr. [44], samaderine E from *Samadera indica* Gaertn. (syn. *Quassia indica*) [45], and sergeolide from *Picrolemma pseudocoffea* Ducke (syn. *Picrolemna sprucei* Hook.f.) [7]. Quassinoids' in vitro anticancer activity has been broadly compiled in excellent reviews [46, 47]. Bruceantin was the first quassinoid introduced in clinical trials (see Sect. 2.2), but the unsatisfactory results obtained led to a decline in interest in research on anticancer activity of quassinoids. Since 2000, new results on bruceantin have suggested that the activity of this molecule towards certain types of cancer (leukemia, lymphoma, myeloma) deserves further investigation [48]. To date, the quassinoids therefore remain a family of molecules with potential in the context of the search for anticancer compounds.

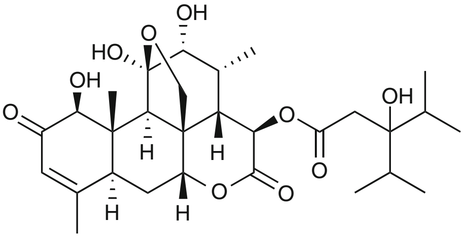


Fig. 125.9 NBT-272

2.2 In Vivo Anticancer Assays with Quassinoids, Clinical Trials

Quassinoids have been widely studied for their anticancer activity in vitro, however, only a few of them have shown interesting activity. Bruceantin was active in animal models against melanoma, colon cancer and leukemia [41, 48]. According to Cuendet et al. (2004), male mice seemed to be more sensitive to bruceantin than females, independent of the age of the treated mice. It was claimed that doses of 2.5 and 5.0 mg kg^{-1} promote the regression of earlier and advanced tumors (multiple myeloma, RPMI 8,226 cells) without apparent toxicity [49]. Phase I and II clinical trials were conducted with this compound. Unfortunately, no objective regression of the proliferative process was observed in humans, whereas a relative toxicity was noticed (hypotension, nausea, and vomiting at low dose, thrombocytopenia at higher dose) [50–53].

NBT-272, a semisynthetic analogue of bruceantin (Fig. 125.9), was found to be two to tenfold more potent than the original compound in inhibiting the cellular proliferation of a variety of cancer cell lines [54]. It also prevented tumor progression in a xenograft model of neuroblastoma cells with coinciding reduction of MYC expression and ERK activation in treated tumors [55].

Peninsularinone extracted from *Castela peninsularis* Rose, has been shown to be very active against pancreatic adenocarcinoma at 4.3 mg kg^{-1} and against colon adenocarcinoma (3.2 mg kg^{-1}) in animal models. The most surprising observation was that a lethal dose of peninsularinone could be administered safely in previously treated animals with low non-toxic concentrations a few days before the injection of the lethal dose. Interestingly, this molecule could be synthesized from glaucarubolone (isolated from *Castela polyandra* Moran & Felger) [56], which also showed activity in this model. Chapparinone, a related compound, also showed potential clinical application according to National Cancer Institute standards in the treatment of colon adenocarcinoma due to its activity against C38 cells implanted in mice [56].

15-desacetylundulatone, isolated from *Hannoa klaineana* Pierre & Engl root bark, which has free hydroxyl functions at C-l, C-11, and C-12 and an ester chain at C-6, was active against P388 leukemia in mice at doses up to 100 mg kg^{-1}. Remarkably, when the carbonyl group in C-2 was reduced, the activity dropped dramatically [57].

Administration of simalikalactone E (SkE) to nude mice implanted with K562-luc human leukemia cells resulted in leukemia regression at 1 mg kg^{-1}

SkE [58]. New assays against leukemia should be conducted with quassinoids as SkE and/or bruceantin are strongly active against leukemia cell lines in which Ras/Raf/MEK/Erk and c-MYC are activated.

2.3 Historical Perspective and In Vitro Antiplasmodial Activities

The emergence of resistance of the human malaria parasite *Plasmodium falciparum* to all commercialized antimalarials is of great concern for mankind. The wide use of Simaroubaceae species against malaria in areas of endemism stimulated the study of quassinoids and derivatives against *Plasmodium species*. As early as 1930, a quassinoid glycoside isolated from the seeds of *Simaba cedron* Planch. demonstrated an interesting potential for the treatment of malaria via the parenteral route, but renal secondary effects at high doses were recorded [59]. In 1947, the pharmaceutical company Merck screened around six hundred plant extracts on bird malaria models in vivo to find new antimalarials [60]. From the Simaroubacea family many plants were found to have excellent activity against *Plasmodium gallinacaeum* in chickens: *Castela spinosa* Cronquist, *Castela tortuosa* Liebm., *Castela tweediei* Planch., *Mannia africana* Hook. f. (syn. *Pierreodendron africanum* (Hook.f.) Little), *Picrolemma sprucei*, *Simaba cedron, Simaba cuneata* A.St.-Hil. & Tul., *Simaba insignis* A.St.-Hil. & Tul., *Simarouba amara*, *Simarouba berteroana* Krug & Urb., *Simarouba glauca* DC., *Simarouba tulae* Urb. Unfortunately, at the end of the World War II, the company stopped the project and no further study was conducted on these plants.

The investigation of the antimalarial properties of quassinoids then restarted at a significant rate in the mid-1980s and has remained relatively constant since then. Review articles on the antimalarial activity of quassinoids were published by Muhammad and Samoylenko in 2007 [59] and Guo et al. in 2005 [47].

The discovery of antiparasitic activities of quassinoids (see Sect. 4) prompted some authors to study the antimalarial potential of these molecules [61]. Among the quassinoids highlighted as potential antimalarials by Trager and Polonsky were simalikalactone D, identified as the most active molecule with complete inhibition of parasite growth at a dose of 2 μg ml^{-1}, as well as glaucarubinone and soularubinone. Other compounds, such as sergeolide [62] and bruceantin [63], also showed significant antiplasmodial activity.

Studies based on traditional use of Simaroubaceae continued in the 1980s, especially by Phillipson et al. [63–67]. Many articles on this subject followed, with, for example, the reisolation of simalikalactone D and the isolation and characterization of gutolactone from the bark of *Simaba guianensis* Aubl., a species used by people in the Amazon Basin [68]. Also, cedronin was isolated from the bark of *Simaba cedron*, a species used in Central and South America for the treatment of malaria [69], and samaderines B, E, X, and Z were isolated from *Quassia indica* branches used in the Indonesian traditional pharmacopeia [70]. In our work, it was chosen to test the traditional remedies as prepared by local people. This approach highlighted the remarkable activity of a decoction made from the leaves of *Quassia amara* [71, 72]. Later, it was shown that the antimalarial activity of this remedy could originate from the presence of two quassinoids: simalikalactone D (SkD) isolated from an optimized young leaf tea and simalikalactone E (SkE) isolated from a mature leaf decoction [73–76]. SkD was shown to be responsible for both the antimalarial activity and the cytotoxicity of the young leaf preparation. Overall, these studies take all their importance within the framework of the World Health Organization's recommendations on the evaluation of traditional medicines.

2.4 In Vivo Antimalarial Activity of Quassinoids in Murine Models

We will focus herein on compounds harboring antimalarial activity in vivo in a mouse model. Because that model requires more facilities than culture and larger amount of compound, only a few quassinoids have been studied against murine malaria. Among them, less than 20 quassinoids showed interesting activity. They were isolated from six species (*Ailanthus altissima*, *Brucea javanica*, *Hannoa chlorantha* Engl.& Gilg. (syn. *Quassia undulata* (Guill. & Perr.) D.Dietr.), *Picrolemma pseudocoffea*, *Quassia amara*, *Simaba cedron*) or were semisynthesized from quassinoids extracted from *B. javanica* (Table 125.1).

They can be separated into the following two classes derived from a core C_{20} carbon skeleton as suggested by Muhammad and Samoylenko [59]:

- Class A: quassinoids with the C-8(13)-oxymethylene bridge in the C ring isolated from *Brucea*, *Quassia*, *Simaba*, *P. pseudocoffea*, and semisynthetic bruceolide derivatives.
- Class B: quassinoids with the C-8(11)-oxymethylene bridge reported from *Ailanthus* and *Hannoa*.

The authors claimed that the quassinoids with a C-8(13)-oxymethylene group were five to tenfold more potent than C-8(11)-oxymethylene analogs in vitro. In vivo, this scheme does not appear so clear.

In class B derivatives, the activity seems to be influenced by the presence or absence of a hydroxyl group or a side chain at the C-15 position. Hence, 15-hydroxy-ailanthone isolated from *A. altissima* is very active (ED_{50} 0.76 mg kg^{-1} day^{-1} when administered orally); while chaparrine, which lacks function in the C-15 position, is inactive [66]. Interestingly, carbonyl group in C-2 is also important; when position 2 is occupied by a hydroxyl substituent (chaparrine) instead of a ketone (chaparinone) the activity disappears. The difference between in vivo activity of glaucarubin (with a C-2 hydroxyl group) inactive and glaucarubinone (=O) very active (ED_{50} 0.86 mg $kg^{-1}day^{-1}$ upon oral administration) is another example. It seems that the length of the C-15 substituent does not influence the antimalarial activity because ailanthone, ailanthinone, and glaucarubinone present the same antimalarial potential. Montjour et al. [77] showed that glaucarubinone was ineffective against *P. berghei* when administrated once by oral route at 2.5 mg kg^{-1} and was effective by intraperitoneal route at 0.5 mg kg^{-1} day^{-1} for

Table 125.1 In vivo antimalarial activity of natural and semisynthetic quassinoids against murine *Plasmodium*

Origin	Molecule	*Plasmodium* species	Route	ED$_{50}$ mg kg^{-1} day^{-1}	Ref
Ailanthus altissima (Mill.) Swingle (stem)	Ailanthone	*P. berghei*	Oral	0.76	[66, 77]
	Ailanthinone			1.25	
	Glaucarubinone			0.86	
Brucea javanica (L.) Merr. (fruits)	Brucein A	*P. berghei*	Oral	3.36	[65]
	Brucein B			0.9	[79]
	Brucein C			Inactive	[80]
	Brucein D			2.79	
	Brusatol			1.27	
	Bruceolide			0.46	
				1.1	
Semisynthetic from bruceolide	3,15-Di-*O*-ethylcarbonyl	*P. berghei*	Intraperitoneal	0.49	[80]
	3,15-Di-*O*-isopropylcarbonyl			1.4	
	3,15-Di-*O*-methylcarbonyl			1.3	
	3,15-Di-*O*-acetyl			0.46	
	3,15-di-*O*-acetyl	*P. berghei*	Intraperitoneal	0.46	[79]
Hannoa chlorantha Engl. & Gilg. (syn. *Quassia undulata* (Guill. & Perr.) D.Dietr.) (seeds & roots)	Chaparrinone	*P. berghei*	Subcutaneous	<50	[78]
	14-Hydroxychaparrinone			<50	
	15-Desacetylundulatone			<50	
Picrolemma pseudocoffea Ducke (syn. *P. spruce* Hook. f.) (roots)	Sergeolide	*P. berghei*	Subcutaneous	0.2	[62]
Quassia amara L. (leaves)	Simalikalactone D	*P. yoelii*	Oral	3.7	[73, 76]
	Simalikalactone E	*P. vinckei*	Subcutaneous	0.5	
			Oral	1	
Simaba cedron Planch. (Stem bark)	Cedronin	*P. vinckei*	Intraperitoneal	1.8	[71]

12 days. The authors also observed toxicity at doses > 2.5 mg kg^{-1} (oral) and 0.5 mg kg^{-1} (intraperitoneal). According to François et al. [78] among the quassinoids isolated from *Hannoa chlorantha*, the most active one was 15-desacetylundulatone but the EC$_{50}$ was not calculated. Nevertheless, the administered doses (50 mg kg^{-1}) were high and close to the toxic range. Authors proposed to split the treatment in several lower doses, but further such experiments have not been described so far. Interestingly, 15-desacetylundulatone is functionalized in position C-6 as SkE, a very active class A compound (see below).

In class A, brucein derivatives are almost all equally potent; the presence of either a hydroxyl or a short acyloxy substituent in C-15 does not influence the antimalarial activity. Nevertheless, with a too long/bulky acyloxy group, the activity drops dramatically (brucein A vs brucein C) [65]. Semisynthetic derivatives of bruceolide [79] were found more potent than chloroquine, increasing by four times the life span of treated mice without noticeable signs of toxicity even at doses of 3 mg kg^{-1}. For example, ED_{50} for 3,15-*O*-diacetylbruceolide was 0.46 mg kg^{-1}, but this compound was unable to remove malaria parasites in the blood stream of infected mice completely [80]. Authors suggested that the 15-acetoxy substituent was critical to the in vivo antimalarial efficiency. Nevertheless, to the best of our knowledge, no other semisynthetic quassinoid derivative has been studied for antimalarial purposes.

SkE seems to be almost four times more active in vivo than SkD [73, 76], but the *Plasmodium* species used for the test were different: *P. vinckei* for SkE and *P. yoelii* for SkD, *P. vinckei* being usually almost ten times more sensitive to treatment than *P. yoelii* [81].

Sergeolide isolated from roots of *Picrolemma pseudocoffea* had an original structure with a butenolide function attached to the ring A. It had been shown to be active in vivo against *P. berghei* (ED_{50} 0.2 mg $kg^{-1}day^{-1}$), unfortunately with a poor therapeutic index (median lethal dose LD_{50} 1.8 mg kg^{-1}) [62].

Compared with the plethoric studies on antiplasmodial activity against *P. falciparum* in vitro, in vivo antimalarial assays dedicated to quassinoids are scarce and hardly comparable. Nevertheless, the excellent antimalarial activities highlighted in those studies should open the way to complementary studies of their pharmacokinetics and toxicity. Association with other antimalarials should also be investigated in order to define new therapeutic schemes and to lower the administered doses, increasing drugs' tolerability.

3 Mechanisms of Action

Anticancer activity is one of the most striking biological properties of quassinoids. It has been particularly well documented and is still a source of discoveries. Many mechanisms of action have been proposed, including inhibition of phosphoribosyl pyrophosphate aminotransferase of the de novo purine synthesis pathway [82], inhibition of aerobic respiration [83], mitochondrial membrane depolarization, activation of caspase-3 [84], and alteration of microtubules [85]. It is also generally well accepted that quassinoids restrain protein synthesis, inhibiting the polypeptide chain elongation that prevents the first round of peptide bond establishment prior to polysome formation [86–88], this inhibition being reversible [87]. In particular, bruceantin binds to the peptidyl transferase center on ribosomes [89]. Brusatol has been claimed to decrease c-MYC oncoprotein expression at the post-transcriptional level [90] and to up-regulate mRNA levels of transcription factor NF-kB [91] and phosphorylate NF-kB inhibitors promoting NK-kB translocation into the nucleous. On the contrary, brucein D has been shown to inhibit NF-kB, increasing the protein level of IkB-alpha, known to sequester NF-kB in the cytoplasm and prevent its nuclear translocation. It has also been shown to generate oxidative stress depleting GSH and to activate p38-MAPK pathway. Some authors suggested that discrepancies between these related molecules are due to different methodologies [46].

Quassinoids also impair the protein synthesis mediated by the translation initiation factor 4E [92]. A liphophilic extract of *Nothospondias staudtii* Engl. inhibited Activator Protein-1 (AP-1), a transcription factor found in cellular nucleus, known to promote tumoral progression under certain conditions [93]. A quassinoid analogue, NBT-272, was reported to induce down-regulation of c-MYC in medulloblastoma-derived cells [54]. Castelletti et al. [55] showed that this molecule interferes with AKT and MEK/extracellular signal-regulated kinase pathways. The authors suggested that the depleting effect of NBT-272 on MYC protein expression occurred via indirect mechanisms, rather than selective inhibition. Crude extract and fractions of *Eurycoma longifolia* have been shown to induce apoptosis via a caspase-9-independent pathway in MCF-7 breast cancer cells [94]. This finding was corroborated by Zakaria et al. [95], who showed that an eurycomanone-enriched fraction was able to induce apoptosis via the p53 pathway in HepG2 liver cancer cells. More recently, Wong et al. [96] found that eurycomanone reduced the abundance expression of the following lung cancer cells markers: heterogeneous nuclear ribonucleoprotein A2/B1, p53 tumor suppressor protein and other cancer-associated genes including prohibitin, annexin 1, and endoplasmic reticulum protein 28 but not the housekeeping genes. Another bruceantin analog has been shown to inhibit the phosphorylation of upstream elements of HIF-1alpha (ERK1/2, MNK1 and eIF4E), a clue mediator of cellular responses to low oxygen, over-expressed in certain cancers [97]. It has been suggested that its effect was the consequence of the inhibition of this dependent MAPK cascade (HIF-1 alpha upstream elements) and/or the inhibition of the phosphorylation of eIF4E, which inhibits HIF-1 alpha translation.

In the case of *Plasmodium*, the main accepted mechanism of action is that quassinoids target plasmodial protein synthesis [98]. Arnot and Gull [99] showed that throughout *P. falciparum* growth inside the red blood cell, protein synthesis increases rapidly while the DNA synthesis peaks between the 20 and the 38 h of its blood cycle. Afterwards, DNA, RNA, and protein synthesis decrease dramatically at the schizont stage announcing the end point of the cycle. Interestingly, when SkE was pulsed every 6 h in a *Plasmodium falciparum* synchronous culture, it was found more effective at stages when DNA synthesis occurs [76]. This finding was corroborated by Bertani et al. [100], who showed that when SkD was pulsed every 4 h, the half-inhibitory concentration (IC_{50}) dropped to 10 nM at the 30 h, when the production of plasmodial DNA is maximal and rate of protein synthesis still elevated. SkD targets a very particular moment of *Plasmodium* growth. It is almost inactive on young and old stages while it is strongly active on mature cells at the DNA replication stage. Mata-Greenwood et al. [101] showed in 2001 that some quassinoids are able to inhibit DNA synthesis with greater efficacy when these molecules possess a C-15 ester side chain; that is the case for SkD.

Bertani et al. [100] also showed that SkD was inactive against heme biomineralization process and did not affect permeability pathways induced by a parasite in the host erythrocyte membrane. They also showed that SkD enhanced the activity of atovaquone on *Plasmodium* mitochondrial membrane potential, while it had an additive effect when combined with other commercial antimalarials.

4 Other Biological Activities and Ethnopharmacological Relevance

An extensive search for antimalarial compounds among the quassinoids was inspired by the discovery in the 1950s of the antiparasitic properties of these compounds, especially towards *Entamoeba histolytica* [61]. Glaucarubine's amoebicide property, isolated from *Simarouba glauca*, had been discovered a few years earlier [102]. Many antiparasitic activities would later be identified for extracts and molecules from the Simaroubaceae: amoebicidal (bruceantin [103], *Castela texana* (Torr. & A. Gray) Rose (syn. *Castela tortuosa*) [104]), nematicide (chaparrinone, klaineanone, and glaucarubolone [105], samaderins B and E [106]), antibabesial (*Brucea javanica* extract, and brusatol, bruceantin, brucein, abruceantinol, dehydrobrusatol, and dehydrobrucein A isolated from this species [107, 108]).

Many other activities were also recorded for quassinoids: insecticidal, antiviral, anti-inflammatory, and so on. These were extensively reviewed by Guo et al. and Almeida et al. [47, 109], thus justifying the interest in drawing parallels between the biological activities identified in vitro or in vivo for these molecules and the traditional uses of species from which they come.

Species belonging to the Simaroubaceae family are widely referenced in the pharmacopoeias of different medical systems around the world, whether they be of contemporary or ancient use, transmitted orally, or from written sources. For example, *Ailanthus altissima*, the area of natural distribution of which extends from Manchuria to Malaysia, is one of the most cited species in ancient Chinese medical treatise for a wide range of indications. One of the oldest recipes for this species has been recorded in a book in China dating back to 732 AD for the treatment of mental illness [110]. Its bark is still registered in actual Chinese and Asian pharmacopoeias, and it is traded across China. The same applies to *Brucea javanica* fruits in Southeast Asia and to *Ailanthus excelsa* Roxb. bark, mentioned in ancient and contemporary ayurvedic medicine books [111]. The use of *Brucea antidysenterica* bark has also been documented since the sixteenth century in ancient Arabic medical pharmacopoeias [112].

Moreover, some species have been introduced, naturalized, cultivated, and marketed outside their original distribution area with respect to their therapeutic value. This is the case for *Simarouba amara*, introduced in India; *Ailanthus altissima*, introduced as an ornamental and medicinal plant in North America, then in Europe; *Brucea javanica*, now cultivated in Africa; and *Quassia amara*, a few specimens of which were introduced in Africa and India. In their country of origin, *Ailanthus altissima*, *Ailanthus excelsa*, *Brucea javanica*, *Castela tortuosa*, *Eurycoma longifolia*, *Picrasma excelsa*, *Quassia amara*, *Quassia indica*, *Simaba cedron*, and *Simarouba amara*, among others, are still prepared under more or less advanced galenic forms and some are even for sale on the Internet.

Within the 102 Simaroubaceae species, an exhaustive bibliographic investigation (Table 125.2) highlights about twenty of most used species in traditional medicine. The most cited genera worldwide are *Ailanthus*, *Brucea*, *Castela*, *Eurycoma*, *Hannoa*, *Picrasma*, *Picrolemma*, *Quassia*, *Simaba*, and *Simarouba*. Despite the multiplicity of species, their geographic distribution range, and therefore the extreme cultural diversity that underlies their use, there is a marked homogeneity in the uses made of these species. Much of the uses of Simaroubaceae are first centered on the gastrointestinal sphere and the corresponding organs (stomach, liver, small intestine, colon). *Ailanthus triphysa* (Dennst.) Alston, *Brucea antidysenterica*, *Eurycoma longifolia*, *Picrasma excelsa*, *Quassia amara*, and *Simarouba amara* are recommended for their invigorating "tonic" and stimulating effect and used to increase appetite, in cases of dyspepsia, and for indigestion. *Ailanthus altissima*, *A. excelsa*, *Hannoa klaineana*, *Picrasma crenata* Engl. in Engl. & Prantl., *Picrasma quassioides* (D. Don) Benn., *Quassia africana*, *Q. gabonensis* Pierre, *Simaba cedron*, and *Simarouba amara* are prescribed as intestinal antispasmodics and in cases of acute colic with or without diarrhea.

Simple diarrhea, without blood, can be treated with *Ailanthus altissima*, *A. excelsa*, *Brucea antidysenterica*, *B. javanica*, *Castela coccinea* Griseb., *Eurycoma longifolia*, *Quassia amara*, *Q. undulata*, and *Simarouba amara*; bloody dysenterical diarrhea is treated by the administration of *Ailanthus altissima*, *A. excelsa*, *A. triphysa* (Dennst.) Alston., *Castela coccinea*, *C. erecta* Turpin, *C. tortuosa*, *Eurycoma longifolia*, *Picrasma excelsa*, and *Simaba cedron*. The most widely used species, the effectiveness of which has been demonstrated for this indication, is *Simarouba amara* (bark and seeds), together with the fruits of *Brucea antidysenterica* and *Brucea javanica*. Stomach pains are treated with *Brucea antidysenterica*, *Castela tortuosa* (syn. *Castela texana*), *Picrasma excelsa*, *Picrolemma sprucei*, *Quassia amara*, *Q. gabonensis*, *Q. indica*, *Q. undulata*. The seed kernels of *Simaba cedron* have an effect on hepatic colic and liver tropism. This is justified in the context of traditional medicine by their bitterness. Indeed, most Simaroubaceae are extremely bitter [72], as it is the case for *Brucea antidysenterica*, *Castela tortuosa*, *Eurycoma longifolia*, and *Quassia amara*, used in cases of jaundice and other chronic liver diseases. Antidiabetic and/or lipolytic remedies *Castela tortuosa*, *Picrasma crenata*, *Picrolemma sprucei*, *Quassia amara*, and *Simaba orinocensis* Kunth. may also target liver.

Ailanthus altissima, *Brucea antidysenterica*, *Picrasma excelsa*, *Quassia africana*, *Quassia undulata*, *Simaba cedron*, and *Simarouba amara* are also used as anthelmintics.

Simaroubaceae are broadly used against dermatological conditions from bacterial origin (furuncles, superinfected wounds, erysipelas, acne, abscesses), parasitic origin (leishmaniasis), related to the presence of dermatophytes (ringworm), to those described as warts or "cancerous tumors of the skin," tropical ulcers, and

Table 125.2 Medicinal uses of Simaroubaceae species (Part of plant used: *AP* aereal part, *Bd* bud, *Ft* fruit, *L* leaf, *Bk* bark, *RBk* root bark, *R* root, *Res* resin, *S* seed, *SBk* Stem bark, *St* stem, *Wd* wood)

Species and main geographic distribution of registered uses	Uses (part of plant used)
Ailanthus altissima (Mill.) Swingle (China, South east Asia)	Anthelmintic (Bk), cutaneous parasitic ulcers (L, Bk, Rbk), kala azar (Bk), intestinal hemorrhage (Bk, RBk), spermathorrea (Bk) [113]; Cardiovascular disease (L), asthma (Bk, R), epilepsy (L), antispasmodic (gastro-intestinal) (L) [114]; Dysentery (Bk, L, RBk) [113–115]; Diarrhea (Bk, RBk) [113, 115]; Leucorrhea (Bk) [115, 116];
	Menstrual disorders (Bk, RBk) [113, 116];
	Uterine hemorrhage (Bk, RBk) [115]
Ailanthus excelsa Roxb. (India)	Asthma (SBk), antispasmodic (gastro-intestinal) (SBk) [117];
	Affection of the mouth (L), cough (L), leucorrhea (L), skin ulcers (BK, L), uterine hemorrhage (L) [116];
	Contraceptive effect (L, SBk) [118];
	Diarrhea (Bk, L), dysentery (BK, L), menstrual disorders (Bk, L) [111, 116]
Ailanthus integrifolia subsp. calcynia (Pierre) Noot. (Nepal, North India)	Furunculosis (Res) [119]
Ailanthus triphysa (Dennst.) Alston (South East Asia)	Dysentery, dyspepsia, bronchitis (Bk, Res) [113]
Brucea antidysenterica J.F.Mill.	Asthmatic disorders (L, R), digestive (Bk, Ft, L, R, S), cancerous tumor of the skin (L, S) [120];
Southern and Eastern Africa, Tropical Africa	Diarrhea (Bk, Ft, L, R, S) [112, 114, 120];
	Dysentery (Bk, Ft, R) [114, 120, 121];
	Fever (Bk, Ft, R) [114, 120];
	Hepatitis (AP, Ft) [121, 122];
	Leprosy (L) [112];
	Ringworm (L) [122];
	Skin disease (L, St), wounds (L, St) [112, 120];
	Snake bite (Ft), teeth problem (Ft) [121];
	Stomachic (Bk, Ft, L, R, S) [120, 122]
Brucea javanica (L.) Merr.	Animal bites (L), diarrhea (Ft), furunculosis (L), dysentery (Bk, Ft, RBk), intestinal hemorrhage (Bk, Rbk), piles (Ft), poison (antidote) (R) [113] ;
China, South East Asia	
	Malaria (Ft, L) [113, 123, 124];
	Ringworm (L) [113, 125];
	Skin corn (Ft), wart (Ft), wounds, skin ulcers (Ft) [124]
Castela coccinea Griseb.	Dysentery, diarrhea (Fr, L) [126]
Bolivia	
Castela emoryi (A.Gray) Moran & Felger	Skin disease (Bd) [127]
Castela erecta Turpin	Skin disease, dysentery, fever [128]
Central America	

(*continued*)

Table 125.2 (continued)

Species and main geographic distribution of registered uses	Uses (part of plant used)
Castela tortuosa Liebm.	Diabetes (Bk, L, St) [129, 130];
Mexico	Dysentery, fever (AP), stomachic (AP) [129];
	Liver problem (L, St) [130]
Eurycoma longifolia Jack	Aphrodisiac (R) [131];
South East Asia	Diarrhea, (Rbk, R), dropsy (R), dysentery (Fr), fever (RBk, R), hepatitis (R, Rbk), indigestion (Bk), antidote (poison) (R), skin disease (L) [113];
	Malaria (R, Rbk) [113, 123]
Hannoa klaineana Pierre et Engl.	Antispasmodic (gastro-intestinal) (R) [132];
Tropical Africa	Cough (Bk) [133]
	Intestinal disease, malaria (R) [134]
Hannoa undulata	Cf. uses under *Q. undulata*.
Picrasma crenata Engl. in Engl. & Prantl	Antidote (poison), malaria (Bk), wound (L) [113]
Tropical America	Anthelmintic, cardiovascular disease, diabetes, digestive, dysentery, fever, intestinal disease, malaria, tonic (Wd), syphilis (St) [20]
Picrasma excelsa (Sw.) Planch.	Alcohol weaning (Wd) [27];
Caribbean islands	Anthelmintic, digestive, dysentery, fever, malaria, stomachic, tonic (Wd) [20]
Picrasma javanica Blume	Antidote (poison), malaria (Bk), wound (L) [113]
South East Asia	
Picrasma quassioides (D. Don) Benn.	Skin disease (L) [135];
Nepal, South East Asia	Fever, stomachic (Bk) [113]
Picrolemma sprucei (Hook.) f.	Diabetes, malaria (L, St) [136];
Tropical South America	Stomach pain [137]
Quassia africana (Baill.) Baill.	Anthelmintic (Bk, L), wounds, skin ulcers (RBk), menstrual disorders (L) [132];
Tropical Africa	Intestinal disease, antispasmodic (gastro-intestinal) (Bk, L), sexually transmitted disease (Bk), bronchitis (R), fever (Rbk) [132, 133];
	Malaria (L, R) [138]
Quassia amara L.	Anthelmintic, cholagogue (StBk), snake bite (R) [136];
Tropical America	Diabetes (L, StBk, Wd), stomach pain (L, Wd) [20][a];
	Diarrhea (Wd) [20];
	Liver, tonic (StBk, Wd) [20, 136];
	Malaria (L) [71]

[a]Bourdy G. 2003, unpublished data from French Guiana

other skin diseases (psoriasis, eczema, pruritus, rash), together with affections of the oral mucosa. Seventeen species are applied locally to treat these conditions: *Ailanthus altissima*, *A. excelsa*, *A. integrifolia* subsp. *calcynia* (Pierre) Noot., *Brucea antidysenterica*, *B. javanica*, *Castela emoryi* (A.Gray) Moran & Felger, *C. erecta*, *Eurycoma longifolia*, *Picrasma javanica* Blume, *P. quassioides*, *Quassia africana*, *Q. amara*, *Q. gabonensis*, *Q. indica*, *Q. undulata*, *Simaba cedron*, and S*imarouba amara*.

The Simaroubaceae find their field of election in the treatment of malarial fevers, which makes these species a major therapeutic recourse in places where the disease is endemic. The species most used against malaria worldwide are *Brucea javanica*, *Eurycoma longifolia*, *Hannoa klaineana*, *Picrasma excelsa*, *Picrasma javanica*, *Picrolemma sprucei*, *Quassia africana*, *Q. amara*, *Q. indica*, *Simaba cedron*, and *Simarouba amara*. As a corollary to the antibacterial and antiparasitic activities of quassinoids, the Simaroubaceae *Brucea antidysenterica*, *Castela erecta*, *C. tortuosa*, *Eurycoma longifolia*, *Picrasma excelsa*, *P. quassioides*, *Quassia africana*, *Q. amara*, *Q. indica*, *Q. undulata*, *Simaba cedron*, and *Simarouba amara* in particular are widely used against febrile illnesses of various etiologies.

Another major indication of the Simaroubaceae also focuses on the respiratory system, and *Ailanthus altissima*, *A. excelsa*, *A. triphysa*, *Brucea antidysenterica*, *Hannoa klaineana*, *Quassia africana*, *Q. gabonensis*, and *Q. undulata* are prescribed in case of serious pulmonary infections (bronchitis, asthma, cough, emphysema, bronchopneumonia).

Some Simaroubaceae also display therapeutic actions on the male or female reproductive system: *Simaba cedron* and *Simarouba amara* are used during childbirth and in the postpartum period; *Ailanthus altissima* and *Simarouba amara* are known to stop uterine bleeding; *Ailanthus excelsa* is contraceptive; and *A. altissima*, *A. excelsa, Quassia indica*, *Q. africana*, *Picrasma crenata*, and *Simarouba amara* regulate menstruation and are sometimes used in cases of vaginal leucorrhea, some of which may be due to the presence of vaginal parasites. For the male reproductive tract, *Ailanthus altissima* cures spermatorrhea; *Eurycoma longifolia* and *Quassia undulata* are reportedly aphrodisiacs; and *Picrasma crenata* and *Quassia africana* are used against syphilitic venereal diseases.

Lastly, three Simaroubaceae species are described as antivenom (*Brucea antidysenterica*, *Quassia amara*, and *Simaba cedron*, with a very strong convergence of use for the cotyledons of *Simaba cedron* seeds in South America). *Brucea antidysenterica*, *Brucea javanica*, *Eurycoma longifolia*, *Quassia undulata*, and *Picrasma javanica* are known as antidotes to food poisoning or other types poisoning. Central nervous system troubles, such as dementia, nervousness, and epilepsy, and alcohol addiction are treated with preparations of *Ailanthus altissima*, *Quassia undulata*, *Simarouba amara*, or *Picrasma excelsa*.

Used for centuries to treat frequent, sometimes life-threatening, medical tropical conditions (malaria, amoebic dysentery, infectious diarrhea in young children), the Simaroubaceae have demonstrated a real therapeutic interest with remarkable convergence of practice undoubtedly linked to the strong antiparasitic, antibacterial, and antiproliferative activities of quassinoids. Moreover, functional activities justifying organ tropisms also contribute to the effectiveness of Simaroubaceae medicinal plants, making them an invaluable resource and justifying their inscription in national pharmacopoeias.

5 Conclusion

Quassinoids are a family of molecules with a broad range of remarkable pharmacological activities (anticancer, antimalarial, ameobicide, nematicidal, anti-inflammatory, antiviral, and so on), the most of which is being made through the still active use of the Simaroubaceae in traditional medicines. Quassinoids' pharmacological potential has been largely demonstrated in the laboratory both in vitro and in vivo. Nevertheless, no drug has been developed so far from these substances. With respect to their anticancer activity, these compounds have not been up to expectations against cancers for which they were tested (breast cancer and melanoma). They are likely to be more efficient against hematological cancers but this remains to be demonstrated. Also, the antimalarial activity seems to be associated with some toxicity, and these compounds have never been placed into clinical trials for their antimalarial potential. However, the results described in the literature speak in favor of the search for new active and less toxic quassinoids and the parallel development of further structure-activity relationship studies aiming at improving the antimalarial selectivity of these natural products. The discovery of synergistic interactions between quassinoids and other clinical antimalarial drugs can also contribute to circumvent the obstacle of their toxicity. Finally, considering the structural complexity of these highly oxygenated molecules containing multiple stereocenters, quassinoids' total synthesis also remains an interesting challenge.

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Glycyrrhizic Acid: Biological Effects on Glucose and Lipid Metabolism 126

So Ha Ton, Chanchal Chandramouli, and Khalid BAK

Contents

1 Introduction 3805
1.1 Glycyrrhizic Acid: A Natural Product from Licorice Roots 3805
2 Pharmacokinetics of GA 3806
3 Biological Effects of GA 3808
3.1 11β-Hydroxysteroid Dehydrogenase (11β-HSD) 3808
3.2 Lipoprotein Lipase (LPL) 3808
3.3 Peroxisome Proliferator-Activated Receptor Class of Nuclear Receptors (PPAR) 3809
3.4 Effects of GA on the Aforementioned Parameters 3809
4 GA on Blood Pressure 3810
5 GA on Liver and Muscle Enzymes 3810
6 GA on Glucose Metabolism 3810
7 GA on Lipid Metabolism 3811
8 Reported Side Effects 3812
9 Discussion 3812
9.1 High-Sucrose Diet and Glucose and Lipid Metabolism 3812
9.2 High-Fat Diet and Glucose and Lipid Metabolism 3817
10 Conclusion 3820
References 3821

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Abstract

Glycyrrhizic acid (GA), an active compound extracted from *Glycyrrhiza glabra*, has been demonstrated to specifically lower blood glucose and improve insulin sensitivity and lipid profile in normal lean rats and rats on high-sucrose or high-fat diet. Blood pressure was not affected by GA during the duration of the experiment. Aspartate aminotransferase, alanine aminotransferase, and γ-glutamyltransferase in the liver and creatine kinase in muscles were normal as well in lean rats given GA. 11β-Hydroxysteroid dehydrogenase activities were lower in the kidneys, liver, adipose tissues, and muscles in treated rats, while glucose-6-phosphatase activities and phosphoenolpyruvate carboxykinase activities in the liver and kidneys were reduced ($p < 0.05$) but induced in the visceral and subcutaneous tissues. Upregulation of lipoprotein lipase was seen in both the high-fat-fed and high-sucrose-fed rats, while peroxisome proliferator-activated receptor γ was also upregulated in high-fat-fed rats.

In conclusion, this work showed that GA could improve hyperglycemia and hyperlipidemia in lean, high-fat-fed, and high-sucrose-fed rats.

Keywords

Glycyrrhizic acid • Metabolic syndrome • Dyslipidemia • Lipoprotein lipase • Lipid profile • Gluconeogenic enzymes

Abbreviations

11β-HSD	11β-Hydroxysteroid dehydrogenase
ALT/AST	Alanine aminotransferase/aspartate aminotransferase
AM	Abdominal muscle
CK	Creatine kinase
FFA	Free fatty acid
G6Pase	Glucose-6-phosphatase
GA	Glycyrrhizic acid
GC	Glucocorticoids
H	Heart
H6PDH	Hexose-6-phosphate dehydrogenase
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
K	Kidney
L	Liver
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
Met S	Metabolic syndrome
PEPCK	Phosphoenolpyruvate carboxykinase
PPAR	Peroxisome proliferator-activated receptor
QF	Quadriceps femoris
SAT	Subcutaneous adipose tissue

T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
VAT	Visceral adipose tissue
VLDL	Very-low-density lipoprotein
γGT	γ-Glutamyltransferase

1 Introduction

1.1 Glycyrrhizic Acid: A Natural Product from Licorice Roots

Plants are abundant sources of natural products with biologically active molecules that play an important role in pharmacology. Licorice (*Glycyrrhiza glabra*) is a tall shrub of the Leguminosae family. Commercial licorice products are often derived from extracts of the licorice root. Glycyrrhizic acid (GA) which is also known as glycyrrhizin or glycyrrhizinate is considered the primary active ingredient in licorice root extract and constitutes 10–25 % of the root extract [1]. It is also known as Gan Cao in Chinese (Fig. 126.1).

Licorice is mainly used as a condiment in food and confectionery industries. It is widely used as a sweetener in diet coke, candies, chewing gum, gelatin, pudding, cream, and alcoholic beverages [2]. The sweetness of GA can mask the bitter taste

Fig. 126.1 Root of licorice shrub

of medicines. Hence, GA in ammoniated form is also used in the production of cough syrups, linctus, and other medicines [3].

Therapeutic uses of licorice have been demonstrated for over 4,000 years in ancient Assyrian, Egyptian, Chinese, and Indian culture. A Greek botanist and pharmacologist, Theophrastus, used licorice to treat respiratory problems such as asthma and dry cough [4]. Apart from that, in China and India, it was used to treat pharyngitis, cough, palpitation, gastric pain, and gastric ulcer. GA was also prescribed as a folk medicine to diabetic patients [5] consistent with current findings that GA improves glucose tolerance in noninsulin-dependent diabetes model mice [6]. Additionally, GA also exhibits antineoplastic, anticarcinogenic, antidote, and antioxidant properties. Licorice was observed to cause apoptosis in melanoma and gastric cancer cells [4]. A registered Japanese medicine, Stronger Neo-Minophagen C (SNMC), is used to treat chronic viral hepatitis [7]. Other Asian nations use GA for treatment of allergic dermatitis and urticaria [8].

2 Pharmacokinetics of GA

GA is a saponin compound, which contains one molecule of triterpenoid aglycone (glycyrrhetic acid, GE) conjugated to two molecules of glucuronic acid (Fig. 126.2).

GE exists as 18α- or 18β-stereoisomeric forms [1]. However, GA has poor oral bioavailability in both rats and humans. Upon oral ingestion of GA into the gastrointestine, it undergoes presystemic deglucuronidation by specialized β-glucuronidase from intestinal flora (*Eubacterium* sp., *Ruminococcus* sp., and *Clostridium innocuum*) and is completely absorbed as GE [1]. GA is only detectable in rats and human plasma after high oral doses of 50–500 mg/kg and 100–1,600 mg/kg, respectively [1, 9].

GE binds extensively to both specific and nonspecific binding sites of human and rat plasma albumin. GE is distributed mainly into the liver and minimally to other body compartments such as heart, spleen, pancreas, kidney, muscle, adipose tissue, and small intestines in rats. Such distribution profile may be similar in human as well [3].

In the liver, a saturable capacity-limited carrier protein mediates rapid hepatic uptake of GE which subsequently undergoes hepatic biotransformation, producing glucuronyl and sulfate conjugates such as 18β-glycyrrhetyl-3-O-hydrogensulfate, 18β-glycyrrhetyl-3-O-monoglucuronide, or 18β-glycyrrhetyl-30-monoglucuronide [10]. GE may also undergo hepatic dehydrogenation to form 3-ketoglycyrrhetic acid.

These metabolites are later excreted into the bile via the canalicular multispecific organic anion transporter (cMOAT) and subsequently re-metabolized by the intestinal flora. This enterohepatic recirculation is responsible for the slow terminal plasma clearance of GE. However, the major elimination pathway for GA is in feces [1, 2]. The summary of the pharmacokinetics of GA is depicted in Fig. 126.2.

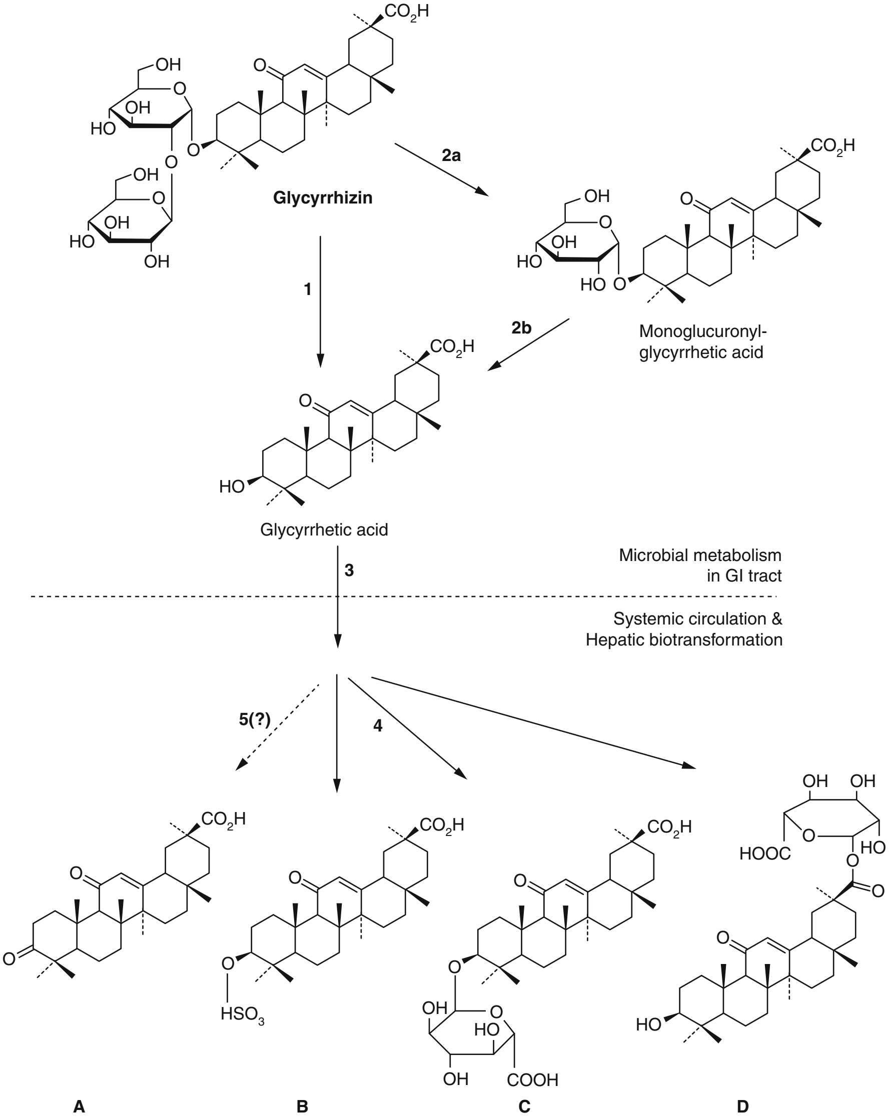
Glycyrrhizin
2a
Monoglucuronyl-glycyrrhetic acid
1
2b
Glycyrrhetic acid
3
Microbial metabolism in GI tract
Systemic circulation & Hepatic biotransformation
5(?)
4
A
B
C
D

Fig. 126.2 Metabolism of glycyrrhizic acid (*GA*); GA is converted to GE either completely (*1*) or through a two-step process (*2a*, *2b*). Glycyrrhetic acid (*GE*) is absorbed (*3*) and undergoes hepatic biotransformation (*4*) to produce 18β-glycyrrhetyl-3-O-hydrogen sulfate (*B*), 18β-glycyrrhetyl-3-O-monoglucuronide (*C*), or 18β-glycyrrhetyl-30-monoglucuronide (*D*). GE may also undergo hepatic dehydrogenation (*5*) to form 3-ketoglycyrrhetic acid (*A*) [1]

3 Biological Effects of GA

3.1 11β-Hydroxysteroid Dehydrogenase (11β-HSD)

Glycyrrhetinic acid (GE), which is deglucuronidated from GA by intestinal flora upon ingestion, has been found to inhibit 11β-HSD and was known to have modulation on the regeneration of glucocorticoids and confers ligand specificity to mineralocorticoids. It is therefore recognized to have antidiabetic properties in terms of its ability to inhibit the enzyme 11β-HSDs [11].

Substantial experimental evidence pointed out that both GA and GE have inhibitory effects on 11β-HSDs but GE is 200–1,000 times more potent than GA [1]. Both 11β-HSD1 and 11β-HSD2 are suppressed by GA and GE since they are nonselective inhibitors of this enzyme. However, GE exerts higher degree of inhibitory effect on 11β-HSD1, favoring the reaction direction that drives the conversion of active GCs into inactive GCs [12].

As elevated 11β-HSD activities and the resulting increased GC production have been reported to impair the glucose and lipid homeostasis and lead to the development of insulin resistance, inhibitory effect of GA could represent a potential therapeutic intervention in the treatment of type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS) [13–18].

11β-HSD is an enzyme complex that catalyzes the reversible conversion of active glucocorticoid (cortisol in human, corticosterone in rodents) to inactive glucocorticoids (cortisone in human, 11-dehydrocorticosterone in rodents) [2]. Clinical studies on obese individuals have demonstrated elevated levels of 11β-HSD1 mRNA, decreased levels of 11β-HSD2 mRNA, and increased GC levels in the adipose tissues [17]. Hyperinsulinemia and elevated FFA levels induce an acute increase in 11β-HSD activity in the adipose tissues of humans [19]. The association between elevated levels of 11β-HSD1 mRNA and metabolic abnormalities such as visceral obesity, IR, and hypertension has prompted the design of 11β-HSD inhibitors for the treatment of MetS [20].

3.2 Lipoprotein Lipase (LPL)

LPL is the major enzyme responsible for the hydrolysis of circulating TAG moiety of both classes of TAG-rich lipoproteins: the chylomicrons and VLDL, generating FFA, that are either oxidized in the muscles or reesterified in the adipose tissues, and glycerol that is returned to the liver. LPL plays a central role in overall lipoprotein metabolism, where (1) the successive interaction of VLDL with LPL generates the LDL that are involved in forward cholesterol transport and (2) the remnant lipoprotein particles so formed from LPL catalysis contributes to the maturation of HDL precursors, the latter of which is then involved in reverse cholesterol transport [21, 22]. Perturbation in LPL activity could therefore lead to significant metabolic consequences, and LPL has been implicated in pathophysiological conditions characterized by marked hypertriglyceridemia, such as that observed in the metabolic syndrome (MetS).

Stimulation of LPL activity by either the use of transgenic overexpression or the administration of LPL-raising drugs has been shown to ameliorate the observed dyslipidemia and hyperglycemia. Interests over GA studies rose further as triterpenoids had been discovered to act as PPAR agonists, and this may suggest that GA could also potentially act to activate the PPAR class of nuclear receptors and may therefore be proposed to be a candidate for raising LPL [23, 24].

3.3 Peroxisome Proliferator-Activated Receptor Class of Nuclear Receptors (PPAR)

In addition, GA has also been suggested to possibly transactivate nuclear receptor peroxisome proliferator-activated receptor (PPAR) class of nuclear receptors; triterpene compounds (such as 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and dehydrotrametenolic acid) have been found to bind to and transactivate PPARγ [25, 26]. This may suggest that GA, a triterpenoid compound, could also possibly transactivate (PPAR) class of nuclear receptors.

Studies of the past few years have identified the therapeutic utility of PPARγ agonists in T2DM and MetS treatment due to their ability to improve hyperglycemia and insulin resistance [27–29]. PPARγ and were also reported to be transcriptional factors that control several genes for the key enzymes involved in lipid and glucose metabolism, for example, LPL, PEPCK, and G6Pase. The gene promoters of these enzymes have also been found to contain PPARγ response element (PPRE) [30, 31]. Therefore, the potential ability of GA as a ligand for PPARγ transactivation may provide another additional molecular mechanism for the GA-induced improvement in the glucose and lipid metabolism other than the GA-induced 11β-HSD inhibition.

3.4 Effects of GA on the Aforementioned Parameters

The metabolic syndrome is a cluster of metabolic disorders characterized by hyperglycemia, dyslipidemia, obesity, hypertension, and insulin resistance. These are risk factors for the development of diabetes mellitus type 2 and cardiovascular diseases [32]. Dyslipidemia is a hallmark of the syndrome and is associated with a reduction in LPL activities, an enzyme involved in lipid metabolism. Functionally, GA inhibits 11β-HSD1 that catalyzes the activation of GC. GC plays a role in the regulation of PEPCK and G6Pase [33, 34]. Our earlier studies have indicated that GA could improve dyslipidemia and insulin sensitivity in lean rats fed on normal rat chow. As obesity is one of the risk factors in the development of diabetes mellitus type 2, we decided to investigate the effects of GA on PEPCK, H6PDH, and G6Pase; 11β-HSD and LPL involved in lipid and carbohydrate metabolism in lean rats; as well as rats on a high-fat or high-sucrose diet.

The following synopsis summarizes the observed enzyme activity involved in the lowering of glucose and improvement in insulin sensitivity and lipid profiles in GA-treated rats under various physiological conditions.

To determine the effects of GA on different tissues, the following tissues were examined, namely, liver (L), kidney (K), visceral and subcutaneous adipose tissues (VAT and SAT), quadriceps femoris (QF), and abdominal muscles (AM).

4 GA on Blood Pressure

No effect on blood pressure was seen between the lean rats given GA and control rats even for a period of three months. Rats on a high-fed diet showed increased blood pressure between the test groups and the control rats. However, no difference was noted between rats on high fat and given GA and rats on high-fat diet only, thus indicating that GA did not cause the rise in the observed blood pressure [35].

Similar observations were seen in rats on high-sucrose diets [36].

5 GA on Liver and Muscle Enzymes

Determination of liver enzymes in the lean rats given GA showed no increase in ALT, AST, and γ-GT. No increase was also observed in CK activity. This may indicate that 100 mg kg^{-1} of GA within the studied period did not affect the liver or muscle [37].

6 GA on Glucose Metabolism

Elevated intracellular GC level is one of the factors in the development of MetS. Studies in our laboratory on lean rats have revealed that administration of 100 $mgkg^{-1}$ of GA orally improved glucose metabolism in rats with effects on enzymes such as PEPCK and H6PDH which reduce blood glucose by increasing insulin sensitivity through a reduction in HOMA-IR [38, 39]. Improved insulin sensitivity HOMA-IR (<0.05) and blood glucose reduction ($p < 0.05$) were observed with the administration of GA. HSD1 and 2 activities decreased significantly in all tissues ($p < 0.05$) except in the AM and QF ($p > 0.05$) [38]. Gluconeogenic enzymes like PEPCK showed a reduction in the L ($p < 0.05$) and K ($p > 0.05$) but an increase in VAT ($p < 0.05$) and SAT ($p > 0.05$). However, H6PDH activities were significantly reduced only in the liver ($p < 0.05$).

Oral administration of 100 mg kg^{-1} of GA to high-fat- or high-sucrose-induced obese rats led to significant reduction in blood glucose concentration and improvement in insulin sensitivity as indicated by improvement in HOMA-IR ($p < 0.05$) [35, 36].

11β-HSD1 activities were significantly higher in SAT, VAT, AM, QF, and L for the high-fat-fed rats compared to the control. When treated with GA, the 11β-HSD1 activities in these tissues were lower compared to the high-fat-fed rats. GA inhibited 11β-HSD2 activities in AM, QF, and L in the GA-treated rats compared to the high-fat-fed rats. However, in the SAT, VAT, and K of the high-fat-fed GA-treated rats, no 11β-HSD2 activities inhibition was found [40].

In both the high-fat- and high-sucrose-fed rats, significant (1) decrease in H6PDH activities was seen in the aforementioned tissues ($p < 0.05$), (2) reduction in G6Pase activities in the L and K ($p < 0.05$), (3) decrease in PEPCK activities in the L and K ($p < 0.05$), and (4) increase in SAT and VAT ($p < 0.01$) was also observed in the GA-treated rats [36, 41].

In GA-treated high-sucrose-induced obese rats, significantly lower blood glucose, serum insulin, and HOMA-IR ($p < 0.05$) and improved lipid parameters ($p < 0.05$) with no elevation in blood pressure were seen. LPL activities were upregulated in all tissues ($p > 0.05$) with significant upregulation in the L ($p < 0.01$). 11β-HSD activities were lower in all tissues in GA-treated rats compared to those on high sucrose alone ($p < 0.05$). A smaller increase in H6PHD activities was observed in the L, SAT, AM, and QF ($p > 0.05$). PEPCK activities were elevated in the L and K ($p < 0.05$) but increased more in the SAT and VAT ($p < 0.01$), while increases were seen in the hepatic and renal G6Pase activities ($p < 0.05$) [36].

Histological analysis on all the studied tissues using hematoxylin and eosin as well as periodic Schiff stain indicated that GA-administered lean rats on normal diet had more intense magenta coloration compared to the control rats indicating increased deposition of polysaccharide, glycogen in the cells. Similar observations were seen in tissues from rats on high-sucrose or high-fat diet with GA [35, 36].

7 GA on Lipid Metabolism

In lean rats given GA, significant increase in LPL expression in the QF ($p < 0.05$) but nonsignificant increase in AM, K, L, heart, SAT, and VAT were seen ($p > 0.05$). Consistent improvement in serum lipid parameters was also observed with decrease in serum FFA, TAG, and total cholesterol and elevated LDL cholesterol ($p > 0.05$). Histological staining using Oil Red O showed significant decrease in lipid deposition in the AM and QF ($p < 0.05$) but nonsignificant decrease in the H, K, and L ($p > 0.05$) [42].

Upregulation of total PPARγ, PPARγ1, and PPARγ2 expressions in VAT, SAT, AM, QF, L, and K was seen in rats given GA ($p > 0.05$). Significant upregulation of total PPARγ ($p < 0.01$) was observed in the QF. Upregulation, albeit nonsignificantly, of total PPARγ, PPARγ1, and PPARγ2 expression in all the studied tissues with improved insulin sensitivity was seen [43, 44].

GA was found to be effective in improving dyslipidemia in rats on high-fat diet via increased tissue PPARγ and LPL expression, together with a positive shift in lipid profile and serum FFA as well as reduced tissue lipid accumulation [35, 45].

Also, GA given to high-fat-induced obese rats led to significant reduction in blood pressure and improvement in HOMA-IR ($p < 0.05$). LPL expression was upregulated in the K, H, QF, AM, VAT, and SAT but downregulated in the L – a condition seen in reverse to that seen in high-fat diet-induced obese rats without GA. Lipid profile showed significant hypotriglyceridemic and HDL-raising effects ($p < 0.05$) with a consistent reduction in serum FFA, total cholesterol and LDL cholesterol and significant decrease in lipid deposition across all tissues studied ($p < 0.01$).

In the high-sucrose-fed rats, consistent decrease in FFA, TAG, total cholesterol, and LDL cholesterol ($p > 0.05$) and an increase in HDL cholesterol ($p < 0.05$) were seen [36]. LPL expression was upregulated in the K, H, QF, AM, VAT, and SAT ($p > 0.05$) but downregulated in the L ($p > 0.05$) [36].

GA administration to high-fat-induced obese rats increased PPARγ2 expression significantly in the K, AM, QF, VAT, and SAT ($p < 0.05$) but insignificantly in the liver ($p > 0.05$). PPARγ2 expression was induced in the L, K, QF, and AM in both the high-fat-fed rats with and without GA with the VAT and SAT showing the highest expression in both groups [46].

Serum leptin levels were also significantly higher in rats fed on high-sucrose diet compared to the rats fed on high-sucrose diet with GA administration ($p < 0.01$) [43]. No significant difference was seen between the normal control and rats fed on high sucrose with GA.

Analysis on both the subcutaneous and visceral adipocytes indicated a decrease in the mean cell area in the rats given GA compared to the control rats with a larger reduction in the subcutaneous adipocytes compared to the visceral adipocytes [38].

8 Reported Side Effects

Chronic consumption of GA has been reported to be associated with corticosteroid-like effects and changes in cortisol metabolism in tissues which may lead to hypermineralocorticoid-like effects such as electrolyte imbalance, hypertension, and depression of the renin-angiotensin-aldosterone system [1, 2]. However, the effects are reversible upon withdrawal of GA [1].

Interest in GA arose following findings that have implicated the role of increased activation of GC receptors in the development of MetS symptoms such as central obesity and hyperlipidemia. Overexpression of 11β-HSD type 1 (11β-HSD1) in white adipose tissue of mice, for example, resulted in increased intracellular GC level, abdominal obesity, insulin resistance, hypertension, hyperglycemia, and dyslipidemia [47].

9 Discussion

9.1 High-Sucrose Diet and Glucose and Lipid Metabolism

It has been widely accepted that high-sucrose diets lead to undesirable metabolic abnormalities such as hypertension, hyperglycemia, insulin resistance, and dyslipidemia. Several studies have utilized animal models such as rabbits [48], mini-pigs [49], mice [50], and rats [51] fed on high-sucrose diet to examine mechanisms underlying diabetes-accelerated dyslipidemia and atherosclerosis. It has been established that overfeeding of animals with more than 50 % of calories from sucrose for approximately 4 weeks is sufficient to initiate moderate obesity and often results in dyslipidemia and T2DM [52]. In our studies, rats were fed a high-calorie diet with high (60 %) calories from sucrose obtained from cane sugar and low amount of calories from fat and protein for 4 weeks. These rats on high-sucrose diet consumed significantly more calories "per day" compared to the controls which were fed on standard rat chow.

The increased blood pressure observed in high-sucrose-fed subjects may be attributed toward the association between hyperinsulinemic state and sympathetic nervous system, accumulation of intracellular glyceraldehyde and dihydroxyacetone phosphate, and increased level of angiotensin-converting enzyme (ACE) [53]. GA administration has also been implicated with mineralocorticoid-like effects characterized by sodium retention, hypokalemia, hypertension, suppression of renin-angiotensin system, and low aldosterone level. This is due to nonselective nature of GA which inhibits both 11β-HSD1 and 11β-HSD2 [3]. However, in our studies, no elevation in the blood pressure in rats on high-fat diet with GA administration was seen. This suggests that the increase in blood pressure in rats from this group is due to the high-sucrose intake and therefore GA administered at 100 mg/kg for 28 days did not contribute toward this increase.

The positive shift in lipid parameters following GA treatment in sucrose-induced obese rats was similar to that previously reported by Lim et al. [42] in lean rats and Eu et al. [35] in high-fat-induced obese rats. The hypertriglyceridemia observed in patients with the MetS and T2DM originates from (1) lipolysis of TAG store from adipose tissue that causes elevated FFA flux to the liver and, hence, increased hepatic TAG synthesis and (2) inhibition of lipolysis of chylomicrons and VLDL due to decreased LPL levels [54]. Our present study indicated that GA treatment has effectively prevented such development through induction of LPL expression in all tissues which promotes catabolism of circulating TAG-rich lipoproteins. More importantly, GA induced a significant increase in HDL levels in the sucrose-induced obese rats. Esterification of free cholesterol is mediated by lecithin-cholesterol acyltransferase (LCAT) which is bound to HDL cholesterol. This key antiatherogenic mechanism facilitates removal of excess free cholesterol from the cells in the peripheral tissues and is returned to the liver and excreted in the bile [55]. Hence, various pharmacological interventions have been focused on raising HDL cholesterol levels [56]. Thus, the increase in HDL concentration could possibly be a more promising avenue to hinder lower coronary heart disease (CHD) instead of lowering LDL cholesterol [57].

Fructose, the monosaccharide component of sucrose, is highly lipogenic. Fructose provides large amount of hepatic triose-phosphate as precursors for fatty acid synthesis. It has indeed been observed in several studies that hepatic de novo synthesis is stimulated after acute fructose ingestion, with fructose contributing to the synthesis of both the glycerol- and the fatty-acyl parts of VLDL triglycerides [58]. Fructose may, in addition, increase the expression of key lipogenic enzymes in the liver [59].

Reduced LPL activities in the kidney, heart, and SAT were observed in high-sucrose-fed subjects. In agreement with our studies, LPL activities in muscle and adipose tissues were seen to be reduced with the onset of IR [60]. The absence of insulin-mediated suppression of lipolysis in adipocytes by hormone-sensitive lipase (HSL) promotes the release of fatty acids which in turn inhibit LPL activity [52]. Fatty acids, when in excess, are postulated to bind to LPL and displace it from its binding sites, thereby rendering them nonfunctional [53]. Therefore, the responses of both LPL and hormone-sensitive lipase (HSL) are blunted [61].

Fructose may also modulate intracellular lipid deposition known as "ectopic lipids," that is, deposition of TAG in the cytoplasm of nonadipose cells, such as hepatocytes and muscle fibers. In rodents, a high-sucrose diet rapidly increased intrahepatic fat deposition within 1 week and intramyocellular lipids on prolonged feeding [62]. This effect of fructose may involve stimulation of de novo lipogenesis through an enhanced intrahepatic synthesis of triose-phosphate precursors, an increased expression of lipogenic genes and LPL in the liver and muscle tissues [63]. Overfeeding promotes increased malonyl-CoA which in turn serves as an immediate precursor to promote de novo fatty acid synthesis. Malonyl-CoA also acts as an allosteric inhibitor of the rate-limiting enzyme, carnitine palmitoyl-transferase-1 (CPT-1), in the transport of long-chain acyl-CoAs (fatty acids) into the mitochondria for β-oxidation. As a result, they are diverted away from mitochondrial oxidation toward biosynthetic enzymes such as glycerol phosphate acyltransferase (GPAT1), diacylglycerol acyltransferase (DGAT1), and serine palmitoyltransferase (SPT1) which promote fatty acid reesterification into TAGs [64].

Inhibition of 11ß-HSD1 by GA caused a reduction in active glucocorticoids, and PPAR agonism properties of GA could be the reasons for the increased LPL expression in all tissues following GA administration. PPARα, once activated, leads to a direct upregulation of LPL expression and also downregulates apolipoprotein C-III (apoC-III), a protein which inhibits TAG hydrolysis by LPL. This results in increased fatty acid uptake and thus reduced serum TAG level as shown by the results from our studies [65, 66]. Elevation of PPARα was observed in 11ß-HSD1 knockout mice by Morton et al. [67]. PPARα is physiologically induced by glucocorticoids, and its elevation following 11ß-HSD1 inhibition may have arisen from increased circulating plasma glucocorticoids due to impaired negative feedback from the hypothalamic-pituitary-adrenal axis [67]. Therefore, GA-mediated direct or indirect activation of PPAR in concert with inhibition of 11ß-HSD1 may have contributed toward the upregulation of LPL expression in all tissues.

Contrary to fructose-mediated increase in LPL expression which led to tissue lipid accumulation, this GA-mediated increase in LPL expression in all tissues neither leads toward ectopic lipid depositions nor insulin resistance. In addition, the significant upregulation of LPL activities in the liver is probably because the liver is centrally involved in metabolism. This may have the highest distribution of GA. Studies on distribution of intravenous (IV) administration of GA in rats have confirmed that GA distribution is highest in the liver [68], thus further supporting this postulation. Similarly, LPL expression in the liver was significantly higher in GA-administered rats fed on high-fat diet as well [35].

In our studies, the mean blood glucose concentration was markedly raised with sucrose feeding. This was in accordance with studies on prolonged feeding on high-sucrose diet which increased glucose and insulin responses to a sucrose load [69] and increased fasting glycemia, thereby leading to hepatic insulin resistance in healthy men [70]. Insulin resistance is closely linked to lipid metabolism disorders. Elevated circulating FFA level and high ectopic lipid deposition in IR subjects lead to increased toxic lipid-derived metabolites, such as diacylglycerol, fatty acyl-CoA, and ceramides. The presence of these metabolites in the intracellular environment leads to higher serine/threonine phosphorylation of insulin receptor substrate-1 (IRS-1), which has been shown to reduce insulin signaling by decreasing GLUT4 translocation to plasma membrane. This in turn reduces insulin-stimulated glucose uptake in peripheral tissues [71].

However, this increase was moderated down in GA-treated rats on high-sucrose diet. The lower fasting blood glucose may be due to the increased glucose uptake into the adipose tissues and muscles by transporter GLUT4. Saltiel and Kahn [72] reported that insulin sensitizing PPARγ agonist increased the expression of Cb1-associated protein (CAP) which mediates downstream signaling molecules in activating GLUT4 protein mainly in the adipose tissue due to its high expression in this tissue. The enhanced GLUT4 activity in muscles was found to be predominantly mediated by 11β-HSD1. According to Vegiopoulos and Herzig [73], GC-induced IR in muscles resides in the suppression of glucose uptake mainly through inhibiting translocation of GLUT4 to the cell surface. Studies on 11β-HSD1 gene knockout mice also suggest that 11β-HSD1 inhibition could decrease blood glucose concentrations without risk of hypoglycemia. Besides increasing insulin-stimulated glucose uptake in tissues, blood glucose concentration was greatly reduced due to the decrease in hepatic glucose production. In T2DM, as much as 90 % of the hepatic glucose output can be due to accelerated gluconeogenesis. Both the inhibition of 11β-HSD1 and activation of PPARγ have been demonstrated to decrease expression of gluconeogenic enzymes [12]. Both actions promote gene expressions of fatty acid binding protein (FABP), LPL, acyl-CoA synthase (ACoAS), and PEPCK. This will lead to increased reesterification of FFA and enhance fat storage in the adipose tissues. Both 11β-HSD1 inhibition and PPARγ activation also suppress the genes that induce lipolysis and the release of FFAs, such as β3-adrenergic receptor, leptin, and TNF-α. This causes reduced circulating FFA levels and amelioration of FFA-induced hepatic and peripheral IR, resulting in increased hepatic and peripheral insulin sensitivity and improved glucose disposal [73].

Results from our studies have revealed that high-sucrose diet resulted in higher H6PDH activities in all six tissues. This result is in agreement with London et al. [74] who observed increased H6PDH mRNA levels in the liver and adipose tissues of Sprague-Dawley rats. High sucrose increases the substrate glucose-6-phosphate (G6P) and the availability for H6PDH, thereby leading to increased production of cofactor NADPH via the pentose phosphate pathway. The increased NADPH subsequently promotes the reductase activity of 11β-HSD1, resulting in elevated production of active GCs [75]. This postulation is further confirmed by elevated 11β-HSD1 activity observed in all tissues. Moreover, fructose enhances glucose phosphorylation in the liver. Fructose, derived from high-sucrose diet, is converted to fructose-1-phosphate which then binds to glucokinase regulatory protein (GKRP) and causes the dissociation of glucokinase (GK) from the glucokinase binding protein (GKBP). GK is translocated from the nucleus to the cytoplasm, resulting in an increase in the conversion of glucose to G6P [76]. This increases the substrate availability for H6PDH activity [77]. Lower H6PDH activities observed in rats with GA administration are probably due to the inhibitory effect of GA on 11β-HSD1. This leads to a reduction in the generation of cofactor $NADP^+$, thereby decreasing the H6PDH activity [36]. GA administration leads to reduced PEPCK and G6Pase expression, which subsequently decrease the rate of hepatic gluconeogenesis. GA's activation of PPARγ also improves hepatic and peripheral insulin sensitivity and glucose uptake. Both effects of GA lead to reduced hepatic glycogen store and circulating blood glucose, leading to a decrease in the production of G6P from glucose and glycogen through glycolysis and glycogenolysis. As a consequence, the availability of G6P for H6PDH decreases, contributing to a reduction in H6PDH activity [36].

As aforementioned, PEPCK is a key regulatory enzyme involved in two metabolic processes: gluconeogenesis, which occurs in the liver and kidney, and glyceroneogenesis, which occurs in the adipose tissues [78]. Our studies showed elevated PEPCK activities in all tissues with sucrose feeding. Increased expression of hepatic PEPCK gene was also observed in diabetic mice, characterized by elevated hepatic glucose output [79]. This could be due to increased production of GCs which stimulates the hepatic and renal PEPCK activities by promoting the assembly of several transcription factors (TFs), hepatocyte nuclear factors (HNF-3β and HNF-4α), Ccaat-enhancer-binding proteins (C/EBP β), and forkhead box protein O1 (FOXO1), on the PEPCK gene promoter [80]. However, Cassuto et al. [81] found that PEPCK activities are only stimulated by GCs in the liver and kidneys but are suppressed in adipose tissues. Our results are contrary to the above, where significantly increased PEPCK activities were observed in all tissues of the group on high-sucrose diet alone. Increased PEPCK activities in SAT and VAT in this group could be attributed to the FFA-induced activation of PPARγ. PPARγ is predominantly expressed in adipose tissues. During high-sucrose feeding, elevated GC-induced lipolysis causes activation of reesterification to prevent an all-out release of FFA into the bloodstream. As a consequence, glyceroneogenesis is activated to produce G3P, which is required for fatty acid reesterification. Under this condition, elevated circulating FFA would activate PPARγ, which binds as a heterodimer complex with RXRα to the PPAR response element (PPRE) on the PEPCK gene promoter. This results in increased PEPCK gene transcription and a corresponding increase in its activities in the adipose tissues [82].

The group on high-sucrose diet and given GA had increased hepatic and renal PEPCK activities compared to the control rats on normal diet, but the elevations were smaller compared to the rats on high-sucrose diet alone. This indicated the ability of GA to improve hyperglycemia through GC-stimulated PEPCK activity in gluconeogenesis. GA administration leads to reduced production of active GCs. This results in a downregulation in the PEPCK gene transcription, thereby decreasing PEPCK activity and gluconeogenesis rate [12]. In addition, activation of PPARγ was also found to downregulate the gene expression of PEPCK in the liver and kidneys, resulting in decreased gluconeogenesis [27]. GA, which is a triterpenoid saponin, has been found to be an agonist of PPARγ [43]. Therefore, GA's activation of PPARγ would lead to downregulation of PEPCK gene transcription, and this would also account for the reduced PEPCK activities observed in GA-treated rats compared to non-GA-treated rats.

G6Pase is mainly expressed in gluconeogenic tissues such as the liver and kidneys. It is a membrane-bound enzyme with a multicomponent system consisting of G6PC and G6PT which are located in the ER [83]. Similar to H6PDH, G6Pase requires G6P as substrate. It catalyzes the final step of gluconeogenesis and glycogenolysis, which is the hydrolysis of G6P to glucose [84]. Following high-sucrose feeding, an increase in six-carbon sugars (glucose and fructose) would increase the availability of substrate G6P for G6Pase. This would lead to increased G6Pase activities and elevated glucose output [85]. In addition, increased production of active GCs during high-sucrose feeding has been found to induce the gene transcription of G6Pase. This involves the binding of GR and cofactors to G6Pase gene promoter and increased coactivation of PGC-1α with GR, FOXO1, and HNF-4, which promote the G6Pase gene transcription [86]. High circulating FFA has also been associated with increased G6Pase mRNA and activities in diabetic condition. Elevated circulating FFA exerts a PPAR-dependent allosteric stimulation on G6Pase, resulting in increased G6Pase expression and gluconeogenesis [87]. Smaller elevation in G6Pase activities were observed in GA-treated rats compared to nontreated rats on high-sucrose diet. Besides the reduction in active GCs, another possible mechanism which might account for reduced G6Pase activities in the liver and kidneys could be the actions of PPARα in improving glucose and lipid metabolism and enhancing whole-body insulin sensitivity. Treatment with PPARα agonist was found to significantly reduce the mRNA level of G6Pase, leading to decreased gluconeogenesis. This suggests the possible role of GA as a PPARα agonist in the modulation of G6Pase activities [74].

Despite significantly higher serum insulin level in sucrose-fed rats, insulin failed to suppress the PEPCK and G6Pase activities in all the studied tissues. This could be due to reduced responsiveness of the tissues to insulin action due to increased circulating FFA levels. This will impair the insulin-stimulated suppression of PEPCK and G6Pase activities [87].

9.2 High-Fat Diet and Glucose and Lipid Metabolism

Numerous studies have revealed that high-fat diets promote hyperglycemia and whole-body IR. It is generally accepted that high-fat diets can be used to generate rodent model for MetS with IR [88]. Overfeeding of animals with high fat of more than 50 % of calories as fat for approximately 5 weeks is sufficient to initiate moderate obesity and often results in IR [89, 90]. Our work on rats fed on high fat showed that these rats consumed significantly more calories on a per day basis compared to the controls which were fed only standard rat chow.

In the insulin-resistant state, the decrease in insulin-mediated suppression of lipolysis in adipocytes promotes the release of fatty acids which inhibit LPL activities [91]. When supply of fatty acids exceeds tissue demand, fatty would probably bind to LPL and displace it from its binding sites, thereby rendering them nonfunctional [92]. LPL expression of rats fed on high fat without GA was downregulated in all nonhepatic tissues compared to the control rats on normal diet. The downregulation of LPL in the adipose tissues, muscles, and kidney may be due to inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) which are found to be elevated in obesity and insulin-resistant states [93]. Its inhibition of LPL gene transcription is suggested to be mediated in part by blocking the nuclear-factor Y/CCAAT interactions with LPL promoter [94, 95]. However, the increase in TNF-α levels renders the opposing effect in the liver. According to Wang and Eckel [96], LPL is not normally expressed in the adult liver of animals but can be expressed under specific physiological and pathological conditions. A single dose of TNF-α can cause a significant increase in LPL mRNA levels in the liver. However, the detailed mechanism of such induction is not well understood. The increase in hepatic LPL activities and their concomitant decrease in the nonhepatic tissues could have resulted in greater partitioning of plasma TAG and apolipoprotein B100 (apoB100) from the liver [97] and hence may account for the observed hypertriglyceridemia in the rats on high fat without GA compared to the control rats on normal diet.

In rats on high-fat diet given GA, LPL expression was upregulated in all nonhepatic tissues, condition that opposes that seen in rats on high-fat diet without GA. It was postulated that GA results in upregulation of LPL via the activation of PPAR class of nuclear receptors since LPL gene is found downstream of the transcriptionally active PPRE [98]. Of the tissues in which LPL expression was upregulated, the highest upregulation occurred mainly in the muscles (AM, QF, and H) compared to SAT and VAT. This suggests that GA may exhibit a higher potency in activating PPARα than PPARγ; PPARα is highly expressed in the heart, liver, and kidney in which it has a crucial role in controlling fatty acid oxidation [99], while PPARγ is highly expressed in the adipocytes which trigger adipocyte differentiation and lipogenesis [94]. The activation of PPARα may lead to a direct upregulation of LPL expression and also downregulate apo-III, an inhibitor of LPL [65] that is upregulated in the insulin-resistant state [54]. The downregulation of LPL expression in the liver might be due to the reduction in macrophage-derived TNF-α in the adipose tissues. According to Jeong and Yoon [100], PPARα activation in adipose tissue mRNA levels of TNF-α production subsequently downregulates LPL in the liver. The end effect of these is that GA promotes partitioning of lipids away from the liver into the oxidative tissues.

Improvement in lipid profile following GA treatment was seen in all GA-treated rats. In the high-fat-fed rats, more prominent hypotriglyceridemic and HDL-raising effects were seen. The hypertriglyceridemia observed in patients with MetS and T2DM originates from (1) lipolysis of TAG store from adipose tissue that causes elevated FFA flux from the liver and, hence, increased hepatic TAG synthesis and inhibition of lipolysis of chylomicrons and VLDL due to decreased LPL levels [54]. Our study on high-fat-fed rats indicated that GA given to these obese rats could curb such development by selective induction of LPL expression in the nonhepatic tissues to promote catabolism of circulating TAG-rich lipoproteins and prevent further uptake of FFA into the liver by downregulating hepatic LPL expression. More importantly, GA induced a significant increase in HDL levels in the obese rats. Elevating HDL cholesterol may serve as a more attractive treatment alternative instead of lowering LDL cholesterol as dyslipidemia is often characterized by a normal range of serum LDL cholesterol but with a predominance of the more atherogenic small, dense LDL rather than the less atherogenic large, buoyant LDL particles [101]. The atheroprotective effect of HDL is exerted through its ability to counteract LDL oxidation, the major initiating event that prompts the development of the atherosclerosis. The HDL particle, by virtue of the antioxidative properties of its attached apo A-1 paraoxidase and glutathione peroxidase, reduces the oxidative modification of LDL by quenching the oxygen-derived free radicals generated from LDL oxidation [56]. Hence, various pharmacological interventions have been focused on raising HDL-induced levels [56].

Obesity-induced IR results in profound dysregulation in the glucose homeostasis and produces elevations in fasting and postprandial glucose levels [54]. The mean glucose levels increased in rats on high-fat diet alone compared to the control rats which are on normal rat chow. With the development of visceral obesity, the high circulating FFA leads to IR that promotes a dual effect to enhance hyperglycemia by downregulating the insulin-sensitive glucose transporter 4 (GLUT 4) via the Randle cycle and thus promotes an accumulation of glucose in the circulation and (2) stimulating hepatic gluconeogenesis by antagonizing the action of insulin in the liver (hepatic IR) [54]. GA-treated rats on high-fat diet demonstrated a significant decrease in fasting blood glucose compared to the rats on high-fed diet alone. The reduction in fasting blood glucose of the high-fat diet rats given GA is proposed to be accounted for by increased tissue glucose uptake via GLUT 4. PPARγ activation in the adipose tissue has been shown to increase the expression of c-Cbl-associated protein (CAP) that is important for the translocation of GLUT4 to the cell surface [54], and inhibition of 11β-HSD1 may also exhibit similar effect by attenuating the inhibition muscle GLUT4 translocation by active glucocorticoids [73]. These effects may therefore increase glucose disposal, giving a decrease in circulating glucose level. More importantly, both 11β-HSD1 inhibition and PPARγ agonism have also been associated with the reduced expression of PEPCK and G6Pase [12, 74], the two rate-limiting enzymes of the gluconeogenesis pathway that are aberrantly induced in T2DM patients [73]. Uncontrolled accelerated gluconeogenesis accounts for 90 % of hepatic glucose output in T2DM patients and is thus a significant contributor to hyperglycemia [12].

Besides a significant reduction in blood glucose concentration in rats on high-fat diet given GA, mean serum insulin level was also reduced compared to rats on a high-fat diet alone. This may be due to improved glucose-sensing proteins in the pancreatic β-cells since β-cells control insulin secretion in response to blood glucose levels [102]. Both GLUT2 transporter and glucokinase are components of the glucose-sensing apparatus of the β-cells whose expressions are both decreased in diabetes. With this, the glucose threshold for insulin secretion is also decreased, leading to aberrant insulin secretion and hyperinsulinemia. PPARγ activation is shown to restore both GLUT2 and glucokinase expression [36]. Thus, the glucose threshold for insulin secretion is increased, thereby reducing insulin secretion. The HOMA-IR is used for assessment of insulin sensitivity from basal (fasting) glucose and insulin levels, a higher value indicating lower insulin sensitivity (higher insulin resistance) and vice versa [103]. The HOMA-IR index decrease in rats on high-fat diet given GA was significant compared to those on high-fat diet alone – indicating an improvement in insulin sensitivity in rats on high-fat diet given GA.

Chromic obesity has been associated with nonadipose tissue lipid accumulation – a condition known as tissue steatosis [54, 104]. During conditions of chronic caloric excess, a compensatory mechanism first occurs in the leptin-responsive state where the surplus FFA upregulates PPARα and promotes the compensatory oxidation of the surplus FFA, with the excess energy dissipated as heat. As such process continues, such caloric excess is no longer compensated, and in the leptin-unresponsive state, the surplus FFA activated PPARγ instead, leading to upregulation of the lipogenic enzymes that causes ectopic TAG accumulation [104]. Leptin resistance has been shown to occur in late phases in both rat and human diet-induced obesity [104]. In our work, lipid deposition in all tissues from rats on high-fat diet given GA was significantly reduced compared to the rats on high-fat diet alone. Thus, it could be that the GA activation of PPARα expression which in turn induced the expression of lipid-catabolizing genes such as carnitine palmitoyltransferase-1 CPT-1, acyl-CoA oxidase (AC), and uncoupling protein (UCP-2) that is induced normally in the state of compensated caloric excess in the above tissues. Tissue lipid accumulation has been associated with obesity-related IR [105, 106], and these are mediated by TAG-derived metabolites and inhibit signal transduction [64]. Thus, our observation of GA-mediated improvement in insulin sensitivity may be related to such decrease in tissue lipid as well.

10 Conclusion

In conclusion, our group has demonstrated that glycyrrhizic acid:

- Not only lowered blood glucose significantly but also improved insulin sensitivity and caused a positive shift with respective to lipid parameters in rats under various physiological conditions. Besides, blood pressure was not affected during the studied period, with the given dosage of GA at 100 mg kg^{-1}
- Did not affect liver enzymes AST/ALT, γ-GT, and muscle CK as their activities were similar to that of the control rats.
- Exerted selective induction/repression of LPL expression, upregulation of PPARγ, increased/decreased of 11β-HSD types 1 and 2, and the activities of PEPCK, G6Pase, and H6PDH in the studied tissues.

The above observations therefore suggested that GA acts via the above regulatory gluconeogenic enzymes as well as LPL and HSD1 to give the observed glucose and lipid effects. GA could therefore be considered as a potential compound in ameliorating dyslipidemia and hyperglycemia in rats.

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Phytoecdysteroids: Phytochemistry and Pharmacological Activity 127

Gert Laekeman and Arnold Vlietinck

Contents

1 Introduction 3828
2 Distribution of Phytoecdysteroids 3829
3 Diversity of Phytoecdysteroids 3830
4 Biosyntheses of Phytoecdysteroids 3836
5 Functions of Phytoecdysteroids 3839
6 Pharmacology of Phytoecdysteroids 3840
6.1 Pharmacology of Phytoecdysteroids 3840
6.2 Toxicology of Phytoecdysteroids 3840
6.3 Adaptogenic Activities 3840
6.4 Stimulation of Protein Synthesis 3841
6.5 Effect on Hyperglycemia 3841
6.6 Cholesterol Homeostasis 3842
6.7 Immunomodulatory Activity 3842
6.8 Antiradical/Antioxidative Properties 3843
6.9 Synergism with Vitamin D 3843
6.10 Antitumoral Activity 3843
6.11 Dermatology and Cosmetology 3844
6.12 Anabolic Effects 3845
7 Conclusions 3845
References 3845

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Abstract

At this moment, more than 450 ecdysteroids are identified, most of them found in different plant species. They are biosynthesized via the mevalonic acid pathway as C-27, C-28, and C-29 steroid structures. The isolation of new phytoecdysteroids is a still ongoing process. Phytoecdysteroids can play a taxonomic role. They are thought to play a role as growth regulators, and they may protect plant species against phytophagous insects. They can be considered as possibly interesting structural templates for different therapeutic indications. However, up to now, no real therapeutic applications have been materialized. They are actually used for dermatological purposes in cosmetology, and putative anabolic activity is highly promoted via Internet, without too much evidence. The large variety of substances may hamper the making of synthetic derivatives that can be patented as original therapeutic entities.

Keywords

Biosynthesis • ecdysteroid • phytoecdysteroid • plant • therapeutic

1 Introduction

Ecdysteroids (also called ecdysones) are a group of natural polyhydroxysteroids present in plants (phytoecdysteroids) and animals (zooecdysteroids). Some ecdysteroids, such as ecdysone, 20-hydroxyecdysone (ecdysterone), and ajugasterone C, can be found in plants and animals [1]. The phytoecdysteroid profile varies in amount and composition between plant species but also depends on plant organs, season, origin, and habitat [2].

The chemical structure of α-ecdysone was elucidated by physiochemical studies and chemical transformations by Karlson et al. [3], and the stereochemical structure was determined by Huber and Hoppe [4] by X-ray analysis. Closely related molecules were discovered in plant species soon thereafter. Galbraith and Horn [5] isolated ecdysterone, a phytoecdysone with insect-molting properties from the fern *Polypodium elatus*. Simultaneously, Nakanishi et al. [6] identified ponasterone from another gymnosperm, namely, *Podocarpus nakaii*. Jizba et al. [7] isolated crustecdysone from *Polypodium vulgare*, whereas Takemoto et al. [8] extracted insect-molding substances from mulberry leaves. These first discoveries triggered further efforts to investigate the distribution of ecdysones in plants. They were also discovered in red algae which contain closely related pinnasterols [9], [10] and later in fungi [11]. Dinan and Lafont [12] made a compilation of the literature for the screening of plant species, among others vascular plants, algae, and fungi. In the beginning, the focus was on the discovery of possible natural antitumoral agents, but very soon, the target moved from human therapy to insecticidal activity. As these molecules interfered with insect development and reproduction, there was hope for development of a new class of natural pesticides as alternative for purely chemical agents. However, the complexity of the molecules makes them difficult to synthesize. They hardly can penetrate in the living insects, and their stability is limited in a natural environment. Up to now, no natural ecdysteroids have made their way in pest control [13].

This chapter deals with a review of the biodiversity of phytoecdysteroids, the possible biosynthetic pathways of these molecules, and the hypothesized functions of this class of secondary metabolites for plants and humans.

2 Distribution of Phytoecdysteroids

Ecdybase (http://www.ecdybase.org) is a good resource for chemical and biological data for ecdysteroid analogues and literature data on the occurrence of phytoecdysteroids in plants. Ecdysteroids seem to occur not only in a wide variety of species but also as a wide variety of structures. Glucosyl-ferulate conjugates are among the newer classes of conjugates discovered in *Microsporum membranifolium* [14]. The question emerges whether phytoecdysteroids are synthesized by the plant species themselves or whether they are taken up and subsequently modified by species like fungi [13].

Another question is related to the capacity of a larger group of plants to synthesize ecdysteroids. In the beginning, ferns were thought to be the species representative for ecdysteroid synthesis, but this was due to the high number of ferns screened [13]. Ecdysteroids were also found in species like *Arabidopsis thaliana*, originally considered as ecdysteroid negative [15]. The actual concept is that few plant families do not contain ecdysteroid species within their members. Some species contain huge amounts of phytoecdysteroids, i.e., 2–3 % of their dry weight (e.g., seeds of *Rhaponticum carthamoides* and stalks of *Diploclisia glaucescens*, inflorescences of *Serrulata inermis* and roots of *Cyanothis arachnoides*), but in general, the steroid content in plants is normally in the order 0.01–0.1 % of the plant dry weight [16]. There may be a relationship between the presence or absence of phytoecdysteroids and the taxonomic position of plant species, e.g., the within the family of the *Chenopodiaceae* and within the genus *Silene* [13].

Therefore, it has been suggested that ecdysteroids could be taken as an indicator for taxonomic purposes, even when fluctuations due to organ type, season, and geographical location may be confounding factors. Presence or absence of phytoecdysteroids may be of help in the taxonomic study of mushrooms (e.g., *Paxillus* and *Tapinella*) [17]–[19]. Presence of phytoecdysteroids in parts of higher plants has been linked to their survival efforts. For annual plants depending upon wind pollinating, this means that ecdysteroids will be present in the pollen-containing parts, the seeds, and the young leaves [20].

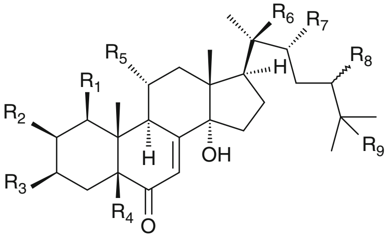
3 Diversity of Phytoecdysteroids

Several large-scale surveys have been conducted in the past, yielding hundreds of phytoecdysteroid structures. Imai et al. [21] did the first large-scale survey on 1,056 diverse well-identified species. They identified ecdysteroids in 61 of them. Hikino et al. [22] concentrated their efforts on Japanese ferns. Their screening was based upon less specific biological activity testing (instar larvae). This may be the explanation for the high number of positives: 170 of 283 species tested gave positive reactions.

The findings of these screenings are incorporated in the Ecdybase (http://www.ecdybase.org): ecdysteroid agonist as well as antagonist containing plants is listed. In July 2012, the database contained 463 ecdysteroids. Apart from the geographical distribution, also the ecological distribution has been focused upon [13]. The search for phytoecdysteroids in a large variety of plant species remains a research target. Publications about isolation of new entities or known ecdysteroids in newly investigated plant species are published in a large variety of scientific journals. We include here recent examples. *Limnanthes alba* or the white meadowfoam is an established oilseed crop and originated from southern Oregon. Ecdysone, 20-hydroxyecdysone, ponasterone A, and muristerone A were identified by LC-MS/MS in the plant seeds by Stevens et al. [23]. Ecdysteroids were already reported from other species of the *Limnanthaceae*, more particularly *Limnanthes bakeri*, *L. douglasii*, *L. floccosa*, *L. gracilis*, and *L. montana* [24]–[26]. Some other plant genera like *Ajuga* have been particularly studied as a rich source of structurally different phytoecdysteroids. Castro et al. [27] isolated three new phytoecdysteroids from the roots of *Ajuga macrosperma var. breviflora*: ajugacetalsterones C and D and breviflorasterone. Another new phytoecdysteroid, ajugatide E, was isolated from *Ajuga taiwanensis* [28]. New compounds reptanslactone A, reptanslactone B, and sendreisterone were isolated from *Ajuga reptans* as well as the known dehydroprecyasterone and breviflorasterone [29]. Also in *Ajuga remota*, 20-hydroxyecdysone is present. *Ajuga remota* is used as an herbal remedy against malaria in Kenya [30].

Wang et al. [31] isolated five new phytoecdysteroid glycosides from the whole plant *Froelichia floridana*. They were tested against human DNA topoisomerase, but did not show any inhibitory activity. Kumpun et al. [32] recently studied their presence in *Chenopodium quinoa*, an ancient Andean crop with high nutritional value. Besides large amounts of 20-hydroxyecdysone, they found also lower amounts of makisterone A, 24-*epi*-makisterone A, 24(28)-dehydromakisterone A and polypodine B. They also identified three new natural compounds, more particularly 24,25-dehydroinokosterone, 25,27-dehydroinokosterone, and 5β-hydroxy-24(28)-dehydromakisterone A. Ecdysteroids were concentrated in the bran of *Chenopodium quinoa* [32]. Tan et al. [33] identified a less common phytoecdysteroid with a 5α-cholestane skeleton in *Cyanotis arachnoidea*. New phytoecdysteroids were also found in the roots of *Achyranthes bidentata*. These steroids contain a furan ring on an acetonide moiety and are named niuxixinsterone A, B, and C [34]. Brainesterosides A–E were isolated as phytoecdysteroid glycosides from the rhizomes of *Brainea insignis*, along with three known phytoecdysteroids

Table 127.1 Diversity of C27-phytoecdysteroids within the same structural entity [13], [16]



R1	H/OH
R2	OH/OCOCH$_3$/OCOC$_2$H$_2$C$_6$H$_5$/β-glucoside
R3	OH/OGlc/OCOCH$_3$/Gal*p*α(1-6)-Gal*p*α*/OCO-C$_2$H$_2$CH$_3$/OCOC$_2$H$_2$C$_6$H$_5$/OCOC$_2$H$_2$C$_6$H$_5$-OH/glycosylferulate/β-glucoside/β-xyloside
R4	H/OH/$\Delta^{4(5)}$/C19OH/C7H$_2$,C8H
R5	H/OH/$\Delta^{8(9),\ 12(13),\ 14(15)}$
R6	H/OH/OCOC$_6$H$_5$
R7	H/OH/OGlc/OCOCH$_3$/CHOH-CH$_2$-CO-OCH$_3$/OCOC$_6$H$_5$/O-SO$_3^-$/O-PO-(OH)$_2$/β-glucoside
R8	H/OH/OCOCH$_3$/OGlc/$\Delta^{24(25)}$/β-glucoside
R9	H/OH/GlcO/OCOCH$_3$/OCH$_3$/OCOC$_6$H$_5$/β-glucoside/β-rhamnoside

*Galactoside

(ponasteroside A, ponasterone A, and 20-hydroxyecdysone) [35]. In *Serrulata wolffii*, a wide variety of structurally different ecdysteroids were found: compounds with an extra double bound at position 20(22) (1-hydroxy-20,21-didehydrotaxisterone and 20,22-didehydrotaxisterone), with a furan ring in the side chain (serfurosterone A and B) or with an intramolecular ether function in the side chain (shidasterone derivatives) [36]–[39]. In *Serrulata wolffii*, Ványolós et al. [40] identified still new ecdysteroids with an α-hydroxyl group, structures that are relatively rarely seen among the class of ecdysteroids (3-epi-shidasterone and 3-epi-22-deoxy-20-hydroxyecdysone), as well as ponasterone-22-apioside [40].

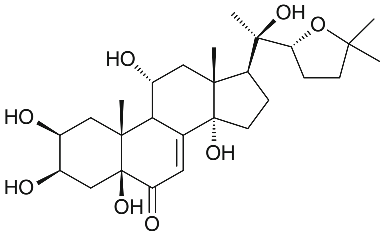
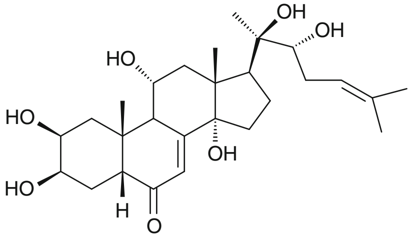
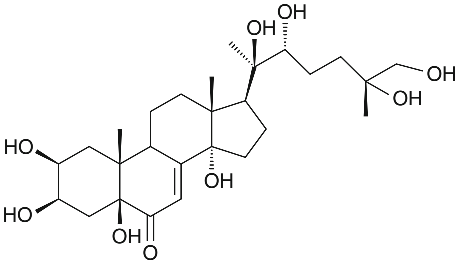
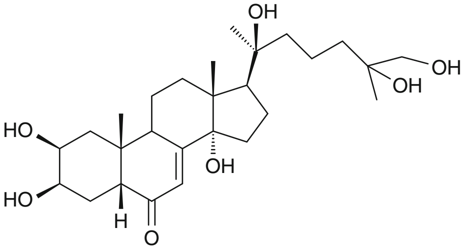
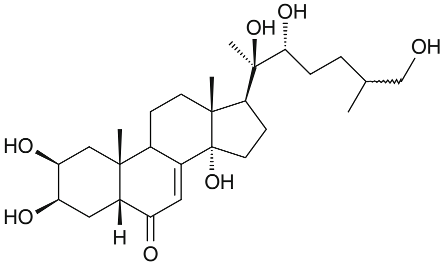
All these investigations confirm the position of phytoecdysteroids as important secondary metabolites in plants. The same Hungarian research group found also three new ecdysones in *Polypodium vulgare*: 5-hydroxyecdysone, 20-deoxyshidasterone, and polypodine B 2-β-D-glucoside [41].

The diversity of C27 phytoecdysteroids is illustrated in Tables 127.1 and 127.2 and examples of C28- and C29-phytoecdysteroids are shown in Tables 127.3 and 127.4.

Phytoecdysteroids have common structural features such as C24 to 29C atoms, several hydroxyl substituents, a Δ^7-6-keto grouping in the B ring, a cis junction of the A/B rings, and a side chain usually containing a (R)-C22-OH group. In most cases, phytoecdysteroids are isolated in the free state, although many derivatives such as ethers, esters, and glycosides have been isolated as well.

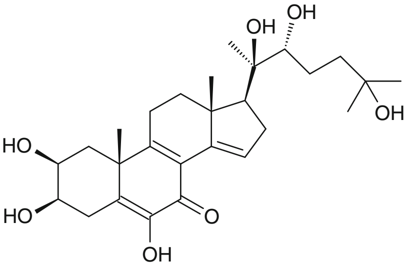
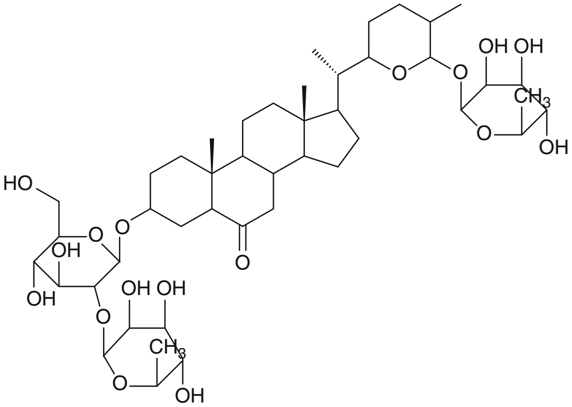
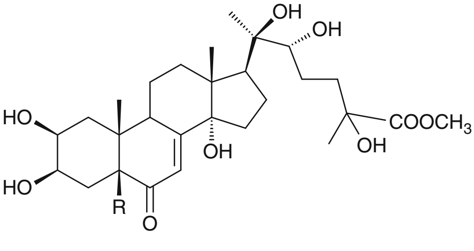
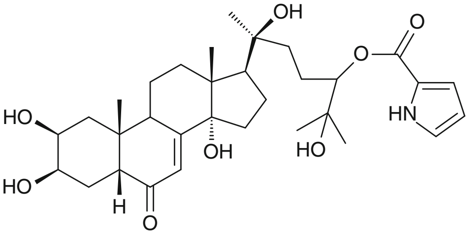
Most commonly, 1–3 major ecdysteroids will represent about 95 % of the total ecdysteroid in one plant species. As shown in the tables, the minor ecdysteroids form a mixture of ecdysteroid structural analogues.

Table 127.2 Examples of C27-phytoecdysteroids with other functional groups [16]

Ajugasterone D	
Vitexirone	
26-OH-polypodine	
22-deoxy-20,26-diOH-ecdysone	
Inokosterone	

(*continued*)

Table 127.2 (continued)

Calonysterone	
Osladin	
Carthamasterone A and B	R = OH (A) R = H (B) 
Pinnatasterone 24-(pyrrol-2-carboxylate)	

(*continued*)

Table 127.2 (continued)

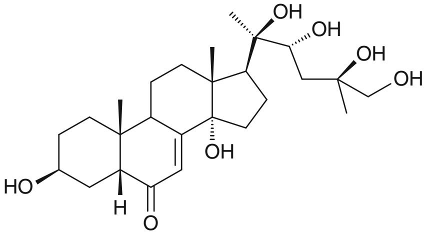
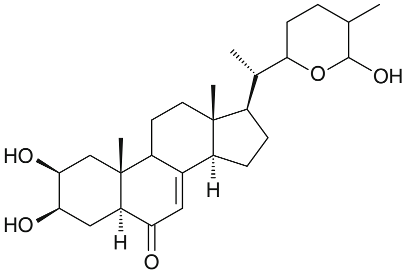
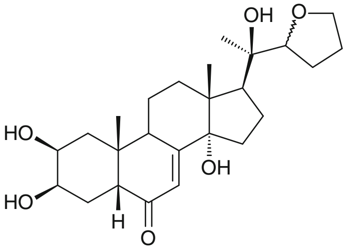
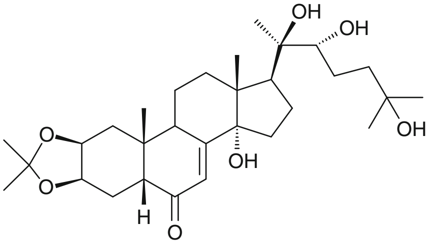
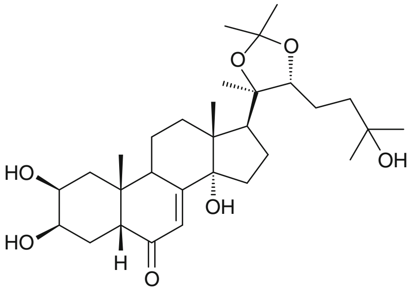
Podecdysone C	
Polypodosaponin	
Stachysterone D	
Ecdysterone 2,3-acetonide	
Ecdysterone 20,22-acetonide	

Table 127.3 Examples of C-28 phytoecdysteroids [16]

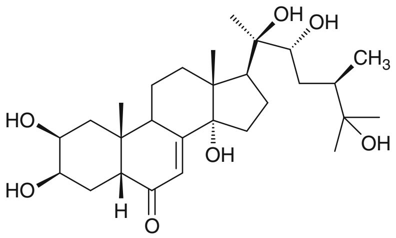
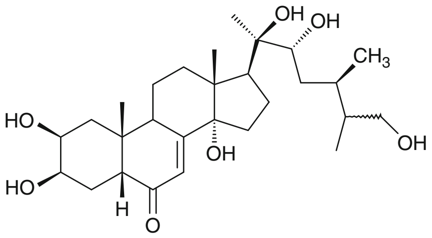
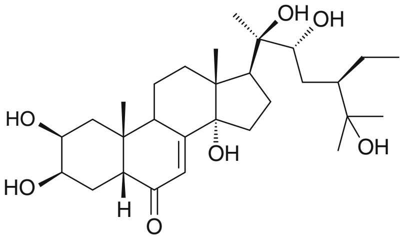
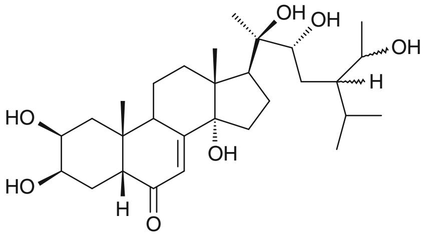
Makisterone A	 OH OH CH3 HO HO OH OH H O
Makisterone B	 OH OH CH3 HO HO OH OH H O

Table 127.4 Examples of C-29 phytoecdysteroids [16]

Makisterone C = podecdysone A	 OH OH HO HO OH OH H O
Makisterone D	 OH OH OH H HO HO OH H O

Tóth and Báthori M [42] described a relatively simple separation procedure for phytoecdysteroids from *Silene viridiflora*. By a purification procedure in four steps, phytoecdysteroids were made available in adequate amounts.

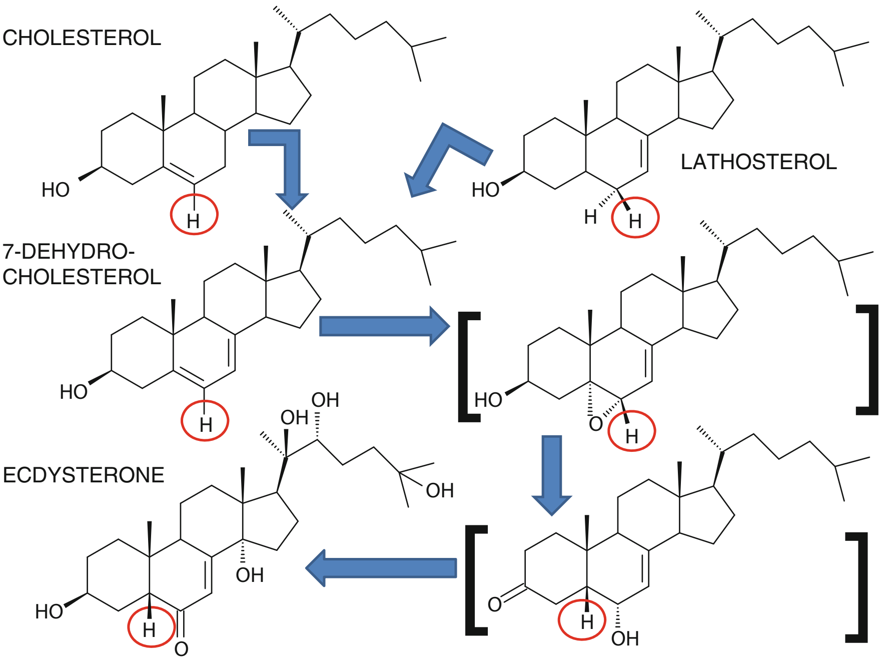


Fig. 127.1 Ecdysteron biosynthesis in *Polypodium vulgare* and *Ajuga reptans*: proposed early steps [13], [45]

4 Biosyntheses of Phytoecdysteroids

At present, the pathway for the biosynthesis in plants may differ from that in animals. The large number of ecdysteroids found in plants makes it unlikely that we are dealing with a single pathway. In *Polypodium vulgare*, cholesterol is converted to ecdysterone, α-ecdysone, and 5-β-OH-ecdysterone [43]. It is approved that the Δ^7-6-keto grouping and the A/B-cis junction arise at the first stages of the biosynthesis of cholesterol, the hydroxylation in position 14-α proceeding next [44]. It is suggested that in the few ecdysteroids with trans-A/B junctions, an isomerization of the chiral center 5 has occurred [16]. The order of the subsequent introduction of hydroxyl groups into the steroidal moiety and side chain, as well as the side-chain alkylation, does not seem to be strictly determined and changed from one plant family to another [45] (Fig. 127.1).

The *Ajuga* genus is unique for the great variety of phytoecdysteroids, with a broad spectrum of biological and pharmacological actions. They accumulate in various plant organs such as flowers, stems, leaves, roots, and fruits, usually present in small quantities of the order of 0.01–0.1 % of the plant dry weight. The major phytoecdysteroid is 20-hydroxyecdysone, which is also the active molting hormone of insects [46–48].

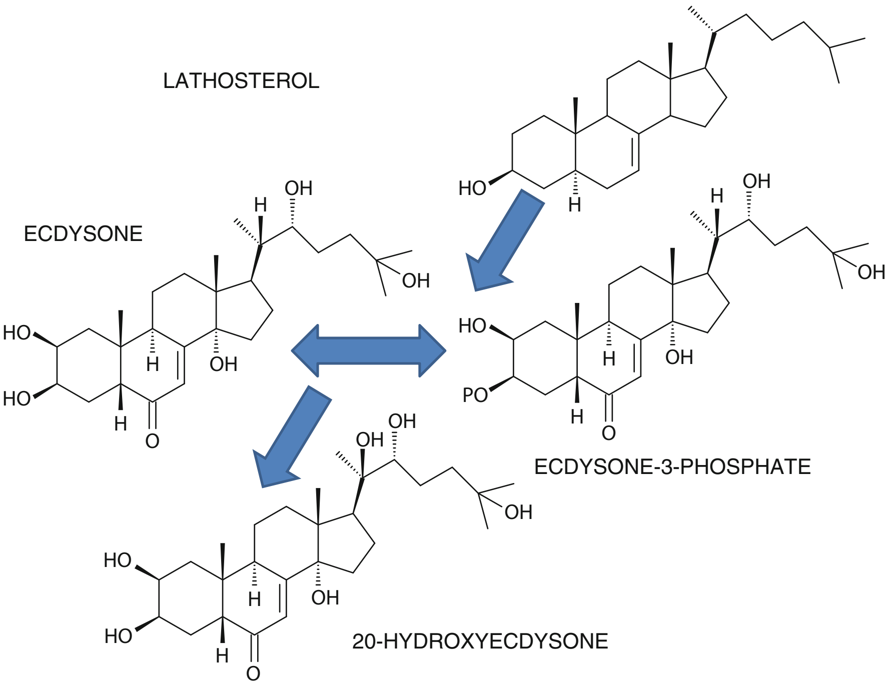


Fig. 127.2 Biosynthesis of spinach (*Spinacia oleracea*) ecdysteroids: the endogenously biosynthesized ecdysone and ecdysone-3-phosphate are converted to 20-hydroxyecdysone [49]

In this biosynthetic scheme, both cholesterol and lathosterol are involved and produce the ecdysteron moiety via 7-dehydro-cholesterol. The intermediate 5,6-epoxide would then be a plausible explanation for the formation of the 6-keto group (Fig. 127.1). Recent evidence suggests that phosphate conjugates are important in the processing or targeting of sterols and ecdysteroids in their biosynthesis in spinach. In animals, ecdysteroids are phosphorylated for excretion for the parent ecdysteroid from the animal gut. In plants, the phosphorylated ecdysteroids appear to be involved in the biosynthetic pathway as shown in Fig. 127.2.

Devarenne et al. [50] incubated radiolabeled [22,23-^{3}H]ecdysone with *Zea mays* leaves. Post-incubation, between 57 and 75 %, of the radiolabeled material recovered was present as unmetabolized [^{3}H]ecdysone. The remaining radioactivity was recovered as [^{3}H]ecdysone diphosphate, [^{3}H]ecdysone phosphate, and polyphosphorylated ecdysone. The same authors found lathosterol and not cholesterol as the preferred precursor to the ecdysteroids in spinach (*Spinacia oleracea*) and hypothesized it being the same situation in *Zea mays*, because it took considerable more time to convert cholesterol into ecdysteroids. Ecdysteroid polyphosphates have a downregulatory effect on the biosynthesis of ecdysteroids. This may lead to a sequestration of lathosterol into the steryl ester pool and subsequently the slow release of this lathosteryl ester for additional biosynthesis of ecdysteroid polyphosphates [50].

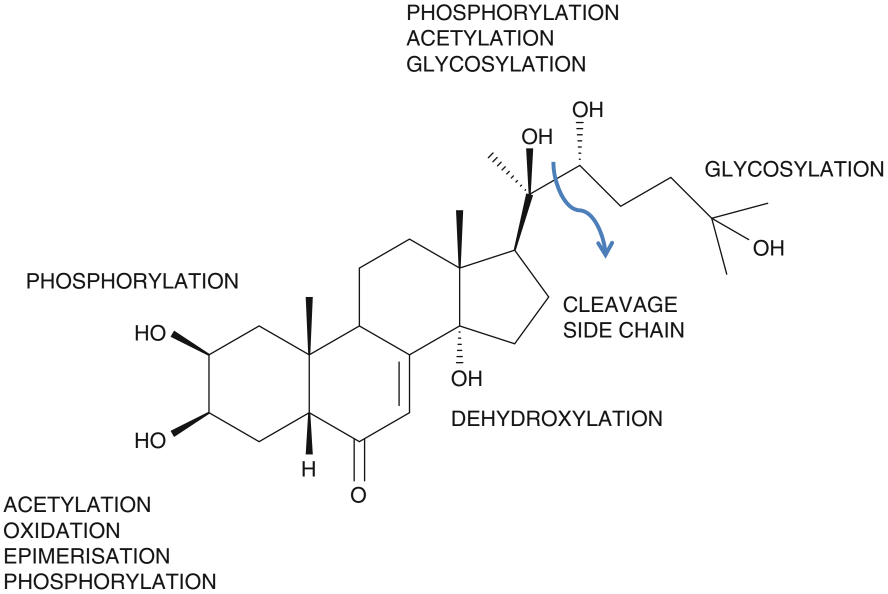
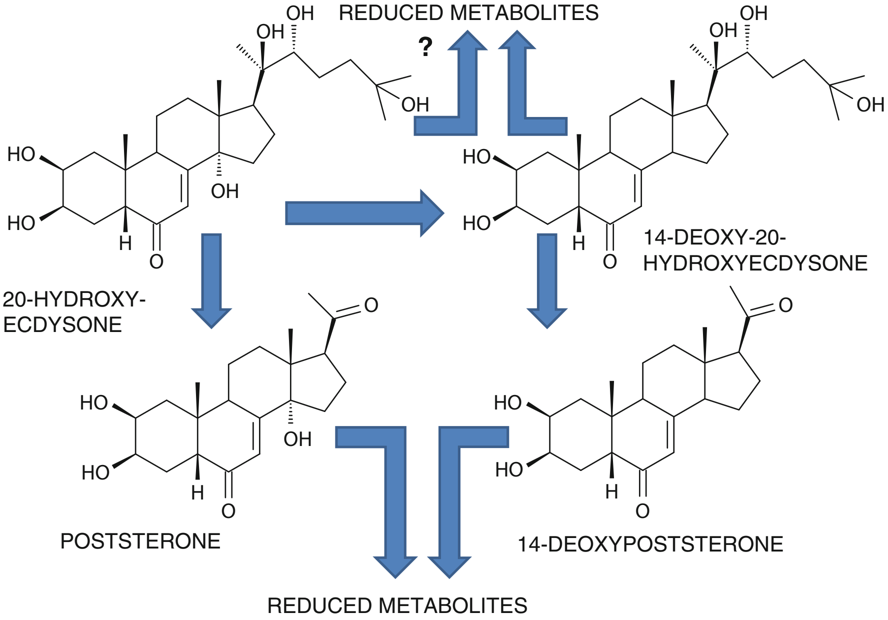

PHOSPHORYLATION
ACETYLATION
GLYCOSYLATION
OH
OH
GLYCOSYLATION
OH
PHOSPHORYLATION
HO
CLEAVAGE
SIDE CHAIN
OH
HO
DEHYDROXYLATION
H
O
ACETYLATION
OXIDATION
EPIMERISATION
PHOSPHORYLATION

Fig. 127.3 Detoxification mechanisms for phytoecdysteroids in insects [13]


OH
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REDUCED METABOLITES
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14-DEOXY-20-
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14-DEOXYPOSTSTERONE
REDUCED METABOLITES

Fig. 127.4 Major metabolic pathways of 20-hydroxyecdysone in mice [32], [53]

5 Functions of Phytoecdysteroids

A number of suggestions have been put forward, but the hypothesis that phytoecdysteroids act as potent deterrents and anti-feeding agents against insects received probably the greatest support.

As secondary plant metabolites, phytoecdysteroids stimulate protein synthesis in plants, activate cell mitosis, and possibly act as plant growth regulators. Ecdysteroids influence signal transduction pathways, similarly to anabolic steroids. They should not bind to cytosolic steroid receptors, but act by membrane-bound receptors [51].

They also protect plants against phytophagous insects either by feeding deterrence or endocrine disruption upon ingestion, which leads to death. The Indian meal moth *Plodia interpunctella* can be taken as an example. A concentration of 200 ppm of phytoecdysteroids in their diet manifested signs of toxicity as a decrease in larval weight, induction of cannibalism, and an increase in mortality, as well as a disruption of development. Minimal structural differences significantly affected the toxicity of phytoecdysteroids, makisterone A being the most toxic [52]. *Bombyx mori* or the silkworm is an example on which phytoecdysteroids have detrimental effects like inhibition of growth and death with or without promoted molting and prothetely. Strangely enough, when silkworms are exposed to low doses of 20-hydroxyecdysone at particular times during their development, improved synchrony of cocoon formation and higher silk yield could be seen. Also in honeybees fecundity of wax improved by the same effect. The mode of action is unknown. It is hypothesized that low levels of phytoecdysteroids may enhance the resistance against stress [46, 48, 53].

However, some insect species remain unaffected by phytoecdysteroids present in their food even at concentrations of 400 ppm and more (e.g., *Heliothis virescens* or the tobacco budworm, *Heliothis armigera* or the cotton bollworm/corn earworm, *Locusta migratoria* or the migratory locust, *Manduca sexta* or a moth known as the tobacco hornworm, *Spodoptera littoralis* or the African/Egyptian cotton leafworm, *Lacanobia oleracea* or a moth called the bright-line brown-eye, *Acherontia atropos* or the death's-head hawk moth). As shown in Figs. 127.3 and 127.4 the following detoxification and inactivation mechanisms, respectively in insects and mice, have been described to detoxify phytoecdysteroids:

- Conjugation to fatty acids, thereby blocking the C-22 hydroxyl group. This mechanism is dependent on the amount of fatty acids in the environment. In case of low concentration, insects will be exhausted sooner by spending their own energetic capacity.
- Instead of fatty acids, glycosidation (C-22, C-25), phosphorylation (C-2 or C-22), acetylation (C-3), and 3-oxo/3-epi derivatization can take place.
- Side-chain cleavage is done between C-20 and C-22.
- Excreting unmetabolized hydroxyecdysone is also seen.

The phytophagous behavior of the insect plays also an important role. Insects tolerant to phytoecdysteroids will not change their phagous behavior. Polyphagous insects can avoid plants containing higher levels of phytoecdysteroids, whereas insect monophagous for ecdysteroid-containing plants will be the most sensitive. Insects can even defend themselves by developing taste receptors, which facilitate avoiding ecdysteroid-containing plants in their diet, at least when they have the choice [53, 54].

Overall, the above-described metabolizations weaken considerably the position of plant ecdysteroids as exogenous pest control agents and divert the attention to possible therapeutic applications in higher species.

6 Pharmacology of Phytoecdysteroids

6.1 Pharmacology of Phytoecdysteroids

The literature reporting the effects of ecdysteroids on mammals has been reviewed by several researchers and summarized by Dinan [56] and Lafont and Dinan [53], and it can be concluded that beneficial pharmacological activities can lead to possible therapeutic applications. They have a higher safety margin as compared to chemical steroid derivatives [51].

6.2 Toxicology of Phytoecdysteroids

Although detailed toxicological data are lacking for phytoecdysteroids, their acute toxicity to mammals seems to be extremely low [46]. In mice, LD_{50} values of 20-hydroxyecdysone after i.p. injection and oral application were 6.4 g/kg body weight and 9 g/kg body weight, respectively. Furthermore, 20-hydroxyecdysone was not embryotoxic when injected into developing chicken eggs [55], [56].

6.3 Adaptogenic Activities

Ecdysteroids are believed to possess adaptogenic, antidepressive, tonic, and roborant properties, i.e., they enhance the ability to cope with stress and enhance resistance to tiredness. Certain plants (*Achyranthes* spp., *Cyathula* spp., *Leuzea carthamoides*) have been used as tonics, diuretics, and adaptogens in traditional Chinese and Asiatic medicines long before they were known to contain large amounts of ecdysteroids [57].

Koudela et al. [58] studied adaptogenic activity in Japanese quails. The animals were fed with a diet containing different amounts of pulverized seeds of the plant species *Leuzea carthamoides*. These seeds contain 1.8 %–2.1 % of 20-hydroxyecdysone and some smaller amounts of other ecdysteroids. In an additional experiment, the quails were administered whole seeds of *L. carthamoides* ad libitum. The basic experimental setup involved testing of (0) a standard diet (SD), (1) SD supplied with 0.2 % *L. carthamoides* seed powder, (2) SD supplied with 1 % *L. carthamoides* seed powder, (3) SD supplied with 5 %

L. carthamoides seed powder, (4) SD supplied with 5 % *L. carthamoides* seed powder + 5 % Biostrong (= biostimulating preparation for birds), (5) SD supplied with 5 % *L. carthamoides* seed powder + 5 % Ecovit (= biostimulating preparation for birds), and (6) SD supplied with whole seeds of *L. carthamoides* ad libitum.

After 37 days, the living mass increase was measured and expressed toward the control group (= 100 %) as follows: (1) 102.8 %, (2) 109.5 %, (3) 120.4 %, (4) 101.9 %, (5) 104.9 %, and (6) 103.5 %. These results show a dose-dependent anabolic effect in the birds. Anabolic effects of *L. carthamoides* were also seen in in vitro studies with preparations from liver and other organs of mice. However, it cannot be excluded that other secondary metabolites are also contributing to the effect. The results of radioimmunoassay analyses revealed that the amount of 20-hydroxyecdysone circulating in the blood of Japanese quails was proportional to the amount of dietary ecdysteroid. However, a direct correlation between these circulating levels and the anabolic effect remains unsure, as the highest concentrations were found in the quails which consumed the whole seeds of *L. carthamoides*, whereas this group did not have the highest increase in living mass [58].

Leuzea carthamoides is the basis of a green "tea," maralan, consumed extensively in Central Europe and said to improve general well-being, increase appetite, and improve digestion. Although these effects have been repeatedly ascribed to the ecdysteroid content of the plant, there is little evidence to directly associate them with this particular class of compounds [56].

6.4 Stimulation of Protein Synthesis

Otaka et al. [59] investigated the effect of 20-hydroxyecdysone on protein synthesis in mouse liver. The ecdysone increased markedly the incorporation of ^{14}C-chlorella hydrolysate into hot-acid insoluble protein, within 2 h after treatment. This effect is comparable to 4-chlorotestosterone. The effect was exerted on microsomes or polysomes. The stimulation induced by 20-hydroxyecdysone was partly insensitive to inhibitors of DNA-dependent RNA synthesis like actinomycin, whereas the stimulation by 4-chlorotestosterone was completely repressed by the same actinomycin. This may indicate other mechanisms involved or a partial protection against actinomycin.

6.5 Effect on Hyperglycemia

Ecdysterone, isolated from *Achyranthes fauriei*, has been recognized to have a suppressive effect on induced hyperglycemia. The administration of ecdysterone did not alter the blood glucose of normal animals, but pretreatment with ecdysone prior to hyperglycemic agents suppressed the hyperglycemia induced by glucagon at low levels. The effect of ecdysterone was also shown in alloxan-diabetic mice, with blood glucose levels reduced to about one-half of the value observed before the administration of ecdysterone. Ecdysterone stimulated the incorporation of ^{14}Cglucose into protein of normal mice liver and into glycogen of normal and mildly diabetic mouse liver [60]. Ecdysterone had a positive influence on insulin resistance induced by injections of hydrocortisone or insulin insufficiency caused by alloxan treatment in rats. The sensitivity toward intravenous administration of insulin was enhanced, but this enhancement was only indirectly measured by nonspecific synthesis of total proteins in cells, rather than by an increase in insulin secretion [61].

Sundaram et al. [62] more specifically described the influence of 20-OH-ecdysone on hepatic key enzymes of carbohydrate metabolism. The ecdysone was isolated from the plant species *Vitex negundo*. Oral administration of 5 mg ecdysone per kg body weight per day to streptozotocin diabetic rats for 30 days decreased the plasma glucose with more than 50 % and the glycosylated hemoglobin (HbA1c) with about 40 %. At the same time, the levels of insulin doubled and the hemoglobin normalized as compared to the control rats. The glycogen content of the liver and the skeletal muscles was enhanced, but not completely normalized by 20-OH-ecdysone. There was also an increase in the enzyme activity of hexokinase and glucose-6-phosphate dehydrogenase. These activities point to an interesting molecular mechanism of 20-OH-ecdysone, altering hormone secretion in case of induced hyperglycemia [62].

6.6 Cholesterol Homeostasis

The influence of phytoecdysteroids on cholesterol biosynthesis is more speculative. It is known that there exist negative feedback control mechanisms by steroids. Possible targets are the hydroxymethylglutaryl-CoA reductase or cholesterol catabolism. The presence of hydroxyl groups may play an important role in the activity [63].

6.7 Immunomodulatory Activity

Ecdysterone is able to stimulate the primary immune reaction. The activity is based upon a cellular activity, with enhanced T cell immunity and phagocyte activity when doses between 5 and 20 mg/kg are given to mice. When the dose is increased to 50 mg/kg, the number of antibody cells in the mouse spleen was marked [64]. These doses, however, are huge when calculated as the amount of extract to be made of phytoecdysteroid-containing plants.

In *Ajuga remota*, they can contribute to a possible antiplasmodial activity by their anabolic, adaptogenic, and immunoprotective activities. According to Dinan [56], ecdysteroids may show some immunomodulatory effects in mice and rats and have anti-inflammatory activity in rodents.

The immunomodulatory effect of 20-hydroxyecdysone has been studied in humans. It acts as a lymphocyte and neutrophil modulator in vivo. In vitro, it activates T cell CD2 presentation that is suppressed both in secondary immunodeficient persons and pharmacologically by increased cAMP levels [65].

6.8 Antiradical/Antioxidative Properties

Studying of and reporting on antioxidative properties of plants and their components are very popular. So it seems quite obvious also to investigate phytoecdysteroids in this field. Several ecdysterone concentrations (10^{-6}–10^{-3} M) were tested in a photochemical system, containing phosphatidylcholine liposomes challenged with Fe^{2+} and Fe^{3+}. The antioxidative activity of ecdysterone was comparable to diethylparaphenylenediamine and ethylenediamine tetra-acetate, both known as inhibitors of peroxide oxidation of lipids [66].

6.9 Synergism with Vitamin D

Experimental vitamin D hypovitaminosis induces changes in the structural and functional characteristics of liver nuclear chromatin fractions. DNA-polymerase activity increases, as well as the activity of the fraction enriched with RNA-polymerase I. Free-radical lipid peroxidase-mediated reactions are preferentially modified in the chromatin fraction with low activity. Moreover, the protein and lipid components of chromatin are changed. Administration of ecdysterone preparations partially restores the structural and functional organization of chromatin, whether it is separately or concomitantly given. Lipid peroxidase is normalized. In general, administration of ecdysterone mimics the activity of vitamin D3 with regard to the normalization of the biochemical indices [67].

Dittrich et al. [68] sought a suitable dosage form with continuous release of 20-hydroxyecdysone from a device implanted in animals. They made a binary mixture of poly-lactic acids and the ecdysone. Release of the active compound was tested in static in vitro conditions, comparable with those at the site of possible implantation. Prolonged drug release of nearly zero-order kinetics was obtained with hollow implants. These types of implants appear most appropriate for testing in vivo [68].

6.10 Antitumoral Activity

A lipophilic root extract of *Leuzea carthamoides* (Maral root), as well as 20-hydroxyecdysone, was tested in human breast adenocarcinoma MCF-7 cells. Cell proliferation was inhibited by the extract with an IC_{50} of 30 μg/mL; 20-hydroxyecdysone did not show inhibitory activity. It is not clear to what extent phytoecdysteroids from *Leuzea carthamoides* were responsible for the activity. Products derived from the roots of this plant are promoted as dietary supplements with adaptogenic and anabolic activity, because of their ecdysteroid content [69].

6.11 Dermatology and Cosmetology

Use of phytoecdysteroids in dermatology seems to be most promising. This sector offers perspectives by its more easy therapeutic access, especially in the field of topical applications.

Meybeck et al. [70] took a patent on the use of ecdysteroids as active substances incorporated into liposomes. The patent contains a non-limitative list of plants that can be used: *Achyranthes aspera*, *Achyranthes bidentata*, *Ajuga decumbens*, *Ajuga iva*, *Bombyx mori*, *Cyanotis arachnoidea*, *Cyathula officinalis*, *Cyathula capitata*, *Leuzea carthamoides*, *Lychnis flos-cuculi*, *Paris axialis*, *Paris dunniana*, *Paris fargesii*, *Paris polyphylla*, *Paris vietnamensis*, *Pfaffia iresinoides*, *Pfaffia paniculata*, *Polypodium vulgare*, *Rhaponticum integrifolium*, *Serratula sogdiana*, *Serratula tinctoria*, *Sesuvium portulacastrum*, *Silene brahuica*, *Silene dioica* (*Melandrium rubrum*), *Silene nutans*, *Silene otites*, *Silene scabrifolia*, *Silene tatarica*, *Silene traemixta*, *and Vitex glabrata*. Preferential plant extracts are those of *Achyranthes bidentata*, *Ajuga decumbens*, *Cyanotis arachnoidea*, *Leuzea carthamoides*, and *Polypodium vulgare*. The species *Rhaponticum integrifolium* and *Serratula sogdiana* have the advantage that they can be cultivated. Extraction procedures are described according to Imai et al. [71]. Examples of formulations are given with different indications: restoring the water barrier of the epidermis, treatment of psoriatic skin, hydration of the epidermis, re-equilibration the desquamation of the cornified layer of the epidermis and restoring the smoothness of the epidermis, and preventive treatment of dry skin treatment of ichthyotic skins. The formulations are prepared with pure ecdysterone, ecdysterone esters, or plant extracts [70]. Some of the properties are documented in the public domain, like experimental findings on in vitro keratinocyte differentiation by 20-OH-ecdysone [72].

Inaoka et al. [73] studied the effects of 80 herbs on hair growth, using normal C-3H/He mice from which telogen hair on the back had been removed. *Polyporus umbellatus* emerged as one of the active species that promoted hair regrowth. A 50 % ethanol extract of *Polyporus umbellatus* was fractionated by consecutive column chromatography. Testing of the fractions on the hair regrowth assay resulted in the isolation of three active principles: acetosyringone, polyporusterone A, and polyporusterone B [74]. The structure of the hair regrowth polyporusterone A was further studied. The NMR data indicated that the side chain has the same conformation in both the crystalline state and solution [75].

Meybeck and Yang [76] took a patent on compositions containing an ecdysteroid, an ecdysteroid derivative, or a natural plant or animal extract containing the same compounds used in cosmetics, pharmaceutics, or as a natural supplement. The patent is related to the use of the compositions to stimulate the natural defenses of cells, such as protein p53, against the consequences of being exposed to ultraviolet rays, in particular solar radiation.

6.12 Anabolic Effects

The backbone of phytoecdysteroids is identical to that of steroid hormones, which led to the comparison of their anabolic effects. As summarized by Sláma and Lafont [57], 20-hydroxyecdysone increased physical performance without training in a forced swimming test in mice and also caused anabolic increase of body mass. Stimulation of protein synthesis by up to 20 % in mouse and human skeletal muscle cells by ecdysteroids (20-hydroxyecdysone, polypodine B, and ponasterone) has been demonstrated [77].

According to other researchers, 20-hydroxyecdysone increased body mass and muscle fiber size in rats and may provide an alternative to anabolic–androgenic steroids in the treatment of muscle atrophy [42]. In contrast to anabolic vertebrate steroid hormones, the anabolic actions of 20-hydroxyecdysone are supposedly not associated with the adverse androgenic, antigonadotropic, or thymolytic side effects. Phytoecdysteroids also appear not to have androgenic or (anti)estrogenic effects [57].

Increase in muscle size is one of the most cited aspects of phytoecdysteroids on the Internet [78]. However, stringent research is needed, as in a double-blind, placebo-controlled trial 30 mg/day of 20-hydroxyecdysone did not significantly affect anabolic or catabolic response to resistance training, body composition, or training adaptations [79]. On the other hand, it cannot be excluded that phytoecdysteroids may have an anabolic influence on the long term. Kumpun et al. [80] found that they are concentrated in the bran of *Chenopodium quinoa*, an Andean crop plant. Most of the 20-hydroxyecdysone was recovered undegraded from the seeds, even after 20 min boiling. Humans who eat quinoa regularly could have some anabolic benefit from their eating habits [80].

7 Conclusions

Although promising therapeutic agents, phytoecdysteroids remain in the experimental pharmacological field. The steroid skeleton is a basic template on which nature continuously varies to make new entities. This may be a hampering factor for chemically synthesizing new substances that can be patented.

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Brassinosteroids and their Biological Activities

128

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Contents

1 Introduction 3852
2 Chemical Structures of the Brassinosteroids 3853
3 Biosynthetic Pathway 3854
4 Brassinosteroid Signal Transduction 3856
5 Biochemical Analysis of Natural Brassinosteroids 3858
5.1 Bioassays 3858
5.2 Gas Chromatography–Mass Spectrometry (GC–MS) 3859
5.3 High-Performance Liquid Chromatography (HPLC) 3859
5.4 Liquid Chromatography–Mass Spectrometry (LC–MS) 3860
6 Biological and Pharmacological Activities 3861
7 Agronomical Uses 3864
8 Conclusion 3866
References 3866

Abstract

Brassinosteroids are endogenous plant polyhydroxysteroids that are essential for normal plant growth and development. They induce cell elongation and division, increase DNA and RNA polymerase activity, interact synergistically with

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auxins, stimulate ethylene production, and increase tolerance to temperature, water, and salinity stress. They are observed in many plant species and are present in nearly every part of the plant, although the highest concentrations occur in the reproductive organs. In mammalian cells, brassinosteroids inhibit cell growth and affect cell cycle progression; as such, they have potential for development as anticancer agents. Moreover, brassinosteroids and their synthetic derivatives possess antiangiogenic properties that could be effective in the treatment of any carcinoma. As such, they are an interesting source of lead compounds for developing novel natural product-derived anticancer drugs, and their properties have attracted the attention of many specialists in the fields of chemistry, biochemistry, pharmacology, plant physiology, and agriculture.

Keywords

Anticancer activity • apoptosis • bioassay • biosynthesis • brassinosteroids • HPLC/MS • signaling pathway • stress protection

Abbreviations

24-epiBL	24-epibrassinolide
28-homoBL	28-homobrassinolide
28-homoCS	28-homocastasterone
BL	Brassinolide
BRs	Brassinosteroids
CS	Castasterone
FW	Fresh weight
GA_3	Gibberellic acid

1 Introduction

The role of steroids as mammalian hormones has been known since 1930, and steroidal hormones have also been found in insects and fungi. Plants can biosynthesize a large variety of steroids, but it was not known until 1979 that steroids with plant growth-promoting activity were discovered. In that year, Grove et al. [1] reported the discovery of a new steroidal lactone called brassinolide from the pollen of *Brassica napus* L. To date, more than 70 structurally and functionally related steroids have been isolated from plant materials [2]. These compounds have been identified as members of a new group of plant hormones – the brassinosteroids (BRs).

BRs have been detected in all plant organs, including pollen, anthers, seeds, leaves, stems, roots, flowers, and grains. They have also been found in other interesting tissues such as insect and crown galls, notably the galls of *Castanea crenata, Distylium racemosum*, and *Catharanthus roseus*. Pollen and immature seeds in particular tend to be especially rich source of BRs, whereas their concentrations in vegetative tissue are very low compared to other plant hormones. Pollen and immature seeds contain about 1–100 $ng.g^{-1}$ FW of BRs, while shoots and leaves usually have lower amounts (0.01–0.1 $ng.g^{-1}$ FW) [3].

BRs control many developmental and physiological processes in plants, including regulation of gene expression, cell division and expansion, germination, vegetative and reproductive development, vascular differentiation, root growth, programmed cell death, and homeostasis [4, 5]. In addition to their growth-regulating activities, BRs have been shown to have dynamic roles in protecting plants against biotic and abiotic stresses [6, 7]. Treatment with exogenous BRs raises the inner potential of treated plants, which promotes survival under stressful conditions and also reduces biotic stress caused by pathogens [5, 8].

The physiological concentrations of BRs in plants are extremely low ($ng.Kg^{-1}Fw$), and it can be very difficult to analyze their abundance in plant tissues. A wide range of methods are currently employed for the determination and quantification of brassinosteroids in plants, including bioassays, diverse chromatographic procedures, radioimmunoassays [9], and enzyme-linked immunosorbent assays [10–13]. The most widely used bioassays are the second bean internode bioassay and rice–lamina inclination test. These bioassays have been used in the isolation of brassinolide from rape pollen [1] and castasterone from chestnut insect galls [14]. Gas chromatography–mass spectrometry (GC–MS) analysis is the current standard technique for instrumental analysis of BRs [15–17].

Some medically oriented applications of BRs have been reported [8, 18, 19]. Wachsman et al. [8, 20] observed that some natural BRs (28-homoCS, 28-homoBL) and their synthetic analogs exhibit antiviral activity in vitro against several RNA and DNA viruses, including herpes simplex virus type 1 (HSV-1), arenaviruses, and measles virus (MV). Moreover, natural and synthetic brassinosteroids have been reported to cause growth inhibition, cell cycle arrest, and initiation of apoptosis in many different cancer cell lines. It has also been demonstrated that BRs can inhibit the proliferation and migration of human endothelial cells, and there is evidence that BR treatment triggers the initiation of cell death by apoptosis. Brassinosteroid analogs have proven to be more effective than natural BRs in this context. Importantly, BRs inhibit cell migration and tube formation, suggesting they may possess antiangiogenic activity [21–23].

2 Chemical Structures of the Brassinosteroids

All known brassinosteroids have a 5α-cholestane, 5α-ergostane, or 5α-sitostane steroidal skeleton with mono- to trioxygenation on ring A and 22α-, 23α-dihydroxylation in the side chain. Ring B may be fully saturated or may contain a ketone or lactone at carbon 6 (Fig. 128.1).

All natural BRs consist exclusively of carbon, hydrogen, and oxygen and have:

1. A normal or B-homo cholestane, ergostane, or sitostane steroidal skeleton
2. An oxygen-containing functional group at carbon 3 (α- or β-hydroxyl, ester, ether, or ketone)
3. An all-*trans* fusion between rings A/B, B/C, and C/D

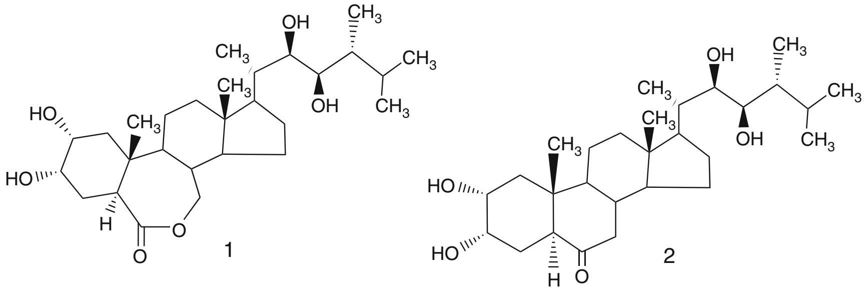


Fig. 128.1 Structures of brassinolide (1) and castasterone (2)

4. A 20R (or 20 β) configuration
5. α –*cis* (R,R) vicinal hydroxyls at carbons 22 and 23

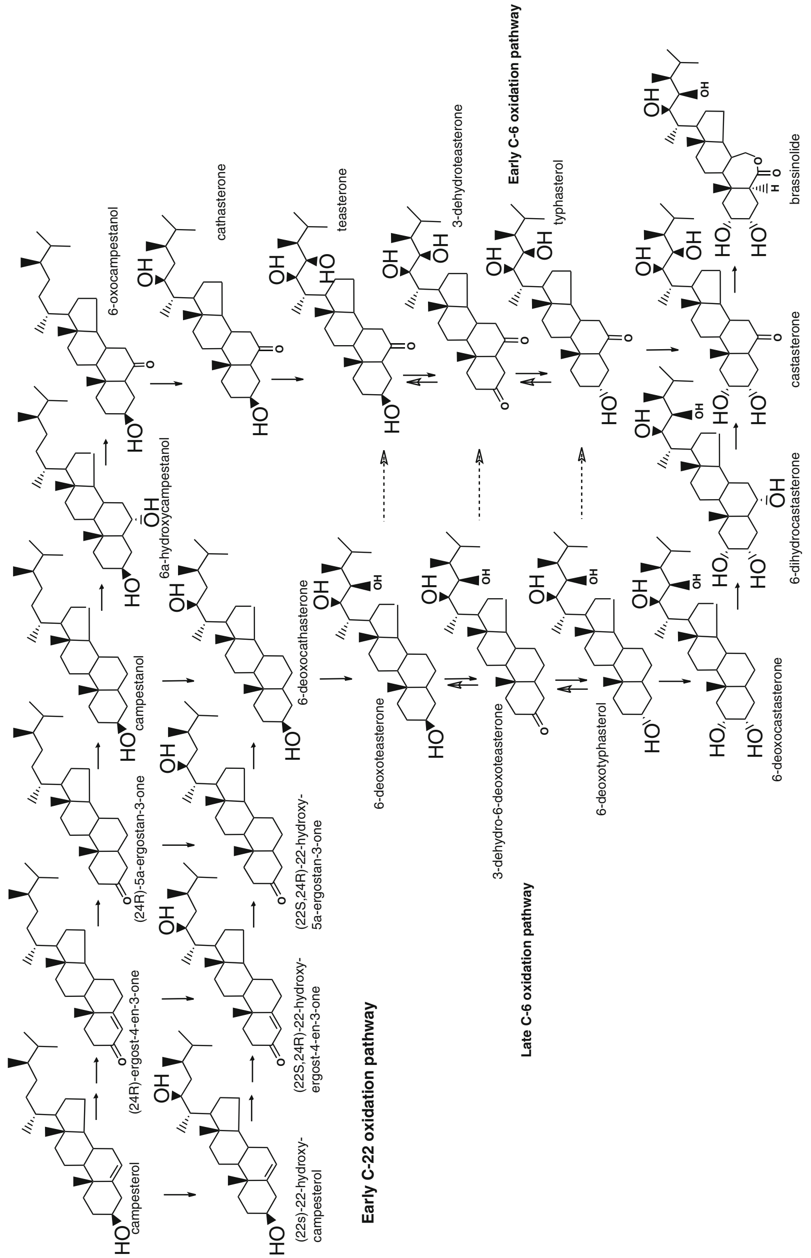
All of these compounds have an oxygen-containing functional group at carbon 3, none exhibit A/B *cis* ring fusion or have a carbon–carbon double bond between rings A and B, and all have at least 27 carbon atoms [24, 25].

3 Biosynthetic Pathway

BRs belong to the class of molecules known as triterpenoids. Because BRs are a group of modified sterols, the BR biosynthetic pathway can be divided into two major parts: the sterol-specific pathway (which converts squalene to campesterol) and the BR-specific pathway (which converts campesterol to the BR). In the sterol-specific pathway, mevalonic acid (which is the starting material in terpenoid biosynthesis) is condensed and cyclized to produce 2,3-oxidosqualene. This precursor is further modified to form the major plant sterols such as sitosterol and campesterol. To become bioactive BRs, sterols must be processed by the BR-specific pathway.

The biosynthetic pathways leading to brassinolide were initially elucidated using cultured *Catharanthus roseus* cells. Extensive metabolic studies suggested the operation of a series of parallel branched biosynthetic pathways that were named the early and late C-6 oxidation pathways (Scheme 128.1) [26–28]. Recent studies suggest that there is cross-talk between these parallel pathways, implying that they are not totally autonomous. The existence of an early C-22 oxidation branch in the BR biosynthetic pathway has also been demonstrated. It thus appears that BR biosynthetic pathways are highly networked [29–31].

As shown in Scheme 128.1, campesterol is first converted to campestanol, which is then converted to castasterone via either early or late C-6 oxidation. Finally, castasterone is converted to brassinolide. However, some of the steps involved have only recently been clarified. One such step, the conversion of 6-oxocampestanol to cathasterone, was demonstrated in cultured *Catharanthus roseus* cells [32], fully substantiating the pathway from campestanol to


campesterol
(24R)-ergost-4-en-3-one
(24R)-5a-ergostan-3-one
campestanol
6a-hydroxycampestanol
6-oxocampestanol
(22s)-22-hydroxy-campesterol
(22S,24R)-22-hydroxy-ergost-4-en-3-one
(22S,24R)-22-hydroxy-5a-ergostan-3-one
6-deoxocathasterone
cathasterone
Early C-22 oxidation pathway
6-deoxoteasterone
teasterone
3-dehydro-6-deoxoteasterone
3-dehydroteasterone
Late C-6 oxidation pathway
Early C-6 oxidation pathway
6-deoxotyphasterol
typhasterol
6-deoxocastasterone
6-dihydrocastasterone
castasterone
brassinolide
HO
OH
O
H

Scheme 128.1 Pathways of brassinosteroid biosynthesis (Modified by Fujioka S, Yokota T [105]) brassinolide via early C-6 oxidation. The campestanol → 6-deoxocathasterone → 6-deoxoteasterone sequence in the late C-6 oxidation pathway has also been established in *Arabidopsis* [31, 33], completing the elucidation of the late C-6 oxidation pathway. Ohnishi and coworkers proposed a novel shortcut in BR biosynthesis in *Arabidopsis*, which allows the direct conversion of early 22-hydroxylated intermediates to 3-dehydro-6-deoxoteasterone and 6-deoxotyhpasterol via C-23 hydroxylation [34]. In tomato and tobacco, the late C-6 oxidation pathway appears to be the primary route used because the only endogenous BRs in these species are produced via the late C-6 oxidation pathway [35, 36].

4 Brassinosteroid Signal Transduction

The perception of signals at the cell surface and the transduction of these signals to the cell's interior are essential in all life forms. In plants, this process is mediated by membrane-integral receptor kinases [37]. Brassinosteroid signaling (Fig. 128.2) and the resulting genomic response is initiated by the binding of a BR molecule to a receptor kinase, brassinosteroid-insensitive 1 (BRI1), which is localized in the plasma membrane. BRs bind to the extracellular leucine-rich repeat receptor kinase (LRR-RK) domain of BRI1. This triggers the phosphorylation of the intercellular serine–threonine kinase domain of BRI1, which in turn causes BRI1 to dissociate from the membrane-bound BRI1 kinase inhibitor 1 (BKI1) and to form a complex with a second receptor kinase, BRI1-associated receptor kinase 1 (BAK1). The active BRI1/BAK1 receptor kinase pair then propagates the signal downstream by inactivating a soluble kinase, brassinosteroid-insensitive 2 (BIN2), which is a negative regulator of BR signaling [39–42]. BKI1 is phosphorylated at a tyrosine residue in response to brassinosteroid perception; this mechanism is conserved in higher organisms and controls protein localization. The phosphorylated residue is part of a membrane-targeting motif and its modification releases BKI1 into the cytosol, allowing it to form an active signaling complex [43]. The structure of the *Arabidopsis thaliana* BRI1 ligand-binding domain has recently been published; it features a superhelix of 25 twisted leucine-rich repeats (LRRs) that is strikingly different to the assembly of LRRs in animal Toll-like receptors [37, 44]. A 70-amino-acid island domain between LRRs 21 and 22 folds back into the interior of the superhelix to create a surface pocket that contains the binding site for the plant hormone brassinolide. It seems that steroid binding to BRI1 generates a docking platform for a coreceptor that is required for receptor activation [37]. Irani et al. [45] used chemical and genetic approaches to interfere with the trafficking of the BRI1–BR complexes and examined their effect on BR signaling. They developed a bioactive, fluorescent BR analog (AFCS) and used it to visualize the endocytosis of BRI1–AFCS complexes in living *Arabidopsis* cells. These studies revealed that interference with clathrin- or ARF-GEF-mediated endocytosis of BRI1 enhanced BR signaling [45].

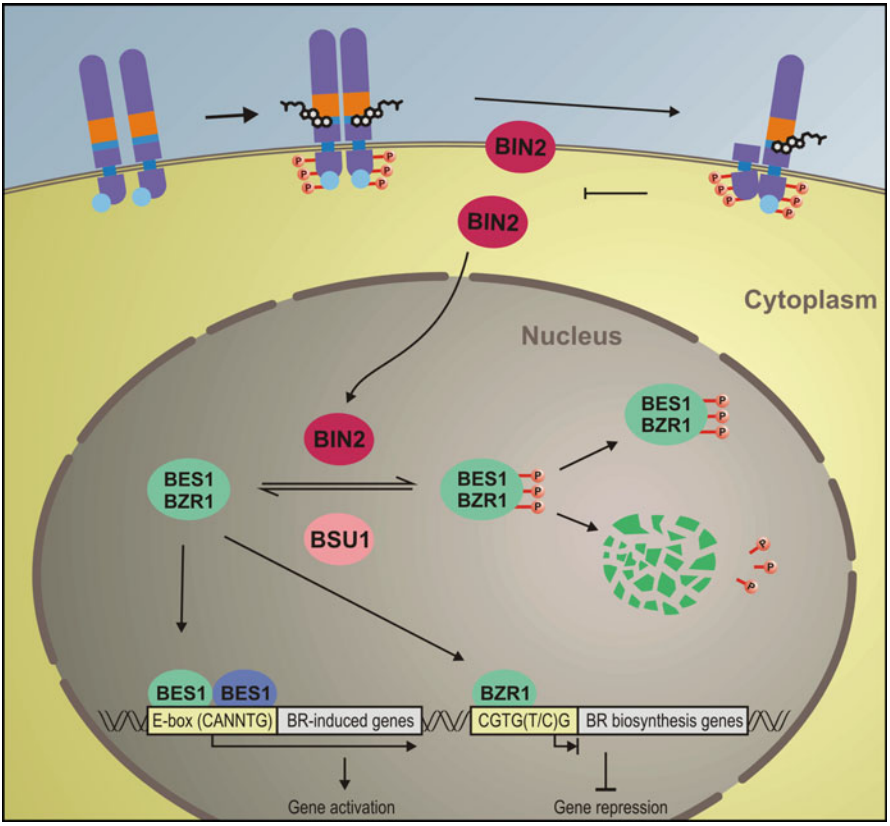


Fig. 128.2 Brassinosteroid signaling in the plant cell. BRs bind to the extracellular domain of brassinosteroid-insensitive 1 (BRI1), a leucine-rich repeat receptor kinase (LRR-RK) that is localized in the plasma membrane. This leads to phosphorylation of the intracellular serine–threonine kinase domain of BRI1, causing it to dissociate from the membrane-bound BRI1 kinase inhibitor 1 (BKI1) and to form a complex with a second receptor kinase, BRI1-associated receptor kinase 1 (BAK1). The active BRI1/BAK1 receptor kinase pair then propagates the signal downstream by inactivating a soluble kinase, brassinosteroid-insensitive 2 (BIN2), which is a negative regulator of BR signaling. BES1 (bri1-EMS-suppressor 1) and BZR1 (brassinazole-resistant 1) are phosphorylated by BIN2 and are closely related transcriptional activators of BR-induced genes. BSU1 (bri1 suppressor 1) counteracts the effects of BIN2 [38]

24-Epibrassinolide (24-epiBL) has also been shown to upregulate the transcription of an important positive cell cycle regulator gene, cyclin D3, which is involved in the activation of cell division by cytokinins in *Arabidopsis thaliana* L. [46]. No plant gene encoding an intracellular BR receptor has yet been identified in plants, although a chaperone heterocomplex that resembles an animal intracellular steroid receptor has been identified in wheat germ lysate [47]. Nevertheless, it is likely that there is an intracellular steroid hormone signaling pathway in plants that regulates gene transcription in a similar way to that found in animals [46].

5 Biochemical Analysis of Natural Brassinosteroids

5.1 Bioassays

The development of bioassays for isolating bioactive compounds from natural sources has played an important role in recent studies of natural BR phytochemistry. The development of highly sensitive and specific bioassays was essential for the isolation and purification of BRs from plant tissues because of the very low physiological concentrations of these hormones. The bean second internode assay was used to isolate BL from rape pollen [1], and the rice–lamina inclination test was used to isolate CS from chestnut insect galls [14]. Following the publication of these results, the rice–lamina inclination test has been widely used to isolate many BRs from various plant sources because of its simplicity, high sensitivity, and specificity for BRs [25].

5.1.1 The Bean Second Internode Bioassay

This test was first used for monitoring the biological activity of separated fractions during the isolation and purification of the "brassin-complex" [48–50] and later also for isolating pure BL [1]. Both gibberellins and BRs are active in this test, but the former cause only elongation, whereas BRs also cause swelling, increased curvature, and splitting of the internodes. Responses of this kind are considered indicative of "brassin activity" and are only observed for fractions containing BRs. The effect depends on the amount of BR applied. For example, swelling and increased curvature are only observed after treatment with at least 0.01 μg BL per plant. The sensitivity of the assay can be increased by omitting nitrate from and adding Ca^{2+} or Mn^{2+} to the solution in which the bean seedlings are grown [51].

5.1.2 The Rice-Lamina Inclination Bioassay

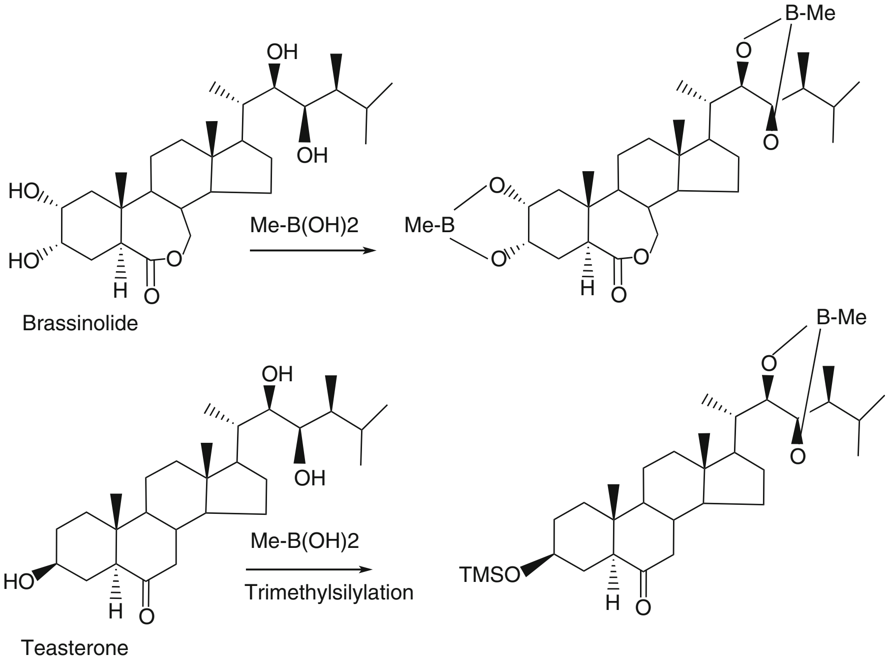
This bioassay is one of the most popular and useful tests for evaluating brassinosteroid activity. It was originally developed by Maeda [52] for gibberellins and indole-3-acetic acid (IAA). Two modifications were developed for use with brassinosteroids, one involving intact plants [53] and the other involving cuttings [54]. The assay is based on measuring the angles between the laminae and the sheaths after a period of incubation with the tested fraction; in the control segments, these angles are approximately 90°. In the presence of 0.0005 μg/ml BL, the angles between laminae and sheaths increased to around 140°; 0.005 μg/ml BL caused pronounced bending at the lamina joints to the abaxial side to the extent that some of the laminae came into contact with the sheaths. The activity of 28-homobrassinolide in this assay was comparable to that of BL, but the response induced by IAA was very modest even at 50 μg/ml [54]. Gibberellins also have only a modest effect on the inclination of the lamina in rice seedlings at a concentration of approximately 100 μg/ml. The sensitivity of the test depends on the rice cultivars used; out of 60 cultivars that were tested during its development, Arborio J-1 and Nihinbare were found to be the most reliable [55].

5.2 Gas Chromatography–Mass Spectrometry (GC–MS)

Brassinosteroids are highly polar and nonvolatile compounds. As such, it is generally necessary to convert them into more volatile derivatives prior to conducting gas-phase analyses. One such gas-phase analytical procedure that has proven useful when trying to identify BRs in a partially purified bioactive fraction is gas chromatography coupled to mass spectrometry in selected ion-monitoring mode (GC–MS-SIM). To facilitate their analysis by gas chromatography, BRs are typically converted into volatile bismethaneboronate (BMB) derivatives by treating them with methylboronic acid, which reacts specifically with vicinal diols (Scheme 128.2). This derivatization process makes it easy to separate the target compounds from contaminants that do not contain vicinal diols. The BMB derivatives are suitable for gas-phase analysis and also for the analysis of fragment ions in electron impact (EI) mass spectra. Furthermore, 24-epimers of BL and CS are also completely separated by capillary GC [15, 16]. In the case of 2-deoxy BRs such as teasterone and typhasterol, which have a vicinal diol in the side chain and single hydroxyl group on the A-ring, the diol moiety is first converted to the methaneboronate and then the remaining 3-hydroxyl group is trimethylsilylated with an appropriate silylating reagent to give a methaneboronate–trimethylsilyl (MB–TMS) derivative [56] (Scheme 128.2). The MB–TMS derivatives of teasterone, typhasterol, and their 28-homo and 28-nor analogs are well separated by capillary GC [56]. These derivatives have limit of detection (LOD) values in the nanogram range for full-scan EI-MS analyses and subnanogram LOD values for analyses by EI-GC in SIM mode.

5.3 High-Performance Liquid Chromatography (HPLC)

HPLC is one of the most frequently used analytical methods for the separation and analysis of BRs [57]. Because BRs have no suitable chromophore for detection, they are derivatized with prelabeling reagents that are suitable for use with ultraviolet (UV), fluorometric, or electrochemical detectors. Several boronic acid reagents that react with the vicinal diol groups of BRs have been used as prelabeling reagents, including naphthaleneboronic acid for UV detection [58], 9-phenanthreneboronic acid [59], 1-cyanoisoindole-2-m-phenylboronic acid [60] and (dansylamino)phenylboronic acid [61] for fluorometric detection, and ferroceneboronic acid for electrochemical detection [62]. The limits of detection for these methods range from 25 to 100 pg per injection, depending on the derivative used. The most effective of these reagents seems to be (dansylamino) phenylboronic acid because its chromophore can be detected at longer wavelength (excitation 345 nm/emission 515 nm) than other boronates, and so its signal is less susceptible to interference from matrix contaminants. The derivatized BRs are effectively separated by HPLC using ODS columns with acetonitrile–water as the mobile phase.



Scheme 128.2 Conversion of brassinolide to its bismethaneboronate derivative and teasterone to its methaneboronate–trimethylsilyl ether

Gamoh et al. [63] developed a HPLC method based on a combination of precolumn labeling and postcolumn fluorescence detection that can be used to separate and detect 24-epimers of BL and CS. Related reverse-phase HPLC methods have also been used successfully to analyze BRs in the pollen of broad bean, corn, sunflower, buckwheat, and orange [64–66].

5.4 Liquid Chromatography–Mass Spectrometry (LC–MS)

Microanalytical LC–MS methods for the analysis of BR-derived boronates have also been developed, using either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI). APCI-based LC–MS has been used to analyze naphthaleneboronate derivatives of BRs; in this case, optimal results were obtained by using a reverse-phase HPLC acetonitrile–water gradient to elute a C18 column [67]. Typical ions observed in the positive-ion spectra of the naphthaleneboronates included a pseudomolecular ion $[M + H]^+$ and a fragment ion $[M + H\text{-}H_2O]^+$. The most abundant ion from the fragmentation of BL derivatives is the parent ion, while the $[M + H\text{-}H_2O]^+$ ion is the most abundant in the mass spectra of CS, teasterone and typhasterol. Full-scan mass spectra were readily obtained from 400 ng of free BRs, whereas the limit of detection in SIM mode was around 2 ng. The most effective mobile phase for naphthaleneboronates was found to be a mixture of acetonitrile and water (9:1) containing 0.5 % HCOOH. Svatoš et al. [68] reported a highly sensitive and selective LC–(ESI)-MS method that was developed for analyzing BRs in plant extracts. The method involves using a microbore C18 column (1.0 mm) in conjunction with chemical derivatization of free BRs to dansyl-3-aminophenylboronates. This gives it a much lower limit of detection than can be obtained with previous analytical methods: its limit of detection for derivatized BRs in selected ion-monitoring (SIM) mode is 125 attomoles. The practical utility of the method was demonstrated in *Arabidopsis thaliana* plants that were fed with a deuterium-labeled precursor of CS and converted it to BL [68]. A highly sensitive electrospray ionization mass spectrometry (MS) technique (LOD 50 fmol) has been developed and used for direct analysis of natural brassinosteroids in different plant tissues [12]. This method was successfully used to detect and quantify brassinolide and castasterone in rape pollen and seedlings of *Daucus carota, Phaseolus vulgaris*, and *A. thaliana* [12]. Despite these advances, there is considerable scope for further improvements upon existing analytical methods and for reducing their LODs by using capillary LC systems coupled to a nano-ESI source. Additional improvements could be made by using ultra-performance liquid chromatography (UPLC) or by developing brassinosteroid-specific immunoaffinity chromatography techniques for the analysis of natural plant samples.

6 Biological and Pharmacological Activities

The best known and the most widely studied biological effect of BRs is their ability to stimulate plant growth in a variety of systems such as whole plants, excised segments, cuttings, and seedlings.

Brassinosteroids have been observed to stimulate seedling elongation in many assay systems, including normal and dwarf pea epicotyls, dwarf bean apical segments, mung bean epicotyls, and cucumber and sunflower hypocotyls [69]. In general, BRs stimulate elongation in the light but not in the dark [70]. Kamuro and Inada [71] examined the effects of light on the growth-promoting effects of BL using mung bean (*Vigna radiata)* epicotyls. Cuttings with attached cotyledons underwent epicotyl elongation in the dark and also under monochromatic blue light (452 nm) or far-red light (722 nm). BL did not promote epicotyl growth under any of these conditions. Epicotyl growth was retarded by white light (400–700 nm) and monochromatic red light (660 nm), and the growth-promoting effects of BL were clearly observed under these light regimes. Cuttings that were alternately irradiated with red and far-red light exhibited retarded epicotyl growth if the treatment ended with exposure to red light; BL treatment overcame this retardation. These results indicated that the effects of BRs are related to phytochrome-mediated growth regulation.

Brassinosteroids have also been observed to inhibit adventitious root development in mung bean hypocotyls [72]. Subsequent work reviewed by Roddick et al. [73] indicated that this inhibition might not be a primary action of BRs and may instead be due to differences in the optimal concentration of BRs for adventitious root development and shoot elongation. When soybean (*Glycine max* L.) hypocotyl segments were produced under photoperiods of 16-h light and 8-h darkness, adventitious roots were induced by treatment with 24-epiBL at the very low concentration of 0.0001 μg/ml during the dark periods. There is also evidence that BRs may regulate root hair development: Kappusamy et al. [74] reported that the levels and patterns of expression for two master epidermal patterning regulators (WEREWOLF and GLABRA2) are regulated by BRs and that plants with BR-related mutations exhibit abnormal root hair development.

The effect of BRs on cell division is somewhat controversial. In carrot (*Daucus carota* L.) and tobacco cell cultures, BRs induced cell enlargement without any effect on cell division [75, 76]. In a culture of *Zinnia elegans* L. mesophyll cells, the inhibition of endogenous GA_3 and BR biosynthesis by treatment with uniconazole retarded tracheid differentiation, which could be restored by treatment with BL or 28-homoBL (but not by GA_3) [77]. BL is essential for entry into the final stage of tracheary element differentiation, where secondary wall formation and cell death occur [78]. Using tuber explants of *Helianthus tuberosus*, Clouse and Zurek [79] also showed that BL stimulates xylem differentiation. Under normal conditions, xylem differentiation occurred only after 3 or 4 days' incubation of the explant on xylem-inducing media. However, when BL was added at a concentration of $6.8.10^{-9}$ M, significant xylem differentiation was observed after 24 h. This evidence suggests that BRs may influence cytodifferentiation in plants [80]. While investigating the potential role of BRs in regulating cell division, Hu et al. [46] found that treating *Arabidopsis* seedlings with 24-epiBL can upregulate the expression of CycD3, a D-type plant cyclin gene that may promote cell division.

Many studies have suggested that BRs play essential roles in responding to various stresses such as abnormal temperatures, drought, high osmotic pressure, and pathogen attacks [81, 82]. The occurrence of cross-talk between BRs and stress-responsive hormones such as abscisic acid, jasmonic acid, and ethylene is consistent with the suggestion that BRs play an important role in plant stress responses [83].

The ability of BRs to enhance plant resistance to various external stresses has been investigated with a view to finding applications for them in agriculture. Because temperature changes are likely to occur more rapidly than other stress-causing factors in nature, many studies in this area have focused on temperature stress. Maize seedlings are highly sensitive to chilling stress during germination and the early stages of growth. Treatment with BRs promoted growth recovery in maize seedlings that had previously been chilled. BRs also promoted the greening of etiolated leaves at low temperature in light [84]. Schilling et al. [85] examined the effects of 28-homoBL on sugar beet (cv. Ponemo) under drought stress. Treatment with 28-homoBL caused an increase in taproot mass, sucrose content, and sucrose yield under drought stress; no such effect was observed under non-stress conditions. Brassinosteroids have also been shown to activate total protein synthesis in wheat leaves under high-temperature stress. BR-treated leaves that were maintained at 43 °C exhibited protein synthesis levels similar to those observed at 23 °C.

However, in untreated leaves maintained at 43 °C, protein synthesis decreased by a factor of 2.5 relative to control samples at 23 °C [86]. In plant cells under heat stress, small heat shock proteins aggregate to produce highly ordered cytoplasmic complexes known as heat shock granules (HSGs). In wheat leaf cells, HSGs aggregate further to form small clusters. The average number of HSGs in the clusters and the average cluster size were both higher in BR-treated leaves than in untreated leaves [86].

The ability of BRs to enhance plant resistance to infection by fungal pathogens has also been investigated [87]. It was demonstrated that potato plants sprayed with BR solution were less prone to infection by *Phytophthora infestans*. The increase of resistance in BR-treated potato tubers was associated with elevated abscisic acid and ethylene production as well as increased levels of phenolic and terpenoid substances. In some investigations, BRs proved to be more effective than standard fungicides at protecting against fungal infection. However, certain concentrations of BRs and treatment with BRs at certain plant developmental stages can stimulate fungal growth and disease progression. As such, it would be necessary to carefully consider the timing of BR application, the concentration applied, and the method of application when developing a potential BR-based fungicidal treatment [88].

The brassinosteroids are essential for many growth and development processes in plants. However, relatively little is known about the effects of BRs and their synthetic analogs on animal cells [89]. It has been reported that cultured hybridoma mouse cells treated with 24-epiBL exhibit certain interesting effects, including an increase in the mitochondrial membrane potential, a decrease in the abundance of intracellular antibodies, an increase in the fraction of the cells in the G_0/G_1 phase, and a corresponding decrease in the proportion of cells in S phase. Moreover, treatment with 24-epiBL at concentrations of 10^{-13} and 10^{-12} mol/l significantly increased the density of viable cells relative to controls [90].

It has recently been reported that the natural BRs 24-epiBL and 28-homoCS have significant effects on the growth and viability of various normal and cancerous cell lines [23]. 28-HomoCS and 24-epiBL were shown to affect the viability of human cancer cell lines of various histopathological origins, but not human fibroblasts (BJ). Cell lines tested included the T-lymphoblastic leukemia CEM, breast carcinoma MCF7, lung carcinoma A-549, chronic myeloid leukemia K562, multiple myeloma RPMI 8226, cervical carcinoma HeLa, malignant melanoma G361, and osteosarcoma HOS. Treatment with 28-homoCS and 24-epiBL resulted in potent, dose-dependent reductions in the viability of CEM and RPMI 8226 cells, although the severity of these effects differed between the two compounds.

Estrogen- and androgen-sensitive and -insensitive breast and prostate cancer cell lines were shown to respond differently to treatment with natural BRs. Most breast cancers consist of a mixture of estrogen-sensitive and estrogen-insensitive cells, and it seems that it is necessary to eliminate both cell types in order to control breast cancer. Hormone-sensitive cell lines were more susceptible to BR treatment. This finding may indicate that natural BRs are capable of modulating the steroid receptor response in human cancer cells. Natural BRs were found to be cytotoxic to cancer cells but not to untransformed human fibroblasts, suggesting that they induce different responses in cancerous and normal cells. As such, these plant hormones may be useful leads for developing novel anticancer drugs [23]. There is also evidence that brassinosteroids may perturb cell cycling in breast and prostate cancer cell lines. Using flow cytometry, it was shown that treatment with 28-homoCS and 24-epiBL [104] induced a blockage in the G_1 phase of the cell cycle in breast and prostate cell lines, with concomitant reductions in the percentage of cells in the S phase of the cell cycle [23]. In the MCF7 breast cancer cell model (which is the most widely studied experimental system in this context), this response mirrors the typical response to antiestrogens, which also reduce the proportion of cells that are actively synthesizing DNA (i.e., cells that are in the S phase) and concomitantly increase the proportion of cells in the G_0/G_1 phase [91].

Angiogenesis, i.e., the growth of new blood vessels in animals, is essential for organ growth [92] and for the growth of solid tumors and metastasis [93]. Potent angiogenic inhibitors capable of blocking tumor growth are thus promising leads for the development of a new generation of anticancer drugs [94]. Recently, several steroids (i.e., 2-methoxyestradiol, progestin, medroxyprogesterone acetate, and glucocorticoids such as dexamethasone and cortisone) have been shown to have antiangiogenic activity [95]. Until recently, nothing was known about the effects of natural BRs on angiogenesis or other processes in endothelial cells. A study was therefore conducted to investigate the effects of naturally occurring BRs and their synthetic analogs on cell proliferation and cycling in human microvascular endothelial or umbilical vein endothelial cells, with a particular emphasis on the migration and formation of tubes within these cells [89]. Although the antiangiogenic activity of natural BRs was weak, it was found that the synthetic BR-derivative cholestanone significantly inhibited angiogenesis in endothelial cells in vitro. At a concentration of 30 μM, cholestanone reduced migration to 38 % relative to untreated cells and decreased the number of tubes by 34 % relative to the control treatment. As such, the BR-derivative cholestanone was a significantly more potent inhibitor of migration and tube formation than natural brassinosteroids [96].

Brassinosteroids seem to exert their effects on growth and the cell cycle effects via the cell cycle machinery and apoptosis and may thus be useful leads in the development of novel agents for managing cancer. The antiangiogenic effects of BRs may also prove useful for controlling tumor growth and metastasis, potentially paving the way for the development of multiple novel phytohormone-derived anticancer drugs [89, 96].

7 Agronomical Uses

BRs have strong effects on plant growth and possess a unique combination of physiological actions that have considerable potential for practical applications. Their potential economic value as agents for increasing crop yields has been discussed previously [97]. Their applications in agriculture and horticulture would be based on their ability to stimulate plant growth and enhance plant

Table 128.1 Selected results from brassinosteroid field trials

Plant	BR applied, dose, mode of treatment	Yield enhancement (%)	References
Wheat	24-epiBL, 4 mg/ha, spraying at flowering	16	[100]
Corn	24-epiBL, 50 mg/ha, spraying at emergence of tassel	10	[101]
Soybean	24-epiBL, 50 mg/ha, spraying at flowering	5	[100]
Potato	24-epiBL, 20 mg/ha, spraying at budding	18	[102]
Tomato	24-epiBL, 25 mg/ha, spraying at flowering	33	[103]
Sugar beet	24-epiBL, 50 mg/ha, spraying at 2–3 leaves	7	[102]

resistance to various external stresses. These effects could make it possible or attractive to grow crops under otherwise-unfavorable conditions such as in areas that have high salinity, are undergoing drought, or have an insufficient nutrient supply. Moreover, BRs have some specific properties that could be very valuable in practical applications [87]. These include:

1. BRs are natural products and are ubiquitous in the plant kingdom. As such, they are a long-standing component of the food chains of men and mammals; the biosynthetic and metabolic pathways of plants and mammals have coevolved over a long period of time and are heavily interconnected.
2. Plants respond to very small doses of BRs (5–50 mg/ha) that are comparable to those that occur naturally.
3. As plant growth-promoting substances, BRs have a broad spectrum of stimulatory and protective activities, all of which should have positive effects on the quantity and quality of crop yields.
4. BRs increase plant resistance to phytopathogens and can be used as substitutes (total or partial) for some traditional pesticides. Their use in this way would reduce the need to use pesticides that interact unfavorably with the environment.
5. BRs can be easily applied to plants and seeds using existing equipment and technologies.

The most commonly used BR in field trials is 24-epibrassinolide because of its high biological activity and relatively simple and synthesis. Treatment with 24-epiBL has been reported to increase yields of wheat, tobacco, rape, orange, grape, and sugar beet [98, 99]. Moreover, the application of 28-homoBL and 24-epiBL to potato plants at a dosage of 10^{-20} mg.ha^{-1} increased yields by 20 % and improved the quality of the crop by decreasing its nitrate content and increasing its content of starch and vitamin C [87]. Some other selected results from BR trials with different agricultural plants are presented in Table 128.1.

The influence of BRs on development and crop yield in different crop plants such as cereals, legumes, vegetables, and fruits has been tested under both laboratory and field conditions. For large-scale field applications, there are two viable methods for applying BRs: soaking seeds and foliar spraying. The performance of these two methods has been investigated in depth; the results obtained with foliar spraying proved to be very dependent on the stage in the plants' development at which it was applied. In general, the best results were obtained when young plants were treated [87].

8 Conclusion

Brassinosteroids are now firmly established as essential regulators of plant growth and development that affect a broad spectrum of processes at the molecular, cellular, and physiological levels. BRs are natural nontoxic, non-genotoxic, biosafe, and eco-friendly plant products and can therefore be used in agriculture and horticulture to improve the growth, yields, quality, and tolerance of various plants to biotic and abiotic stress.

Over the past decade, genetic screening has been widely used for identifying and characterizing major components and genes involved in the BR signaling pathway. However, the molecular mechanisms of these processes remain largely unknown. As such, there is a need for additional research to understand the mechanisms by which BRs affect plant and animal cells on the molecular level.

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Part XIV

Terpenes: Parapharmaceutical Uses

Lycopene in Cancer Prevention 129

Kazim Sahin and Omer Kucuk

Contents

1 Introduction 3878
2 Lycopene 3878
2.1 Mechanism of Lycopene in Cancer Prevention 3878
3 The Protective Role of Lycopene in Cancer Prevention 3886
3.1 Lung Cancer 3886
3.2 Gastric Cancer 3888
3.3 Breast Cancer 3890
3.4 Hepatocellular Carcinoma 3892
3.5 Pancreatic Cancer 3893
3.6 Colorectal Cancer 3894
3.7 Bladder Cancer 3896
3.8 Head and Neck Cancer 3897
3.9 Prostate Cancer 3899
3.10 Renal Cancer 3902
3.11 Leiomyoma 3903
4 Chemotherapy and Lycopene 3904
5 Radiation Therapy and Lycopene 3909
6 Conclusion 3910
References 3910

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Abstract

Lycopene, a red-colored carotenoid, present in many fruits and vegetables, including tomatoes and their processed products, watermelon, guava, carrots, pink grapefruit, and sweet potatoes, is inversely associated with the risk of many cancers. Unlike beta-carotene, lycopene lacks a beta-ionone ring and therefore has no provitamin A activity. However, the 11 conjugated and two nonconjugated double bonds in lycopene make it highly reactive toward oxygen and free radicals. Lycopene has been proposed to protect against cancer through various properties including decreased lipid oxidation, inhibition of cancer cell proliferation, and most notably potent antioxidant properties, apoptosis, increased gap-junctional communication, interferences in insulin-like growth factor 1 receptor signaling pathways, and cell cycle progression. Preclinical studies and clinical trials show that lycopene has potent in vitro and in vivo antitumor effects, suggesting potential preventive and therapeutic roles for lycopene. Further mechanistic studies and randomized controlled clinical intervention trials with lycopene in cancer patients are warranted. In this chapter, cancer prevention using lycopene is reviewed and the possible mechanisms of action are described.

Keywords

Cancer • lycopene • molecular mechanism • nutrition • prevention

Abbreviations

4-NQO	4-nitroquinoline-1-oxide
5-LOX	5-lipoxygenase
ABCA1	ATP-binding cassette transporter 1
ACF	Aberrant crypt foci
AFB1	Aflatoxin B1
ALOX5	Arachidonate 5-lipoxygenase
AOM	Azoxymethane
ARE	Antioxidant response element
BPH	Benign prostate hypertrophy
BQE	Betel quid extract
BRCA	Breast cancer
CAT	Catalase
CDK	Cyclin-dependent kinases
CI	Confidence interval
COX-2	Cyclooxygenase-2
CSE	Cigarette smoke extract
Cx43	Connexin 43
DEN	Diethylnitrosamine
DMBA	7,12-dimethyl-benz[a]anthracene
DMH	1,2-dimethylhydrazine

EGCG	Epigallocatechin gallate
EPHX1	Epoxide hydrolase 1
ER	Estrogen receptor
FLAP	5-LOX-activating protein
GJC	Gap-junctional intercellular communication
GnRH	Gonadotropin-releasing hormone
GSK-3β	Glycogen synthase kinase-3β
HCC	Hepatocellular carcinoma
HepG2	Human hepatocellular liver carcinoma cell line
HFD	High-fat diet
HGPIN	High-grade prostatic intraepithelial neoplasia
IGF-1	Insulin-like growth factor
IGFBP-3	Insulin-like growth factor binding protein 3
IL	Interleukin
Keap-1	Kelch-like ECH-associated protein 1
LEC	Long-Evans Cinnamon
LXRα	Liver X receptor alpha
MCF-7	Human breast adenocarcinoma cell line
MMP	Matrix metalloproteinase
MNNG	*N*-methyl-*N′*-nitro-*N*-nitrosoguanidine
MNU	*N*-methyl-*N*-nitrosourea
NASH	Nonalcoholic steatohepatitis
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHANES	National Health and Nutrition Examination Survey
NNK	4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridal)-1-butanone
Nrf2	Nuclear factor-E2-related factor 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
ORs	Odds ratios
PanIN	Pancreatic intraepithelial neoplasias
PCB	Polychlorinated biphenyls
PCNA	Proliferating cellular nuclear antigen
PGIS	Prostacyclin synthase
PGST	Placental glutathione-*S*-transferase
PPAR	Peroxisome proliferator-activated receptor
PPARγ	Peroxisome proliferator-activated receptor gamma
PSA	Prostate-specific antigen
RAR	Retinoic acid receptor
RCC	Renal cell carcinoma
RR	Relative risk
SOD	Superoxide dismutase
TE	Tomato extract
TF	The metal-binding protein transferrin
THP1	Human acute monocytic leukemia cell line
TNF-α	Tumor necrosis factor-alpha

1 Introduction

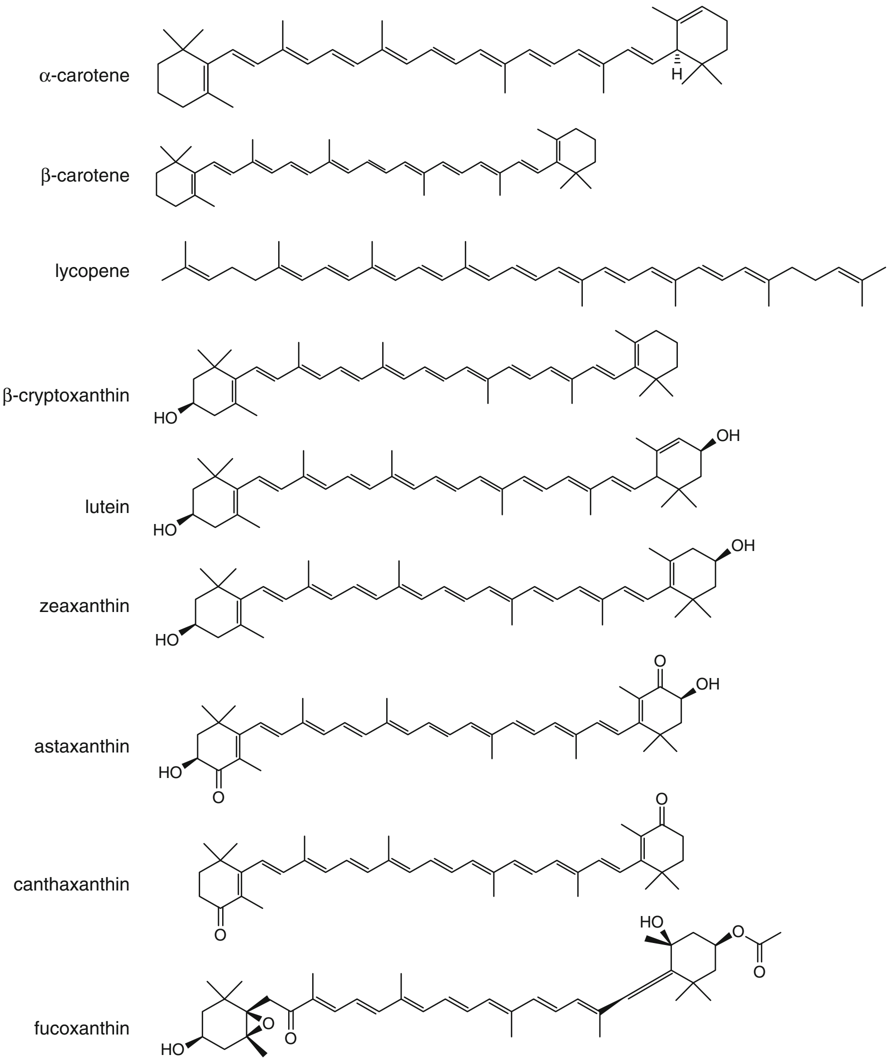
There is a link between nutritional factors in the diet and carcinogenesis in humans [1, 2]. Dietary risk factors have ranked higher than smoking and much higher than pollution or occupational hazards in their association with death due to cancer [3]. Intake of a number of compounds naturally occurring in foods, particularly antioxidant compounds in plants, is associated with a lower risk of cancer [4]. However, it is not clear which dietary chemical compounds account for this benefit. Most epidemiological studies suggest that vegetable and fruit consumption has constantly been associated with a reduced incidence of a variety of cancers [1, 2, 4], and dietary carotenoid intake from these sources has similarly been correlated with a reduced cancer risk [4]. Vegetables contain carotenoids such as β-carotene, lycopene, lutein, zeaxanthin, β-cryptoxanthin, and capsanthin. Citrus fruits contain β-cryptoxanthin and marine carotenoids include astaxanthin, β-carotene, lycopene, zeaxanthin, canthaxanthin, and fucoxanthin (Fig. 129.1) [4].

2 Lycopene

Lycopene is a carotenoid, an acyclic isomer of beta-carotene. It is a non-provitamin A carotenoid which gives tomatoes their red color. Humans and animals do not synthesize lycopene and, thus, depend on dietary sources. Tomatoes and tomato products, watermelon, pink grapefruit, apricots, guava, and papaya are the dietary sources of lycopene [5, 6]. Lycopene, one of more than 600 carotenoids synthesized by plants and photosynthetic microorganisms, is a tetraterpene hydrocarbon containing 40 carbon atoms and 56 hydrogen atoms [7]. Lycopene contains 11 conjugated and 2 nonconjugated double bonds, which make it highly reactive toward oxygen and free radicals [8]. The latter feature makes it exist in both the cis- and trans-isomeric forms (Fig. 129.2). In natural plants, it exists predominantly in an all-trans configuration. However, induced by light, thermal energy, and chemical reactions, it can also form cis–trans isomers including 15-, 13-, 11-, 9-, 7-, 5-cis isomers [9]. A recent finding showed that the 5-cis isomer of lycopene is the most stable one followed by the all-trans, 9-cis, 13-cis, 15-cis, 7-cis, and 11-cis isomers [5]. It is absorbed better from heat-processed food sources and lipid-rich diets than from raw food. Lycopene is permitted as a food colorant in the EU and was also approved for use as a food supplement in the USA in July 2005. The only permitted source is tomatoes (*Lycopersicon esculentum*, Lycopersicon, meaning wolf peach). Besides lycopene, tomato oleoresin also contains appreciable amounts of β-carotene, phytoene, and phytofluene [4].

2.1 Mechanism of Lycopene in Cancer Prevention

The mechanisms underlying the chemopreventive activities of lycopene may involve changes in pathways leading to cell growth or cell death (Fig. 129.3 and Table 129.1).


α-carotene
H
β-carotene
lycopene
β-cryptoxanthin
HO
lutein
HO
OH
zeaxanthin
HO
OH
astaxanthin
HO
O
O
OH
canthaxanthin
O
O
fucoxanthin
HO
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HO
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O

Fig. 129.1 Chemical structures of carotenoids

These include (a) inhibition of growth and induction of differentiation in cancer cells by modulating the expression of cell cycle regulatory proteins [22–26], (b) modulation of the insulin-like growth factor (IGF-1)/insulin-like growth factor–binding protein 3 (IGFBP-3) system [27–29], (c) upregulation of gap-junctional gene connexin 43 (Cx43) and increased gap-junctional intercellular communication (GJC) [30–34], (d) modulation of redox signaling [35], (e) prevention of oxidative DNA damage [36, 37], (f) inhibition of interleukin- (IL) 6 and androgen [38], (g) inhibition of 5-lipoxygenase

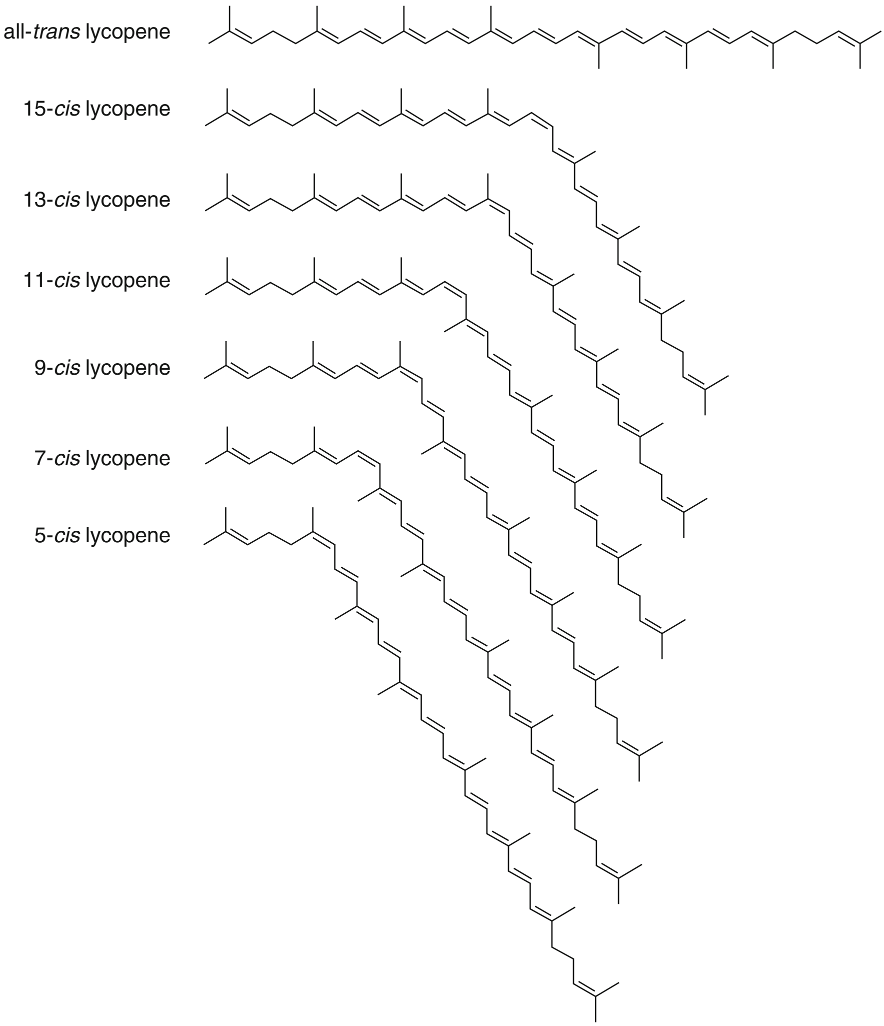
all-*trans* lycopene
15-*cis* lycopene
13-*cis* lycopene
11-*cis* lycopene
9-*cis* lycopene
7-*cis* lycopene
5-*cis* lycopene

Fig. 129.2 Chemical structures of of trans- and cis-isomers of lycopene

(5-LOX) [39], (h) modulation of carcinogen-metabolizing enzymes [40], and (i) modulation of immune function [41] (Fig. 129.4).

Lycopene has been demonstrated to be the most potent antioxidant with the ranking: lycopene > α-tocopherol > ß-carotene > ß-cryptoxanthin > zeaxanthin = ß-carotene > lutein [42]. Lycopene's oxygen-quenching ability protects against oxidative DNA damage in vitro and in vivo, thereby preventing potential mutations that may be associated with cancer initiation and progression [43]. Due to its extended system of conjugated double bonds, lycopene can quench singlet oxygen and free radicals and has been reported to be the most effective singlet oxygen quencher among

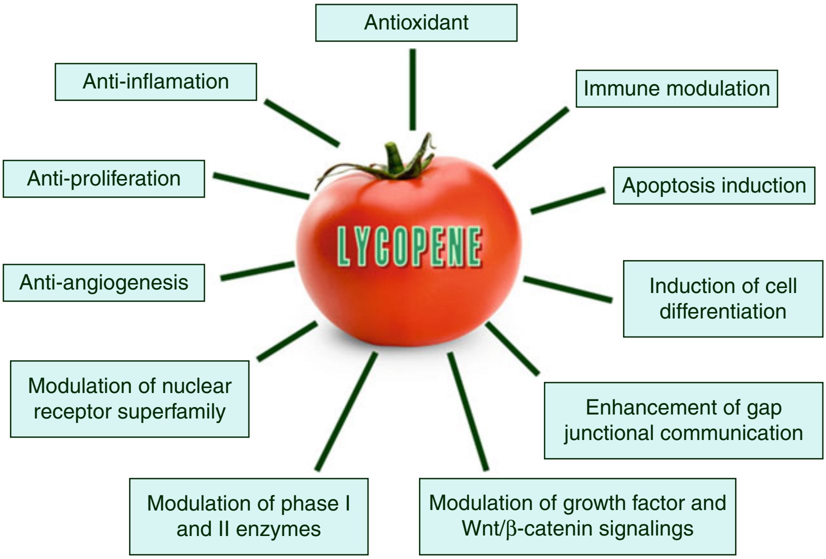
Antioxidant
Anti-inflamation
Immune modulation
Anti-proliferation
Apoptosis induction
LYCOPENE
Anti-angiogenesis
Induction of cell differentiation
Modulation of nuclear receptor superfamily
Enhancement of gap junctional communication
Modulation of phase I and II enzymes
Modulation of growth factor and Wnt/β-catenin signalings

Fig. 129.3 Mechanisms of cancer chemoprevention by lycopene

approximately 600 naturally occurring carotenoids [8]. In addition, lycopene can upregulate the antioxidant response element (ARE) and thereby stimulate the production of cellular enzymes such as superoxide dismutase, glutathione-*S*-transferase, and quinone reductase that protect cells from reactive oxygen species and other electrophilic molecules [8]. In human hepatocellular liver carcinoma cell line (HepG2) and human breast adenocarcinoma cell line (MCF-7), lycopene upregulates the ARE through the Nuclear factor-E2-related factor 2 (Nrf2) nuclear transcription pathway [44] (Figs. 129.4 and 129.5). Goo et al. used quantitative proteomics to show that lycopene upregulates the expression of proteins regulated by the ARE in the androgen-sensitive human prostate cell line LNCaP [45]. These proteins included epoxide hydrolase 1 (EPHX1), superoxide dismutase-1 (SOD-1), catalase (CAT), and the metal-binding protein transferrin (TF).

One of the mechanisms of action of lycopene is to increase gap-junctional intercellular communication (GJC) by increasing expression of a widely expressed gap-junctional gene, connexin 43 [32–34, 46] (Fig. 129.4). Increased expression of Cx43 and resulting increases in GJC have previously been shown to occur after treatment of human and murine cells in culture with diverse carotenoids, including lycopene [34]. This action correlates strongly with the ability of these carotenoids to suppress neoplastic transformation in model cell culture systems [32, 34], an action which is shared by retinoids [31]. Decreased expression of connexins, including Cx43, has been reported in human tumors in comparison to normal tissue [47], and connexins are regarded by many as putative tumor suppressor genes [48, 49]. Upregulated junctional communication has in turn been linked to decreased proliferation in normal and preneoplastic cells [50]. This action of

Table 129.1 In vitro and in vivo studies for mechanism of lycopene in cancer prevention

References	Cancer	Mechanism	Findings
Uppala et al. [10]	Breast cancer	Inhibition of cell proliferation	Lycopene was shown to modulate cell cycle proteins such as beta tubulin, CK8/18, CK19, and heat shock proteins in MCF-7 breast cancer cells
Al-Malki et al. [11]	Breast cancer	Antioxidant defense	MDA↓, NO↓, SOD↑, CAT↑, GPx↑
Sahin et al. [12]	Breast cancer	Antioxidants	MDA↓, 8-isoprostane↓, 8-OhdG↓ and Bcl-2↓, increased Bax↑, caspase 3↑ and caspase 9↑
Yang et al. [13]	Hepatocarcinoma	Antimetastatic effects	apo-8′-lycopenal: (1) decreased the activities and protein expression of metalloproteinase-2 (MMP-2) and -9; (2) increased the protein expression of nm23-H1 and the tissue inhibitor of MMP (TIMP)-1 and -2; (3) suppressed protein expression of Rho small GTPases; and (4) inhibited focal adhesion kinase–mediated signaling pathway, such as ERK/p38 and PI3K-Akt axis
Yang et al. [14]	Prostate cancer	Anti-proliferative effect	Lycopene on LNCaP cells involves the activation of the PPARγ-LXRα-ABCA1 pathway, leading to reduced cellular total cholesterol levels
Yang et al. [15]	Prostate cancer	Inhibition of cell Proliferation	Lycopene can inhibit DU145 cell proliferation via PPARγ-LXRα-ABCA1 pathway
Ford et al. [16]	Prostate cancer	Reduce cell proliferation and alter cell cycle progression	Levels of the gap junction protein, connexin 43, were unaltered by lycopene or apo-lycopenal treatment while cell apoptosis rates significantly decreased
Lin et al. [17]	Colon cancer	Suppress MMP-7 expression and leptin-mediated cell invasion	Lycopene could effectively inhibit the phosphorylation of Akt, glycogen synthase kinase-3β (GSK-3β), and ERK 1/2 proteins in human colon cancer HT-29 cells
Luo et al. [18]	Gastric cancer	Enhances antioxidant enzyme activities and immunity function	Lycopene increased blood IL-2, IL-4, IL-10, TNF-α levels and reduced the IL-6 level and enhanced blood IgA, IgG, and IgM levels in gastric cancer rats
			Decreased MDA and increased blood and gastric antioxidant parameters (SOD, CAT, and GSH-Px)

(*continued*)

Table 129.1 (continued)

References	Cancer	Mechanism	Findings
Ettorre et al. [19]	Leukemia	Trigger apoptosis	Lycopene phytocomplex triggered apoptosis via a mitochondrial pathway. Redox state of cells was measured in terms of glutathione (GSH) content, but only a small percentage of cells showed GSH depletion, suggesting that the loss of GSH may be a secondary consequence of ROS generation. Moreover, LycoC pretreatment effectively increased apoptosis induced by photodynamic therapy
Palozza et al. [20]	Prostate cancer	Apoptosis and cell growth inhibition by altering mevalonate pathway and Ras signaling	Lycopene may alter mevalonate pathway through inhibition of HMG-CoA reductase. This may induce a decreased prenylation of Ras, which, in turn, may modulate redox-sensitive molecular pathways responsible for NF-κB activation and consequently, for cell cycle arrest and apoptosis induction, as evidenced by decreased cyclin D1 and pAKT levels; increased p21, p27, and p53 levels; and changes in Bax:Bcl-2 ratio
	Colon cancer		
Salman et al. [21]	Colon cancer	Anti-proliferative and apoptotic effect	Lycopene exerted on the proliferation capacity of K562, Raji and HuCC lines, whereas this effect was observed in EHEB cells
	Lymphocytic leukemia		
	Erythroleukemia		
	Burkitt lymphoma		Increased apoptotic rate was found after incubation of HuCC cells with 2.0 and 4.0 μM of lycopene and in Raji cells following incubation with 2.0 μM

carotenoids has been proposed to have mechanistic significance by enabling the transfer of growth-regulatory signals between normal growth-inhibited cells and preneoplastic cells. Indeed, when neoplastic cells were forced into junctional communication with quiescent normal cells, the neoplastic cells became growth arrested in direct proportion to their extent of junctional communication [30]. Furthermore, recent studies have shown that in human carcinoma cells, genetically engineered to be inducible for Cx43 expression, gene induction leads to decreases in their neoplastic potential as measured by changes in anchorage-independent growth and by growth as tumors in the nude mouse [51]. Consistent with the

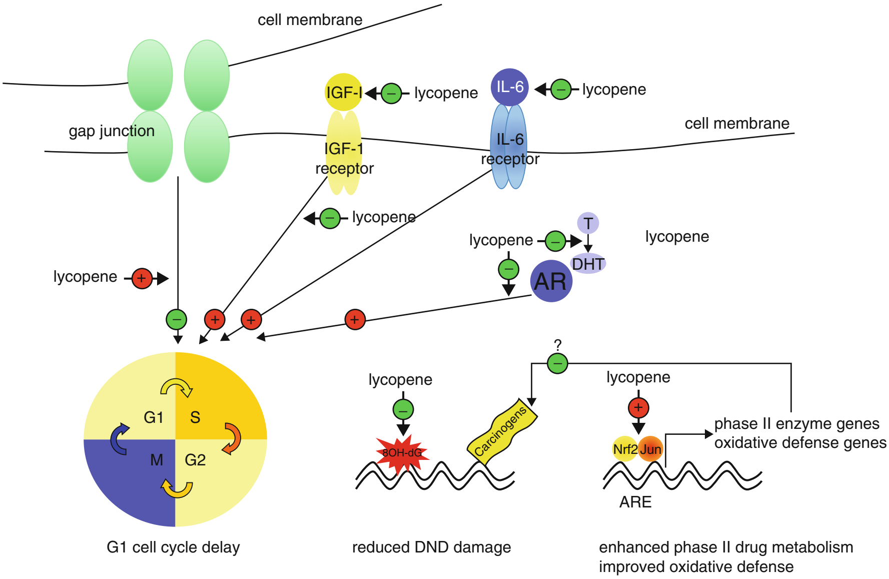
cell membrane
gap junction
IGF-I
lycopene
IGF-1
receptor
IL-6
lycopene
IL-6
receptor
cell membrane
lycopene
lycopene
T
DHT
AR
lycopene
lycopene
G1
S
M
G2
?
lycopene
8OH-dG
Carcinogens
lycopene
Nrf2
Jun
phase II enzyme genes
oxidative defense genes
ARE
G1 cell cycle delay
reduced DND damage
enhanced phase II drug metabolism
improved oxidative defense

Fig. 129.4 Mode of action of lycopene to contribute to reduced cancer risk. *IGF-I* insulin-like growth factor I, *IL-6* interleukin 6, *AR* androgen receptor, *T* testosterone, *DHT* 5-α-dihydrotestosterone, *8OH-dG* 8-hydroxy-2′-deoxyguanosine, and *ARE* antioxidant-response element (From Wertz et al. [33])

hypothesis of growth control via junctional communication, first proposed by Loewenstein [52], have been multiple reports that connexin expression and/or junctional communication is severely impaired in most solid tumors [47]. Progressive decreases with disease severity in the expression of Cx43 have been reported in the human prostate [53], implying that Cx43 expression is negatively selected during tumor progression. Similar reductions have been seen in tumor versus normal prostate cells [54]. When functional communication was restored in a human prostatic carcinoma cell line, cells had more normal differentiation, reduced proliferation, and suppressed tumorigenicity [55]. There is evidence in prostatic carcinoma cell lines that some of this loss of junctional communication may result from defects in assembly of Cx43 protein into gap junctions [56].

Another mechanism of action of lycopene is the modulation of the IGF-1/IGFBP-3 system (Fig. 129.4). Several studies show that lycopene administration to humans with colon cancer for 1–5 weeks prior to surgery significantly reduces serum IGF-1 levels [57]. Insulin-like growth factors have mitogenic and antiapoptotic effects on normal and transformed prostate epithelial cells [27, 58, 59]. As mitogens and antiapoptotic agents, IGFs may be important in carcinogenesis, possibly by increasing the risk of cellular transformation by enhancing cell turnover. Whereas IGF-1 is an important mitogen for prostate cells, IGFBPs have opposing actions, in part by binding IGF-1, but also by direct inhibitory effects on target cells [27]. In recent epidemiologic studies, relatively high IGF-1 and low IGFBP-3 plasma levels have been independently associated with greater

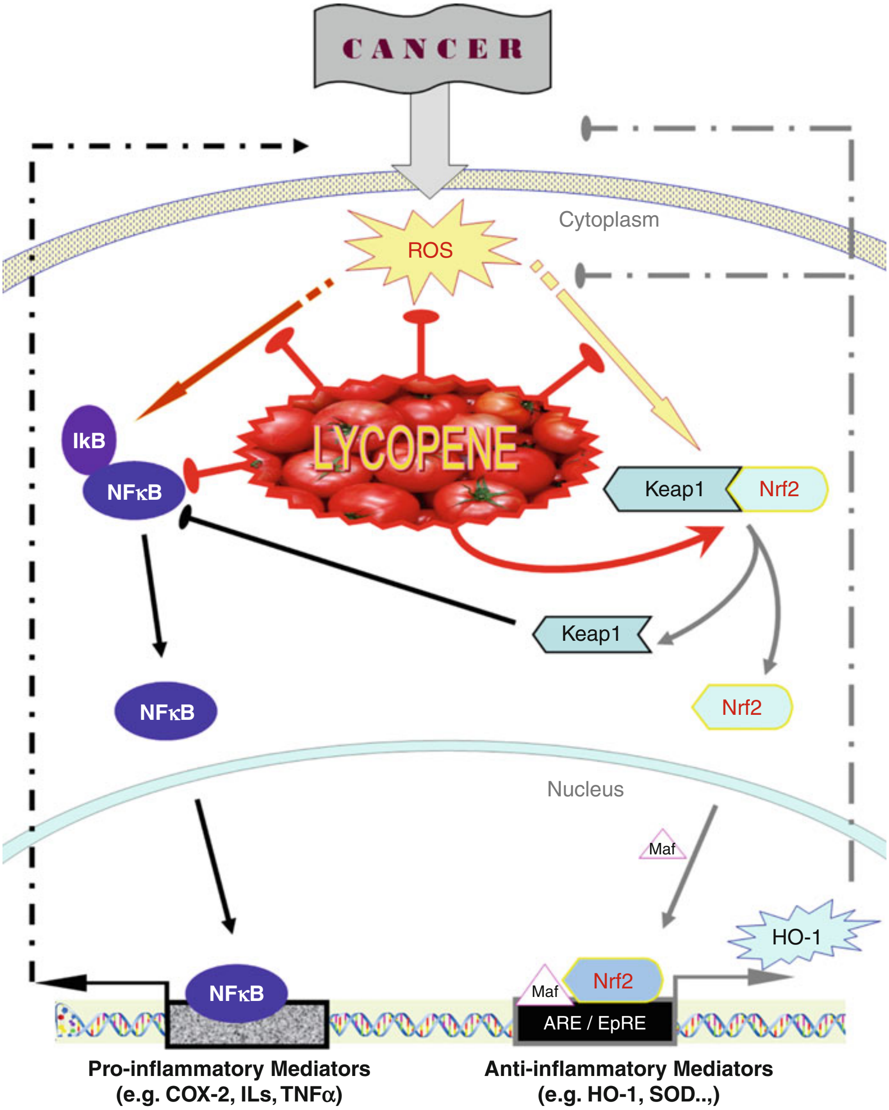
CANCER
Cytoplasm
ROS
IkB
NFκB
LYCOPENE
Keap1
Nrf2
Keap1
NFκB
Nrf2
Nucleus
Maf
HO-1
NFκB
Maf
Nrf2
ARE / EpRE
Pro-inflammatory Mediators
(e.g. COX-2, ILs, TNFα)
Anti-inflammatory Mediators
(e.g. HO-1, SOD..,)

Fig. 129.5 The effects of lycopene on NF-κB and Nrf2 pathways

risk of prostate cancer [28, 29]. A two- to fourfold elevated risk has been observed for prostate cancer in men in the top quartile of IGF-1 relative to those in the bottom quartile, and low levels of IGFBP-3 were associated with an approximate doubling of risk [28]. Siler et al. tested lycopene (200 ppm) and vitamin E (540 ppm) in the MatLyLu Dunning prostate cancer model to gain insight into their in vivo action in humans for 4 weeks [38]. They reported that both compounds accumulated in tumor tissue and macroscopic evaluation of the tumors by magnetic resonance imaging showed a significant increase in necrotic area in the vitamin E and the lycopene treatment groups. Microarray analysis of tumor tissues revealed that both compounds regulated local gene expression. Lycopene interfered with local testosterone activation by downregulating 5-alpha-reductase and consequently reduced steroid target genes expression (cystatin-related protein 1 and 2, prostatic spermine-binding protein, prostatic steroid-binding protein C1, C2, and C3 chain, probasin). In addition, lycopene downregulated prostatic IGF-I and IL-6 expression.

Lycopene may suppress nonphosphorylated β-catenin protein levels and Akt activation, and augment the phosphorylated form of β-catenin, which were associated with reduced protein expression of cyclin D1 [60]. Hence, lycopene may inhibit Wnt/β-catenin signaling via the connection along the Akt/GSK3β/β-catenin [17]. Another potential target for the anticancer activity of lycopene is the 5-LOX pathway. Hazai et al. tested the acyclic tomato carotene lycopene (in all-trans and 5-cis isomeric configurations), its natural dihydroxy analogue lycophyll (also present in tomato fruit), and two bioactive oxidative metabolites of lycopene (4-methyl-8-oxo-2,4,6-nonatrienal and 2,7,11-trimethyl-tetradecahexaene-1,14-dial) and subjected them to molecular modeling calculations in order to investigate their predicted binding interaction(s) with human 5-LOX [39]. Lycopene and lycophyll were predicted to bind with high affinity in the superficial cleft at the interface of the beta-barrel and the catalytic domain of 5-LOX. Carotenoid binding at this cleavage site provided the structural rationale by which polygenic compounds could modify the 5-LOX enzymatic function via an allosteric mechanism, or by radical scavenging in proximity to the active center. In addition, the two bioactive metabolites of lycopene were predicted to bind to the catalytic site with high affinity, suggesting potential direct competitive inhibition of 5-LOX activity that should be shared by both lycopene and lycophyll after in vivo supplementation [61]. Simone et al. reported that lycopene regulates cigarette smoke-driven inflammation in human macrophages, THP-1 [62]. They have shown that lycopene inhibits the production of the proinflammatory cytokine IL-8 induced by cigarette smoke. Yang et al. [14] demonstrated that the anti-proliferative effect of lycopene on human prostate cancer cells (LNCaP) involves the activation of the peroxisome proliferator-activated receptor gamma (PPARγ)-liver X receptor alpha (LXRα)-ATP-binding cassette transporter 1 (ABCA1) pathway.

3 The Protective Role of Lycopene in Cancer Prevention

3.1 Lung Cancer

Lung cancer is one of the most common cancers in the world. The important risk factors for lung cancer are smoking, air pollution, and diet. Several studies have indicated that smokers and lung cancer patients tend to have lower plasma concentrations of retinol, α-tocopherol, β-carotene, lycopene, β-cryptoxanthin, selenium, and zinc [63]. This observation was explained by cigarette smoking impairing the uptake of the fat soluble antioxidants or that smokers have differing diets compared to nonsmokers, smoke being an appetite suppressant [64]. In a study, authors have treated lycopene solutions, human plasma, and isolated LDL with cigarette smoke and monitored all-(E)-lycopene, 5(Z)-lycopene, and beta-carotene depletion. In plasma, the depletion of all-(E)-lycopene (15.0+/−11.0%, $n = 10$) was greater than 5(Z)-lycopene (10.4+/−9.6%) or beta-carotene (12.4+/−10.5%). In LDL, both all-(E)- and 5(Z)-lycopene were more susceptible than beta-carotene (20.8+/−11.8%, 15.4+/−11.5%, and 11.5+/−12.5%, $n = 3$ respectively). The effects have been compared with Sin-1 reactions and isomerization of all-(E) lycopene is common to both treatments [64]. Consumption of tomato or tomato products such as lycopene has been associated with a lower risk of lung cancer [5]. Additionally, Choi et al. reported that Hel299 lung cells, A549 lung cancer cells, and HeLa cervical carcinoma cells were highly susceptible to inactivation by glycoalkaloid-rich green tomato extracts [65].

Recently, in vitro and in vivo experimental data have demonstrated lycopene's notable anticarcinogenic effect in lung carcinogenesis. Results from the combination of the Nurses' Health Study and Health Professional Follow-Up Study [66] and from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study [67] showed that high intake of lycopene was associated with an approximately 20–30% lower risk of lung cancer. Cohort studies failed to show an inverse relation between lycopene intake and lung cancer prevalence [68, 69]. However, in almost all case–control studies, odds ratio of developing lung cancer with high lycopene or tomato intake was below one [70–74]. A case–control study on lung cancer in Spanish women matched 103 cases to 206 hospital controls by age and residence. High intake of lycopene was the carotenoid with the strongest, although not significant, inverse association with the risk of lung cancer (Odd ratio [OR] = 0.56, confidence interval [CI] = 0.26–1.24, trend $p = 0.15$) when adjusted for smoking status, total pack-years smoked, vitamin E and vitamin C intake, total flavonoid intake, and intake of other specific carotenoids [75]. These results were confirmed by Ito et al. [76]. In their nested case–control study, a marginally significant inverse association between serum lycopene level and lung cancer death was found (0.46, 95% CI 0.21–1.04) after adjusting for smoking and serum levels of other carotenoids. Furthermore, a 40% reduction in risk of mesothelioma was observed for those consuming tomato or tomato juice 16 or more times a month versus nonconsumers [77]. Only 1.7% of control subjects reported not consuming tomatoes or tomato juice as opposed to 9% of case subjects, suggesting that people who do not consume tomato products are at a high risk for mesothelioma.

In a cell culture study, Lian et al. observed that apo-10′-lycopenoic acid inhibited the growth of NHBE normal human bronchial epithelial cells, BEAS-2B-immortalized normal bronchial epithelial cells and A549 non-small cell lung cancer cells [78]. This inhibitory effect of apo-10′-lycopenoic acid was associated with decreased cyclin E, inhibition of cell cycle progression from G(1) to S phase, and increased cell cycle regulators p21 and p27 protein levels. In addition, apo-10′-lycopenoic acid transactivated the retinoic acid receptor beta (RARβ) promoter and induced the expression of RARbeta. They also examined the effect of apo-10′-lycopenoic acid treatment on 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridal)-1-butanone (NNK)-induced lung tumorigenesis in the A/J mouse model.

They found that the lung tumor multiplicity was decreased dose-dependently from an average of 16 tumors per mouse in the NNK injection alone group, to an average of 10, 7, and 5 tumors per mouse in groups injected with NNK and supplemented with 10, 40, and 120 mg/kg diet of apo-10′-lycopenoic acid, respectively. Lian et al. also reported that treatment with apo-10′-lycopenoic acid, in a time- and dose-dependent manner, results in the nuclear accumulation of transcription factor Nrf2 protein in BEAS-2B human bronchial epithelial cells [79]. The activation of Nrf2 by apo-10′-lycopenoic acid is associated with the induction of phase II detoxifying/antioxidant enzymes including heme oxygenase-1, NAD(P)H:quinone oxidoreductase 1, glutathione-*S*-transferases, and glutamate-cysteine ligases in BEAS-2B cells. Furthermore, apo-10′-lycopenoic acid treatment increased total intracellular glutathione levels and suppressed both endogenous reactive oxygen species generation and H(2)O(2)-induced oxidative damage in BEAS-2B cells. Animal experiments also showed that lycopene might play a role in prevention of lung cancer. In a study, the incidence of lung adenoma and carcinoma in male mice that received 50 ppm lycopene in addition to diethylnitrosamine (DEN), *N*-methyl-*N*-nitrosurea (MNU), and 1,2-dimethylhydrazine (DMH) was found to be lower than the incidence in those that did not receive lycopene (75.0% vs. 18.8%) [80]. It was also reported that lycopene supplementation prevented downregulation of apoptosis in ferrets exposed to smoke while increasing the IGFBP-3 levels [81]. Taken together, these findings demonstrated that lycopene could suppress the proliferation and promote the lung cancer cells, which may be involved in the action mechanisms of lycopene in the prevention of lung cancer.

3.2 Gastric Cancer

Gastric cancer is the fourth most frequent cancer and the second leading cause of cancer death worldwide. Epidemiologic, experimental, and animal studies indicate that diet plays an important role in the etiology of gastric cancer. High intake of fresh fruits and vegetables, lycopene, and lycopene-containing food products may reduce the risk for gastric cancer [82]. In the meta-analysis of cohort studies, the summary relative risks of gastric cancer were 0.82 (95% CI 0.73–0.93) for fruits and 0.88 (95% CI 0.69–1.13) for vegetables [83]. There is great interest in lycopene in gastric cancer because it is a leukotriene inhibitor [61] and the products of the arachidonic acid–metabolizing enzyme, 5-LOX, stimulate the growth of several cancer types. Inhibitors of 5-LOX and 5-LOX-activating protein (FLAP) induce apoptosis in some cancer cells. Fan et al. [84] investigated the effect of a FLAP inhibitor, MK-886, on the inhibition of proliferation and induction of apoptosis in gastric cancer. They found that MK-886 inhibited cell growth in a dose- and time-dependent manner. Apoptosis was induced in gastric cancer cells and was characterized by upregulation of p27kip1 and Bax, with release of cytochrome c from mitochondria into cytosol, which initiated caspase-3 activation. Specific caspase-3 inhibitors partially blocked MK-886-induced apoptosis.

In a large cohort of 120,852 men and women, no association was found between lycopene intake and gastric carcinoma [85]. However, in at least six case–control studies to date, an inverse association between lycopene or tomato intake and gastric carcinoma incidence was reported [86–91]. In a case–control study conducted in Italy with 723 cases of stomach cancer and 2,879 matched controls, a significant trend in decreased risk associated with raw tomato intake consumption was demonstrated (odds ratio for quartiles: 1.00, 0.59, 0.45, 0.43) after adjusting for age, sex, study center, education, caloric intake, alcohol consumption, and smoking [88]. In another case–control study recently published including 191 cases and 570 age-, neighborhood-matched controls from China, odds ratio for gastric carcinoma in the highest versus lowest quartile of prediagnostic lycopene levels was found to be 0.55 (95% CI 0.30–1.00) [92]. Serum lycopene levels were significantly different between two groups. Pelucchi et al. found decreased odds ratios for the highest versus lowest quartile of vitamin E (OR = 0.50), alpha-carotene (OR = 0.52), and beta-carotene (OR = 0.42) intake [93]. They also reported that gastric cancer was directly associated with sodium, with ORs of 2.22 for the second, 2.56 for the third, and 2.46 for the fourth quartile of intake. However, no significant relation emerged with iron, calcium, potassium, zinc, vitamin C, thiamin, riboflavin, niacin, vitamin B6, folate, vitamin D, retinol, beta-cryptoxanthin, lycopene, and lutein plus zeaxanthin.

Several studies reported that lycopene was shown to inhibit gastric carcinogenesis induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and saturated sodium chloride [94–96]. In that studies, lycopene's chemopreventive activity was suggested to be due to modulating the expression of apoptosis-associated proteins Bcl-2, Bax, Bim, caspase 8, and caspase 3 and its antioxidant activity [94–96]. Study by Luo and Wu investigated the chemopreventive effect of lycopene on the oxidative injury and immunity activities of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-induced gastric cancer rats [18]. Administration of lycopene (50, 100, and 150 mg/kg body weight) to gastric carcinoma-induced rats largely upregulated the redox status and immunity activities to decrease the risk of gastric cancer. In an animal model, the effects of lycopene administration on cigarette-smoke-induced changes in protein levels of p53 tumor suppressor gene, p53 target genes (p21 $^{\mathrm{Waf1/Cip1}}$ and Bax-1), cell proliferation, and apoptosis in the gastric mucosa were examined [97]. In that study, lycopene levels were significantly elevated in a dose-dependent manner in the gastric mucosa of ferrets supplemented with lycopene alone, but were markedly reduced in ferrets supplemented with lycopene and exposed to smoke. It was also found that total p53 and phosphorylated p53 levels were higher in ferrets exposed to smoke alone than in all other groups. However, smoke-elevated total p53 and phosphorylated p53 were markedly attenuated by both doses of lycopene. p21(Waf1/Cip1), Bax-1, and cleaved caspase 3 (an index for apoptosis) were substantially decreased, whereas cell proliferation indices like cyclin D1 and proliferating cellular nuclear antigen (PCNA) were increased in ferrets exposed to smoke alone, while lycopene prevented smoke-induced changes in p21(Waf1/Cip1), Bax-1, cleaved caspase 3, cyclin D1, and PCNA in a dose-dependent fashion. In contrary to these results, Persson et al. found no significant association between plasma levels of lutein/zeaxanthin, lycopene, retinol, alpha- or gamma-tocopherol, and gastric cancer risk [98].

3.3 Breast Cancer

Breast cancer is by far the most frequent cancer among women. Environmental factors, particularly dietary factors, have been postulated to play important roles in the etiology of breast cancer. An overall benefit of consumption of fruits and vegetables has been suggested for breast cancer in women in the USA [99]. Carotenoids such as lycopene are hypothesized to reduce the risk of breast cancer due to their capacity for scavenging DNA damaging free radicals, inhibit cell proliferation, induce apoptosis, and suppress angiogenesis [100]. Several case–control and prospective cohort studies have conducted the relationships between the lycopene and breast cancer. However, the results remain inconsistent. Comparing the highest with the lowest intake, dietary intake of lycopene did not significantly reduce the breast cancer risk (RR = 0.99; 95% CI: 0.93–1.06; I2 = 0.00%) when data from cohort studies were pooled [100]. Furthermore, no significant association was observed in further subgroup analyses. When data from case–control studies were pooled, dietary intake of lycopene significantly reduced the breast cancer risk by 29.0% (OR = 0.71; 95% CI: 0.56–0.92; I2 = 84.71%) [100, 101]. A nested case–control study was also done in the same cohort with 508 breast cancer cases and 508 controls and their plasma lycopene levels were not different (10.8 vs. 10.3 mcg/dl). Another nested case–control study including 1,452 breast cancer cases mice and 5,239 women showed that age-adjusted relative risks for breast cancer in increasing quintiles of lycopene intake were 1.0, 1.15, 0.93, 0.97, and 1.01 [102]. In another nested case–control study with 295 cases and 295 matched controls, the risk of developing breast cancer in the highest fifth quintile of plasma lycopene level was approximately half of that in the lowest fifth [103]. In a study comparing lycopene levels in breast adipose tissue between 46 cases and 63 benign controls, lycopene levels were inversely associated with breast cancer risk when adjusted for age, smoking status, and menopausal status [104]. Dorjgochoo et al. prospectively investigated the associations of plasma levels of tocopherols, retinol, and carotenoids with the risk of developing breast cancer among Chinese women [105]. They conducted a study of 365 incident breast cancer cases and 726 individually matched controls nested within a large cohort study of women aged 40–70 years at baseline. They observed no associations between breast cancer risk and any of the tocopherols, retinol, and most carotenoids. However, high levels of plasma lycopene other than trans, 5- and 7-cis, or trans alpha-cryptoxanthin were inversely associated with the risk of developing breast cancer. Moreover, Wang and Leung lycopene administration slightly reduced the 7,12-dimethyl-benz[a] anthracene (DMBA)-induced ethoxyresorufin-*O*-deethylase activity by 20% in MCF-7 cells [106].

Lycopene has been shown to inhibit the incidence and growth of the chemically induced breast tumors in laboratory animal studies [12, 107]. A protective effect of lycopene was found on DMBA-induced rat mammary tumors. When compared with placebo or control rats, the lycopene fed rats had the smallest average size of tumors, but the difference was not statistically significant. The number of tumors that developed over 135 days was much lower in the lycopene-treated group than the control group [107]. In a study, using rats, we have demonstrated that inhibition of mammary cancer incidence by lycopene (70%), genistein (60%), and their combination (40%) was observed [12]. In the same study, tumor weight decreased by 48%, 61%, and 67%, and mean tumor volume decreased by 18%, 35%, and 65% with lycopene, genistein, and lycopene + genistein, respectively. Animals administered DMBA developed breast cancer, which was associated with increased expression of Bcl-2 and decreased expression of Bax, caspase 3, and caspase 9 in mammary tissues. Administration of genistein and lycopene in combination was more effective in inhibiting DMBA-induced breast tumors and modulating the expression of apoptosis-associated proteins than the administration of each agent alone [12]. Moselhy and Al-mslmani [108] reported protection against DMBA-induced carcinogenesis was 66.5% in animals that received lycopene, whereas it was 80% in animals that received lycopene with melatonin as compared with nontreated rats. However, Cohen et al. reported that neither pure lycopene nor lycopene in the form of a mixed carotenoid oleoresin exerted an inhibitory effect on tumor incidence, latency, multiplicity, volume, nor total tumors per group compared with unsupplemented controls [109]. Differences in routes of administration (intraperitoneal injection, gavage, intra-rectal instillation, drinking water, and diet supplementation), species and strain differences, form of lycopene (pure crystalline, beadlet, and mixed carotenoid suspension), varying diets (grain-based and casein based), and dose ranges (0.5–500 ppm) resulted in no prevention effect on development of chemically induced mammary cancer. Additionally, Hu et al. reported that no significant association between dietary intake of β-cryptoxanthin, lutein/+zeaxanthin, and lycopene and breast cancer was observed in a comprehensive study [100]. In an another study, the combination of lycopene and tocopherol treatment showed a potential reduction of malondialdehyde and nitric oxide more than lycopene alone in serum and breast tissues of DMBA-injected rats. Formation of tumor and angiogenesis in DMBA-induced rats and these abnormal changes were ameliorated by combined treatment with combination of lycopene and tocopherol [11].

In a cell study on breast tumor cell lines, cells were cycle-arrested at G1/S phase after treatment with 10 microM lycopene for 48 h [110]. Expressions of breast cancer-1 (BRCA1) and breast cancer-2 (BRCA2) genes were increased in estrogen receptor (ER)-positive cell lines (MCF-7 and HBL-100) and were decreased in ER-negative cell lines (MDA-MB-231), suggesting an indirect effect of lycopene on the estrogen pathway [110]. In another cell study on MCF-7 cell line, bcl-2 mRNA expression was decreased by 88% and cell cycle progression was blocked at G(2)/M phase after treatment with lycopene [111]. Nahum et al. suggested that lycopene inhibited cell cycle progression via reduction of the cyclin D level and retention of p27 in cyclin E-cdk2, leading to inhibition of CDK activities [112]. Uppala et al. have shown that lycopene inhibits cell proliferation in MCF-7 human breast cancer cells but not in the MCF-10 mammary epithelial cells [10]. Lycopene was shown to modulate cell cycle proteins such as beta tubulin, CK8/18, CK19, and heat shock proteins.

3.4 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the fifth leading cause of death from cancer in males and the eighth leading cause in females in the USA [113]. Worldwide, liver cancer is the third leading cause of death from cancer [114, 115]. The incidence of and mortality from HCC are variable worldwide, with the Far East and sub-Saharan Africa having the highest incidences. The primary risk factors for HCC are infection with hepatitis B and hepatitis C viruses, and long-term exposure to aflatoxin. Chronic alcoholism leading to chronic liver disease is a significant risk factor [115, 116].

Phytochemicals have been examined for their effects including anticancer with its single oxygen-quenching ability on carcinogenesis in the liver [115, 117]. Lycopene is known to have a significant anticancer effect with its single oxygen-quenching ability. Several studies have reported that lycopene exhibits multifunctions in inhibition of metastasis in hepatoma SK-Hep-1 cells both in vitro and in nude mice [118, 119], upregulation of kelch-like ECH-associated protein 1 (Keap1)-Nrf2-antioxidant response element system in HepG2 cells [44]. Although experimental evidence from in vitro studies has suggested a beneficial effect of lycopene against hepatocarcinogenesis, [117, 120], results from human and animal studies are inconsistent. For example, Tharappel et al. found that lycopene decreased the number of placental glutathione-*S*-transferase (PGST)-positive foci induced by diethylnitrosamine/polychlorinated biphenyls-77 (DEN/PCB-77) but slightly increased their volume [121]. A case–control study has examined the relationship between lycopene and aflatoxin B_1 (AFB_1) initiated hepatocellular carcinoma. Lycopene was thought to influence the binding of AFB_1 to hepatic DNA. The urine level of the major in vivo AFB_1-DNA adduct, AFB_1-N^7-guanine, was significantly higher in subjects with HCC than in controls (HCC OR = 7.52). Plasma lycopene level was inversely related to the presence of AFB_1-N^7-guanine in urine [122]. However, lycopene (1.5 g/kg of 5% lycopene oleoresin) administered during the initiation phase did not show any effect on the size or number of AFB_1-induced rat liver tumors [123]. A separate mouse study gave 30 C3H/He male mice lycopene (0.005% in drinking water) for 40 weeks. Lycopene reduced the number of liver tumor-bearing mice by 49.7% (88.2% for control vs. 38.5% for lycopene-treated mice) and reduced the average number of tumors per mouse by 88% (7.65–0.92) [124]. In an animal model of hepatocarcinogenesis in rats, lycopene decreased the size of preneoplastic foci in liver induced by DEN, but did not reduce the number of lesions [125]. Anticarcinogenic effects of lycopene were demonstrated with the inhibition of toxic effects of aflatoxin B1 [126]. Reddy et al. demonstrated that hepatocytes pretreated with lycopene and beta-carotene are protected from the effects of the carcinogen aflatoxin at both cellular and molecular levels. In contrast, in another study with Long-Evans Cinnamon (LEC) rats, lycopene administration (50 mg/kg diet) for 70 weeks did not reduce the risk of spontaneous liver carcinogenesis [127]. These discrepant observations could be related with different models or lycopene dosages used in these studies.

In a study using Hep3B human hepatoma cell lines, Park et al. have demonstrated that lycopene induced G0/G1 arrest and S phase block and inhibited cell growth in a dose-dependent manner by almost 40% [128]. In another study on SK-Hep1 human hepatoma cell line, lycopene's antimetastatic properties were shown by adhesion and migration assays. Invasiveness of the cells was reduced by 62% after treatment with 10 microM lycopene [118]. It has been recently reported that antimetastatic effects of apo-8′-lycopenal (1–10 μM) in comparison with lycopene (10 μM) in SK-Hep-1 cells were determined by Yang et al. [14]. They found that both apo-8′-lycopenal and lycopene inhibited the invasion and migration of SK-Hep-1 cells, and the effect of apo-8′-lycopenal was stronger than that of lycopene at the same concentration (10 μM). They also reported that apo-8′-lycopenal: (1) decreased the activities and protein expression of metalloproteinase-2 (MMP-2) and -9; (2) increased the protein expression of nm23-H1 and the tissue inhibitor of MMP (TIMP)-1 and -2; (3) suppressed protein expression of Rho small GTPases; and (4) inhibited focal adhesion kinase–mediated signaling pathway, such as ERK/p38 and PI3K-Akt axis. On the other hand, Takahashi et al. also demonstrated that contrary to previous reports of the inhibitory effects of lycopene and EGCG on the development of various carcinogen-induced animal tumors, these compounds exert no chemopreventive effects on spontaneous liver tumorigenesis in C3H/HeN mice [129]. Wang et al. investigated the efficacy of an equivalent dosage of dietary lycopene from either a pure compound or a tomato extract against high-fat diet (HFD)-induced nonalcoholic steatohepatitis (NASH)-promoted hepatocarcinogenesis in a rat model for 6 weeks [130]. They found that both lycopene and tomato extract supplementations significantly decreased the number of altered hepatic foci expressing the placental form of glutathione-*S*-transferase in the livers of HFD-fed rats. This was associated with significantly lower proliferating cell nuclear antigen–positive hepatocytes and cyclinD1 protein, as well as decreased activation of ERK and nuclear NF-κB. Although both lycopene or tomato extract supplementations reduced HFD-induced lipid peroxidation in the livers, they observed significantly decreased cytochrome P450 2E1, inflammatory foci, and mRNA expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-12) in the HFD + tomato extract–fed group but increased nuclear NF-E2-related factor-2 and heme oxygenase-1 proteins in the HFD + lycopene–fed group, relative to HFD feeding alone.

3.5 Pancreatic Cancer

Chronic pancreatitis has long been thought to be mainly associated with immoderate alcohol consumption, tobacco smoke, and obesity, diets high in animal protein and fat, as well as antioxidant deficiencies. Diets high in processed or red meat, diets low in fruits and vegetables, phytochemicals such as lycopene and flavonols, have been proposed and refuted as risk or protective factors in different trials [131]. In a case–control study of 462 cases and 4,721 controls, there was a significant inverse association between lycopene intake and pancreatic cancer, after adjustment for age, body-mass index, smoking status, total energy intake, education, and dietary folate [132]. However, no difference was found in women. Another prospective case–control study comparing frozen serum samples from 22 pancreatic cancer cases to 44 matched controls demonstrated that mean levels of lycopene were significantly lower in cases (0.70 ± 0.07 μmol/L) than in control subjects (0.93 ± 0.06 μmol/L), after adjusting for smoking status, educational level, and serum levels of other carotenoids and retinoids [133]. Seo et al. investigated whether lycopene protects oxidative stress–induced cell death of pancreatic acinar AR42J cells by preventing the loss of Ku70 in the nucleus [134]. The cells received oxidative stress caused by glucose oxidase acting on beta-D-glucose (glucose/glucose oxidase) and were cultured in the absence or presence of various concentrations of lycopene. Lycopene inhibited glucose/glucose oxidase–induced cell death by preventing nuclear loss of Ku70 and a decrease in Ku-DNA-binding activity of AR42J cells.

3.6 Colorectal Cancer

Colorectal cancer is one of the most common causes of cancer-related mortality in Western societies, and the death rate from colon cancer has also been stably increasing in Asia [135]. According to American Cancer Society, more than 50,000 people die from colorectal cancer each year. Although the causes are not completely understood, it is generally accepted that the hereditary genetic component combined with low intake of fibers, proteins, fruits, and vegetables as well as diets with high red and processed meat and fat levels are among the greatest risk factors [136]. Although many people are diagnosed with colorectal cancer every day, there is no reported preventative treatment for this deadly disease. However, it has been suggested that a diet loaded with natural antioxidant-rich foods may reduce the risk of developing colorectal cancer [137]. Malila et al. reported that no significant association was found between colorectal cancer risk and dietary lycopene intake in a large cohort study of middle-aged male smokers during an 8-year follow-up [138]. However, case–control studies reported about a 60% reduction in risk of colorectal cancers associated with higher tomato consumption [139, 140]. In an Italian case–control study with 955 colon cancer and 629 rectal cancer patients, a significant trend in decreased colon and rectum cancer risk associated with raw tomato intake consumption was identified [141]. In another study, when subjects who had colorectal adenomas were compared to those who had no adenomatous polyps, the median plasma lycopene level was 35% lower than that in the adenoma group [142]. Also, plasma lycopene concentration <70 mcg/l was found to be a risk factor for adenomatous polyps in logistic regression (OR: 2.31, 95% CI: 1.12–4.77). Tissue lycopene level in colon, along with levels of lutein, tocopherols, carotenes, and beta-cryptoxanthin, was found to be significantly lower in 10 patients with adenomatous polyps than in 15 patients with normal colonic mucosa [143]. In cell culture study, Lin et al. demonstrated that lycopene significantly inhibited leptin-mediated cell invasion and matrix metalloproteinase-7

(MMP-7; matrilysin) protein expression in human colon cancer HT-29 cells [17]. Lycopene could augment the expression and stability of E-cadherin proteins. They showed that MAPK/ERK and PI3K/Akt signaling pathways played important roles in leptin-mediated MMP-7 expression and cell invasion. Lycopene could effectively inhibit the phosphorylation of Akt, glycogen synthase kinase-3β (GSK-3β), and ERK 1/2 proteins.

Several studies have evaluated the protective effects of lycopene supplementation against rat and mouse colon carcinogenesis induced by carcinogens such as azoxymethane (AOM), 1,2-dimethylhydrazine (DMH), or *N*-methyl-*N*-nitrosourea (MNU) with some contradictory results. In rats pretreated with MNU, those who were treated with a daily intragastric lavage of lycopene had a significant reduction in both size and number of colonic aberrant crypt foci (ACFs), precursor lesions of colon cancer [144]. Furthermore, ACFs following treatment with AOM were reduced by 58% in rats treated with lycopene compared to the controls [145]. However, in another study, addition of lycopene into drinking water had no effect on number of ACFs in mice treated with DMH [146]. In male Fischer 344 rats initiated with AOM, treatment with this carotenoid during the initiation phase inhibited the early formation of ACF, but not during the post-initiation phase [147]. Also, post-initiation treatment with lycopene did not reduce the development of ACF in male B6C3F1 mice initiated with MNU or in male and female B6C3F1 mice initiated with a combination of the carcinogens DEN, MNU, and DMH [148]. In contrast, treatment with lycopene during the post-initiation phase reduced ACF development but not colon tumors in female Fischer 344/NSlc rats, respectively [149]. A rat study evaluated whether a synergy exists for the combined treatment with lycopene (300 mg/kg) and symbiotic (*Bifidobacterium lactis* plus oligofructose/inulin) on early biomarkers of colon carcinogenesis by DMH. Treatment with lycopene, symbiotic or their combination, significantly increased apoptosis, reduced the PCNA and p53 labeling indexes, and the development of classical ACF and mucin-negative ACF. Furthermore, a lower genotoxicity of fecal water was also detected in the groups treated with the chemopreventive agents. An additive/synergistic effect of the combined treatment with lycopene/symbiotic was observed only for the fecal water genotoxicity and mucin-negative ACF parameters [150].

Study from our laboratory has shown that tomato powder added to feed at 5% rate decreases the rate of ACF and reduces the development of adenocarcinoma and growth of AOM-induced colorectal cancer in rats (Fig. 129.6). In addition, we demonstrate that tomato powder supplementation shows its chemopreventive activities through inhibition of cyclooxygenase-2 (COX-2) expression via NF-κB pathway and promotion of apoptosis, as well as regulating Nrf2/HO-1 signaling pathway in colorectal tissue of AOM-treated rats (Fig. 129.7). Our findings identify an intimate connection between dietary supplementation of tomato powder and the decreased risk of colorectal cancer in rats, and suggest that consumption of tomato powder would be a natural candidate for the prevention of colorectal cancer in men [151]. Tang et al. investigated whether consumption of lycopene could prevent the growth and progression of colorectal tumor in a mouse xenograft model [152].

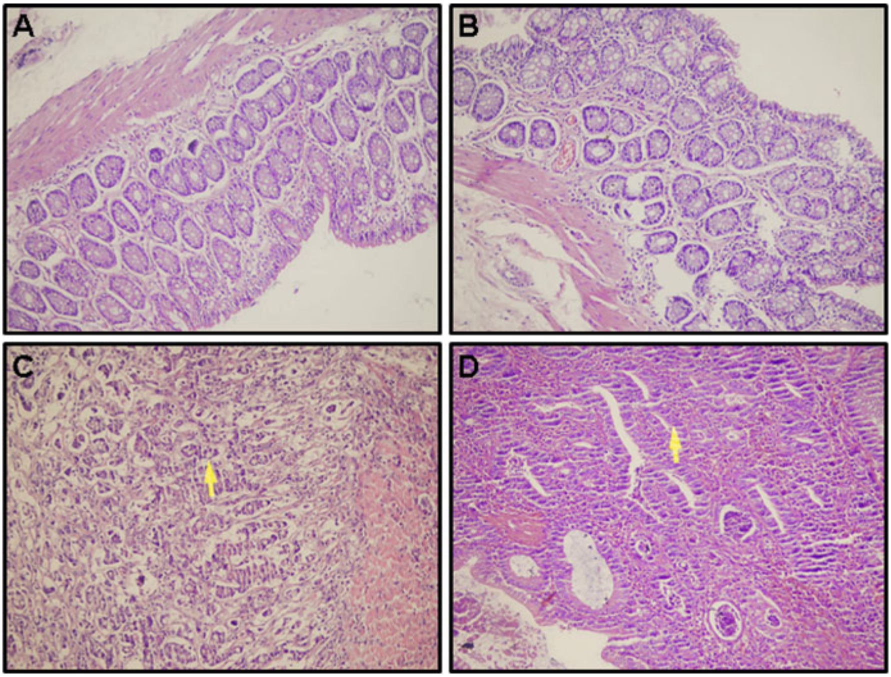

Fig. 129.6 Representative histopathology of H&E stained colorectal tissue sections (**a**) control group fed with a standard diet (**b**) control group + tomato powder fed with a standard diet +5 % tomato powder (**c**) AOM-treated group fed with a standard diet (**d**) AOM + tomato powder group fed with a standard diet +5 % tomato powder (×400 magnification) [151]

The results indicated that lycopene could effectively suppress the growth and progression of colon cancer in tumor-bearing mice. They also demonstrated that lycopene significantly suppressed the nuclear expression of PCNA and β-catenin proteins in tumor tissues. Consumption of lycopene could also augment the E-cadherin adherent molecule and nuclear levels of cell cycle inhibitor p21(CIP1/WAF1) protein. The chemopreventive effects of lycopene were associated with suppression of COX-2, PGE(2), and phosphorylated ERK1/2 proteins. Furthermore, the inhibitory effects of lycopene were inversely correlated with the plasma levels of matrix metalloproteinase 9 (MMP-9) in tumor-bearing mice.

3.7 Bladder Cancer

A relative risk of 1.40 (95% confidence interval = 1.08–1.83) was reported for a diet low in fruit intake in a meta-analysis of diet and bladder cancer [153]. However, no associations were observed for intakes of total fruits or vegetables [154, 155]. In a nested case–control study of 569 bladder cancer cases and 3,123 controls, relative risk for bladder cancer was 1.08 comparing highest to lowest

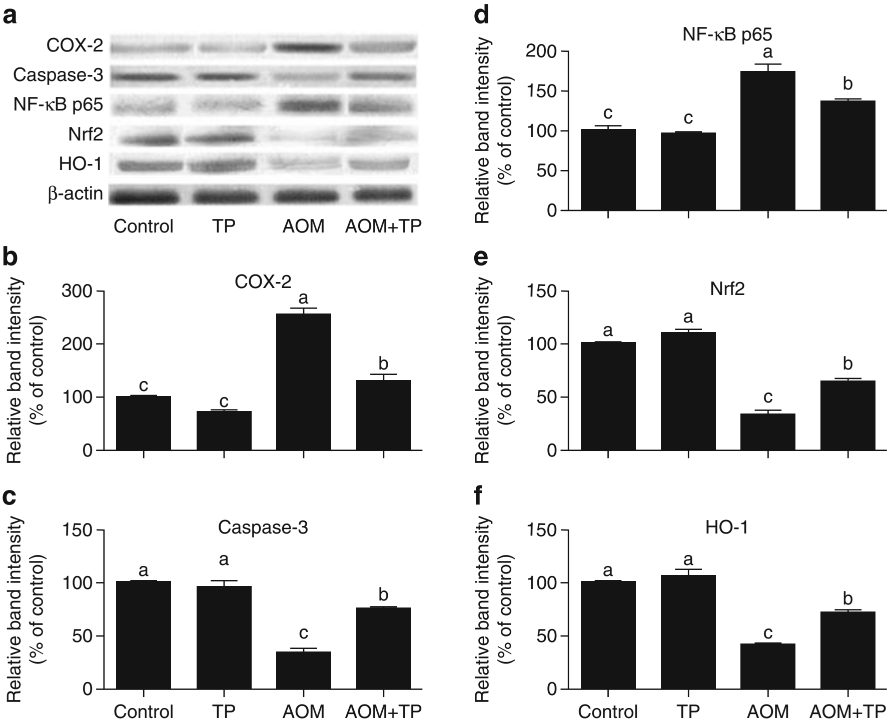

Fig. 129.7 The effects of tomato powder on COX-2, Caspase-3, NF-κB, Nrf2 and HO-1 expressions in the colon of rats [151]

quintile of lycopene intake [156]. However, in a previous cohort study, serum lycopene level was found to be lower in bladder cancer cases than in matched controls [157]. In another case–control study with 84 cases and 173 controls, OR for bladder cancer was found to be 0.94 (95% CI 0.89–0.99) in the highest quartile of plasma lycopene intake when compared to the lowest after controlling for age, sex, education, and pack-years of smoking [158]. None of the other case–control studies found statistically significant associations with risk of bladder cancer, although tendencies for inverse associations were reported [159–161]. In an animal study, lycopene administered in drinking water exhibited a nonsignificant tendency to decrease the number of transitional cell carcinomas of the bladder in rats after the administration of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine [162].

3.8 Head and Neck Cancer

Epidemiologic studies, in vitro, and in vivo animal studies have suggested that tomato and lycopene have beneficial effects in the treatment of head and neck cancer [9, 163–165]. Mayne et al. evaluated the association between plasma b- and a-carotene, lycopene, lutein⁄zeaxanthin, total carotenoids, retinol, a-tocopherol, and subsequent mortality in a cohort of 259 patients with prior oral, pharynx, or larynx cancers [164]. They reported that only plasma lycopene was significantly inversely associated with total mortality and mortality in nonsmoking patients. In a case–control study including 754 oral cancer cases and 1,775 controls, dietary lycopene measured by food frequency questionnaires was not found to be significantly associated with oral cancer risk [166]. Another case–control study including 201 larynx cancer cases failed to show an association between cancer risk and tomato intake [167]. A similar result was found in another case–control study in Italy, in which reduced risk of cancers of oral cavity and pharynx emerged in subjects reporting more frequent consumption of fresh tomatoes [168]. However, Zheng et al. reported in a case–control study that OR for oral cancer was 0.49 (95% CI 0.26–0.94) in subjects with high tomato intake [169]. Additionally, lycopene strongly and dose-dependently inhibited proliferation of a human oral cancer cell line KB-1 while upregulating expression of gap-junctional protein connexin 43 [165].

Animal experiments also showed that lycopene might play a role in prevention of oral cancer. In a study, an inverse association was observed between lycopene (2.5 mg/kg lycopene orally) and buccal pouch squamous cell carcinomas in hamsters [170]. It was also reported that the daily lycopene intake improved oral leukoplakia lesions in a group of patients [171]. The chemopreventive efficacy of lycopene with regard to oral carcinogenesis was investigated using 4-nitroquinoline-1-oxide (4-NQO)–induced tongue squamous cell carcinoma in rats by El-Rouby [172]. Lycopene treatment at a dose of 2.5 mg/kg body weight by intragastric intubation once a day significantly decreased the incidence of 4-NQO induced tongue carcinogenesis. A decreased percentage of PCNA-positive nuclei was associated with lycopene treatment. Increased E-cadherin and β-catenin immunoexpression was recorded in the lycopene-treated group in comparison to the carcinogen group [172]. Cheng et al. investigated the chemopreventive effect of lycopene and other carotenoids in betel quid extract–induced hamster oral cancer model [173]. They reported that no carcinoma was found in the lycopene or mixed carotenoid groups, whereas apparent ones appeared in the control group. In dysplastic lesions, the expressions of PCNA by the lycopene were less than that of control group. In order to study the relationship between tomatoes, tomato products, and lycopene and cancers of the upper aerodigestive tract (oral cavity, pharynx, larynx, and esophagus), De Stefani et al. conducted a case–control study in Uruguay [174]. Two-hundred and thirty eight cases and 491 hospitalized controls were frequency matched on age, sex, residence, and urban/rural status. Both series were submitted to a detailed questionnaire, including tobacco smoking, alcohol drinking, and queries on 64 food items. Tomato intake was associated with a reduction in risk of 0.30 (95% CI, 0.18–0.51), whereas tomato sauce–rich foods displayed a protective effect of 0.57 (95% CI, 0.33–0.96 for the highest quartile of intake). The food group composed of raw tomato and tomato-rich foods showed a strong inverse association with head and neck cancer (OR, 0.23; 95%

CI, 0.13–0.39 for the highest quartile of intake). Lycopene was also strongly associated with a reduced risk of 0.22 (95% CI, 0.13–0.37). Adjustment of tomato intake for several phytochemicals explained almost completely its protective effect, which disappears in this model.

3.9 Prostate Cancer

Prostate cancer is the second leading cause of cancer deaths in males in the USA. It accounts for about 30% of all cancers that are diagnosed in men. The incidence of prostate cancer has increased dramatically in the last decade mainly due to the increase in screening using prostate-specific antigen. The prevalence of the precursor lesion, high-grade prostatic intraepithelial neoplasia (HGPIN), and carcinoma of the prostate increases with aging, starting in men in their early 30s. Lycopene has been postulated to be the protective compound against prostate cancer [175–178]. The epidemiologic studies on dietary lycopene intake or level and prostate cancer based on observational studies have been inconsistent overall. Several studies had appeared more promising but some studies are not supportive [163, 176, 177, 179–181]. In a review, Ilic and Misso reported that meta-analysis of four studies identified no significant decrease in the incidence of benign prostatic hyperplasia (RR =0.95, 95% CI 0.63, 1.44) or prostate cancer diagnosis (RR = 0.92, 95% CI 0.66, 1.29) between men randomized to receive lycopene and the comparison group [182]. Meta-analysis of two studies indicated a decrease in PSA levels in men diagnosed with prostate cancer, who received lycopene (mean difference = −1.58, 95% CI −2.61, −0.55). A strong inverse dose–response relationship between lycopene intake and prostate cancer risk was reported in a case–control study of Chinese men [183]. For lycopene intakes of 1,609–3,081, 3,081–4,917, and >4,917 μg/day, the relative risks of prostate cancer compared with lycopene intake <1,609 μg/day were 0.47 (95% CI: 0.25–0.86), 0.40 (95% CI: 0.21–0.77), and 0.17 (95% CI: 0.08–0.39), respectively. A significant inverse association between tomato intake and prostate cancer was similarly reported in a case–control study of 617 Canadian men with prostate cancer and 636 age-matched controls conducted between 1989 and 1993. The RR of prostate cancer was 0.64 (95% CI: 0.45–0.91) for tomato intake >73 g/day compared with <24 g/day. However, there was no significant association reported for lycopene intake and prostate cancer [184]. As per results from a recent meta-analysis [180], high serum lycopene levels were associated with significant decreased risk of prostate cancer: RR = 0.78 (95% CI: 0.61–1.00). Subsequent studies on serum lycopene levels conducted in the post-PSA era [185, 186] have not reported significant inverse associations with total prostate cancer risk. Rao et al. observed significantly lower serum and prostate tissue lycopene levels (44%, 78%, respectively) in patients with PCa than in controls in a case–control study [37]. Lycopene and beta-carotene were the predominant carotenoids in human prostate glands obtained by radical prostatectomy from patients with PCa [187]. Also, some preliminary data suggest that persons with a variant in the XRCC1 gene are at higher prostate cancer risk if their lycopene intake is low [188]. This may suggest that effects of lycopene intake can differ by an individual's genetic characteristics [189].

In in vitro studies, using PCa LNCaP and PC-3 cells, Ivanov et al. [190] reported that treatment of cells with lycopene-based agents (100 nM) resulted in mitotic arrest, with cells accumulating in G0/G1 phase. There was block in G1/S transition mediated by decreased levels of cyclins D1, E, cdk-4, and suppression of retinoblastoma phosphorylation. These responses correlated with decreased IGF1R expression and activation, increased IGFBP-2 expression, and decreased AKT activation. Exposure to lycopene also induced a profound apoptotic response in LNCaP cells.

Several studies have been published supporting an inhibition of prostate tumorigenesis by lycopene in transplantable model systems [191]. Remarkably, few carcinogenesis studies have been reported [192]. Siler et al. studied the effects of lycopene consumption in the MatLyLu Dunning prostate tumor model on rats. The three treatment groups were fed diets containing 200 mcg lycopene, 540 mcg vitamin E, or both. The other two groups were control groups [193]. After 4 weeks of supplementation, tumors were induced by injection of 10^5 MatLyLu prostate tumor cells into the ventral prostate lobe. Both vitamin E and lycopene single treatment substantially increased the necrotic area of the tumors which were examined in vivo by MRI, to 36.4% and 36.0%, respectively, compared to 20.0% and 23.3% in the two control groups. There was no significantly increased necrosis rate (27.5%) in the vitamin E and lycopene combination group. Also, the influence of lycopene and/or vitamin E on gene expression in prostate tumors was investigated. The hallmark of both lycopene and vitamin E effect was suppression of genes involved in steroid metabolism and signaling, mechanisms known to be important in prostate cancer development. Moreover, a set of androgen target genes was consistently downregulated in both lycopene-treated groups**.** These investigators also showed more pronounced anti-androgen and anti-inflammatory effects of lycopene in normal prostate tissue compared to prostate tumor tissue. In a xenograft model of BALB/c nude mice implanted with DU145 prostate cancer cells, administration of 100 and 300 mg/kg of lycopene reduced tumor growth to 55.6% and 75.8%, respectively [191], while, in a different model represented by PC-346 C orthotopic mouse, no effect was observed at a lower dose (5 or 50 mg/kg) [194]. In other study using rats, Boileau et al. did not observe any anticancer effect of lycopene compared to the control group, but risk of death was lower for rats fed with the tomato powder diet than for rats fed with the control diet [195]. Although there is considerable interest in the role of lycopene as a therapeutic agent in prostate cancer, only a few small clinical trials have been reported. For example, in a randomized two-arm clinical trial, patients with a diagnosis of prostate cancer who were scheduled to undergo radical prostatectomy were randomly assigned to either 30 mg of oral lycopene supplementation or no intervention for 3 weeks prior to surgery. The study reported that the plasma PSA level decreased by 18% in the intervention group, while it increased by 14% in the control group over the study period. In the intervention group, 11 of 15 patients (73%) had no involvement of surgical margins and/or extra-prostatic tissues with cancer, compared to 2 of 11 patients (18%) in the control group. Twelve of 15 patients (80%) in the lycopene group had tumors that measured 4 cc or less, compared to 5 of 11 (45%) in the control group [196]. In the same study, Kucuk et al. noted that the expression of Cx43, in the malignant part of the prostate glands, was higher in the lycopene group than the control group. Prostatic tissue lycopene levels were 47% higher in the intervention group compared to control group, which was significantly different [196]. Chen et al. conducted a similar clinical trial to examine the effects of consumption of tomato sauce–based pasta dishes in patients with prostate cancer [197]. Thirty-two patients with localized prostate adenocarcinoma consumed a lycopene-rich diet for 3 weeks (30 mg of lycopene per day) preceding their scheduled radical prostatectomy. After the dietary intervention, serum and prostate lycopene concentrations were significantly increased. Serum PSA levels decreased from 10.9 ng/mL (95% CI = 8.7–13.2 ng/mL) to 8.7 ng/mL (95% CI = 6.8–10.6 ng/mL). Furthermore, leukocyte oxidative DNA damage was significantly reduced, from 0.61 8-OHdG/10^5 dG to 0.48 8-OHdG/10^5 dG. Prostate tissue oxidative DNA damage was significantly lower in men who had consumed the lycopene-rich diet than in the randomly selected patients (0.76 8-OHdG/10^5 dG and 1.06 8-OHdG/10^5 dG, respectively). The efficacy of lycopene plus orchiectomy with orchiectomy alone in 54 patients with metastatic prostatic cancer was investigated by Ansari and Gupta [198]. After 6 months of follow-up, there was a significant reduction in PSA level in both groups, but more marked in the lycopene plus orchiectomy group (mean 9.1 and 26.4 ng/mL). After 2 years, these changes were more consistent in the lycopene group (mean 3.01 and 9.02 ng/mL). Eleven (40%) patients in orchiectomy and 21 (78%) patients in the lycopene plus orchiectomy group had a complete PSA response. Bone scans showed that in the orchiectomy arm, only four (15%) patients had a complete treatment response, whereas in the lycopene plus orchiectomy group, eight (30%) patients had a complete response. Additionally, there was a significant improvement in the peak urine flow rate in the lycopene group. Twelve (22%) patients in the orchiectomy group and seven (13%) in the lycopene group died of prostate cancer.

In a study, total of 40 patients with high-grade PIN (HGPIN) were randomly assigned to receive 4 mg of lycopene twice daily for 1 year or no treatment [199]. The serum PSA level in the treatment group changed from a mean level of 6.07–3.5 ng/ml, while in the control group, PSA increased from a mean value of 6.55–8.06 ng/ml. During follow-up, six patients had increased PSA in treatment group and nine in control group. Subsequent prostate biopsy in these patients with increased PSA showed four patients with benign prostate hypertrophy (BPH) and two with adenocarcinoma in study treatment group, whereas the total was three with BPH and six with adenocarcinoma in the control group. In a non-randomized dietary intervention study, patients with histological diagnosis of BPH and plasma levels of serum PSA between 4 and 10 ng/ml had a decrease of 11% in their serum PSA levels after consuming 50 g of tomato paste daily for 10 weeks [200]. In the same way, Clark et al. conducted a phase I–II trial of lycopene supplementation in 36 men with biochemically relapsed prostate cancer after definitive local therapy [201]. Six consecutive cohorts of six patients each received daily supplementation with 15, 30, 45, 60, 90, and 120 mg/day for 1 year. The primary endpoints were PSA response (defined as a 50% decrease in serum PSA from baseline), pharmacokinetics, and the toxicity/tolerability of the regimen. No serum PSA responses were observed and 37% of the patients had PSA progression. The plasma levels of lycopene were similar for a wide dose range (15–90 mg/day) and reached a plateau by 3 months. In a case–control study, an inverse association was found for *cis*-lycopene-1 only. *cis*-Lycopenes 2 through 5 individually and in sum as total *cis*-lycopene and trans-lycopene were not associated with prostate cancer risk [202]. The authors suggest that the structural type of lycopene measured may influence the ability to detect an association, as one cis isomer but not total cis- or trans-lycopene was associated with decreased prostate cancer risk. The multicenter case control Third National Health and Nutrition Examination Survey (NHANES III) of US Caucasian and African-American men aged 40–79 years reported a significant inverse association between serum lycopene and aggressive prostate cancer (highest compared with lowest quartile RR = 0.37; 95% CI: 0.15–0.94; Ptrend = 0.04) and nonsignificant association between serum lycopene and prostate cancer (highest compared with lowest quartile RR = 0.65; 95% CI: 0.36–1.15; Ptrend = 0.09) [203]. In a double-blind randomized placebo-controlled trial, 105 African-American male veterans recommended for biopsy to detect prostate cancer were administered tomato sauce containing 30 mg/day of lycopene or placebo over 21 days [204]. The group randomized to lycopene had an increase in serum lycopene and decrease in PSA while the placebo group had the reverse, with a decrease in serum lycopene and increase in PSA. This study did not report a significant decrease in prostate cancer risk for individuals administered lycopene, but the study duration of 21 days was likely inadequate to significantly influence prostate cancer risk. Yang et al. demonstrated that lycopene can inhibit the proliferation of androgen-dependent prostate LNCaP cancer cells through the activation of the peroxisome proliferator-activated receptor gamma (PPARγ)-liver X receptor alpha (LXRα)-ATP-binding cassette transporter 1 (ABCA1) pathway [14]. They also showed that lycopene significantly increased protein and mRNA expression of PPARγ, LXRα, and ABCA1 and cholesterol efflux (i.e., decreased cellular cholesterol and increased cholesterol in culture medium). Lycopene (10 μM) in the presence of a specific antagonist of PPARγ (GW9662) or of LXRα (GGPP) restored the proliferation of DU145 cells and significantly suppressed lycopene-induced protein and mRNA expression of PPARγ and LXRα and cholesterol efflux. Liver X receptor α knockdown by siRNA against LXRα significantly promoted the proliferation of DU145 cells, whereas si-LXRα knockdown followed by incubation with lycopene (10 μM) restored the proliferation to the control level [14].

3.10 Renal Cancer

Renal cell carcinoma (RCC), the most common type of kidney cancer in adults, has been increasing worldwide, at least until the mid-1990s. RCC will account for approximately 3.8% of adult malignancies and 90–95% of neoplasms arising from the kidney in 2010 [205]. There will be an estimated 58,240 new cases of and 13,040 deaths from renal cancer in 2010 in the USA, accounting for 2.3% of all cancer deaths in the USA [205]. Dietary factors have been linked to RCC [206]. For example, several studies indicate an increased risk of RCC with increased consumption of meat [207], fried meats [208], dairy products [209], margarine, and oils [210]; reduced risks of RCC have been observed with increased intake of vegetables and fruit [207]. On the other hand, micronutrients with anticancer and antioxidant capacities such as carotenoids were associated with a reduced risk of renal cancer [208]. In Eker rats, we found that presence of tumor ranged from 94% to 65% among the experimental group, which was independent of dietary lycopene, but the differences were not significant. Mean numbers of renal carcinomas were decreased in lycopene-treated rats when compared to untreated control. Moreover, tumor numbers linearly decreased and tumor number tended to decrease linearly as supplemental lycopene increased from 0 to 200. Control rats fed only with basal diet had a greater length of smooth muscle tumors (23.98) than rats fed with lycopene supplement groups (12.90 and 11.07). Moreover, tumor length linearly decreased and tumor length tended to decrease linearly as supplemental lycopene increased from 0 to 200. All tumors showed strong staining with antibodies against mTOR, phospho-S6, and EGFR [211].

3.11 Leiomyoma

Uterine leiomyomas arise from the uterine myometrium and are the most common benign tumors in women of reproductive age and are the leading cause of hysterectomy in the USA, accounting for 1.2 billion dollars in hospital expenditures annually [212, 213]. Clinically significant leiomyoma occurs in 30% of these women [214], resulting in a menorrhagia/anemia, pelvic pain/pressure, dyspareunia, and, in some cases, reproductive dysfunction**.** Etiology of fibroids is not well understood although studies have shown that these tumors are hormonally dependent, similar to breast and ovarian cancers [215]. Treatment options depend on the size and location of the tumor, patient's symptoms, age, and future reproductive plans. Medical treatment involves gonadotropin-releasing hormone (GnRH) agonists, GnRH-antagonists, oral contraceptive pills whereas surgical approaches are myomectomy, hysterectomy, endometrial ablation, or uterine artery occlusion [214]. Terry et al. reported that total lycopene intake was not associated with diagnosed uterine leiomyoma risk [213]. They reported that compared to women in the lowest quintile of cumulative average lycopene intake, the corrected HRs for women in the second, third, fourth, and fifth quintiles of cumulative lycopene intake were 1.01 (95% CI = 0.86–1.18), 1.11 (95% CI = 0.94–1.31), 0.90 (95% CI = 0.75–1.08), 0.97 (95% CI = 0.79–1.20) and with regard to baseline intake of lycopene, women in the highest quintile had a 7% lower risk of diagnosed uterine leiomyoma of borderline significance, but there was no association with other levels of lycopene use and no trend in the association. However, in a previous study [216], we reported that tomato powder and lycopene supplementation decreased the number of leiomyoma compared with control subjects in quail. We also found that no measurable lycopene could be detected in the serum of control birds, whereas a dose-dependent increase was observed in the serum of lycopene-supplemented birds. Similar to tumor size results, we reported that the administration of a lycopene supplement to quail results in reduced size of leiomyoma in the oviduct compared to quail who did not receive the lycopene supplement. In Eker rats, we found that the incidence of leiomyoma in control and lycopene-treated uteri were 39%, 48%, and 21%, respectively, but the differences were not significant. The presence of tumor was 7/18 in the control, while the presences were 10/21 in 100, and 4/19 in 200 groups, respectively. Lycopene did not significantly reduce the mean numbers of leiomyoma compared to the control value. The average diameter of tumors was smaller in the group supplemented with higher dose compared to the group supplemented with lower dose of lycopene, indicating a dose response. The mean diameter of tumors was 7.00 mm in the control, while the diameters were 5.00 in 100, and 2.80 cm in 200 groups, respectively [217].

4 Chemotherapy and Lycopene

Chemotherapy is a common method of treatment for many types of cancer. The side effects of chemotherapy come about in part because cancer cells are not the only dividing cells in the body. Chemotherapeutic agents cause increased production of free radicals that can be harmful for normal cells, and these free radicals can be bound by antioxidant vitamins and supplements, including lycopene. A large number of studies have reported the beneficial effects of a variety of antioxidants in antineoplastic agents-induced nephrotoxicity, hepatotoxicity, ototoxicity, and peripheral neuropathy [218, 219]. Chemoprotective activities of lycopene [219, 220], and other dietary components that scavenge free radicals induced by exposure to antineoplastic agents [218, 220] have been well documented (Table 129.2).

Administration of antineoplastic agents during cancer chemotherapy causes oxidative stress as evidenced by increased lipid peroxidation products but decreases total radical-trapping capacity of blood plasma, plasma levels of antioxidant vitamins, and glutathione levels and activity of antioxidant enzymes [218, 219, 221]. In a study, Erhola et al. showed that anthracycline-based chemotherapy caused decreased plasma total antioxidant capacity after chemotherapy treatment in small cell lung cancer patients [222]. The high level of oxidative stress during chemotherapy may overcome the antioxidant defenses of cancer cells, with the resulting lipid peroxides reducing or halting cancer cell proliferation and interfering with antineoplastic activity. Thus, the antioxidant status of cancer patients may play an important role in their response to chemotherapy, with individuals having an impaired status being relatively unresponsive. However, supportive nutritional therapy with antioxidants during chemotherapy, which reduces the generation of lipid peroxides that results from the treatment [218], may overcome the growth-inhibiting effects of oxidative stress and maintain responsiveness to antineoplastic

Table 129.2 Potential mechanisms of action of lycopene in chemotherapy [219]

Observed effect	Potential mechanisms
Reduced lung coefficients as well as the extent of alveolitis and pulmonary fibrosis	Decreased oxidative stress and inflammation markers and expression of TNF-a in lung
	Increased antioxidant enzymes activities
Protective effects against cisplatin-induced nephrotoxicity and adriamycin-induced cardiotoxicity	Decreased oxidative stress and expression of renal Bax, HSP60, and HSP70 proteins
	Reduction in the number of abnormal metaphases

agents. Block et al. have previously reviewed randomized controlled trials in which antioxidants were given with chemotherapy and survival and tumor response outcomes were measured [223]. They reported that no indication was found that antioxidants were associated with decreased survival or tumor response, although further research with very large sample sizes would be required to definitively reject the hypothesis of interference. Their analysis suggested, in fact, that concurrent use of supplements and chemotherapy treatments might produce better tumor response rates and increased chances of survival, although small sample sizes and low quality of studies precluded firm conclusions. It is reported that antioxidants might protect cancer cells against the oxidative damage induced by chemotherapy, which would mitigate against their use or they may enhance drug-induced cytotoxicity by blocking reactive oxidant species [224].

Several studies have demonstrated that lycopene, a strong antioxidant, can influence the response to chemotherapy as well as the development of adverse side effects that results from treatment with antineoplastic agents (Table 129.3) [225, 229, 230]. Zhou et al. reported that lycopene can partially reduce the extent of pulmonary fibrosis induced by bleomycin in rats [227]. They found that the lung coefficients in group lycopene were reduced (day 14) as well as the extents of alveolitis (day 7 and 14) and pulmonary fibrosis (day 14 and 28) compared with bleomycin group. The concentrations of TNF-alpha (day 7, 14, and 28), NO (day 14), and malonyldialdehyde (day 3) in plasma as well as the expression of TNF-alpha in lungs decreased, whereas the plasma superoxide dismutase activities increased (day 28) in lycopene-supplemented group compared with bleomycin group. Lycopene supplementation has also been shown to enhance the antitumor activity of cisplatin and ameliorate cisplatin-induced renal oxidative stress in rats [225]. To test the protective effect of lycopene against cisplatin-induced oxidative damage in renal tissue, we treated cisplatin-administered rats with tomato lycopene complex (6 mgkg^{-1}, daily; 6% lycopene, 1.5% tocopherols, 1% phytoene and phytofluene, 0.2% b-carotene). Cisplatin administration (7 mgkg^{-1} i.p., single dose) resulted in a significant increase in serum urea-N (171 vs. 37 mg/dl) and creatinine (1.80 vs. 0.42 mg/dl) and decrease in body weight in comparison with the control rats. Serum creatinine and urea-N levels were lower in rats treated with tomato lycopene complex + cisplatin compared with rats treated with cisplatin alone. Significantly higher malondialdehyde (172 vs. 93 nmol/g) and 8-isoprostane levels (1,810 vs. 610 pg/g) was observed in the renal tissue of cisplatin-treated rats in comparison to the control rats. Tomato lycopene complex prevented the rise of >malondialdehyde and 8-isoprostane. No measurable lycopene could be detected in the serum of animals that are not supplemented while lycopene was detected in the serum of rats supplemented with tomato lycopene complex (0.19 and 0.11 µmol/L). Expression of Bax protein in renal tissue was significantly higher in cisplatin-treated rats compared with control rats, and tomato lycopene complex treatment significantly reduced the levels of Bax in renal tissue. Tomato lycopene complex did not affect the level of Bcl-2 expression in cisplatin-administered rats.

Table 129.3 In vivo and in vitro studies with lycopene and chemotherapy [219]

First author (References)	Agents	Toxicity	Treatment	Results
Dogukan et al. [225]	Cisplatin	Nephrotoxicity	Male Wistar rats; groups: control rats, tomato lycopene complex rats (6 mg kg^{-1}, daily); cisplatin-injected rats (7 mg kg^{-1} i.p., single dose); cisplatin-injected + tomato lycopene complex–treated rats	*Cisplatin*
				Increased serum urea-N and creatinine, MDA production, 8-isoprostane levels
				Increased renal Bax protein expression
				Lycopene supplementation
				Decreased serum creatinine and urea-N levels
				Prevented the rise of MDA and 8-isoprostane
				Reduced the levels of Bax
				No difference in the level of Bcl-2 between tomato lycopene complex–treated/cisplatin-administered rats and cisplatin-injected rats
				Lowered the expression of renal HSP60 and HSP70
Rios et al. [226]	Cisplatin	Human DNA	Cell-free experimental model by the bixin and lycopene	Inhibition of ROS generation by DNA–cisplatin interaction by both lycopene and bixin in a concentration-dependent manner
				At a concentration of 100 lM, lycopene inhibited superoxide anion generation at 90 %, the total ROS generation at 44 %

(*continued*)

Table 129.3 (continued)

First author (References)	Agents	Toxicity	Treatment	Results
Zhou et al. [227]	Bleomycin (BLM)	Pulmonary fibrosis	Sixty Sprague–Dawley rats; groups; control group, BLM-treated (intratracheal instillation) group, lycopene group (administered at a dose of 5 mg/kg body weight once a day) + BLM-treated group	Reduced by lycopene
				Lung coefficients as well as the extents of alveolitis and pulmonary fibrosis
				Decreased by lycopene
				TNF-alpha ($P < .001$),
				NO (day 14, $P < .05$),
				MDA (day 3, $P < .01$)
				Expression of TNF-alpha
				Increased plasma superoxide dismutase
Ferreira et al. [228]	Doxorubicin	Cardiac myocyte	Wistar male rats, groups: control (C), lycopene (L; tomato oleoresin 5 mg/kg body wt. day for a 7-week period), doxorubicin (D; 4 mg/kg body wt., i.p. at the 3rd, 4th, 5th, and at 6th week) and doxorubicin + lycopene (DL) groups	Higher cardiomyocyte levels of SBs, SBs FPG, and SBs Endo III in rats from D when compared to other groups
				No differences in DNA damage levels in cardiomyocytes from DL when compared to C and L groups
				The viability of cardiomyocytes from D or DL was lower than C or L groups
				Similar lycopene levels (mean+/−S.D.nmol/kg) in hearts between L (47.43+/−11.78) and DL (49.85+/−16.24) groups
Sendão et al. [229]	Cisplatin	Chromosomal aberrations	Rats, three lycopene doses in the acute treatment (2, 4, and 6 mg/kg b.w.), three lycopene doses in the subacute treatment (0.5, 1.0, and 1.5 mg/kg b.w.) with and without cisplatin (5 mg/kg b.w. i.p.)	Lycopene is neither cytotoxic nor clastogenic when compared with the negative controls
				Cisplatin-treated animals submitted to acute and subacute treatments with different lycopene doses and showed a significant reduction in the number of abnormal metaphases when compared with the animals treated only with cisplatin

(*continued*)

Table 129.3 (continued)

First author (References)	Agents	Toxicity	Treatment	Results
Karimi et al. [230]	Doxorubicin	Cardiotoxicity	Mice; doxorubicin toxicity(15 mg/kg; i.p) tomato extract (1.2 and 2.4 g/kg, i.p.) and lycopene (1.7 and 3.5 mg/kg, i.p.)	Lycopene supplementation Prevented the rise in serum creatine kinase-MB Ameliorated cardiac cell injury
Puri et al. [231]	Radiotherapy + Paclitaxel	High-grade gliomas	50 patients with anaplastic astrocytoma or GBM. All of them received RT to a dose of 60 Gy in 30 fractions over 6 weeks with concomitant Paclitaxel, 60 mg/m^2 i/v weekly. Two groups: Group A: (n = 25) received 8 mg of oral lycopene daily, Group B: (n = 25) received placebo; along with RT	Lycopene levels in patients of Group A were 152 and 316 ng/ml and in Group B were 93 and 98 ng/ml Among Group A (lycopene group): 10 (40 %) patients had complete response (CR), 10 (40 %) had partial response (PR), 1 (4 %) had stable disease (SD), 3 (12 %) had progressive disease (PD) while response was not available for 1 patient (4 %) Among Group B (placebo group); 5 (20 %) patients had CR, 6 (24 %) had PR, 1 (4 %) had SD, 8 (32 %) had PD while response was not available for 5 patients (20 %)

The expression of renal HSP60 and HSP70 was significantly lower in tomato lycopene complex + cisplatin-treated rats compared with rats treated with cisplatin alone [225]. The protective effect of tomato extract (1.2 and 2.4 g/kg, i.p.) and lycopene (1.7 and 3.5 mg/kg, i.p.) to reduce acute doxorubicin (15 mg/kg)-induced myocardial toxicity has been demonstrated in mice by preventing the rise in serum CPK(MB) and ameliorate cardiac cell injury [230]. Sendão et al. investigated the cytogenetic effects of a single acute and four daily gavage administrations of lycopene, and to examine possible protective effects on chromosomal damage induced by the antitumor drug cisplatin in rat bone marrow cells and reported that lycopene is neither cytotoxic nor clastogenic when compared with the negative controls [229]. Cisplatin-treated (5 mg/kg b.w. i.p.) animals subjected to acute (2, 4, and 6 mg/kg b.w.) and subacute (0.5, 1.0, and 1.5 mg/kg b.w.) treatments with different lycopene doses showed a significant reduction in the number of abnormal metaphases when compared with the animals treated only with cisplatin. Ferreira et al. has shown that tomato oleoresin at 5 mg/kg body weight enhances the chemotherapeutic effect of doxorubicin [228]. Lycopene reduced the cardiotoxic effect of doxorubicin. They also found that cardiomyocyte levels of SBs, SBs FPG, and SBs Endo III were higher in rats from doxorubicin when compared to other groups. DNA damage levels in cardiomyocytes from doxorubicin+lycopene were not different when compared to control and lycopene groups. The viability of cardiomyocytes from doxorubicin or doxorubicin + lycopene was lower than control or lycopene groups. In another study, Rios et al. tested the modulation of reactive oxygen species produced by the cisplatin–human DNA interaction in a cell-free experimental model by the carotenoids bixin and lycopene, extracted from natural dietary sources and purified through luminol- and *Cypridina* luciferin methoxy-analogue (MCLA)–enhanced chemiluminescence assays and reported that results showed that the ROS generation by DNA–cisplatin interaction was inhibited by both lycopene and bixin in a concentration-dependent manner [226]. At a concentration of 100 μM, lycopene and bixin inhibited superoxide anion generation at 90% and 82%, respectively, and the total ROS generation at 44% and 42%, respectively. We also showed that cisplatin-induced decrease in the levels of Nrf-2 and HO-1 was counteracted by lycopene. On the other hand, cisplatin-mediated increase in NF-kappaB p65 was brought down by lycopene [221].

5 Radiation Therapy and Lycopene

An ideal radioprotective agent would be easily administered, inexpensive, nontoxic, and would selectively protect normal tissues, but not the tumor, from radiation. Prevention of radiation toxicities may improve patient's quality of life and may allow higher doses of radiation administered, which may lead to higher disease response and/or cure rates. In a randomized placebo control study [231], 50 patients with high-grade gliomas were treated with surgery followed by adjuvant radiotherapy and concomitant paclitaxel. Patients were randomized to receive either oral lycopene 8 mg daily with radiotherapy or placebo. They found that pre- and posttreatment plasma lycopene levels in the patients in lycopene A were 152 ng/ml and 316 ng/ml and in the patients in control group were 93 ng/ml and 98 ng/ml ($P = 0.009$). There were nonsignificant differences in favor of lycopene between Group A and Group B with higher overall response at 6 months, response at last follow-up, and time to progression (40.83 vs. 26.74 weeks). Andic et al. evaluated whether lycopene could reduce acute gastrointestinal side effects in Wistar albino rats receiving abdominopelvic irradiation [232].

They reported that rats receiving radiotherapy, using 8 Gy single fractions, lost weight within 4 days after radiotherapy and weight loss rates were significantly higher in the radiotherapy-only group compared to lycopene + radiotherapy group. Diarrhea ratio was 72.7% (8/11) in the radiotherapy-only group and 46.7% (7/15) in lycopene + radiotherapy group. Plasma TBARS levels, 4 and 6 days after radiotherapy, were significantly higher in radiotherapy-only group than in lycopene + radiotherapy group. As a result, lycopene reduced oxidative stress, diarrhea, and weight loss seen after abdominopelvic irradiation in Wistar rats. Since lycopene has activity against prostate cancer, it may be particularly beneficial in prostate cancer patients receiving pelvic radiation therapy by preventing radiation toxicity in the bowel and bladder while exerting an anticancer effect on the prostate.

6 Conclusion

In this chapter, we have summarized the potential effects of lycopene in prevention and treatment of cancer. Lycopene intake and plasma levels of lycopene have been inversely associated with cancer risk in most of the case–control studies and animal studies. Clinical trials need to be conducted to investigate lycopene in prevention of cancer and as an adjunct to standard cancer therapy. Until more data is available regarding lycopene supplementation, it is suggested that the potential health benefits of lycopene can best be achieved through a diet rich in a variety of fruits and vegetables, which include tomatoes.

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Xanthophylls and the Eye 130

Hannah E. Bartlett

Contents

1 Introduction 3925
  1.1 Retinal Anatomy 3925
  1.2 Structure and Function of the Photoreceptors 3926
  1.3 The Retinal Pigment Epithelium, Bruch’s Membrane, and Choriocapillaris 3927
  1.4 The Macula 3928
  1.5 Lutein, Zeaxanthin, and Meso-zeaxanthin: The Macular Pigment 3928
  1.6 Lutein and Zeaxanthin Uptake and Bioavailability 3929
  1.7 Meso-zeaxanthin 3929
  1.8 Spatial Profile and Distribution of Xanthophylls and Macular Pigment 3930
  1.9 Measurement of Macular Pigment 3931
  1.10 Normal Values of Macular Pigment 3932
  1.11 Functions of Macular Pigment 3933
  1.12 Effect of Nutritional Supplementation or Dietary Modification on MPOD 3933
2 Xanthophylls and Normal Visual Function 3935
  2.1 Chromatic Aberration 3936
  2.2 Visibility 3937
3 Relating the Function of Macular Pigment to Retinal Structure and Disease Pathogenesis 3937
4 Age-Related Macular Degeneration and the Xanthophylls 3937
  4.1 Terminology 3938
  4.2 Pathogenesis of AMD: Retinal Pigment Epithelium Structure 3939
  4.3 Pathogenesis of AMD: Lipofuscin Formation 3940
  4.4 Clinical Evidence Regarding the Proposed Role of Macular Pigment in the Prevention of Age-Related Macular Disease 3941

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5 Macular Pigment as an Antioxidant 3943
5.1 In Vitro Studies on Lipofuscin 3943
6 Macular Pigment as a Blue-Light Filter 3944
6.1 Filtering Characteristics of the Eye 3944
6.2 Retinal Effects of Blue Light 3945
6.3 The Role of Photooxidation in the Onset of Disease 3946
6.4 Photooxidation and the Role of Antioxidants 3947
7 Conclusion 3948
References 3948

Abstract

Lutein (L) and zeaxanthin (Z) are the only carotenoids found within the retina, and although they are not essential micronutrients, they have antioxidant and photoprotective functions that are thought to be useful in the prevention of onset or progression of age-related macular degeneration (AMD). This condition is the leading cause of visual loss in the developed world, and the number of people affected by it is predicted to increase dramatically as the proportion of the population aged over 65 years increases. Epidemiological studies suggest that people who consume high levels of L and Z are at lower risk of AMD than those who consume low levels. Intervention studies have shown that increasing dietary intake of foods that contain L and Z or consuming L and Z supplements can increase serum and retinal levels of L and Z. L and Z supplementation is thought to increase macular pigment optical density (MPOD, the amount of L and Z within the macular region of the retina), although clinical methods for MPOD assessment can be unreliable. Small randomized controlled trials have demonstrated improvements in visual function in people who have age-related macular disease and have taken nutritional supplements that contain more than 10 mg L. The results of a large multicenter trial, AREDS 2, are due in December 2012. These results will inform clinical practice with regard to the recommendations that are made about L and Z supplementation for the prevention of progression of AMD.

Keywords

Age-related macular degeneration • lutein • macula • meso-zeaxanthin • retina • xanthophylls • zeaxanthin

Abbreviations

AMD	Age-related macular degeneration
ARM	Age-related maculopathy
ARMD	Age-related macular disease
HFP	Heterochromatic flicker photometry
L	Lutein
MP	Macular pigment
MPOD	Macular pigment optical density

MZ	Meso-zeaxanthin
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
Z	Zeaxanthin

1 Introduction

Carotenoids are a family of pigments that are divided into two main groups: carotenes and xanthophylls. They are introduced to the human body through dietary means alone, and although not considered to be essential micronutrients, they have important antioxidant and photoprotective properties. These functions have prompted interest in their potential role in prevention of disease.

Oxidative damage may be an important factor in the development of age-related diseases [1, 2]. Chemically, oxidation refers to the removal of electrons, and when it occurs within the body, it can result in the formation of cytotoxic chain reactions. Reactive oxygen species (ROS) is a term used to describe some types of free radicals, hydrogen peroxide, and singlet oxygen, which are all capable of damaging membrane lipids, proteins, nucleic acids, and carbohydrates via oxidation [3].

The eye is particularly prone to ROS damage. The transparency of the cornea, aqueous humor, lens, and retina allows continuous exposure to light, which along with aging, inflammation, air pollutants, and cigarette smoke has been shown to increase production of ROS [4, 5]. Polyunsaturated fatty acids are abundant in the retina, predominantly found in photoreceptor outer membranes, and are readily oxidized [4, 6, 7]. Phagocytosis, a process that produces ROS, occurs within the retinal pigment epithelium.

Lutein (L) and its stereoisomers, zeaxanthin (Z) and meso-zeaxanthin (MZ), are xanthophylls. They are relevant to human ocular health and as they are the only carotenoids present in the lens [8] and retina [9]. It has been suggested that they play a similar role in humans as in plants, as antioxidants and screeners of high-energy blue light [10]. Within the central retinal area, these xanthophylls are known as the macular pigment (MP). The MP may prevent some of the biological processes within the retina that can result in the development of sight-threatening macular diseases [11–16] and subsequent visual loss. In addition, it has been shown that those at very high genetic risk of macular disease may not exhibit retinal conditions that promote the accumulation and stabilization of MP [17] (Figs. 130.1–130.3).

1.1 Retinal Anatomy

The retina is a highly differentiated neuroectodermal tissue that can be divided into the central retina (the macula), which is specialized for detailed vision, and the peripheral retina, which is specialized for night vision. The human retina is

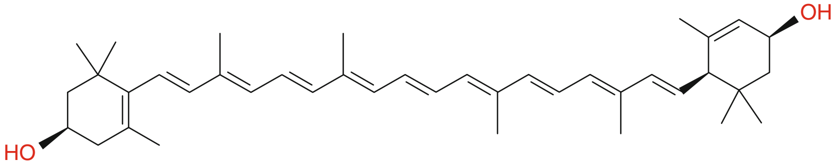


Fig. 130.1 Chemical structures of lutein

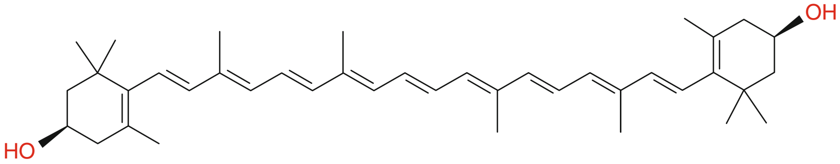


Fig. 130.2 Chemical structures of zeaxanthin

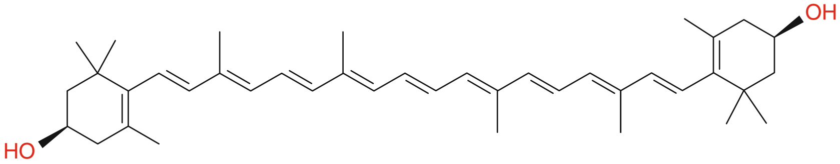


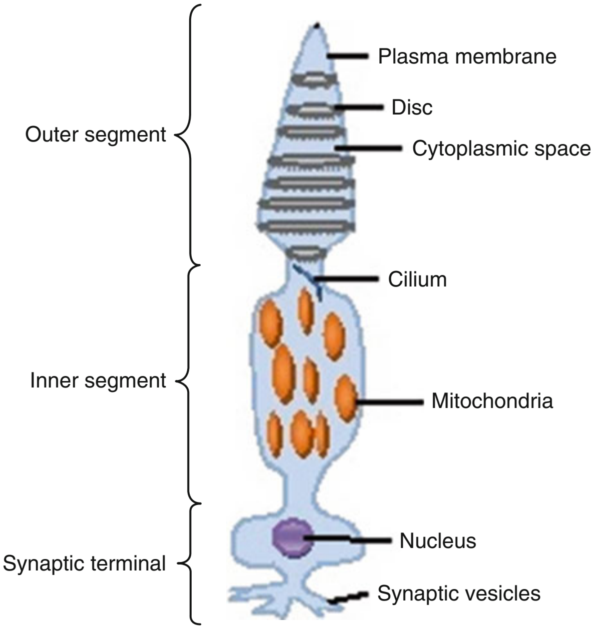
Fig. 130.3 Chemical structures of meso-zeaxanthin

comprised of three layers of nerve cell bodies and two layers of synapses. The outer nuclear layer contains photoreceptor cell bodies. The inner nuclear layer contains bipolar, horizontal, and amacrine cell bodies. The ganglion cell layer contains ganglion cells and displaced amacrine cells [18].

1.2 Structure and Function of the Photoreceptors

Within the human retina, there are two types of photoreceptors – rods and cones. There are approximately 92 million rods, predominantly found at the periphery of the retina, which contain the visual pigment rhodopsin and are required for night vision. There are approximately 4.6 million cones found predominantly in the macula, which contain the red, green, or blue opsin pigments necessary for color vision and sharp visual acuity [19]. These visual pigments are located within the outer segment discs of the photoreceptors and are essential for light absorption (Fig. 130.4).

Fig. 130.4 Schematic diagram of a cone photoreceptor (kindly provided by Dr. Emma Berrow)



1.3 The Retinal Pigment Epithelium, Bruch's Membrane, and Choriocapillaris

The retinal pigment epithelium (RPE) plays an important role in maintaining the health and efficiency of the tissues within which phototransduction occurs [20]. Each RPE cell is associated with around 30 photoreceptor cells. One important function of the RPE is the phagocytosis of outer segments that are shed by photoreceptors, allowing for constant turnover of photoreceptor membrane discs [21]. The RPE consists of a nonrenewing monolayer of cuboidal-shaped cells, positioned between the photoreceptors and the Bruch's membrane. The correctly functioning RPE also provides an outer blood-retinal barrier [22].

The RPE cells contain melanosomes that provide pigment to absorb stray light – essential for image sharpness by minimizing light scatter [23].

Within the visual cycle, the RPE is involved in the processing of the vitamin A derivative, retinal, which is transported to the photoreceptors and combined with opsin to form the visual pigments. Retinal pigment epithelial cells reduce uniformly over time with increasing age within the retina and at a greater rate equatorially than centrally [24]. Melanosome number also consistently decreases with increasing age, possibly through the damaging effects of blue-light irradiation [25].

Bruch's membrane lies between the RPE and the choriocapillaris. It provides a semipermeable membrane through which major metabolic transfer occurs [26].

The choriocapillaris is directly adjacent to Bruch's membrane and is comprised of an intricate network of blood vessels which provide oxygen and nutrients to the RPE. It is among the most highly perfused tissues in the body [27].

1.4 The Macula

The macula is the central portion of the retina. If you were to look straight through an individual's pupil toward the back inner surface of their eye, you would be looking at the macula. Within the macular region, photoreceptors are densely packed in order to facilitate high-detailed vision. The macula is defined as the portion of the posterior retina that contains xanthophylls and two or more layers of ganglion cells [28]. The macula is approximately 6 mm in diameter and is centered on the cone-dominated fovea, which is a depression in the inner retinal surface in the center of the macula measuring 0.8 mm in diameter. The parafovea encircles the fovea and is dominated by rod photoreceptors [19]. The central floor of the fovea is called the foveola, and this is 0.35 mm thick. The foveola lies within a capillary free zone and thus has no retinal circulation [28].

1.5 Lutein, Zeaxanthin, and Meso-zeaxanthin: The Macular Pigment

Lutein and Z are two of around 600 plant pigments in the carotenoid group and are both xanthophylls. They have the structural formula $C_{40}H_{56}O_2$, are not made within the body, and so can only be obtained from the diet. Carotenoids are synthesized in plants, where they are essential for photosynthesis [29] and photoprotection. L and Z are found in most fruits and vegetables, although Z is found in much smaller quantities than L [30, 31]. The highest mole percentages of L and Z are found in egg yolk and maize [30], although good sources are also spinach [32], collard greens, and kale [33]. A full list of L- and Z-containing foods can be found via the US Department of Agriculture website (www.nutriton.gov) by clicking on the "What's in food" link.

Although humans consume a wide range of carotenoids, L, Z, lycopene, beta-carotene, alpha-carotene, and beta-cryptoxanthin account for 90 % of circulating carotenoids [34]. Carotenoids are water-insoluble molecules and are transported around the body by water-soluble lipoproteins. Inside cells, they are solubilized in membranes or lipid vesicles, or bound to proteins. The transport and localization of L and Z is thought to be mediated by carrier proteins [35–37]. The human retina, and more specifically the macula, is the single richest site of carotenoid accumulation within the human body. Postmortem retinal analysis has shown that the total L and Z concentration at the macular is 100 times more than at the peripheral retina. The assumption that L and Z within the retina is of dietary origin is supported by fundus photographs of rhesus monkeys on carotenoid-depleted diets that demonstrate an absence of macular pigmentation [38].

In the foveal region of the macula, L and Z concentrations are at their highest [39], and in this location, they are known as the MP. The MP acts as optical filter, filtering out incoming blue light. The absorbance spectrum of MP peaks at 460 nm, which is the same as short-wavelength blue light. This is thought to protect the retina against the actinic effects of blue light that include damage to the RPE and overlying photoreceptors [40, 41].

1.6 Lutein and Zeaxanthin Uptake and Bioavailability

Although possible binding proteins have been identified, the mechanism for uptake of xanthophylls into the bloodstream is still not clear. The efficacy of absorption from the gut depends on its original source, for example, L from egg yolk [42] is more readily absorbed than that derived from green leafy vegetables [43]. This relatively low absorption from green leafy vegetables may be caused by complexing with proteins in chloroplasts within cell structures [44]. Xanthophylls that are associated with oil or fat, such as those found in egg yolk, may be more readily extracted during digestion [44].

Unesterified xanthophylls such as lutein in its pure form are absorbed by mucosal cells and subsequently appear unchanged in the circulation and peripheral tissue. Esterified xanthophylls must be de-esterified to their pure form before uptake [34, 45]. The xanthophylls are packaged as plasma lipoproteins by the liver, released into the systemic circulation, and absorbed by a range of tissues including liver, lung, adipose, skin, prostate, and macula [34, 46]. Their major storage site is adipose tissue [34, 47], so much so that a negative correlation between the adipose tissue L concentration and the amount of L and Z in the retina has been reported in women [48].

Although some tissues, such as the skin, are not particularly selective about carotenoid uptake and compositions (they are determined by serum levels), other tissues such as the macula are highly selective [49–51]. It is likely that specific binding proteins are involved when tissues exhibit such highly selective uptake and deposition of biological molecules.

Primates fed with carotenoid-free diets have no detectable yellow pigmentation of the macula [38], and studies have shown that macular pigment levels can be raised in humans using dietary supplementation [52–54]. Serum concentrations of L and Z are also reported to be responsive to dietary modifications [48, 53, 54].

1.7 Meso-zeaxanthin

Lutein and Z are produced as a single stereoisomer by plants [55]. However, the central retina also contains a high concentration (between 25 % [56] and 30 % [57]) of MZ. Meso-zeaxanthin has been found in the human macula, retina, and RPE but has not been detected in the plasma or liver [49]. Within the central macula, L, Z, and MZ are found in equal quantities, but the ratio of MZ to

Z decreases with increasing eccentricity [56]. This forms the basis for the assumption that MZ is formed via isomerization of L [56], and it is thought that the conversion mechanism is concentrated at the macula. The xanthophyll-binding protein may also act as an enzyme for the conversion of L to MZ. Supplementation with 16 mg MZ and 4 mg L is reported to increase MPOD levels by 18 % in 120 days [58]. This percentage increase is similar to that found when supplementing with only 20 mg L daily. This further supports the L to MZ conversion theory.

Meso-zeaxanthin is a xanthophyll that is not found within most foods but is present in some types of seafood [30, 55]. It is found to reach a maximum level in the central macula, where L levels reach a minimum [9]. This suggests that it may have some specific function at the macula. There may be a functional relationship between L and MZ within the central macula, as there is an inverse relationship between the two xanthophylls in terms of concentration at that location. Meso-zeaxanthin may be more effective than L at some essential role within the central macula and may not be needed in the peripheral retina. This suggests some kind of protective function in ARMD. It may also be that L is oxidized within the central retina, and then reduction results in its conversion to MZ [9]. The evidence suggests that there are specific mechanisms or biological pathways in place for the conversion of L to MZ within the central macula.

1.8 Spatial Profile and Distribution of Xanthophylls and Macular Pigment

The concentration of L, Z, and MZ is higher at the macula than anywhere else in the human body [39]. The distribution of MP generally peaks at the central macula (the fovea) [51, 59, 60]. The ratio of L to Z and MZ within 0.25 mm of the fovea is approximately 1:2.4 [60, 61], but the situation reverses at the retinal periphery, where the ratio is 2:1 [60]. In other words, Z and MZ are present in higher concentrations within the fovea, whereas L predominates in the more peripheral macula. It is proposed that retinal L is converted to MZ in the central macula [62].

Macular pigment has been located in the inner axons of the photoreceptors and the rod outer segments. Within the central fovea, the carotenoids are most concentrated within the photoreceptor axons of the Henle nerve fiber layer [59]. It may be that L and Z are transported here from the choroid, passing across the RPE and the photoreceptor outer segments.

In the perifoveal region, L and Z are present in the outer segments of rod photoreceptors [63, 64], where there is a high concentration of polyunsaturated fatty acids. These are particularly prone to oxidative attack. Within the rod outer segments, the concentration of L and Z is highest in the perifoveal region, where it is approximately 2.5 times higher than in the peripheral retina [64] (Fig. 130.5).

There is a 100 fold drop in the concentration of MP in the peripheral retina compared with the fovea, although levels vary considerably between donors [51, 65]. The MP is optically undetectable at a distance of 2 mm from the fovea,

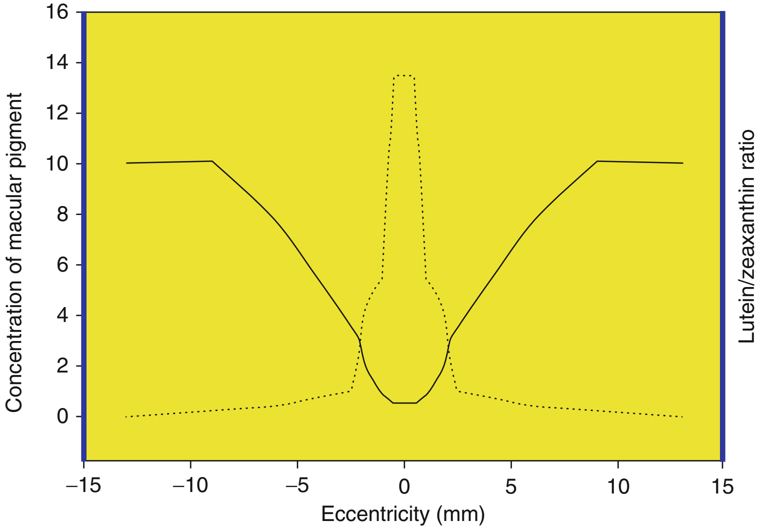


Fig. 130.5 Graph showing spatial profile of xanthophylls within the macular region (Adapted from [9])

although L and Z are present in very small quantities beyond this eccentricity (their combined concentration beyond 8.7 mm from the fovea is 1/300 of that within 0.25 mm of the fovea) [60]. The ratio of L to Z and MZ varies linearly with the ratio of rods to cones with increasing eccentricity up to approximately 6° from the fovea [60]. The hypothesis that Z is only found in the rods is refuted by the fact that the fovea contains predominately cones, as well as by the fact that squirrel monkey and macaque retinae have their highest concentration of L and Z in the central fovea [61].

1.9 Measurement of Macular Pigment

The measured amount of MP within a retina is known as macular pigment optical density (MPOD). The measurement of MPOD can be divided into two categories: subjective psychophysical techniques and objective optical techniques. Objective techniques include fundus reflectance and autoreflectance, and subjective techniques are psychophysical in nature. Heterochromatic flicker photometry (HFP) is the best known and most widely used subjective technique and involves the calculation of MPOD based on the luminance ratio of short-wavelength blue light presented in the central retina (where it is assumed to be partly absorbed by the MP) compared to that presented at a more peripheral retinal point (where MP levels are assumed to be minimal). When the short-wavelength light is alternated at an appropriate frequency with a wavelength that is not absorbed by MP and luminance of the two wavelengths is not perceived to be equal, then the combined stimulus will appear to flicker [66–70] (Fig. 130.6).

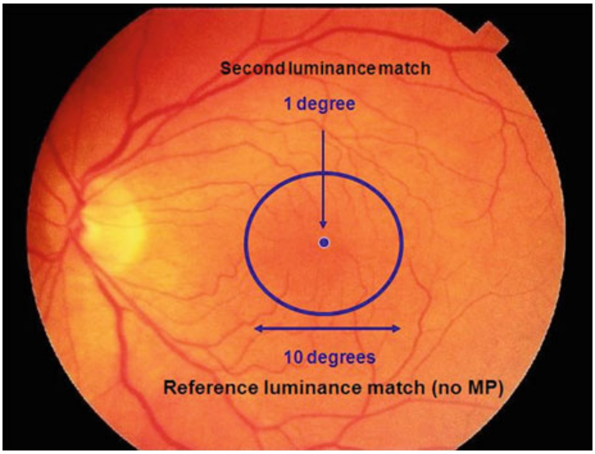


Fig. 130.6 Luminance matches in heterochromatic flicker photometry

Although there are various ways in which HFP can be used to measure MPOD, in all techniques, the observer is required to flicker match using one wavelength of light that is absorbed by MP and one wavelength of light that is not. These flicker matches are made in the central macula and at a peripheral point at which the MP is assumed to be absent. Conventional HFP requires the observer to adjust the luminance ratio of the two wavelengths of light at both retinal locations until the flicker is perceived to disappear, or be reduced to a minimal amount [71–74]. This can be conceptually difficult to achieve, and so more recent variations of HFP involve the subject indicating when the flicker is first noticed, which may be more intuitive, particularly for naïve subjects [75]. Comparison of HFP with objective measurement of MP using spectral fundus reflectance reports a high correlation between the two techniques ($r = 0.72$, $p < 0.001$) [76].

1.10 Normal Values of Macular Pigment

Studies that used HFP to measure MPOD have reported average central values in normal cohorts of 0.211 ± 0.13 (n = 280, age range 18–50 years) [77], 0.28 ± 0.21 (n = 280, age range 18–50 years) [78], 0.289 ± 0.156 (n = 46, age range 21–81 years) [79], 0.319 (n = 100, age range 22–60 years) [80], 0.43 ± 0.23 (n = 1,648, age range 53–86 years) [81], 0.47 ± 0.14 (n = 38, age range 19–46 years) [82], 0.50 ± 0.24 (n = 76, age range 18–74 years) [83], and from 0.58 ± 0.29 to 0.72 ± 0.27 (n = 24, mean age 38.1 ± 10.6 years) [84]. The wide range of mean MPOD values may be the result of differing test protocols and stimulus characteristics.

The relationship between MPOD and age has also been investigated using HFP; some studies have reported a negative correlation between the two variables [82, 85, 86], while others reported no relationship [60, 78, 83, 87, 88].

Ethnic differences in MPOD have also been reported, with South Indians displaying higher mean MPOD values (0.64 ± 0.23) [86] than Africans (0.59 ± 0.14) [89], Asian Chinese (0.48 ± 0.23) [90], and white non-Hispanics (0.36 ± 0.13) [89].

The relationship between gender and MPOD is also conflicted within the literature with several studies reporting no difference [77, 79, 82, 83, 90–93] and others reporting that females have lower MPOD than males [88, 94–96].

1.11 Functions of Macular Pigment

Carotenoids are also able to quench singlet oxygen (a potent oxidant) [97], scavenge reactive oxygen species [98], limit peroxidation of membrane phospholipids [99], and reduce lipofuscin formation [100]. The presence of MP in the rod outer segments and RPE [63, 64] is suggestive of a ROS-quenching function. The fact that L and Z have been found in higher concentration in the rod outer segments of the perifoveal retina than the peripheral retina lends support to their proposed protective role in age-related macular disease [63].

The absorbance spectrum of MP peaks at 460 nm, and it is purported to act as a broadband filter, reducing the sensitivity of the macular region to short-wavelength light which is most damaging in the 440–460 nm range [101, 102] (see Fig. 130.3). The presence of MP in the inner retinal layers [66] supports a photoprotective role (Fig. 130.7).

Blue light is the highest energy form of visible light and is known to induce photooxidative damage by generation of ROS. Lutein is reported to be a superior filter [103] due to the fact that it is orientated both parallel and perpendicular to the plane of the membrane [104]. Zeaxanthin is orientated perpendicular to the membrane plane only and so may not be able to absorb the excitation beam from all directions. Zeaxanthin, however, is reported to be a superior photoprotector during prolonged light exposure; the shorter timescale of protective efficacy of L has been attributed to oxidative damage of the carotenoid itself [104].

As well as reducing the potential for photooxidation, the blue-light filtering by MP may also reduce glare, increase contrast reduce chromatic aberration, and improve visual acuity.

1.12 Effect of Nutritional Supplementation or Dietary Modification on MPOD

Several studies have provided information about the relationship between dietary intake of L and Z and MPOD. Macular, peripheral retina, and crystalline lens levels of L, Z, and their metabolites were chemically measured in 228 eyes of 147 human donors in one study [105]. The results were then correlated with retrospective supplement histories from families of selected members of the study population. Investigators found that those eyes with unusually high levels of macular carotenoids tended

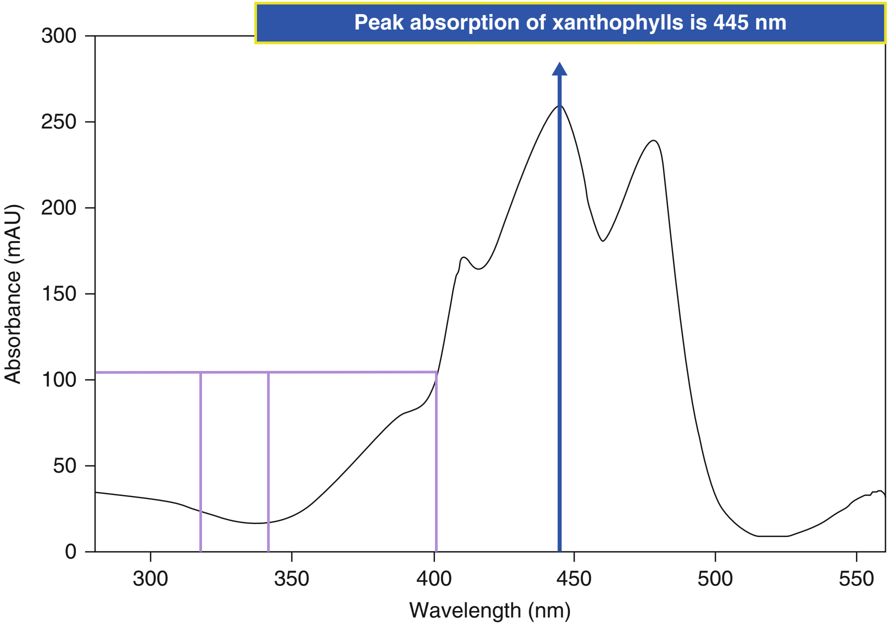


Fig. 130.7 The absorbance spectrum of xanthophylls

to come from donors who consumed high-dose L supplements before death and that in these eyes, carotenoid levels were also unusually high in the peripheral retina and lens. Lutein supplementation was less common in the eyes of donors whose macular carotenoids were at normal levels [106]. The investigators concluded that their findings support the hypothesis that L supplementation can increase levels of MP.

A study of dietary, serum, and retinal carotenoids in couples found that of 20 male-female married couples between the ages of 29 and 70 years who had been married for an average of 21.6 years (SEM = 2.4), the dietary intake and serum concentrations were correlated between partners but that MPOD levels (measured using HFP) were not [107]. There was, however, a statistically significant relationship between dietary intake and serum levels of carotenoids, and MPOD for the cohort.

Another study investigated the effect of consumption of two and four egg yolks per day on MPOD measured using HFP. Of 37 participants, those with low MPOD (defined as MPOD $\leq$0.50 at 0.25°, $\leq$0.4 at 0.5°, and $\leq$0.35 at 1°) showed increases of $\leq$50 % ($p < 0.05$) at the three retinal eccentricities when consuming four egg yolks per day for 5 weeks. Those consuming two egg yolks per day demonstrated a nonsignificant increase of 31 % in MPOD at 0.5° [108].

More recently, supplementation for 6 months with a combination of L, Z, and MZ was found to significantly increase MPOD in healthy subjects [109].

The results of these studies support the hypothesis that MPOD can be augmented by supplementation with L and Z, or by increasing dietary intake of these carotenoids by consuming foods rich in L and Z. Having said this, there is some variability in retinal response between subjects [48, 53, 110]. Short-term feeding studies have reported increases in retinal L and Z levels following a diet of L- and Z-rich foods [48, 53] and supplements [52, 110–112] for at least 3 months. The variability in retinal response may be related to differences in the bioavailability of carotenoids from different foods [113]. Cooking, chopping, or ingesting carotenoid-containing foods with dietary fat can all increase bioavailability of L and Z [114]. In other words, eating chopped spinach leaves with a little olive oil would make it easier for L and Z to be extracted during digestion than eating whole spinach leaves without oil. Also, choosing a nutritional supplement that contains L and Z bound in oil may also be more beneficial than choosing a dry powder formulation.

It has also been suggested that retinal levels of L and Z may be more easily affected by dietary modification and nutritional supplementation in men than women. Higher MPOD levels in men have been reported in some studies [94, 96, 115] but not others [80, 91]. MPOD levels are also reported to be lower in those people with higher levels of body fat [77, 96, 116]; this may be explained by the fact that adipose tissue is a preferred storage site for L and Z, and so the retina has to compete for the xanthophylls. MP is known to be lower in those with lighter iris color [116] and in those who smoke [77, 80, 115, 117]. Cultural differences in L and Z intake have been reported; for example, Hispanic and White Americans consume around half as much L as African Americans [118].

There have been no large-scale studies looking at the effect of L and Z supplementation in MPOD. The results of several small studies suggest that MPOD can be modified by nutritional supplementation and dietary modification but only for some people. For example, MPOD increased by 4–5 % in eight males supplementing with 10 mg L daily [111]. Additionally, an average increase in MPOD of 19 % was found in people supplementing with spinach (providing 10.8 mg L and 0.3 mg Z) or sweet corn (providing 0.4 of L and 0.3 mg Z) for up to 15 weeks, although three out of 13 participants were nonresponders [53]. The fact that some people do not appear to have a retinal response to L and Z supplementation may be related to the xanthophyll-binding protein [36]. It could be that some people are genetically predisposed not to have the binding protein, which would mean that they are unlikely to respond to any amount of supplementation or dietary modification.

A recent review found that supplementation with 10 mg or 20 mg of L does not increase MPOD by a value of more than 0.1 in either healthy or diseased eyes [119]. The same review found a wide range of reliability values for clinical MPOD assessment devices. It was concluded that the chance of eliciting an increase in MPOD during 6 months of daily supplementation with between 10 and 20 mg L that is of sufficient magnitude to be detected by using HFP techniques on an individual basis is small.

2 Xanthophylls and Normal Visual Function

The specific uptake of L and Z at the macula may also be suggestive of a functional role in normal visual function. The macula is specialized for high spatial resolution and also for color vision, and so it may be that L and Z play a part in these processes.

With respect to healthy eyes, the blue-light filter effect of L and Z may reduce longitudinal chromatic aberration [120–123]. In addition, the acuity hypothesis states that these nutrients may improve visual acuity for images that are illuminated by white light by absorbing poorly focussed short wavelengths before this light is processed by the retina [124–128]. In theory, if an emmetropic eye views a mid-wavelength object (approximately 550 nm) in blue-dominated sunlight, shorter wavelengths will focus in front of the retina and longer wavelengths will focus behind such that there is a range of focus of approximately 1.20 diopters [122]. The fact that images are not degraded may be explained in part by the preretinal filtering effect of L and Z.

2.1 Chromatic Aberration

When any optical system defects in the formation of the image occur, these aberrations can be classified as either chromatic or monochromatic. In the human eye, longitudinal chromatic aberration results from the dispersion characteristics of the ocular media, and a dioptric interval of around 0.9 D between the paraxial foci for 656.3-nm red light and 486.1-nm blue light has been reported [129]. Transverse chromatic aberration results in long-wavelength light being deviated less than short-wavelength light, which has the effect of producing a red blur around the edge of an image. When viewing white light, the combined effect of transverse and longitudinal chromatic aberration would be to create a purple penumbra to the image.

In 1866, it was proposed that MP might reduce longitudinal chromatic aberration through absorption of short-wavelength light [130]. It has since been shown that a filter covering a similar spectral range to MP can reduce the radiance of the short-wavelength blur circle to a subthreshold value [102]. The hypothesis that the MP reduces short-wavelength chromatic blur and therefore enhances spatial vision has been termed the acuity hypothesis by Wooten and Hammond [126]. Trials provide conflicting evidence for the use of optical filters in enhancement of spatial vision and varying effects across different individuals. It could be that in those with low levels of MP, the filter does improve spatial vision, whereas the effect is less in those with high levels of MP [131]. It has more recently been suggested that image quality is independent of wavelength [132] and is not degraded by longitudinal chromatic aberration for shorter wavelengths [133]. Further work is required to confirm the role of MP in reducing the effect of chromatic aberration and therefore improving visual function, as current evidence is largely associative.

The acuity hypothesis has more recently been tested by resolution acuity and hyperacuity under two different illumination conditions. One condition consisted of mid-wavelength yellow light that is not absorbed by MP, and the other consisted of a perceptual white light that was subject to chromatic aberration because the blue portion would be absorbed by MP [134]. There was no relationship between MP and resolution acuity or between MP and hyperacuity in either the white or yellow conditions.

2.2 Visibility

Short-wavelength light is scattered more than long-wavelength light by air molecules and larger atmospheric molecules. This scattering effect results in the blue coloration of the sky, as well as the blue haze seen around objects viewed in the distance. Wooten and Hammond [126] hypothesize that MP may increase visibility by reducing the luminance of the background with respect to the object itself. This means that the contrast of the object is increased. A person with a MPOD of 0.0 would only be able to see an object at 10 km that a person with an MPOD of 0.5 would be able to see at 11.9 km.

3 Relating the Function of Macular Pigment to Retinal Structure and Disease Pathogenesis

The MP is thought to reduce the amount of light reaching the RPE by 40–90 %, and in turn, this should reduce the rate of formation of A2E and ROS. Lutein and Z have been shown to reduce lipofuscin formation in cultured RPE cells (111). A2E is toxic, appears to reduce mitochondrial integrity, and also exhibits phototoxic behavior when exposed to blue wavelengths of light (112, 113, 114). The phototoxicity of A2E is reduced by L, which suggests that some of the protective effects of carotenoids may occur at the level of the RPE.

It is thought that the carotenoids are transported from the choroid, across the RPE and the photoreceptor outer segments and that L and Z molecules are preferentially orientated to absorb plane polarized light incident perpendicular to the nerve axons.

UV light is a source of potential damage for the cornea and the lens but very little reaches the retina. However, the retina is prone to photochemical damage from visible light between the wavelengths of 400 and 500 nm. The retina is most at risk from blue light between the wavelengths of 430 and 470 nm [40].

In summary, the fact that L and Z are selectively absorbed at the macula and the fact that L is likely to be converted to MZ at the fovea suggest some specific roles and have prompted interest in the possible functions of these carotenoids at that location. These functions may include screening of photooxidation inducing blue wavelengths of light, scavenging of free radicals, and also protection of RPE cells.

4 Age-Related Macular Degeneration and the Xanthophylls

Age-related macular degeneration (AMD) is the leading cause of irreversible visual loss in the developed world [135–140]. In the UK, half of those registered as blind or partially sighted every year (approximately 30,000) have AMD [141]. This pattern in the registered population is reflected in the rest of Europe, North America, and Australia [136, 142–146].

There are estimated to be nearly one million visually impaired people in Britain, 90 % of whom are over 65 years of age [147]. AMD is the most frequently occurring disease that results in permanent vision loss in this age group [137, 148].

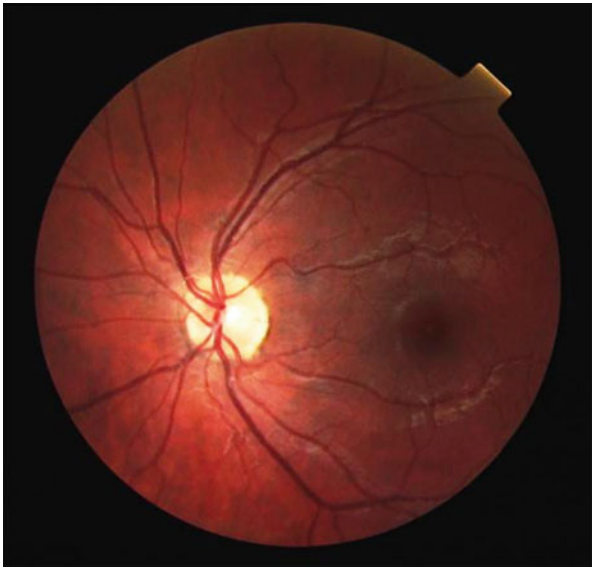


Fig. 130.8 Healthy retina

The proportion of blindness attributable to AMD is expected to increase during the twenty-first century as a result of the aging population [147, 149]. People over the age of 65 represent the fastest growing segment of the US population, and between 2000 and 2020, this group is expected to increase by 53 %. This age group will then make up 16.5 % of the entire US population [150].

Information about risk factors for AMD is limited, and there are no treatment options for most people, despite the prevalence of the condition. It is thought that the condition becomes manifest subject to genetic predisposition and exposure to certain risk factors, including lifestyle and environmental factors [151]. Aging, smoking, and having a positive family history are now undisputed risk factors [152]; other factors such as dietary deficiency of antioxidants thought to be beneficial to retinal health and light exposure are also proposed. Treatments, such as laser photocoagulation and photodynamic therapy, can delay the progression of visual loss in the small proportion with exudative AMD [153] but are unlikely to significantly reduce AMD-related blind- and partial-sight registrations. Developments in genetic methods and molecular biology have been used more recently for the identification of risk factors and may play an important role in reducing the incidence of AMD in the future [154] (Figs. 130.8 and 130.9).

4.1 Terminology

Classification of age-related macular degeneration is required for studies investigating the condition in order to make reliable comparisons between findings. For this reason, the international classification and grading system for

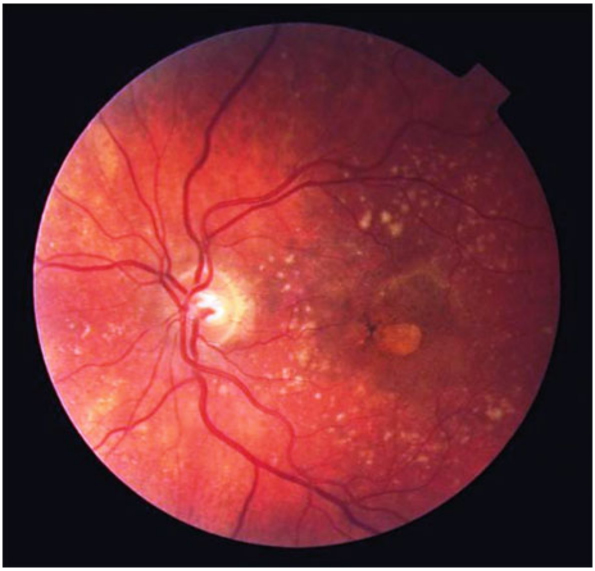


Fig. 130.9 Dry age-related macular degeneration

age-related maculopathy (ARM) and age-related macular degeneration (AMD) has been developed in an attempt to standardize terminology. ARM refers to large soft drusen and pigmentary abnormalities of the retinal pigment epithelium (RPE) and the retina, and AMD refers to later stages of the disease such as geographic atrophy (GA), choroidal neovascularization, pigment epithelium detachment, and fibrous scaring of the macula [155]. This review will use the terms ARM and AMD according to this classification. The term "age-related macular disease," or ARMD, will be used as an umbrella term for all stages of the disease.

4.2 Pathogenesis of AMD: Retinal Pigment Epithelium Structure

The RPE consists of a single layer of cells and is positioned between the photoreceptors and Bruch's membrane. The RPE cells perform several functions, including absorption of light, enabling the turnover of photoreceptor outer segments, and formation of visual pigments by storing and releasing vitamin A. The RPE cells also absorb light and prevent incident light being reflected back to the neural retina, which result in loss of image sharpness. The number of RPE cells per eye can range from 4.2 to 6.1 million, and in younger eyes, these form a highly organized hexagonal pattern. In older eyes, the regular pattern is lost as a result of the low regenerative ability of these cells. Cell loss prompts hyperplasia (abnormal multiplication) of adjacent cells. Interestingly, monkeys that were fed diets free of L and Z had a significantly lower RPE cell density than those fed normal diets [156].

The RPE cells are joined by tight junctions forming a barrier that limits the flow of ions and prevents diffusion of large toxic molecules from the choriocapillaris to the photoreceptors. Each RPE cell supports around 50 photoreceptors. A breach in the connection between photoreceptors and RPE cells, as well as damage to RPE cells, will result in visual loss.

The basal end of each RPE cell rests on a basement membrane that forms part of Bruch's membrane, and the apical end has multiple microvilli measuring 5–7 μm that project between the outer segments of the rods and cones. The apical microvilli continuously erode the outer segments of the rods. Lysozymes within the microvilli contain hydrolytic enzymes that break down the photoreceptor outer segments. In this way, the RPE cell phagocytoses the discs of broken-down visual pigment within the outer segments. Lipofuscin granules are the final products of this process. As we age, lipofuscin collects within the RPE cells [157], and this continual increase in the lipofuscin content of RPE cells between the age of 20 and 70 years has been described as "physiological" [158].

4.3 Pathogenesis of AMD: Lipofuscin Formation

The RPE progressively accumulates a brown-yellow, autofluorescent, electron dense material called lipofuscin. It is a chemically and morphologically polymorphous waste material, originating from a variety of intracellular structures and which accumulates at the primary site of waste disposal, the lysosome. It accumulates within postmitotic cells primarily because it is undegradable and cannot be removed from the cells via exocytosis. Lipofuscin is often called age pigment because the amount of lipofuscin increases with age, and it is composed from a variety of compounds, mainly of protein and lipid origin (30–70 % and 20–50 %, respectively) [159]. The lipofuscin found in the RPE is different to that found in other body tissues because it is mainly derived from the chemically modified residues of incompletely digested photoreceptor outer segments [160].

The accumulation of lipofuscin in the liposomes of the RPE can adversely affect RPE function and may be involved in the development of ARMD. If the RPE is not able to phagocytose the photoreceptor outer segments completely, then remnants of the outer segments accumulate on the inner collagenous layer of Bruch's membrane [161]. These remnants have been morphologically described as basal linear deposit [162] and are clinically recognized as large drusen [158]. Age-related macular disease may also be related to the effects of A2E, which is a lipofuscin-associated chromophore. Chromophore is the name given to a molecule that can exhibit color within a compound. The A2E chromophore requires light for its formation (109, 110), is found within lipofuscin, and is derived from photoreceptor outer segments [163]. A2E is likely to interfere with mitochondrial respiration within RPE cells [164], which is likely to compromise cellular survival. Apoptosis (self-destruction of damaged cells) is four times higher in the center of the macula than in the periphery [165], which supports a role for this mechanism in the development of ARMD. Interestingly, it has been reported that quail that were exposed to bright light and supplemented with Z demonstrated significantly less photoreceptor apoptosis than quail that were exposed to light and fed diets low in carotenoids [166, 167].

Drusen represent the first clinical appearance of ARM. They result from the accumulation of insoluble, lipophilic material between the Bruch's membrane and the RPE. The RPE deforms and thins as it stretches to cover the bulge formed by druse. The thinning of the light-absorbing layer of cells results in more light being reflected back from the retina in these areas and permits the physical examination of drusen [168, 169]. The deformation of the RPE layer compromises the contact between the microvilli of RPE cells and the photoreceptors. Photoreceptor death follows the complete separation of photoreceptors and the RPE. The timescale for this process can vary from individual to individual [168].

Wet or neovascular AMD results when the separation of the RPE and Bruch's membrane is associated with growth of new blood vessels into the subretinal space. These new blood vessels develop from the choriocapillaris, which is responsible for the nutrition and oxygenation of the outer retina.

4.4 Clinical Evidence Regarding the Proposed Role of Macular Pigment in the Prevention of Age-Related Macular Disease

Several studies have investigated possible links between the amount of L, Z, and MZ within the retina (MPOD) and ARMD, using a variety of measurement techniques. Some of these studies have shown that increased levels of MP may delay, avert, or modify the course of ARMD [79, 170, 171] and some have not [11, 78]. However, the fact that MPOD is modifiable means that ongoing research into its role in prevention of the onset or progression of ARMD is important.

Serum concentrations of L and Z and MPOD are reported to be responsive to dietary modifications [48, 53, 54]. A cross-sectional study reported that people with plasma concentrations of L in the lowest third of the distribution have a significant OR for risk of ARMD of 2.0 (95 % CI, 1.0–4.1) compared with those in the highest third after adjustment for other risk factors [172]. In other words, subjects in the study with low-plasma L had a much greater chance of developing ARMD than those with high-plasma L. There were no significant trends between plasma concentrations of L or L combined with Z.

A study of retinal levels of L and Z in donor eyes found an 82 % lower risk of AMD in retinae among the 25 % with the highest L and Z levels compared to the 25 % with the lowest levels [170]. A 70 % reduced risk of AMD has been demonstrated with high (>0.67 μmol/L) versus low (0.25 μmol/L)-L/Z plasma levels [173]. Measurement of MPOD in healthy eyes showed an age-related decline, and healthy eyes considered to be at risk for AMD had significantly less MP than healthy eyes not at risk [79]. This evidence suggests that there is an increased risk of AMD with lower plasma and retinal levels of L and Z.

The research that has been done in this area seems to suggest that the dose of L that is used in supplements is important. Generally speaking, a dosage of

10 mg/day or higher is associated with an increase in MP and, in some cases, an improvement in visual performance. Conversely, supplementation with lower doses does not bring about these changes. A brief overview of the literature follows.

Early work on L and Z supplementation suggested that both serum and macular response is dose dependent [52]. This finding is supported by more recent studies that investigated the impact of supplementation of L and Z in lower doses and did not find a significant increase in MP. For example, daily supplementation with 6 mg L for 1 year did not increase MPOD in a Japanese cohort of 43 adults, some of whom had macular disease. However, the supplementation was associated with an improvement in visual function [174]. Similarly, daily supplementation with 6 mg L as part of a randomized controlled trial did not improve contrast sensitivity in people with ARM [175] and did not improve visual function in young healthy adults [176].

However, L supplementation at achievable dietary levels increased and maintained serum levels, and this was associated with an improvement in glare recovery and VA [177]. A pilot study found that short-term intervention including 15 mg lutein was associated with statistically significant changes in macular focal electroretinogram parameters, suggestive of an improvement in retinal function in ARM [178].

A 35 % increase in L serum levels and a 20 % increase in MPOD were demonstrated in a study supplementing 11 subjects daily with 11 mg of L from 60 g of spinach and 150 g or corn/maize [53]. Supplementation with 10 mg/day of L esters for 12 weeks was shown to increase serum lutein levels by five times and MPOD by approximately 20 % [111]. Serum levels of L doubled over 24 months of taking 15 mg lutein three times weekly [177].

The lutein and antioxidant supplement trial (LAST) was a 12-month RCT designed to evaluate the effect of daily supplementation with 10 mg alone or lutein combined with additional carotenoids and antioxidants/minerals on MP optical density and objective visual outcome measures in 90 subjects with ARMD. Glare recovery and contrast sensitivity significantly improved with both interventions, although it is worth noting that 95.6 % of the study population was male [179].

The Carotenoids in Age-Related Maculopathy (CARMA) Study was a multicenter RCT that investigated intervention with 12 mg L and 0.6 mg Z in combination with other antioxidants in people with AMD. A total of 433 participants were recruited, and at 36 months from baseline, corrected visual acuity was improved in the treated arm of the study compared to the placebo arm. However, it should be noted that the sample size had reduced to 41 at this stage in the study [180].

No positive effect of supplementation with 6 mg in combination with other antioxidants was reported from an RCT that included participants with and without AMD. The outcome measure was contrast sensitivity, which is a "real-world" measure of visual function. However, the sample size was small (25 with AMD0), and it should also be noted that the level of lutein included in the intervention was low (6 mg) compared with other studies [175].

The Age-Related Eye Disease Study (AREDS) 2 is currently underway and is investigating the effect of supplementation with L and Z, with and without omega-3 essential fatty acids, on progression to AMD (www.areds2.org). This is a large multicenter trial, enrolling around 4,000 subjects, that is due for completion in December 2012. The results will undoubtedly inform clinical practice regarding the recommendations for nutritional supplementation and/or dietary modification for the prevention of progression of ARMD.

5 Macular Pigment as an Antioxidant

5.1 In Vitro Studies on Lipofuscin

An in vitro study was carried out to determine whether lipofuscin was detrimental to lysosomal and antioxidant function in cultured human RPE cells. It was concluded that lipofuscin inhibited lysosomal function and reduced antioxidant capacity in RPE cells. It occurred in an in vitro cellular system with its full complement of antioxidants and repair systems and therefore demonstrated the potential for such action inside the eye. The lysosomal system of the RPE is integral to the degradation and turnover of intracellular organelles and ingested photoreceptor outer segments. A decline in the efficiency of this system leads to the buildup of nondegradable material and cellular congestion, culminating in cell dysfunction [181].

A further in vitro study was carried out on cultured RPE cells in order to investigate the involvement of oxidative reactions in lipofuscin formation, the effect of antioxidants on lipofuscin formation, and the effect of lipofuscin accumulation on the phagocytic capacity of RPE cells. The involvement of oxidative reactions in lipofuscin formation was studied by culturing RPE cells fed with photoreceptor outer segments in 8 % and 40 % oxygen [182]. It was shown that significantly more lipofuscin was formed in cells cultured in 40 % oxygen than in cells cultured in 8 % oxygen, indicating an involvement of oxidative mechanisms in lipofuscin formation. When antioxidants were added to the RPE, there was a significant reduction in lipofuscin formation. The antioxidants were found to act as free radical scavengers and chain-breaking agents in peroxidation reactions. The phagocytic capacity of lipofuscin-loaded RPE cells was significantly reduced in comparison to unloaded control RPE cells. The reduced capacity of the lipofuscin-loaded RPE to cope with the needs of the photoreceptors in terms of phagocytosis of their outer segment tips may contribute to the development of AMD [183].

Another in vitro experiment investigated if lipofuscin formation in RPE cells was reduced by antioxidants. It was shown that α-tocopherol and the macular carotenoids, L and Z, have chain-breaking properties in peroxidation reactions of lipid membranes and also play a part in the quenching of free radicals. It was reported that formation of lipofuscin was significantly reduced as a result of the antioxidant substances in the RPE [100].

L, Z, and MZ all contain a high number of double bonds and therefore a high number of free electrons [57]. This means that they form part of a defense mechanism against the production of ROS within the retina. Oxidative stress occurs when the level of ROS overwhelms the antioxidants within a system [184]. The production of ROS occurs as a by-product of oxygen metabolism but can also be promoted by aging, inflammation, smoking, alcohol consumption, and atmospheric pollution. ROIs are unstable molecules that interact with and damage cells and tissues in order to achieve stability. They include singlet oxygen, hydrogen peroxide, and free radicals.

Macular pigment is able to quench or deactivate ROS such as singlet oxygen and free radicals [104, 185, 186]. As such, it has been shown to protect the macula against oxidative stress [50, 187]. Both L and Z are orientated perpendicular to the surface of cell membranes, which means that they are soluble, stable, and can infer significant effects on the membrane itself [104]. It has been proposed that this orientation may also enhance to antioxidant properties of the MP, by changing the properties of the membrane so that it is less sensitive to oxidative damage [188]. In addition, L and Z have been shown to protect RPE cells by increasing the viability in cells exposed to an acute oxidative stress induced by hydrogen peroxide [189]. In the presence of tocopherol and ascorbic acid, zeaxanthin is reported to protect RPE photosensitized cells [189]. L, Z, and MZ are all able to quench singlet oxygen, but when combined in the ratio 1:1:1, they were able to quench 2.4 times more than when L is in isolation [190].

Supplementing with Z has also been shown to reduce light-induced photoreceptor apoptosis in quail [167]; L, Z, and DHA supplementation was found to reduce oxidative stress-induced apoptosis in photoreceptors [191].

The studies above have indicated a potential link between lipofuscin and oxidative reactions which can lead to the dysfunction of RPE cells. This could in turn lead to AMD, with RPE damage being a fundamental part of the onset of AMD. Increased oxygen levels fuel the oxidative stress induced on the RPE; antioxidants help relieve this stress as they act as free-radical scavengers and chain-breaking agents in photoreceptor outer segment peroxidation reactions [3]. However, further research is required to investigate whether this is the case in vivo, as most studies have been carried out in vitro. A potential mechanism is however available, in order for oxidative stress to lead to AMD.

6 Macular Pigment as a Blue-Light Filter

6.1 Filtering Characteristics of the Eye

Light consists of electromagnetic particles which travel in the form of waves. Visible light is the portion of the electromagnetic spectrum which is visible to humans, and a typical eye will respond to wavelengths from 400 to 700 nm [192]. The human eye has unique filtering characteristics that determine in which area of the eye each wavelength of light will be absorbed. Wavelengths $\leq$295 nm are absorbed by the cornea, that is, some of the shortest, most energetic wavelengths of light (all UVC and some UVB) are filtered out before they reach the crystalline lens. In adults, the lens absorbs the remaining UVB and all UVA (295–400 nm), and therefore, only visible light reaches the retina. However, the very young human lens transmits a small window of UVB light (320 nm) to the retina, while the elderly lens filters out much of the short blue visible light (400–500 nm) [193].

6.2 Retinal Effects of Blue Light

There are three types of radiation insult in the spectral range 400–1,400 nm that cause damage to the retina. These are mechanical disruption of retinal structure such as shock waves produced by extremely short pulses of radiation which are absorbed in the RPE and choroid. The second is thermal insult resulting from absorption of energy in the RPE and choroid sufficient to produce temperatures greater than 10 °C above ambient temperature in the RPE, neural retina, and choroid. The final is actinic insult from the photochemical effects of extended exposure to the short wavelengths in the visible spectrum (400–550 nm). The effects produce temperatures of only a few degrees Celsius above ambient [194]. Photooxidative damage, usually simply referred to as photochemical damage, is associated with long-duration exposures to wavelengths of 550 nm or shorter and low to moderate tissue irradiance because of the requirement for relatively energetic photons to drive the chemical reactions which causes the underlying damage [195].

In one experiment, it was established that photochemical damage to the RPE was related to wavelength, with the damage threshold being lowest for short-wavelength blue light and continued to decrease into the UV region of the electromagnetic spectrum [196]. In another experiment by the same researchers, rhesus monkeys were exposed to blue light (441 nm). It was found that the exposure needed to produce the photochemical lesion was much less than that needed to produce a retinal burn. Also, mild photochemical lesions show uniform damage across the entire exposed area and require 48 h to become funduscopically visible. A nonthermal type of photochemical lesion originating in the RPE was found on monkey retina which led to hypopigmentation of the RPE which resembled that seen in the human aging retina [197]. Recent in vitro experiments investigated whether blue light damaged RPE cells. It was concluded that damage induced by blue light to cultured RPE cells may originate in the mitochondria, and the type of cell death seems to be determined mainly by the intensity of the light but is also related to the duration of exposure. Also, the RPE cells can be damaged directly by blue light after excluding the possible influence of phagosomes and lipofuscin [198, 199].

The experiments mentioned show that irradiation to high-intensity short-wavelength light and particularly blue light can accelerate aging of the retina. UV is more damaging to the retina than blue light as the photons of light contain more energy, but most of the UVs are filtered out by the components of the eye, so blue light from the visible spectrum is the most damaging to the human eye. The blue light can induce photochemical damage to the RPE as shown from the studies and lead to hypopigmentation which is a characteristic sign of AMD, hence indicating a potential link between blue light and AMD.

6.3 The Role of Photooxidation in the Onset of Disease

In the retina, the generation of ROS can occur as the by-products of cellular metabolism or as a result of photochemical reactions [41]. Photooxidative damage results when incident light interacts with an endogenous chromophore in the eye, causing a chemical change that is not related to a thermal increase in the irradiated tissue [195]. A chromophore is a substance that absorbs light, and an endogenous chromophore is a chromophore present in the eye [193]. Lipofuscin is an example of an intraocular chromophore in the RPE. Short-wavelength blue light of the visible spectrum is strongly absorbed by lipofuscin, which is photoexcitable. The extent of the photochemical reaction depends on the amount of blue light absorbed in the lipofuscin. When lipofuscin absorbs a photon of blue light, it is typically excited to a triplet state. Triplets are reactive species and readily undergo chemical reactions with other molecules [195]. These chemical reactions can result in damage to the RPE and photoreceptors.

It was concluded from early studies that elevated blood oxygen increases retinal sensitivity to blue-light damage; it lowers the damage threshold and increases the severity of damage at a given radiant exposure [200]. There have been numerous studies carried out indicating how blue light, ROS, and lipofuscin induce RPE cell death, therefore leading to AMD.

An in vitro experiment was carried out to determine whether human RPE exhibits any substantial photoreactivity that may lead to retinal phototoxicity. It was found that illumination of human RPE cells induced a significant uptake of oxygen that was both wavelength and age dependent. It was observed that photoreactivity of human RPE cells was significantly determined by their lipofuscin content. Lipofuscin irradiated with blue light, under aerobic conditions, generates several ROS. It was also established that lipofuscin photosensitized aerobic reactions leading to enhanced lipid peroxidation. It was postulated that lipofuscin is a potential photosensitizer that may increase the risk of retinal photodamage and contribute to the development of ARM [201].

Lipofuscin is known to accumulate with age in secondary lysosomes of RPE cells. The autofluorescent lipofuscin pigment has an excitation maximum within the range of visible blue light, while it is emitting in the yellow-orange area. This property of the pigment indicates it may have a photooxidative capacity and destabilize lysosomal membranes of blue-light-exposed RPE. Thus, an in vitro study was conducted in order to find out if lipofuscin accumulation in cultured RPE cells caused enhanced sensitivity to blue-light irradiation. The cultured lipofuscin-loaded RPE cells exposed to blue light showed a considerably enhanced loss of both lysosomal stability and viability. It was concluded that accumulation of lipofuscin within secondary lysosomes of the RPE sensitizes these cells to blue light by inducing photooxidative alterations of the lysosomal membranes, resulting in a presumed leakage of lysosomal contents to the cytosol with ensuing cellular degeneration of an apoptotic type [202]. The leakage of lysosomal content into the cytosol may indicate a potential system for which RPE cell death can occur. This in turn can lead to photoreceptor death and hence can result in early ARM and ultimately AMD.

N-retinylidene-*N*-retinyl-ethanolamine (A2E) is the major photosensitizing chromophore in lipofuscin that causes reactive oxygen species production [203]. This chromophore is known to strongly absorb in the blue region of the visible spectrum; therefore, a study was carried out to utilize the known susceptibility of the RPE to blue-light damage, in order to investigate A2E as a fluorophore involved in blue-light toxicity. The investigation implicated A2E as a mediator of blue-light damage in the RPE. It was deduced that healthy RPE cells that have amassed A2E at critical concentrations and that are subsequently exposed to blue light can undergo an apoptotic form of cell death. No damage was found in RPE cells irradiated with blue light in the absence of internalized A2E [204]. A further investigation was carried out by the same researchers to evaluate the role of oxidative mechanisms in mediating cell damage. The augmentation of cell death in the presence of a singlet-oxygen enhancer and the protection afforded by quenchers and scavengers of singlet oxygen indicated that the generation of singlet oxygen may be involved in the mechanisms leading to the death of A2E containing RPE cells after blue-light illumination. In the presence of a singlet-oxygen quencher, the photooxidative changes in A2E were diminished. Therefore, it was postulated that singlet oxygen may not only mediate the cellular damage directly but may also serve in the photooxidation of A2E with the products generated from the photochemical changes in A2E, being the ravaging agents [204].

6.4 Photooxidation and the Role of Antioxidants

Both oxygen and blue light in the visible light spectrum are strong oxidative agents; therefore, lipid peroxidation by light and/or free radicals produces damaged photoreceptors that are indigestible by the RPE cells and accumulate as lipofuscin [205]. Strong evidence has been presented that lipofuscin and its major photosensitizing chromophore A2E are responsible for blue-light-induced RPE cell damage. Blue light also results in enhanced ROS in the RPE, thus causing oxidative stress on the RPE. The degree of oxidative stress is restricted by a range of potent antioxidants and the repair of damaged elements [206]. With age, however, the RPE loses its antioxidant protection [207–209], and ROS production in the aging RPE can lead to apoptosis and cell death [193]. The functions of the RPE are to transport nutrients to the photoreceptor cells and phagocytose the outer segments of photoreceptors. With the death of the RPE cells, the photoreceptors are no longer nourished and the outer segments of the photoreceptors cannot be phagocytosed; therefore, they die off, and waste products accumulate near Bruch's membrane [193]. The death of RPE and photoreceptors thus leads to loss of vision and hence AMD.

It has been shown that antioxidants can protect against blue-light damage to inhibit the death of RPE cells, [210] therefore reducing the risk of developing AMD.

7 Conclusion

Although the exact etiology of AMD is unknown, there is substantial evidence confirming a plausible mechanism by which blue light, oxidative stress, and AMD can interrelate. As the buildup of lipofuscin increases with age [159], circumstantial evidence has shown that it may initiate events in the macula that are central to the development of AMD. The main photosensitizing chromophore of lipofuscin A2E has shown that it generates numerous harmful ROS in the presence of blue light, which seems likely to trigger the events leading to AMD.

Lutein (L) and its stereoisomers, zeaxanthin (Z) and meso-zeaxanthin (MZ), are xanthophylls. They are relevant to human ocular health and as they are the only carotenoids present in the lens [8] and retina [9]. It has been suggested that they play a similar role in humans as in plants, as antioxidants and screeners of high-energy blue light [10]. Within the central retinal area, these xanthophylls are known as the macular pigment (MP). The MP may prevent some of the biological processes within the retina that can result in the development of sight-threatening macular diseases [11–16] and subsequent visual loss.

Antioxidants such as L and Z have been shown to have a protective effect over the macular area suppressing the oxidative stress induced by blue light and lipofuscin. However, further research needs to be carried out as the previous studies and experiments performed have remained contentious. Comprehensive in vivo experiments need to be carried out in order to investigate whether blue light and ROS can cause damage to the same extent in the eye, as to that seen from in vitro experiments. Furthermore, additional research needs to be conducted in order to find out if L and Z can help reduce oxidative damage in vivo and whether any systemic or ocular side effects may be caused by increasing macular levels of these xanthophylls. The results of the large multicenter randomized controlled trial, AREDS 2, is likely to inform clinical practice with regard to recommendations about L and Z supplementation for ARMD.

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Triterpenoid Centellosides: Bioactivities and Uses in Healthcare Application 131

Puziah Hashim

Contents

1 Introduction 3960
2 Chemical Composition 3961
3 Pharmacological Studies for Healthcare Applications (In Vivo and In Vitro) 3962
3.1 Antioxidant Activity 3962
3.2 Anti-inflammatory 3963
3.3 Anticancer and Cytotoxic Activity 3963
3.4 Antidiabetic and Hypertension Protection 3964
3.5 Anti-rheumatoid Arthritis Effect 3964
3.6 Wound Healing 3965
3.7 Brain Improvement and Neuroprotective Effect 3966
3.8 Gastric Ulcer Prevention 3967
3.9 Cardioprotection 3967
3.10 Anxiolytic Activity 3968
3.11 Venous Hypertension Improvement 3968
4 Clinical Studies 3968
5 Safety and Toxicology 3969
6 Conclusion 3969
References 3970

Abstract

Triterpenoid centellosides of *Centella asiatica* (CA) have been used as an ingredient in a wide range of applications that include healthcare. The triterpenoid centellosides' specific constituents, namely, asiatic acid, asiaticoside, madecassic acid, and madecassoside, have been extensively researched and reported to have many health and therapeutic benefits.

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Its pharmacology in vitro and in vivo presents potential remedies in antioxidant, anti-inflammatory, anticancer, antidiabetic, hypertension protection, anti-rheumatoid arthritis, wound healing, brain improvement and neuroprotective effect, gastric ulcer prevention, cardioprotection, anxiolytic activity, and venous hypertension improvement. The clinical study of triterpenoid centellosides has shown its potential in venous hypertension-related improvements. Due to its great prospect and potential, this chapter will present the recent application of triterpenoid centellosides in healthcare covering the pharmacological (in vivo and in vitro) and clinical studies as well as its chemical composition and safety.

Keywords

Centella asiatica • clinical study • healthcare • pharmacology • safety • triterpenoid centellosides

Abbreviations

AA	Asiatic acid
AS	Asiaticoside
CA	*Centella asiatica*
CIA	Collagen-induced arthritis
CLP	Cecal ligation and puncture
IL	Interleukin
iPLA2	Phospholipase A2
LPS	Lipopolysaccharide
MA	Madecassic acid
MDA	Malondialdehyde
MS	Madecassoside
NO	Nitric oxide
PGE	Prostaglandin E
ROS	Reactive oxygen stress
SOD	Superoxide dismutase
TECA	Titrated extract of *Centella asiatica*
TNF-α	Tumor necrosis factor-alpha
TTF	Total triterpenic fraction
TTFCA	Total triterpenoid fraction of *Centella asiatica*
WHO	World Health Organization

1 Introduction

Medicinal herbs and their phytochemical components used for pharmacological application have raised interest in research all over the world. One such important medicinal herb is *Centella asiatica* (L.) Urban, belonging to the family Apiaceae or Umbelliferae. The *Centella asiatica* (CA) is a small herbaceous, creeping plant with kidney-shaped green leaves. It is being used as a medicinal herb in Ayurvedic medicine and traditional herbal medicine in Malaysia, China, and other parts of Asia for hundreds of years [1]. The herb is commonly known as pegaga in Malaysia, Indian pennywort and gotu kola in the Western world, and mandookaparni in the Ayurvedic system of medicine in India. This plant is commonly used in these countries for various applications such as traditional medicine, vegetables, salad, and drinks as in tea or juice [2]. CA is widely used mainly as an extract and has been known for many years in treating all kinds of diseases such as wound healing, headache, body ache, insanity, asthma, ulceration, and eczema [1]. It is believed that the biologically active triterpenoids of CA also known as triterpenoid centellosides are responsible for the health benefit claims [3, 4]; the most important are the asiatic acid (AA), madecassic acid (MA), asiaticoside (AS), and madecassoside (MS) [5, 6]. Previous studies reported that triterpenoid centellosides had various health benefits such as memory-enhancing property [7], antidiabetic [8], anti-inflammatory [9], antioxidant [2, 10], wound healing [11, 12], anticancer [13], as well as tonic drinks for general health and blood circulation. The titrated extract of CA (TECA) is a mixture of three triterpenoids containing 40 % of AS and 60 % of AA and MA, which produces a wide range of preventive and therapeutic effects [14, 15]. HMPC [14] has reported that total triterpenoid fraction of CA (TTFCA), TECA, and total triterpenic fraction (TTF) are different acronyms to designate the same extract, commercially known as Madecassol® or Centellase®, containing 40 % of AS and 60 % of AA and MA. TECA is being used in Europe in wound healing drugs and to treat inflammatory skin problems (leprosy, lupus, varicose ulcers, eczema, atopic dermatitis, and psoriasis), intestinal problems, fever, anxiety, and mental diseases [15]. Due to triterpenoid centellosides' importance and wide applications, this chapter will cover the advances achieved in research works of CA triterpenoid centellosides as potential source of treatment and prevention of illness (healthcare). The compilation of recent findings on numerous pharmacological studies on various in vitro, in vivo, and clinical test models as well as the chemical composition and safety of CA is also included.

2 Chemical Composition

The medicinal herb CA is rich in phytochemical components such as alkaloids, tannins, triterpenoid centellosides, flavonoids, and phenols. Among these compounds, triterpenoid centellosides are the primary components of CA with the most potential of preventive and therapeutic effect. Based on previous reports, the concentration of triterpenoid centellosides is in the range of 0.01–2.20 % [10, 16, 17]. It comprises mainly of AA, MA, AS, and MS [5, 6]. Figure 131.1 shows the structure of the triterpenoid centellosides; the difference of the glycosides and the aglycones (AA and MA) is the sugar glucose-glucose-rhamnose; meanwhile for the aglycones, the sugar molecule is replaced by an OH group. The similar triterpenoid centellosides are also found and identified in Malaysian accession [10, 16]. These compounds have been used as biomarker to determine the quality of the plant [18]. However, the triterpenoid centellosides' components are

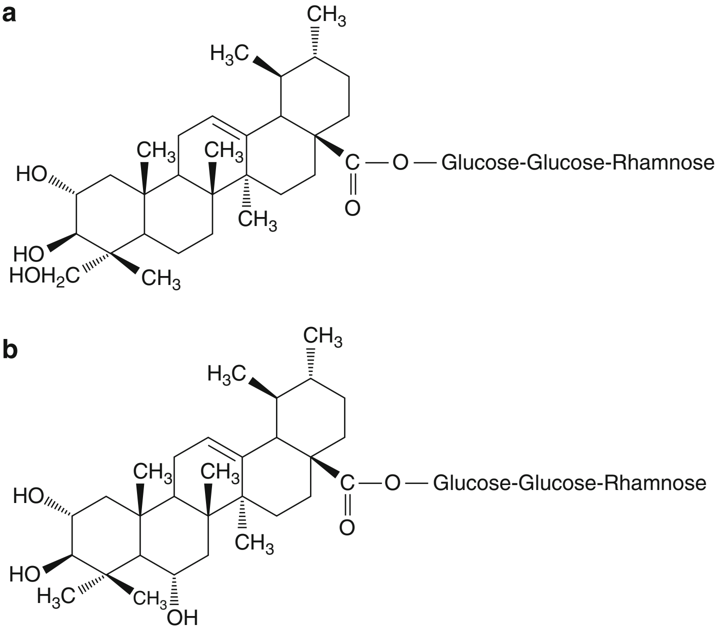
a
CH3
H3C
CH3
CH3
HO
C — O — Glucose-Glucose-Rhamnose
O
CH3
HO
HOH2C
CH3
b
CH3
H3C
CH3
CH3
HO
C — O — Glucose-Glucose-Rhamnose
O
CH3
HO
H3C
CH3
OH

Fig. 131.1 The structure of triterpenoid centellosides. (**a**) AS and AA (without glucose-glucose-rhamnose); (**b**) MS and MA (without glucose-glucose-rhamnose)

varied with regard to its content based on location and diverse environmental conditions [19]. Therefore, it is required to check the source of the plant for their consistent efficacy and quality [20]. Other triterpenoids of CA are composed of brahmoside, brahmic acid, brahminoside, thankiniside, isothankunisode, centelloside, madasiatic acid, centic acid, and cenellic acid [18].

3 Pharmacological Studies for Healthcare Applications (In Vivo and In Vitro)

Healthcare is the treatment and prevention of illness. The CA triterpenoid centellosides provide many health and medicinal benefits. As its primary application has been in promoting wound healing, today, it is being used in many preparations and, hence, is becoming an important compound. The use of triterpenoid centellosides in healthcare applications has become increasingly important in our integral health system today.

3.1 Antioxidant Activity

Studies on the triterpenoid centellosides demonstrated its strong antioxidant properties and can prevent oxidative damage which leads to promoting health benefits.

Antioxidant is useful as it offers an effective and a safe way of increasing the body defense system against free radicals [21] and keeps the reactive oxidative stress (ROS) in a state of balance [22]. The antioxidant in CA (84 %) is comparable to vitamin C (88 %) and grape seed extract (83 %), which may be due to its triterpenoid centellosides and flavonoids [10]. The AS has been proven to affect the levels of certain antioxidants in the wound healing process as it enhanced the production of antioxidant levels at the early stage of healing [23]. During the application of 0.2 % (topical) AS, twice daily for 7 days to excision-type cutaneous wounds in rats, an increase in enzymatic and nonenzymatic antioxidants in newly formed tissue was detected, which included 36 % superoxide dismutase (SOD), 67 % catalase, 49 % glutathione peroxidase, 77 % vitamin E, and 36 % ascorbic acid, whereas lipid peroxidation level decreased by 69 %. Beside flavonoids and selenium, the triterpenoid centellosides in CA might contribute to its powerful antioxidant properties to increase cell rejuvenation and improve mental and physical health [24].

3.2 Anti-inflammatory

CA herbal plant extract has been used in Ayurvedic medicine for the treatment of inflammation. MA, one of the components in triterpenoid centellosides, has been identified to possess antioxidant and anti-inflammatory activities in collagen-induced arthritis (CIA) mice [9]. The anti-inflammatory effect of MA (3, 10, 30 mg kg^{-1}) on CIA mice may be related to its inhibition of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), prostaglandin E_2, expression of cyclooxygenase-1, and upregulation of anti-inflammatory molecule IL-10. The anti-inflammatory study in rat model using alcoholic and aqueous extracts of CA (100 mg kg^{-1}) inhibits edema after 3 h at 71 % and 46 %, respectively. It appears from the study that the carrageenan-paw-induced inflammatory method and the effect may be due to the presence of AS, MA, and triterpenes [25]. The results strongly justify the traditional use of this plant for improvement of inflammatory conditions.

3.3 Anticancer and Cytotoxic Activity

There are several in vitro studies using various cancer cell lines that demonstrated the potential cytotoxicity and anticancer properties of CA triterpenoid centellosides. The protection against chemical-induced carcinogenesis was demonstrated in phenobarbital-induced rat liver microsomes in vitro using the CA233 extract containing 80 % triterpenoid glycoside (43 % MA and 39 % AS). The findings indicate that the components of MA and AS in CA233 exerted an inhibition effect on hepatic xenobiotic drug/metabolism enzymes, particularly cytochrome P459 in a dose-dependent manner with a median inhibition of IC_{50} 523 μg mL^{-1} [26]. In other studies, AA was used to inhibit cell proliferation by the induction of apoptosis, which is a process of programmed cell death [13]. In the studies, AA demonstrated potent cytotoxicity to squamous carcinoma cells SCC4 and SCC5 (oral cancer cells), SK-BR3 breast cancer cells, and HeLa cells as compared to Taxol (commercial leading chemotherapeutic agent). Studies by Lee et al. showed that AA was effective to decrease the viability and induce apoptosis of HepG2 human hepatoma cells through increased intracellular calcium (Ca^{2+}) which led to enhancement of p53 expression in HEpG2 cells [27]. These results demonstrate that AA may exert as potential preventive agent for human hepatomas. Meanwhile, AA also exhibited its ability as candidate for prevention of human skin cancer. It inhibited cell proliferation and induced apoptosis in human melanoma SK-MEL-2 cells through generation of ROS, alteration of Bax/Bcl-2 ratio, and activation of caspase 3, but p53 independent [28]. The latest study on AA also indicated that this triterpenoid centelloside exerts anticancer property by inhibiting breast cancer cell line MCF-7. AA at a concentration of 10 μM induced 95 % cell death in 48 h as compared to 10 μM of tamoxifen, an antiestrogen drug used in breast cancer [29]. These results further suggest that AA may be a good candidate for therapeutic intervention of human cancer.

3.4 Antidiabetic and Hypertension Protection

Diabetes mellitus is a chronic hyperglycemia caused by insufficient insulin in the body and is estimated about 4 % of global health problems today [8]. There is an increasing demand of oral intake of hypoglycemic agents from herbal plant preparations with antidiabetic activity and with hypertension protection for hypertensive people. The latest potential of these triterpenoid centellosides is antidiabetic and hypertension protection. After 14 days, the AS-administered rats showed significantly lower blood glucose level and higher sugar absorption inhibitory effect and decrease in the systolic blood pressure [30]. The activity was lower than the control drug for angiotensin-converting enzyme (ACE) inhibitors, captopril. In this study, AS may be candidates for antidiabetic and hypertensive protection.

3.5 Anti-rheumatoid Arthritis Effect

The intake of MA (20 and 40 mg kg^{-1}) in CIA mice claimed to be useful for rheumatoid arthritis by decreasing joint swellings and erythema (reduced clinical score), increasing infiltration of inflammatory cells and synovial hyperplasia, and protecting the destruction of the joints [31]. In addition, MA at 20 and 40 mg kg^{-1} decreased the serum level of anti-CII IgG antibody by 21 % and 62 %, respectively, and significantly reduced the delayed type hypersensitivity against CII in mouse ears by 21.4 % and 33.3 %, respectively, while the control (dexamethasone) inhibited 56.8 %. It moderately suppressed CII-stimulated proliferation of lymphocytes from popliteal lymph nodes in CIA mice. Moreover, MA exhibited its ineffectiveness in the activation of macrophages caused by lipopolysaccharide. Therefore, the study proved that MA was responsible in preventing mouse CIA and it might be the major clinical uses in rheumatoid arthritis, mainly by regulating the abnormal humoral and cellular immunity as well as protecting the joint.

3.6 Wound Healing

Wounds are physical injuries that cause openings or cuts of the skin. There are many processes in wound healing that involve cell-matrix interactions and overlapping phases, inflammation, cellular proliferation, and skin remodeling [32]. It is also known that angiogenesis plays a very important role in wound healing, whereby the newly formed blood vessels assist the hypoxic wounds to attain normal levels of oxygen, known as normoxic conditions [33]. The CA extract and its triterpenoid centellosides have been used for wound healing applications and many skin disorders for decades as topical agent and oral supplements. It has been reported that AA is the active ingredient in Madecassol®, which is recognized as a medicinal effector and commercially available for the treatment of keloids and the proliferation of connective tissues and hypertrophic scars [13, 34]. Its medicinal effect is to restore a damaged tissue by modifying the fibrosis progress and protect the cells in the tissue. Oral intake of MA, the major triterpenoid centelloside of CA, at 6, 12, and 24 mg kg^{-1} was seen to speed up the healing of burn wound in a time-dependent manner due to the involvement of several mechanisms such as antioxidative activity, collagen synthesis, and angiogenesis [35]. During the burn healing process, MA assists in dermal proliferation of fibroblast by increasing infiltration of inflammatory cells and stimulating epithelization process, decreasing nitric oxide (NO) and malondialdehyde (MDA) in burn skin tissue, but increasing the reduced glutathione and hydroxyproline content, and, hence, facilitates the healing process. The collagen is the key component in wound healing as it involves in the extracellular matrix composition and granulation of wound. The in vitro studies using fibroblast cells on CA ethanol-aqueous extract containing triterpenoid centellosides showed significant stimulation of collagen production in a dose-dependent manner [10]. At 50 mg mL^{-1}, the extract stimulated collagen threefold as compared to the control. The results demonstrated are in agreement with several studies conducted on TECA, wherein the triterpenoids consisting of 40 % of AA and 60 % of AS and MA stimulated collagen synthesis and increased tensile strength of tissues [11, 36]. In other studies, similar constituents of TECA stimulated extracellular matrix accumulation in rat experimental wounds, and these results were demonstrated in vitro by gene expression changes in the human fibroblast [12]. AA was the component responsible for the stimulation of collagen synthesis, whereas MS increased the collagen III production.

Studies by Bian et al. showed that MA exerted a healing effect on myocardial ischemia-reperfusion injury by significantly decreasing the infarct size, lactate dehydrogenase and creatine phosphokinase, SOD, MDA, and C-reactive protein [37]. Previous studies on AS also exhibited that this compound was able to enhance wound healing activity by stimulating collagen synthesis and angiogenesis as well as increasing cell strength of the newly formed skin [23, 38]. It is able to promote fibroblast cell proliferation and extracellular matrix synthesis in wound healing [39]. Application of 0.2 % solution of AS led to a 56 % increase in hydroxyproline and 57 % increase in tensile strength, increased collagen content, and improved epithelization, thereby facilitating the healing process [23]. The AS is said to have curative effect, including wound repair, whereby the treatment of keratinocyte cell line (HaCaT) at 2 × 10^5 cells with AS (10 pg, 1 ng, 100 ng per wound area), after 24 h, significantly increased monocyte chemoattractant protein-1, vascular endothelial growth factor, and IL-1β levels in burn area [40]. The application of AA and AS and its derivatives have shown to be effective to treat pulmonary fibrosis induced by bleomycin in rats [41]. The protective effect of AS (45 mg kg^{-1}) has been studied on septic lung injury induced by cecal ligation and puncture (CLP) in mice [42]. The AS exerted effective protection from septic lung injury induced by CLP. It significantly decreased CLP-induced mortality, lung pathological damage, infiltration of mononuclear and polymorphonuclear leucocytes, and total proteins. The underlying mechanisms might be related to upregulation of peroxisome proliferator-activated receptor-Y, which facilitate the wound closure by inhibits, mitogen-activated protein kinases, and NF-κB pathway.

3.7 Brain Improvement and Neuroprotective Effect

CA extract has been used as herb in Ayurvedic medicine as a nerve tonic in stimulating learning and memory [7]. The intake of the CA extract containing triterpenoid centellosides has been reported to act as micronutrients which could retard brain aging and assist in renewal of neural tissue. It also helps as an effective antistress, is adaptogenic, enhances memory and revitalizes the brain, and increases attention span and concentration [43]. Phospholipase A2 (iPLA2) is an enzyme group that causes irregularity of the central nervous system activity and commonly relates to neuropsychiatric diseases. The in vitro study using brain cells exhibited the CA aqueous extract (500 μg mL^{-1}) containing 84 % AS and inhibited iPLA2 and cytosolic phospholipase (cPLA2) by 96 % and 77 %, respectively [44]. The inhibition of these PLA2 enzymes in the brain by AS demonstrated that it can be useful for prevention of epilepsy, stroke and multiple sclerosis, and other neuropsychiatric disorders. Hence, AS is suggested to be a key component and potentially responsible for brain protection. The study by Krishnamurthi revealed the potential use of AA as a neuroprotective agent in the treatment of cerebral ischemia [45]. In a mouse model of focal cerebral ischemia, AA significantly reduced the infarct volume to 60 % at day 1 and 26 % at day 7 postischemia and improved neurological outcome at 24 h postischemia. It is claimed that the neuroprotective effect of AA might be due to the decrease in blood–brain barrier permeability and mitochondrial injury. The AA (30 mg kg^{-1} of body weight) treatment in male Sprague–Dawley rats showed significant results on memory and learning improvement by prolonging the latency on retention trial [7].

The AA and AS derivatives including AS6 and SM2 demonstrated very promising results in protecting the brain against beta-amyloid neurotoxicity [46]. At 1 μM, they were shown to decrease hydrogen peroxide-induced cell death, reduce free radical concentration, and inhibit beta-amyloid cell death, hence suggesting their potentials in the treatment and prevention of Alzheimer's disease and beta-amyloid neurotoxicity. Following nerve damage or disease-induced axonal degeneration or transaction in the peripheral nervous system, axonal regeneration is very important for functional recovery. However, the recovery is slow because the rate of axonal elongation is slow. In vitro studies using CA extracts and its AA fractions (1 μM) significantly increase in neurite elongation in human SH-SY5Y cells, whereas in vivo, these extracts had shown more rapid functional recovery and increased in axonal regeneration (larger caliber axons and thicker myelin sheaths), indicating that the axons grew at a faster rate [47]. This findings indicate that the components in CA particularly the AA may be responsible for accelerating repair for damaged neurons.

3.8 Gastric Ulcer Prevention

Many studies demonstrated that triterpenoid centellosides exhibited significant protection as anti-gastric ulcers. The CA water extract and its AS exhibited the healing effects on acetic acid-induced gastric ulcers in rats by significantly increasing the activity of myeloperoxidase, promoting epithelial cell proliferation and angiogenesis, and upregulating expression of fibroblast growth factor in the ulcer tissues, which led to the strengthening of the mucosal defensive factors [48]. Meanwhile, Guo et al. also demonstrated that CA water extract and AS possessed an anti-inflammatory property that enables the inhibition of NO synthesis and facilitates the healing of ulcers [49]. The ulcer protection effect may be due to the reduction in edema and leucocyte infiltration of submucosal layer.

3.9 Cardioprotection

MA, the major triterpenoid component of CA, has been found to have cardioprotective activity in lipopolysaccharide-mediated sepsis through the blocking of extracellular signal-regulated kinase 1/2 and p38 and NF-κB [50]. The intakes of MA (20 mg kg^{-1} of body weight) in male Sprague–Dawley demonstrated that the extract inhibits the lipopolysaccharide (LPS). The disturbance caused the inhibition of plasma TNF-α, slowed down the drop of the mean arterial blood pressure, and diminished the tachycardia induced by LPS, while, in vitro, the MA significantly inhibited LPS-induced TNF-α in neonatal rat cardiomyocytes in a dose-dependent manner. These results showed that MA can be a potential protective agent in cardiovascular diseases.

3.10 Anxiolytic Activity

CA has been used for centuries in Ayurvedic medicine for psychoactive purposes to control anxiety and help in relaxation and mental calmness. Various studies and evaluations by human and animal models have confirmed the potential claim of CA and its triterpenoid centellosides in anxiolytic activity. In animal studies, the methanolic and ethyl acetate extract of CA as well as the AS of the plant have been reported to possess anxiolytic activity in rat behavioral models [51]. In elevated plus maze study, CA methanol extract (3,047 mg kg^{-1} of body weight) and AS (3 and 5 mg kg^{-1}) significantly reduced the number of closed arm entries and increased the time spent in protected head dips, whereas both CA ethyl acetate extract (111 mg kg^{-1}) and AS (3 and 5 mg kg^{-1}) significantly increased the number of protected head dips and the time spent for this activity. For the open-field test, AS (3–10 mg kg^{-1}) significantly increased the rat spent time (41.3 s) at the center of the arena, while the social interaction test treatment with 1 and 3 mg kg^{-1} of AS significantly reduced the number of non-interaction activity by 29.8 s and 35.0 s, respectively.

3.11 Venous Hypertension Improvement

Restoring tissue firmness and elasticity is also a very important criterion to venous wall. The CA extract containing triterpenoid centellosides is used as venous tonic in the treatment of venous-lymphatic disorders [3]. It improves and maintains the connective tissue by strengthening the weakened veins [33]. The TTFCA, containing 40 % of AS and 60 % of AA and MA, enhanced microcirculatory effect by reducing the capillary filtration rate and increasing the oxygen and carbon dioxide transcutaneous partial pressure [52]. Consequently, due to its modulating action, it improves the connective tissue modulation, stimulates the synthesis of collagen and other tissue protein by modulating the action of fibroblast in the vein wall, and increases collagen remodeling in and around the venous wall [38]. As such, the TTFCA is effective to treat venous wall alterations in chronic venous hypertension and in protecting the venous endothelium.

4 Clinical Studies

To date, very few clinical studies have been conducted using the triterpenoid centellosides of CA. Most of the studies that have been realized with the CA extracts do not determine the concentration of the active components of the triterpenoid centellosides. In some studies, TTFCA showed its effectiveness on microcirculation and capillary permeability, while AS stimulates angiogenesis both in vitro and in vivo [23, 52]. A study with TTFCA combined with the use of vacuum suction chamber device improved the microcirculation and capillary permeability in patients with venous hypertension [53]. In a similar treatment, placebo-controlled, randomized trial, TTFCA improved the microcirculation in venous hypertension microangiopathy [54]. Oral administration of TTFCA (30 and 60 mg day^{-1}) taken for 60 days that was conducted in a double-blind study in 87 patients with chronic venous hypertensive microangiopathy confirmed the results that TTFCA was effective in the treatment of venous hypertensive [55]. In a 3-week treatment with 90 mg TTFCA on patients with post-phlebetic syndrome (PPS), a reduction in circulating endothelial cells was noted compared to the normal patients [56]. An increase in the circulating endothelial cell number was observed in vascular injury, thrombosis, acute myocardial infarction, and other peripheral venous diseases. As such, the reduction of epithelial cell number was indicated by the effectiveness of TTFCA to protect the integrity of vascular intima. In another study involving 52 patients with venous hypertension (pressure greater than 42 mmHg), patients were treated with 60 and 30 mg day^{-1} of TTFCA [57]. After a 4-week treatment, there was significant improvement in the filtration rate, ankle edema, and ankle circumference. Studies on 94 patients suffering from venous insufficiency of the lower limb were conducted for 2 months using 120 and 60 mg day^{-1} of TECA [58]. These results also confirmed the significant improvements of the lower limb and edema for the TECA groups compared to the placebo.

5 Safety and Toxicology

The safety and nontoxicity effect of CA were reflected by its use in traditional Indian Ayurvedic, Asian, and Chinese medicines for hundreds of years [1]. World Health Organization (WHO) recommendation stated that no toxic effects due to the oral administration of CA have been reported so far, and WHO recommends oral dosage for scar healing is 1–2 g day^{-1}, divided into three parts, taken as such or as an infusion [59]. However, concern on safety should be on the use of its triterpenoid centellosides in pure form. The oral administration of standardized CA extracts and AS was safe in experimental animal model [33]. The AS has shown nontoxicity effect even at 1 mg kg^{-1} in oral administration. Meanwhile, the toxic dose by intramuscular application on rabbit and mice was reported at 40–50 mg kg^{-1} [60]. In oral application, AS at 1 g kg^{-1} of body weight has proven to be nontoxic [61]. Studies on CA triterpenoid active components (AS, AA, and MA) with human cytochrome P450 2C9 (CYP2C9) enzymes have shown moderate to strong inhibition with the strongest ($K_i = 9.1$ μg mL^{-1}) being AA [62, 63]. Therefore, these results demonstrated the potential risk of drug-herb interactions when these CA products, particularly when high AA, were taken together with CYP2C9 isoform substrates.

6 Conclusion

The in vitro and in vivo studies and clinical evaluations have shown the healthcare potential of triterpenoid centellosides' bioactivities and uses. However, the clinical evaluation in human is still limited. As such, it is important to substantiate its healthcare claim clinically, especially on the possible interaction with other drugs. With all these claims in healthcare applications, the prospect of triterpenoid centellosides as an alternative to standard drugs should be further explored.

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Essential Oils as Antimicrobial Agents 132

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Contents

1 Introduction 3976
2 An Overview of Essential Oils 3978
3 Chemistry of Essential Oils 3980
4 Action Mechanism of Essential Oils 3981
5 Synergism and Antagonism 3982
6 Legal Regulations on the Use of Essential Oils 3983
7 Future Perspectives 3983
8 Conclusion 3984
References 3984

Abstract

During the last decades, the limit of microbial diseases and infections has been exceeded dramatically. Several types of pathogenic microbes have been reported; however, some are being less infection responsible. Increase in disease infections in human and plants caused by pathogenic bacteria and fungi mainly occurs due to the development of new specific features in microbial majority, making them to adapt in any environmental condition. Resistance of these pathogens to commercial antibiotic drugs has emerged as a great health concern to the consumers. Diseases caused by bacteria and fungi and their resistant nature to antibiotics have played a vital role to increased rate in plant and

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human infectious diseases. Extensive use of antibiotics in several industries against hazardous bacteria and fungi has resulted in additional antibiotic resistance to these pathogens, which has become a matter of great concern to the public health. There has been an increasing concern worldwide on therapeutic values of natural products including essential oils. Hence, there are multitudes of potential useful bioactive substances to be derived from plants. With the recent trend of high percentage of resistance in microorganisms to the present-day antibiotics, search for new and effective drugs is a challenging task. This chapter discusses antimicrobial efficacy and suitability of plant essential oils against diverse range of microbial pathogens causing several diseases in human and agricultural industry. Also, a brief description of the chemical nature of essential oils, mode of antimicrobial action, their legal use in various beneficial industries, and the area for future research has been laid out.

Keywords

Antibiotic resistance • antimicrobials • essential oils • microbial pathogens

1 Introduction

Microbial pathogens including bacteria and fungi have become the major cause of human and plant diseases which has raised a great safety concern to public health. In several geographic regions, a large proportion of bacterial and fungal diseases have been confirmed by the hazardous pathogens [1, 2]. Diseases caused by microbial pathogens including bacteria and fungi can affect human health and economically important plants [3, 4]. In this respect, the infection may initiate and spread dramatically to various body or plant sites if the person or plant is not treated with suitable antibiotics. There are many examples of plant diseases that have made a major impact on society and have even changed human history. More than 70% of all major crop diseases are caused by fungi. The disease cause of fungi as an agent of plants, human diseases, and decomposers has spurred scientists worldwide to control such pathogens through plant-based natural antimicrobials including essential oils [5]. The impact that fungi have in agricultural industry with regard to plant health, food loss, and human nutrition is staggering. Some of the world's great famines and human suffering can be blamed on plant pathogenic fungi. Similar to all other groups of plant pathogens, fungal pathogens have developed ways to survive periods of unfavorable environmental conditions or in the absence of a susceptible host, spread, infect, grow, and reproduce on and within plants [6].

Food industry, although having a number of food preservatives, is also experiencing a lack of potent food preservative agents to secure the safety of food or food products [7]. Although antimicrobial drugs have supported to control the food-borne pathogens or diseases in food system, however, acquired resistance to these antimicrobial agents is a major drawback due to their several side effects [8]. Hence, certain effective antimicrobial drugs are needed for their practical applications to control food-borne bacterial pathogens.

The essential oils are plant secondary metabolites which are biosynthesized in glandular structures of a plant cell. Essential oils are potential antimicrobial agents because of the presence of bioactive volatile components by which they control several pathogenic bacteria and fungi [9–11]. Use of essential oils in several industries has been exploited since decades [9, 12]. Chemistry of essential oils also supports the phenomena that these oils containing volatile components serve as the most potent antimicrobials [13, 14]. The major drawback of food products is that they are easily deteriorated by food-borne pathogens during their processing [15]; this warrants search for new and effective food preservatives for food and agricultural industries [16, 17]. It has been reported that various factors are responsible for the biological efficacy of essential oils to serve as potent antibacterial agents in food system which include temperature, appearance, pH, load of microbial flora, and favorable environment [18]. Essential oils find practical application as potent food preservatives in meat production than commercial antimicrobials [19]. Several essential oils have been used effectively to control the load of microbial pathogens in various food products [20, 21]. Hence, addition of essential oils can serve as green preservatives to food industry against pathogenic bacteria [12, 22]. Due to these findings, focus on the research related to essential oils has changed from their biological efficiency [23–25] to the activity against various pathogenic bacteria [26–28].

In general, the biological efficacy of essential oils is confirmed due to their chemical components that include phenolics or the components from terpene origin [12, 29]. Although all the essential oils have been shown to exert antimicrobial efficacy, variations in their chemical nature and the amount of their volatile components have been observed. This is attributed to variations in the collection time of sample, abundance or lack of mineral components, distribution, changes in genetic levels, environmental conditions, and portion of the plant used for distillation [30, 31]. Although several essential oils, as a whole, show potent biological efficacy [27, 32], the antimicrobial efficacy of essential oils has been credited to the components present in higher amount. Besides, the components present in lower amount have been shown to exert synergistic effect with the major components of the oil [33].

Several concerns have been raised to minimize the load of chemical preservatives in food or agricultural food products. Recently, the use of essential oils has become a focal area of research for their practical applications to control the hazardous pathogens in food and agricultural systems [2, 34–36]. Pathogenic microbes in storage food or food products are responsible to degrade or deteriorate the quality of food or agricultural products, resulting in the emerging plant and human diseases in various regions of the world [7]. In this regard, applications of essential oils, being potent antimicrobials and low toxic in nature, can be a good strategy to control or inhibit the pathogenic bacteria and fungi in marketable food or processed food or agricultural products with higher percentage of consumer acceptability [37, 38].

2 An Overview of Essential Oils

Although food and agricultural industries have been enriched with several modern practices, consumers are still aware about the health problems caused by pathogenic bacteria and fungi [11, 39]. A large proportion of population is suffering from the diseases caused by pathogenic bacteria and fungi in several geographical regions of the world [11, 39]. This has requested an urgent need to develop new and effective natural antimicrobials to combat with the diseases caused by pathogenic microbes including bacteria and fungi as the western society has appeared to experience the concern that corporates the chemical preservatives with a lesser amount of environmental impact [5, 39–43].

In nature, essential oils having aroma and flavor are isolated from the various parts of the plants [39]. The essential oils for commercial utilization can be isolated using various methodologies which include steam distillation, solvent extraction, and expression [39, 44]. As reported previously, most of the plant essential oils exert potent biological efficacy [11, 35, 39, 45–47]. The antimicrobial or other biological activities of essential oils are directly correlated to the presence of their bioactive volatile components [11, 43, 48].

The use of essential oils was exploited in several industries; however, the extreme utilization rate of essential oils was reported to have in aroma and flavor industries [39]. Essential oils have been used widely in industries [39, 44, 49, 50]. Also, the role of essential oil components has been exploited vigorously in several beneficial industries to secure the antimicrobial efficacy in food and agricultural industries [43, 51]. Combinations of various essential oils with natural and herbal forms are also available in the market for their practical utilization [39, 52, 53].

Extension of life span of food or agricultural products for human utilization is needed from the contamination caused by pathogenic bacteria and fungi. Traditional preservative practices cannot control the microbial contamination; hence, other new methodologies are required to maintain the shelf life of food and agricultural products [54]. Besides, concerns on reducing chemical preservatives in food and agricultural industries have been raised due to the adversary effects of chemical preservatives, resulting in the release of toxic materials in food and agricultural systems. Use of essential oils can be effective application in food and agricultural industries naturally due to their potent antimicrobial nature. The essential oils have been thoroughly screened for their potentials using various test assays, and the inhibitory effect is defined directly or by measuring the physical properties [39, 54–57]. Although variations in the antimicrobial activities of the essential oils have been observed, the less sensitivity of negatively charged bacteria is attributed to the extrapolysaccharide layer as compared to positively charged bacteria lacking this outer cell wall coverage. The antimicrobial efficacy of some of the selected essential oils as minimum inhibitory concentrations against bacteria and fungi has been summarized in Table 132.1.

Table 132.1 In vitro antimicrobial activity of some selected essential oils against bacteria and fungi

Essential oil derived from	Bacteria/fungi	MIC	Reference
Cestrum nocturnum	*Salmonella typhimurium KCTC2515*	25.0 μg/ml	[76]
	Staphylococcus aureus ATCC6538	12.5 μg/ml	
	Listeria monocytogenes ATCC15313	12.5 μg/ml	
Cestrum nocturnum	*Fusarium oxysporum ATCC41083*	500 μg/ml	[43]
	Botrytis cinerea ATCC40573	125 μg/ml	
	Colletotrichum capsici ATCC410978	500 μg/ml	
Ziziphus jujube	*S. typhimurium KCTC2515*	250 μg/ml	[77]
	Escherichia coli ATCC8739	500 μg/ml	
	Bacillus subtilis ATCC6633	125 μg/ml	
Lonicera japonica	*S. typhimurium KCTC2515*	125 μg/ml	[78]
	S. enteritidis	250 μg/ml	
Metasequoia glyptostroboides	*S. typhimurium KCTC12021*	1000 μg/ml	[79]
	Pseudomonas aeruginosa KCTC2004	500 μg/ml	
	F. oxysporum KACC41083	500 μg/ml	[11]
	Phytophthora capsici KACC40157	1000 μg/ml	
	F. solani KACC41092	500 μg/ml	
	B. cinerea KACC40573	1000 μg/ml	
Nandina domestica	*S. typhimurium*	500 μg/ml	[10]
	S. enteritidis	1000 μg/ml	
Nepeta rtanjensis	*Alternaria sp. 1*	0.8 μg/ml	[80]
	Alternaria sp. 2	0.6 μg/ml	
	Cladosporium cladosporioides	1.0 μg/ml	
	Trichoderma viride	1.4 μg/ml	
	Bipolaris spicifera	1.0 μg/ml	
Aloysia triphylla	*Candida albicans*	0.80 mg/ml	[81]
Thymus vulgaris	*Bacillus subtilis*	0.49 mg/ml	
Origanum vulgare	*Staphylococcus aureus*	1.0 mg/ml	
Thymus pulegioides	*C. albicans ATCC 10231*	0.64 μl/ml	[82]
	C. tropicalis H18	0.64 μl/ml	
	C. glabrata H30	0.64 μl/ml	
	Trichophyton rubrum FF5	0.32 μl/ml	
	Microsporum canis FF1	0.16 μl/ml	
	Aspergillus niger ATCC 16404	0.32 μl/ml	
	A. niger CECT 2574	0.32 μl/ml	
	A. fumigatus ATCC 46645	0.16 μl/ml	
	A. flavus F44	0.32 μl/ml	

(*continued*)

Table 132.1 (continued)

Essential oil derived from	Bacteria/fungi	MIC	Reference
Moringa oleifera	*T. rubrum*	1.6 µg/ml	[83]
	T. mentagrophytes	0.8 µg/ml	
	Epidermophyton floccosum	0.2 µg/ml	
Ferulago angulata	*S. aureus*	15 µg/ml	[84]
	S. typhimurium	$>2 \times 10^4$ µg/ml	
	Escherichia coli	$>1.9 \times 10^3$ µg/ml	
	Pseudomonas aeruginosa	$>9.5 \times 10^2$ µg/ml	
	Listeria monocytogenes	170 µg/ml	
	C. albicans	$>1.9 \times 10_3$ µg/ml	
Cinnamomum zeylanicum	*S. aureus*	3.2 mg/ml	[85]
	B. subtilis	>1.6 mg/ml	
	Klebsiella pneumoniae	3.2 mg/ml	
	P. vulgaris	>1.6 mg/ml	
	P. aeruginosa	>0.8 mg/ml	
	E. coli	>1.6 mg/ml	
Eugenia caryophyllus	*S. aureus*	>6.4 mg/ml	[85]
	B. subtilis	>3.2 g/ml	
	K. pneumoniae	>6.4 mg/ml	
	P. vulgaris	>3.2 mg/ml	
	P. aeruginosa	>1.6 mg/ml	
	E. coli	>1.6 mg/ml	
Pelargonium graveolens	*S. aureus*	>12.8 mg/ml	[85]
	B. subtilis	>6.4 mg/ml	
	K. pneumoniae	12.8 mg/ml	
	P. vulgaris	>12.8 mg/ml	
	P. aeruginosa	>12.8 mg/ml	
	E. coli	>6.4 mg/ml	
Citrus limon	*S. aureus*	>12.8 mg/ml	[85]
	B. subtilis	>12.8 mg/ml	
	K. pneumoniae	>12.8 mg/ml	
	P. vulgaris	>6.4 mg/ml	
	P. aeruginosa	12.8 mg/ml	
	E. coli	>6.4 mg/ml	

3 Chemistry of Essential Oils

Plant essential oils are the low molecular weight volatile mixtures, biosynthesized in various plant organs. The chemical nature of essential oils belongs to the composition of terpene compounds (mono-, sesqui-, and diterpenes), which are mainly obtained as hydrocarbon compounds or the derivatives of oxygen molecule. A few components of the essential oil are nitrogenous or sulfury in nature which are found as alcohols, acids, esters, epoxides, aldehydes, ketones, amines, and sulfides [58]. The components of the essential oil are divided in two groups: (1) compounds from terpene origin and (2) aroma compounds [59].

Terpene compounds have natural occurrence in plants found as major components of most of the plant essential oils. Based on their structural and functional properties, terpene compounds have been classified according to their basic structural unit isoprene containing five carbons. In the formation of terpenes, prenyldiphosphate serves as a precursor. The terpene compounds exist in the form of mono-, sesqui-, hemi-, di-, tri-, and tetraterpenes. The monoterpenes containing two isoprene units are responsible to construct the major portion of all the essential oils. These compounds work as carbure, alcohol, aldehyde, ketone, ester, ether, peroxyde, and phenols [58]. The sesquiterpene compounds contain three isoprene units, and the functional properties are very close to monoterpene compounds.

The aromatic compounds are the derivatives of phenylpropane, which are aldehydes, alcohols, phenols, methoxy, and methylenedioxy in nature. A few nitrogen and sulfur compounds present in essential oils are also characterized as plant essential constituents. A list of selected and important antimicrobial components of essential oils has been presented in Fig. 132.1.

4 Action Mechanism of Essential Oils

Though a large volume of data is available on biological effect of essential oils, the mechanism of antimicrobial effects is not clearly understood till recently as essential oils are a complex mixture of several diverse components. Besides, different essential oils or their different components show antimicrobial mode of action not only at a particular location but also at different cell sites [21, 60]. Hydrophobic nature of essential oils makes them interact too well with lipid membrane of bacterial pathogens, resulting in the leakage of the inner cell components of the cell as well as affecting potassium ion reflux and eventually leading to cell death [24, 32, 61, 62]. Generally, phenolic nature of essential oils makes them to work effectively against pathogenic bacteria [32] by disrupting the cell membrane as well as effectively inhibiting the functional properties of the cell and eventually leaking the inner materials of the cell [62, 63]. Besides, phenolics contain an OH group, hence working effectively against pathogenic bacteria [22, 64].

Volatiles from the essential oils not only work on a single target site in the cell, but also they bind to protein structures of the cell. Bacterial cell membrane contains the enzymatic proteins to maintain the functional properties, and storage of hydrocarbons to lipid membrane can make the changes in the building block of lipids and proteins, resulting in the permeability of the cell components [62, 65]. Some of the essential oils and their volatiles are found responsible in inhibiting the enzymatic proteins in some bacterial pathogens [66].

In addition to this, terpene compounds have been well known to affect the bacterial and fungal cell membrane by inserting themselves between the fatty acyl chains that make up the membrane lipid bilayers [62]. This results in changes in cell functions, leakage of cell components making starving conditions

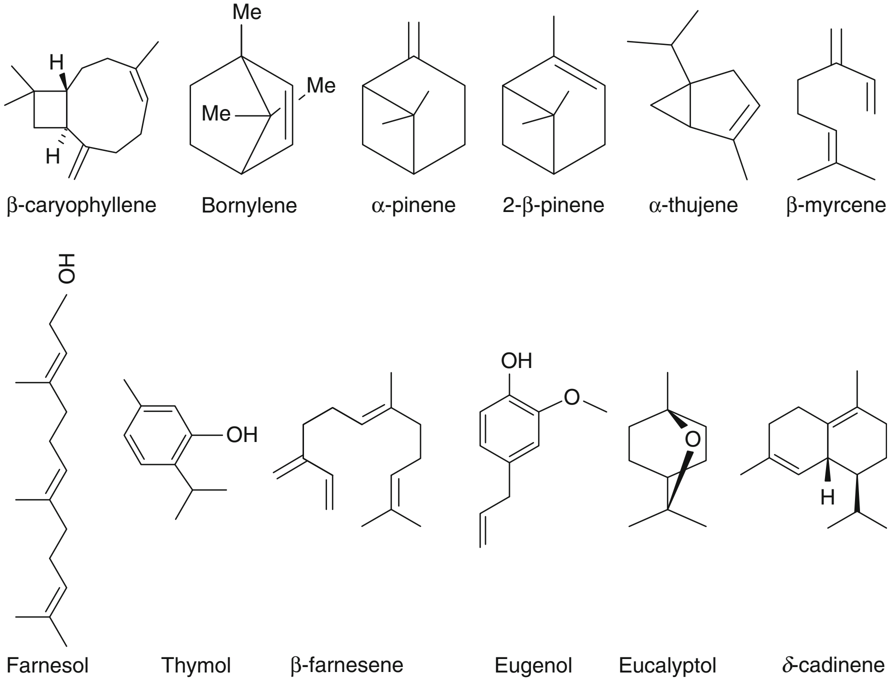

Fig. 132.1 Chemical structures of some selected antimicrobial principles of essential oils

of the cell [67]. In particular, the fungal plasma membrane ATPase may protect cells by maintaining cell homeostasis and by countering the permeabilizing effects of essential oils. On the other hand, the essential oils and their terpene composition have been shown to inhibit respiration in fungal pathogens, suggesting adverse effects on mitochondria [24, 68]. Also, the sesquiterpene dialdehyde polygodial has been shown to inhibit the yeast mitochondrial ATPase, affecting medium acidification indirectly by reducing or eliminating the large amounts of cellular ATP required to fuel the plasma membrane ATPase in fungal pathogens [69]. Hence, the functions of cell components including nucleus can be reduced by the effect of essential oil or their volatiles due to the permeability changes occurring in the membrane of fungal and bacterial cell [67, 70].

5 Synergism and Antagonism

The antimicrobial efficacy of essential oils can be attributed to the volatile majority of essential oil, volatile ratio, as well as their mode of action [22, 34, 39]. Antagonistic efficacy can be visualized upon the supply of two compounds where the sample mixture has low antimicrobial efficacy as compared to using them separately [39]. Additional antimicrobial efficacy can be visualized upon the supply of two compounds where the sample mixture has similar antimicrobial efficacy while using them separately [39]. However, when combinatory effect of two compounds is higher than the effect of individuals, it is known as synergism [39, 71]. Literature survey has demonstrated that an oil as a whole showed better antibacterial efficacy than only a combination of major volatiles of the oil [39, 72]. Hence, it might be said that the minor elements of the oil have crucial role in increasing the biological effectiveness of the oil, resulting in the synergism. Previously, we reported that cone essential oil of *Metasequoia glyptostroboides* exerted potential synergistic effect along with nisin against gram-positive bacteria at varied concentrations in whole, low, and skim milks [73]. Combined fractions of different essential oils such as cilantro, coriander, dill, and eucalyptus have been shown to exert synergistic and additive mode of action [39, 74]. Although proportions of volatile compounds of essential oils have been found to exhibit a complete inhibitory action to some of the bacterial pathogens, however, these volatiles could not be able to exert antibacterial efficacy when applied individually against the tested organisms [39, 75]. The biological efficacy of essential oils not only corresponds to the volatiles present in a higher quantity but also the volatiles present in low amount can influence the biological efficacy of whole essential oil.

6 Legal Regulations on the Use of Essential Oils

The European Union (EU) has laid down recommendations and laws for using plant volatiles and essential oils to retain the quality of the food or food products which are easily hampered by the pathogenic microbes. FDA is very likely to follow the EU over these issues. Several essential oils and volatiles in their marketable forms are sold as flavoring agents. The registration of oil or oil components at EU and FDA is considered the products to be nonhazardous as well as safe to the health of consumers. Besides, a new registration to serve as natural flavoring agent can only be considered if the concerned metabolic and toxicological researches have been performed in order to confirm its daily purpose use in the USA so as to call approved food additives [39]. Interestingly, it will be more beneficial economically to the people of the developing countries to use herbal plant or the plant parts as a whole or a whole essential oil as an ingredient as well as herbal drug than using an individual component of the essential oils [39, 42].

7 Future Perspectives

The most diverse and interesting area of the essential oils and their volatiles for their proper utilization is to suppress hazardous pathogens including bacteria and fungi. The improvement in the quality of food or agricultural products as well as control of multidrug-resistant pathogens might be an important area of further research. The essential oils can also be used in the food stuffs which have not been associated previously with any flavor, if one or more components of the oil together can exert desired antimicrobial efficacy at the particular amount, having no deteriorating effect on the quality of food or agricultural products [39]. In fact, the consumer goods are experiencing an increased influence of essential oils or their volatiles in order to develop new and effective natural antimicrobials of plant origin [39, 41]. These products can also be served as natural in other related industries for their safe use. For large-scale production of essential oils, biotechnological approaches of their synthesis may be useful to obtain the desired volume of essential oils. In addition, standardization of the chemical nature of commercial essential oils will be needed for their safe and practical applications.

In addition to this, the mechanism of action of plant essential oils and volatiles on proteins associated with cytoplasmic membrane as well as on lipid structure has not been completely studied. Therefore, it might be an important aspect of future studies [39]. Moreover, evaluation of the mechanism of action of essential oils will potentially strengthen their practical applications and significance in future research in antimicrobial therapy [39]. In order to optimize the antimicrobial efficacy, synergistic, antagonistic, and additive effects, essential oils can be explored so as to optimize the lowest concentration required for the inhibition of any pathogenic bacteria or fungi which will indeed be a helpful strategy in the practical application of essential oils. Besides, experimental data on various direct or indirect consequences of using essential oils and their volatiles will confirm whether the essential oil or its volatiles are safe for using human purposes.

8 Conclusion

Microbial pathogens including bacteria and fungi are getting hazardous to human society in various aspects of utilization. Food or agricultural products including ready-to-eat products and vegetables are the major source of microbial contamination resulting in various human diseases. Practical applications of essential oils are needed for the complete elimination of hazardous pathogens. Several essential oils and their volatiles have shown a great potential to exert antimicrobial efficacy in several in vitro and in vivo model systems to control the harmful and pathogenic microbes. The volatiles of essential oils from terpene origin are most likely to affect the target pathogen. Based on selectivity and specificity, essential oils or their volatiles can be applied as potent and natural antimicrobial agents to control the pathogenic microbes including bacteria and fungi.

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Menthol: Biological Effects and Toxicity 133

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Contents

1 Introduction 3990
2 Menthol Effects on Nonvascular Smooth Muscle 3991
3 Respiratory Smooth Muscle 3991
4 Gastrointestinal Smooth Muscle 3992
5 Genitourinary Smooth Muscle 3992
6 Actions of Menthol in Vascular Smooth Muscle 3993
7 Assessment of Cytotoxic Effects Caused by D, L-Menthol 3994
8 Menthol Effects on Voltage-Gated Channels 3996
9 Conclusion 3997
References 3997

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Abstract

Natural products are still been explored to provide new molecules that could interact with known and unknown pharmacological targets. Medicinal chemists have put a lot of effort in order to validate highly selective and potent biological agents. The studies focusing on the understanding of how these molecules act are very important and relevant to the field. A number of studies have shown that menthol possesses a large spectrum of biological activities. Interestingly, menthol has been implicated in the generation of an inward cationic current related to temperature sensing and this fact has been the focus of attention of various research groups around the world. In this chapter, we will provide information of menthol's pharmacological profile in different tissues. We also discuss the cytotoxic effects that have been attributed to menthol and many groups have provided evidence to confirm that this cytotoxicity is somehow related to the activation of TRPM8 ion channels and it seems to be dependent on intracellular calcium handling.

Keywords

Natural products • menthol • cytotoxicity • ion channels • TRP channels • voltage-dependent ion channels

1 Introduction

Over the last years, research on natural products have contributed enormously not only to the development of important therapeutic drugs used currently in different areas of modern medicine, but these compounds have also been used as molecular tools to explore new pharmacological targets and uncover intracellular signaling mechanisms involved in their distinct biological functions. However, the still unexplored potential of natural products as sources for new drugs relies on the fact that only a small fraction of plants, animals, and microorganisms have been so far investigated phytochemically or biologically [1]. The goal of this chapter is to collect basic information available in the literature related to menthol and its effects.

Menthol is a plant-derived cyclic terpene alcohol that gives plants of the *Mentha* genus their typical minty smell and flavor. The plant oil, often referred to as peppermint oil (from *Mentha piperita*) or cornmint oil (from *M. arvensis*), is prepared by steam distillation from the fresh parts of the plant or synthesized from other essential oils such as citronella oil, eucalyptus oil, and Indian turpentine oil. Among the optical isomers, (−)-menthol is the predominant chemical form present in nature and it is considered as a fragrance and flavor compound. For this reason, it is widely used as a flavoring for toothpaste, other oral hygiene products, and chewing gum [2]. Either *Mentha* herbs or menthol are mentioned in the Japanese Pharmacopeia, British Herbal Pharmacopeia, and US Pharmacopeia.

In this chapter, we will bring to attention some of the published studies that actually implicate menthol as an important pharmacological agent in different tissues.

2 Menthol Effects on Nonvascular Smooth Muscle

Menthol has been characterized in the literature mainly as an agonist on TRP channels, particularly as an activator of TRPM8, the receptor that makes the transduction for cool sensation [3–5]. Experimental work, however, has demonstrated that menthol has several additional pharmacological activities [6, 7]. Concerning smooth muscles, menthol is reported to act directly on their contractility, either via activity on excitation-contraction coupling or via intracellular signaling mechanisms.

Menthol blocks the excitation-contraction coupling that is mediated via cytoplasmic membrane depolarization (electromechanical coupling) and opening of voltage-operated Ca^{2+} channels (VOCC). On the experiments performed to investigate the effect of menthol on electromechanical coupling high concentrations of extracellular K^+ (20–60 mM) were used to induce depolarization of the cytoplasmic membrane and provoke VOCC opening, which causes increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [8]. Blockade of electromechanical coupling has been demonstrated on the tracheal [9], bronchial [10], intestinal [11], urogenital [12–14], and vascular smooth muscles. This inhibition of electromechanical coupling has been attributed to menthol-induced blockade of VOCC [12–14].

Menthol is also described to block the excitation-contraction coupling that is mediated via the cascade of phenomena triggered by combination of agonists with cytoplasmic membrane receptors, also known as the pharmacomechanical coupling. Blockade of pharmacomechanical coupling has been demonstrated on tracheal [9], bronchial [10], urogenital [13, 14, 15], and vascular smooth muscles. Pharmacomechanical coupling include activity of receptor-operated calcium channels (ROCC) as one of the initial steps of the cascade of events leading to contraction, which causes increase in ($[Ca^{2+}]_i$) [8]. Menthol is also reported to act on the TRP channels located on the sarcoplasmic reticulum with implications on the contractility of the smooth muscle [12, 14]. Due to the diversity of parameters related to contractility that were investigated below, we will give the relevant details of menthol activity on each smooth muscle type.

3 Respiratory Smooth Muscle

The contractions of tracheal muscle of guinea pig induced by high concentrations of extracellular K^+ (20–60 mM) and methacholine (MCh, 0.01–10 μM) on the bath solution were inhibited by menthol [9]. The temperature dependence of this inhibitory effect was tested for the MCh-induced contraction: increase in temperature decreased menthol effect on contraction. Menthol also inhibits the increases of $[Ca^{2+}]$ induced by MCh or high K^+. This diminished increase in $[Ca^{2+}]_i$ was interpreted to be caused by inhibition of Ca^{2+} influx, which was concluded to be an important role on menthol-mediated inhibition of smooth muscle contraction [9].

On bronchial muscle of guinea pig, in experiments done in vitro, menthol caused dose-dependent relaxation of bronchial contraction induced by high concentrations of K^+ and ACh. In experiments in vivo, menthol also attenuates both capsaicin and NKA-induced bronchoconstriction. These effects were interpreted to be, in part, explained by a direct action of menthol on bronchial smooth muscle tissue [10].

4 Gastrointestinal Smooth Muscle

Menthol blocks the contractions induced by high concentrations of K^+ in guinea pig ileal smooth muscle (IC_{50} 7.7 μg/ml). Based on the similarity of IC_{50} for menthol-induced blockade of ileal contraction and Ca^{2+} uptake by synaptosomes it was concluded that menthol blocks Ca^{2+} channels. Additionally, it was suggested that this channel blocking property is one of the mechanisms contributing to the blockade of electromechanical coupling in ileum [11].

5 Genitourinary Smooth Muscle

In vitro experiments demonstrated that menthol (0.1–1.0 mM) inhibited carbachol-induced contraction of the detrusor smooth muscle of rat. Intravesical infusion of menthol (3.0 mM) facilitated the micturition reflex since it caused a reduction in voided volume (by 20%), volume threshold for inducing micturition (19%), and pressure threshold for inducing micturition (31%). This effect on micturition reflex was not modified by pretreatment with capsaicin. This was interpreted to suggest that intravesically infused menthol cannot relax detrusor muscle, and acts on capsaicin-resistant afferents (probably through TRPM8 in urothelium or sensory nerve endings) to facilitate the micturition reflex [15].

In rat vas deferens in vitro experiments menthol (0.1–1.0 mM) inhibited contractions induced by high concentration of K^+, carbachol, or noradrenalin. It was suggested that menthol acts via two mechanisms: partial blockade of Ca^{2+} entry via the voltage-gated, L-type calcium channels and a decrease of the calcium storage capacity of the sarcoplasmic reticulum (SR). It was also suggested that the effect on SR at least in part was mediated by menthol-induced activation of the SR-resident TRPM8 channel, which would lead to the enhancement of passive leak of Ca^{2+} from the SR and reduction in the amount of the releasable calcium during activation of contractions [12]. Investigation of the same research group reported that in the prostatic portion of smooth muscle cells of rat vas deferens higher TRPM8 protein targeted specifically the SR and cytoplasmic membrane, while in the epididymal portion it targeted only the plasma membrane [13]. Based on its localization on membrane of SR, TRPM8 was suggested to be capable of modulating the contractile activity [13]. Comparing the results obtained in normal versus castrated rats and in epididymal versus prostatic portions of the vas deferens it was suggested that TRPM8 modulates contractility of the prostatic portion by primarily decreasing Ca^{2+} storage in the SR, while in the epididymal portion by both decreasing the Ca^{2+} storage in SR and supporting Ca^{2+} entry. It was also suggested that castration and its resultant drop in the circulation of androgens changes the modulation of the rat vas deferens SM contractility mediated by menthol and icilin via the decrease of expression of L-type VOCC and increase in expression of TRPM8 [14].

6 Actions of Menthol in Vascular Smooth Muscle

Despite the use of menthol in culinary and medicinal preparations [2], relatively little is known about its actions on the human cardiovascular system, particularly in vascular smooth muscle. Studies in blood vessels evaluating the effects of menthol demonstrated different actions, particularly related to the precontractile state of the vasculature prior to menthol administration.

In studies using thoracic aorta from rat, menthol induced relaxation in precontracted vessels. This effect was significantly greater when the endothelium was left intact (by 15–20%), compared with denuded vessels suggesting that endothelium mediators participate in menthol-elicited relaxation. However, in relaxed tail artery with endothelium removed menthol addition caused a small but consistent contraction that peaked in the first minute of application. These evidences demonstrate that menthol induces a moderate vasoconstriction in vessels that have low vasoconstrictor tone and a marked vasodilatation when vasoconstriction is set at a high level [16].

Menthol actions in other vessels have also been characterized. As in the aorta, menthol induced relaxation in precontracted femoral, renal, mesenteric, and tail arteries, revealing no significant differences between potency and magnitude of effect between tissue beds. Therefore, menthol causes relaxation of vascular smooth muscle through intracellular Ca^{2+} concentration regulatory mechanisms [16].

However, it is well described that topical menthol application produces an innocuous cooling sensation at low concentrations and a burning pain sensation at high concentrations. Studies have indicated that menthol stimulates membrane depolarization and increased action potential firing on a subpopulation of primary afferent neurons. This depolarization results, as mentioned before, from activation of channels considered to be the main molecular thermosensors in sensory neurons belonging to the family of transient receptor potential (TRP) channels. TRPM8 (Transient receptor potential melastatin 8) is a Ca^{2+} permeable nonselective cation channel [17], predominantly expressed in a subpopulation of thermoceptive/nociceptive neurons found in the dorsal root ganglia and in trigeminal ganglia.

The expression of TRPM8 channels has also been described in the vascular smooth muscle cells [16, 18–20]. TRPM8 mRNA and protein were detected in rat tail, femoral and mesenteric arteries, and thoracic aorta [16, 20]. Additionally, this was confirmed in single isolated vascular myocytes by immunocytochemistry [16]. It has been demonstrated that the vascular effects induced by menthol seems to be related to the activation of TRPM8 channels, and this monoterpene is vastly used as a TRPM8 agonist. The major effect was observed in precontracted vessels, where

TRPM8-specific agonists, including menthol, caused a profound dilatation. These effects were predominantly attributable to direct stimulation of TRPM8 channels associated with vascular smooth muscle cells [16].

Studies using rat aorta demonstrated that the relaxation effect of menthol was abolished in the absence of extracellular Ca^{2+} or in the presence of Ni^{2+} but not in the presence of nifedipine, an voltage-dependent L-type Ca^{2+} channel blocker, suggesting that, at least in part, TRPM8 proteins are functional Ca^{2+} influx channels in vascular myocytes [20].

Additionally, it was found that in human forearm cutaneous vessels, menthol caused a profound dilatation in an assay where cutaneous red cell flux (RCF) was recorded from the ventral surface of the left forearm using a laser Doppler perfusion monitor. The delay in action (8–18 min) in relation to what was observed in other vessels may simply reflect the time taken for menthol to passively diffuse into blood vessels from which RCF measurements were taken, 1 mm below the skin surface [16]. This observation is of particular relevance to demonstrate that experiments conducted in humans seems to reflect similar vasorelaxation effects observed in rats.

In summary, these findings suggest that menthol disturb Ca^{2+} homeostasis on smooth muscle cells and help to reveal that as the common mechanism by which menthol affects various cell properties, although the effects of menthol on $[Ca^{2+}]_i$ are opposite in different tissues and species.

7 Assessment of Cytotoxic Effects Caused by *d, l*-Menthol

It has been reported that the monoterpene menthol presents a variety of biological actions including antibacterial, antifungal, antioxidant, and radical scavenging properties. Moreover, some studies have shown menthol effects on ion channels, the voltage-gated sodium channels [21], and transient receptor potential channels, specifically Transient Receptor Potential Melastatin 8 subfamily (TRPM8) channels, which is a member of the TRP ion channel superfamily [22] and these effects on TRPM8 channels have been associated with menthol's cytotoxic actions. By using a number of experimental approaches different research groups demonstrated that menthol could cause cell death [23, 24].

The relationship between the pharmacological effects elicited by menthol and ion channel dysfunction depends on the interaction of this monoterpene with distinct types of ion channels. In this way, the reported local anesthetic effects attributed to menthol and for some of its structural chemical homologues, such as thymol, carvacrol, and carveol, might be caused by a blockade of voltage-gated sodium channels [21, 25, 26]. On the other hand, a pronounced cytotoxic effect of menthol is correlated with the action of menthol in TRPM8 channels [22, 27]. At this point, we will make a short introduction to TRPM8 channels to further discuss the putative cytotoxic effects of menthol in various cell types.

The TRPM8 channel is a cold- and menthol-sensitive ion channel, as was shown by Reid and Flonta in 2001 [28]. TRPM8 is a nonselective cation channel and calcium permeable which is activated by temperatures below 26°C. This channel has six transmembrane domains and the functional channel is formed by assembly of four identical subunits, homotetrameric protein [29] and its structure is reasonably conserved although evolution from invertebrates to vertebrates. These channels play a key role in regulation of cell cycle and calcium handling [30]. Furthermore, it has been demonstrated that some substances, including menthol has the ability to activate the TRPM8 channel [22, 31].

TRPM8 has been found in neurons although its expression is low, moreover this channel is also expressed in smooth and skeletal muscle, prostate, bladder, genital tract, and lungs [21, 32, 33]. However, it has been shown that there is greater expression of these channels in cancer cells than the corresponding noncancer cells and it could be addressed as a marker for diagnosis and also suggests the possibility of target-directed drug design studies [22, 31, 33].

As the TRPM8 channel seems to be related to events such as proliferation, apoptosis, and cell differentiation, it is reasonable to think of TRPM8 contribution to modulate important cellular processes. It was shown by Thebault and colleagues [33] a TRPM8 channel restrict to the endoplasmatic reticulum (ER) membrane of human prostate cancer epithelial cells (also known as LNCaP an androgen-dependent prostate cancer cell lineage). This menthol-activated channel at subcellular localization provoked calcium release from ER which was associated with the entry of calcium through plasma membrane store-operated channels. These calcium handling processes might be important for cell fate decision (p.ex. apoptosis or proliferation). Menthol also triggered the TRPM8 channel in another human prostate cancer cell, DU145 which is androgen-independent cells and has significant TRPM8 protein expression. It was shown that menthol decreased both, proliferation and motility of DU145 cells [34].

It was also reported that menthol suppressed the viability of human malignant melanoma cell line, G-361 and was proposed that the influx of calcium through activation of TRPM8 channels may be responsible for the regulation of cell cycle events leading to cell death in such melanoma cells [27].

Various authors have demonstrated that menthol acts not only by activating the TRPM8 channel but also in other cellular mechanisms. In line with this statement, human gastric cancer SNU-5 cells menthol reduced the cell proliferation rate by inhibiting the expression of topoisomerase I [24]. In murine WEHI-3 and human promyelocytic HL-60 leukemia cells menthol decreased the Mac-3 and CD11b markers which are involved in processes, such as leukocyte adhesion and migration, phagocytosis and cell-mediated cytotoxicity, and in HL-60 menthol–stimulated cell death [35, 36] Another study showed that menthol was able to change tubulin polymerization as well as apoptosis inhibiting human epithelial colorectal adenocarcinoma cells [37].

In general, some studies have addressed several issues mentioning that menthol has the potential to become a new drug active on cancer cells. However, caution should be exercised here because menthol has a number of different biological actions which could be an actual problem in terms of developing a new class of drugs. Clearly more in vivo and in vitro studies are essential to better certify this prospective.

8 Menthol Effects on Voltage-Gated Channels

Voltage-gated channels have fundamental roles in many cells, excitable or not. Alteration in the regular function of these molecules alters excitability in muscle and neuronal cells and secretory pattern in endocrine cells, for example. Despite the stimulatory action of menthol on TRPM8 channels, in general the reports so far show menthol as an inhibitory molecule on voltage-gated channels.

Voltage-gated calcium channels mediate calcium influx that controls many functions such as contraction, secretion, neurotransmission, and gene expression in different cell types. In neurons and neuroblastoma cells, menthol inhibits calcium currents measured from low voltage-activated (LVA) and high voltage-activated (HVA) calcium channels in the concentration range of 0.1–1 mM [38, 39]. Additionally, menthol also accelerates inactivation phase of the calcium current from HVA calcium channels in a concentration-dependent manner and without major calcium influence [39, 40].

Similar to that observed in neurons, menthol can inhibit cardiac L-type calcium channels. However, another well-known TRPM8 agonist, icilin, had only a minimal effect on these channels suggesting that menthol-induced modification on cardiac voltage-gated calcium channels is independent of activation of TRPM8 [41].

Voltage-gated sodium channels are crucial for the initial phase of action potential in excitable cells and inhibition of these channels is one of the primary mechanisms implicated on local anesthetic effects [42]. Menthol was reported as local anesthetic in vivo and in vitro experiments, strengthening the notion that menthol acts on voltage-gated sodium channels [43]. Corroborating with this study, two more recent reports demonstrate menthol inhibitory effect on tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) voltage-gated sodium channels [21, 44].

Menthol inhibits sodium inward currents generated by heterologously expressing in HEK293 cells rat neuronal (rat type IIA) and human skeletal muscle (hSkM1) sodium channel isoforms [21]. Also, the inhibitory potency of menthol on these channels increases when maintaining the holding potential in more depolarized levels. This fact, per se, indicates that menthol could have more affinity for channels in inactivated state [21].

Menthol also inhibits native TTX-R sodium channel isoforms (Na_V 1.8 and 1.9) in dorsal root ganglia neurons independent of TRPM8 ion channel activation. Similarly, TTX-S sodium channel isoforms inhibitory potency increases at more depolarized holding potentials [44]. In TTX-R and TTX-S sodium currents, menthol induced a negative shift in steady-state inactivation curve indicating a change in inactivation kinetics without major changes on the voltage-dependence of the steady-state activation [44]. Menthol inhibited firing at high-frequency stimulation with minimal effects on normal neuronal activity and, in low concentrations, menthol also cause analgesia in mice alleviating pain produced by Amm VIII, a sodium channel-targeting toxin which slows down sodium channel inactivation [44].

Despite these reports, until now menthol effects on voltage-gated potassium channels were not well explored. Menthol-activated large-conductance

Ca^{2+}-activated K^+ (MaxiK) channels by increasing intracellular calcium levels and stimulated cell migration on human glioblastoma cells [45]. However, in inside-out patch clamp experiments (using pituitary GH3 cells as a model), to avoid any influence of intracellular calcium, menthol did not increase MaxiK channels activity [46].

In a recent review, Araújo and colleagues [26] have pointed out some of the pitfalls and promises in this exciting field of research that is worth reading. The authors argued that we are far from a complete set of experimental approaches that lead us to actually investigate the biotechnological potential of substances isolated from natural sources.

9 Conclusion

Menthol is a natural compound isolated from a variety of plants and it has been mostly used as analgesic. Recently, it was demonstrated by a number of studies that menthol produces cool sensation which is mediated by the activation of TRPM8 channels. Experimental work from different laboratories has demonstrated that menthol has several additional pharmacological activities that compose its pharmacological features. In this chapter, we have pointed out a scenario where menthol is posed as the main actor toward the understanding of its biological and toxicological effects.

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Eugenol as Local Anesthetic

134

Gehoon Chung and Seog Bae Oh

Contents

1 Introduction 4002
2 Definition with Chemical Examples 4002
3 Biosynthetic Pathway and Regulation 4003
4 Biological and Pharmacological Activities 4003
4.1 Analgesic Activity 4003
4.2 Anesthetic Activity and Inhibition of Nerve Impulses 4004
4.3 Inhibition of Synaptic Transmission 4005
4.4 Therapeutic Effects 4006
4.5 Antimicrobial Activities 4007
4.6 Other Aspects 4008
5 Conclusion 4009
References 4010

Abstract

Eugenol is an active ingredient of essential oil extracted from cloves and other herbs. Eugenol is used extensively in dentistry for its analgesic and anti-inflammatory activities. However, the molecular mechanism underlying the pharmacological action of eugenol is only recently investigated. This chapter discusses eugenol's abilities to modulate various ion channels that are responsible for nociception, generation of neuronal spikes, and synaptic transmission. Therapeutic use of eugenol as antibiotic, anti-inflammatory, antitumor, and antioxidant agent is described and then followed by other aspects of eugenol

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such as cytotoxicity, insecticidal activity, allergic reaction, and olfactory response. In conclusion, eugenol is a versatile natural compound that has many potential uses in wide areas.

Keywords
Eugenol • analgesic • anesthetic • anti-inflammatory • antibiotic

1 Introduction

Eugenol (4-allyl-2-methoxyphenol) is an active ingredient of several herb plants that are well known and widely used in traditional medicines, especially for analgesic and antiseptic purpose in treatment of toothache [1]. For example, eugenol is a major constituent of clove oil, which was rubbed on the gums in treatment of toothache [2]. Its therapeutic use in dental clinic is documented in as early as 1873 in its mixture form with zinc oxide [3]. It was more than a hundred years later that the anodyne [4] and the antibacterial effects [5] of the zinc oxide eugenol were assessed by modern scientific method. Eugenol was commonly used as dressings after periodontal surgery in the 1960s [3]. In present days, eugenol is extensively used in dentistry, as an active ingredient of filling materials, endodontic sealers, dental cements, periodontal dressing materials, and dry socket dressings. However, traditional use of eugenol was not limited to analgesics and antiseptics but as versatile as anesthetic, antiemetic, antiseptic, antispasmodic, antiparasitic, healing, and disinfectant effect [1]. The application of eugenol is growing fast as modern scientific methods have revealed antifungal[6], anticarcinogenic [7], antimutagenic [8], antiallergic [9], antioxidant [10], and insecticidal activity [11] of eugenol. This review mainly focuses on the recent advances on the understanding of mechanism underlying the neuronal modulation by eugenol and then discusses other pharmacological effects of eugenol.

2 Definition with Chemical Examples

Eugenol is a member of the phenylpropanoids, a large family of organic chemicals that contain a phenyl ring and a C_3 side chain. Phenylpropanoids are synthesized in plants from the phenylalanine, an essential amino acid, to serve various important roles such as an antibiotics, natural pesticides, signal substances, protective agents against ultraviolet light, an essential component of a cell wall, attractants for pollinators [12]. Other bioactive phenylpropanoids include carvacrol, thymol, cinnamaldehyde, salicylic acid, vanillin, and coumarin [2].

Eugenol is also a phytochemical, meaning it is a naturally occurring compound in plants. While cloves (*Syzygium aromaticum* or *Eugenia caryophyllata*) have the highest percentage of eugenol in its oil extract, eugenol is also found in allspice

(*Pimenta dioica*), sweet basil (*Ocimum basilicum*), holy basil (*Ocimum tenuiflorum*), bayrum tree (*Pimenta racemosa*), carnation (*Dianthus caryophyllus*), and cinnamon (*Cinnamomum verum*) [13].

3 Biosynthetic Pathway and Regulation

The first step of phenylpropanoid biosynthesis is conversion of phenylalanine into cinnamic acid by cleavage of ammonium group by the enzyme phenylalanine ammonia-lyase (PAL). Reduction of carboxylic acid from the cinnamic acid leads to cinnamaldehyde, which is then acylated with acetate from acetyl-CoA to form coniferyl alcohol [14]. Reductive cleavage of coniferyl alcohol by eugenol synthase yields eugenol [15].

4 Biological and Pharmacological Activities

4.1 Analgesic Activity

Historically, eugenol was used primarily as an analgesic agent. However, its molecular mechanism underlying the analgesic effect was studied only in recent years [16–22].

4.1.1 TRPV1 Channels

Transient receptor potential (TRP) channels are large family of cation channels that play roles from insects to humans as key transducers of various physical and chemical stimuli [23]. Among over 30 members of mammalian TRP family, several have been implicated in transduction of nociception. The most extensively studied TRPV1, or transient receptor potential vanilloid 1, is of particular interest because it is activated by noxious heat and algogenic chemicals. The chemical activators of TRPV1, such as capsaicin, resin feratoxin, and olvanil, are called vanilloids because they share vanillyl functional group in their structure [24]. Interestingly, the vanillyl moiety is also included in the structure of eugenol. Based on the vanilloid structure of eugenol and observation of calcium-permeable current induced by eugenol in dorsal root ganglion neurons [25], it was tested and demonstrated that eugenol activates TRPV1 [26] and TRPV3 channels [27].

Activation of TRPV1 induces a robust inward cationic membrane current, but repetitive or prolonged exposure to the activating condition leads to desensitization, and TRPV1-induced currents are gradually reduced [28]. Therefore, the analgesic mechanism of eugenol, especially in its ability to alleviate thermal hyperalgesia after nerve injury [29], might be partly attributed to the desensitization of TRPV1. However, because eugenol does not induce a strong response of TRPV1 as capsaicin, and because desensitization of TRPV1 by repetitive application of eugenol has not been observed, this is yet to be proved in future study.

4.1.2 HCN Channels

Hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels depolarize cell membrane in response to membrane hyperpolarization. The current produced by HCN channels, hyperpolarization-activated current, or I_h, was first identified as a pacemaker current in cardiac muscle cells [30]. I_h in dorsal root ganglion (DRG) neurons, particularly in medium- and large-size neurons, evokes spontaneous firing of action potentials and induces ectopic discharges after peripheral nerve injury [31, 32]. The role of HCN channels in development of neuropathic pain was further confirmed by that pharmacological blockade of I_h reversed both pain behavior and the spontaneous discharge in injured nerve fibers [33]. It was recently shown that eugenol abolished mechanical allodynia induced by chronic constriction injury of infraorbital nerve [16]. The mechanism of the subsidized mechanical allodynia could not be attributed to inhibition of voltage-gated sodium channels because mechanical allodynia was reversed by much lower dose of eugenol than thermal hyperalgesia [16]. Because upregulated HCN channels in neuropathic pain condition contribute more to mechanical allodynia than thermal hyperalgesia, the effect of eugenol on I_h was tested in rat trigeminal ganglion neurons. Whole-cell patch-clamp recording revealed that I_h was successfully inhibited by eugenol at much lower dose than voltage-gated sodium channels. The mechanism underlying inhibition of I_h by eugenol does not seem to be mediated by $G_{i/o}$-protein activation, although intracellular cAMP concentration is somehow related. Although the molecular mechanism is yet to be discovered, it was proposed that eugenol could be potentially used for treatment of mechanical allodynia in neuropathic pain patients [16].

4.1.3 Purinergic Receptors

P2X receptors are cation channels that are activated by extracellular adenosine 5′-triphosphate (ATP). Among seven members of P2X receptor family, $P2X_3$ is predominantly expressed in subpopulation of IB4-positive nociceptive sensory neurons [34, 35]. Since $P2X_3$ is also expressed in trigeminal ganglion neurons that innervate tooth pulp [36], eugenol used in dental treatment might play its analgesic action through inhibition of $P2X_3$ channels expressed in tooth pulp neuron [21].

4.2 Anesthetic Activity and Inhibition of Nerve Impulses

Eugenol has been shown to be effective in anesthetizing fish including rainbow trout (*Oncorhynchus mykiss*) [37, 38], channel catfish (*Ictalurus punctatus*) [39], and rats [40]. A low dose of eugenol as 65 mg/L induced anesthesia successfully and safely in juvenile tambaqui fish (*Colossoma macropomum*) [2]. The anesthetic mechanism of eugenol might involve its ability to block nerve impulses as demonstrated in sciatic nerve of the bullfrog prepared in nerve chamber. Eugenol of 0.01% to 100% concentration extinguished the compound action potential in irreversible manner within time frame of 3 h [41]. Another study observed that eugenol slowed nerve conduction in crayfish and made it less excitable [42]. Reversible inhibition of nerve impulse and compound action potentials by eugenol was recorded in tooth pulp nerve of adult cats [43] and phrenic nerve [44], sciatic nerve, and superior cervical ganglion neurons of rats [45]. A study on rat vagus nerve suggested that eugenol is more effective than lidocaine in inhibiting compound action potential of C nerve fibers [46]. These in vivo and ex vivo results suggest that eugenol might have a direct effect on ion channels in peripheral sensory nerve fibers.

4.2.1 Voltage-Gated Sodium Channels

Sodium channels depolarize cell membrane to initiate action potential [47]. Therefore, it is possible that voltage-gated sodium channels are the molecular targets of eugenol in its analgesic action. When tested in dental primary afferent neurons [18] and dorsal root ganglion neurons [48] of rats, eugenol successfully inhibited voltage-gated sodium channels [18]. The magnitude of inhibition was similar between TTX-resistant and TTX-sensitive sodium channels, suggesting that eugenol might block action potentials in both nociceptive and non-nociceptive afferent fibers. The molecular mechanism underlying the inhibition of sodium channels is not clear. The inhibitory action of eugenol was comparable between neurons with and without transient receptor potential vanilloid 1 (TRPV1) channel expression, suggesting that eugenol does not modulate sodium channels through activation of TRPV1. Eugenol might bind to voltage-gated sodium channels and modulate directly, but the supporting evidence is yet to be found. However, it is clear that modulation of voltage-gated sodium channels is a mechanism that contributes to the analgesic action of eugenol [17].

4.2.2 Voltage-Gated Potassium Channels

In addition to above voltage-gated channels, voltage-gated potassium channels are also inhibited by eugenol [22]. However, voltage-gated potassium channels play roles in repolarization of cell membrane after action potential firing. Therefore, inhibition of voltage-gated potassium by eugenol might result in enhanced neuronal activity. It is not clear how action potential spikes are modified by eugenol together with its inhibition of voltage-gated sodium channels. The inhibition of voltage-gated potassium channel was similar between capsaicin-sensitive and capsaicin-insensitive neurons.

4.3 Inhibition of Synaptic Transmission

The potential use of eugenol in the treatment of epilepsy and cephalic has been proposed. Eugenol reversed epileptiform field potentials and spreading depression induced by KCl microinjection, presumably by inhibition of synaptic plasticity [49]. Depression of synaptic transmission by eugenol is also reported in CA1 region of hippocampal slices [50]. Molecular mechanism underlying reduced synaptic transmission is yet to be discovered.

4.3.1 Voltage-Gated Calcium Channels

Voltage-gated calcium channels play a critical role in exocytosis of neurotransmitter by promoting fusion of synaptic vesicle and presynaptic terminal membrane. Therefore, the molecular mechanism underlying reduced synaptic transmission might be partly explained by inhibition of calcium channels by eugenol [19, 20]. The structural similarity of eugenol to capsaicin might contribute to the inhibition of calcium channels, since capsaicin has been demonstrated to inhibit high-voltage-activated calcium channel (HVACC) currents [51, 52]. However, the inhibition does not seem to be mediated by activation of TRPV1, because calcium current was also inhibited by eugenol in neurons where capsaicin response was negative.

4.3.2 NMDA Receptors

N-Methyl-ᴅ-aspartic acid (NMDA) receptor is an NMDA-sensitive ionotropic glutamate receptor that plays an important role in synaptic modulation and memory function. Antagonism of NMDA receptor by eugenol [53] might result in its presumed inhibition of synaptic plasticity [49] or contribute to its anesthetic ability.

4.3.3 GABA$_A$ Receptors

GABA$_A$ receptor is a receptor channel that takes γ-aminobutyric acid (GABA) as its ligand. The pore of GABA$_A$ receptor channel conducts chloride ion into cell upon activation, making membrane potential hyperpolarized and less excitable in central nervous system. Therefore, GABA$_A$ receptor-mediated synapses are called inhibitory, and pharmacological activation of GABA$_A$ receptor causes sedation or general anesthesia. Interestingly, eugenol has been shown to potentiate GABA$_A$ receptor activation [54], which might be another mechanism that contributes to its ability to cause anesthesia.

4.4 Therapeutic Effects

In addition to the modulation of ion channels in nervous system, eugenol displays a broad spectrum of biological activities.

4.4.1 Anti-inflammatory Activity

Anti-inflammatory action of eugenol is mostly attributed to its ability to inhibit cyclooxygenase (COX) [55]. COX converts membrane phospholipid arachidonic acid to prostanoids, biological mediators that mediate inflammation and pain. Therefore, anti-inflammatory and analgesic effect can be achieved by pharmacological inhibition of COX. Nonsteroidal anti-inflammatory drugs such as aspirin and ibuprofen are the best examples of COX inhibitor. It was reported that prostaglandin and thromboxane production after an intentional damage to incisal teeth of rats was markedly reduced immediately with application of zinc oxide eugenol paste [55]. Inflammatory mediators such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and prostaglandin E2 in LPS-activated macrophage were reduced after eugenol application [56]. An in vivo study with rat liver showed reduced inflammatory cells and necrosis following administration of eugenol [57].

4.4.2 Antitumor Activity

Anticarcinogenic [7] and antimutagenic [8] effect of eugenol has been reported. Reactive-oxygen-species-mediated cytotoxic effect of eugenol on human promyelocytic leukemia cell line HL-60 has been reported [58]. Eugenol in volatile gas form was cytotoxic against human tumor cell lines PC-3 [59]. In vivo effect of eugenol on amelioration of gamma-radiation-induced clastogenic effects [60] and DNA damage induced by genotoxin [61] was also reported. Enhanced activity of the detoxifying enzymes, glutathione S-transferase, by dietary administration of eugenol was observed in rat liver [62]. A recent study of rats revealed that eugenol restricted skin carcinogenesis by attenuation of c-Myc and H-ras and modification of p53-associated gene expression [63].

4.4.3 Antioxidant and Prooxidant Activity

Many plant phenolic molecules found in the fruits, seeds, and leaves serve as natural antioxidants [64], which apply to eugenol as well [10]. The mechanisms by which eugenol and other natural or synthetic phenolic compound act as antioxidants are attributed to their ability to reduce superoxide to H_2O_2 [65] or to scavenge the free radicals through chelation of metal ions [2, 66]. Eugenol showed strong chelating potential against Fe^{3+}, thereby inhibiting the initiation of hydroxyl radicals [67]. Antioxidant effect of eugenol was suggested to be one of the mechanisms by which eugenol rescued liver damage caused by carbon tetrachloride. Coadministration of eugenol with liver-damaging drugs might provide beneficial effect [68]. The antioxidant action, along with its anti-inflammatory and antimutagenesis effect, suggested eugenol as potential liver drug [57].

However, phenolic compounds are double-edged swords. High concentration of eugenol generates free radicals and acts as prooxidant agent that causes tissue damage [66, 69–72]. Photochemical reduction of eugenol might play a role in its prooxidant effect [73]. The joint FAO/WHO committee has permitted daily intake of eugenol of 2.5 mg/kg body weight for humans [69].

4.5 Antimicrobial Activities

4.5.1 Antibacterial Activity

Antibacterial activity of eugenol is well documented by Chaieb et al. [2]. Pathogenic bacteria influenced by eugenol include *Campylobacter jejuni*, *Salmonella enteritidis*, *Escherichia coli* and *Staphylococcus aureus, Staphylococcus epidermidis, Listeria monocytogenes, Bacillus cereus* [2, 59, 74–79]. Bactericidal effect of eugenol on coagulase-negative Staphylococci (CoNS) is of particular interest since CoNS are known to survive on medical devices which can be hazardous for patients with poor immune responses [80]. Eugenol was reported to be effective against both Gram-positive and Gram-negative strains [81, 82]. Bactericidal effect of eugenol against oral bacteria that cause periodontal disease and dental caries is also described [83].

4.5.2 Antifungal Activity

Antifungal activity of eugenol against *Candida albicans*, that causes serious health problem in immunocompromised patients, was demonstrated by in vitro and in vivo experiments [84, 85]. It is also shown that eugenol inhibited growth of a number of dermatophyte fungi that cause onychomycosis, such as *Trichophyton rubrum, Trichophyton mentagrophytes* var. *interdigitale*, and *Epidermophyton floccosum* [1]. Eugenol was also effective in inhibition of several food-borne fungal species including *Fusarium graminearum* [86], *Penicillium islandicum*, *Aspergillus flavus* [87], and *Aspergillus niger* [88]. The antifungal activity seems to be exerted by disruption of cellular membrane [89], which was confirmed by scanning electron microscope image of significant morphological deformity of *Saccharomyces cerevisiae* by clove oil [6].

4.5.3 Antiviral Activity

Viruses are generally sensitive to essential oils [2]. In a research that investigated antiviral activity of several plant extracts that were used in traditional medicine of Sudan, extract of *Syzygium aromaticum*, whose main ingredients is eugenol, showed over 90% inhibition on replication of the hepatitis C virus [90].

4.6 Other Aspects

4.6.1 Olfactory G-Protein-Coupled Receptor

Olfactory system is unique in that there are numerous receptors for each specific kind of odorants [91]. It is very peculiar compared to other sensory systems like vision, touch, or taste in which only a few kinds of receptors exist. Eugenol has a very distinctive odor that is usually remembered as dental clinic scent. The olfactory receptor that eugenol binds was identified in mice and named mOR-EG or Olfr73, a G-protein-coupled receptor with a seven-transmembrane domain that increases intracellular cAMP concentration upon activation [92].

4.6.2 Allergic Reactions

Eugenol is a well-known contact allergen in dermatology [3]. Majority of patients who experienced stomatitis after dental treatment with eugenol-containing dressings were shown to be allergic to eugenol by patch testing [93]. Subsequent study found that as high as 10% of patients who were insensitive to eugenol prior to dental treatment with eugenol became sensitized postoperatively [94]. The allergic reaction to eugenol is usually in association with its use in surgical and periodontal packs [93], root canal sealers [95], mouth rinses [96], and impression pastes [3]. In rare cases, eugenol can induce severe allergic response when applied in the mouth [97]. In one most severe case, patient even experienced anaphylactic-like shock after receiving pulpotomy with zinc oxide eugenol cement [98]. Localized skin irritation and allergic contact dermatitis are also reported in dental professionals [3]. Allergic contact dermatitis reaction against eugenol is increasing and gained focus in perfume industry recently [99–102].

4.6.3 Cytotoxic Activity

Direct contact of tissue to high concentration of eugenol is cytotoxic and reported to have adverse effect on osteoblast-like cells [103], fibroblasts [104], and oral mucosa [105]. Uncontrolled concentration of eugenol can induce necrosis and reduce healing [3]. Application of eugenol in its mixture with zinc oxide is beneficial in terms of concentration control. Eugenol chelates zinc oxide and forms zinc eugenolate by hardening reaction. This compound readily undergoes hydrolysis with the presence of water. As a result, free eugenol is gradually released from the surface of the zinc oxide eugenol mixture, and the intraoral eugenol concentration stays relatively within certain range [106].

4.6.4 Insecticidal Activity

Eugenol has been shown to be effective against several parasites and insects such as larvae of common house mosquitos (*Culex pipiens*) [107], malaria mosquitos (*Anopheles dirus*) [108], head lice (*Pediculus capitis)* [109], red flour beetle (*Tribolium castaneum*), and maize weevil (*Sitophilus zeamais*) [110]. Acaricidal activity by direct contact or by vapor gas of eugenol was observed against a few species of mites including *Dermatophagoides farinae, D. pteronyssinus* [111], and *Psoroptes cuniculi* [112]. Eugenol could be used as a fumigant agent against Japanese termite (*Reticulitermes speratus*) [113]. A careful consideration has to come before using drugs to control parasitic insects in terms of environmental damage and drug resistance [114]. Using eugenol could be beneficial since it is a naturally available insecticidal agent.

5 Conclusion

Eugenol is a versatile natural compound with many beneficial effects and wide range of safe dose. It has been used most widely in dentistry as analgesic and anti-inflammatory agent. However, its usage is growing wide rapidly. Its greatest advantage is that it is a natural molecule that is already being used by plants as an insect attractant or repellent. Its ability to anesthetize fish can be used in aquarium for research and management. Eugenol can be used as supplementary additive to drugs that could harm liver because of its antioxidant, anti-inflammatory, and antimutagenetic effect. Its ability to inhibit growth of bacteria, virus, and fungi can be adopted in topical drugs that control appropriate infections, from dermatophytosis of a healthy human to opportunistic infection of an immunosuppressed patient. Further research on application and safety of eugenol will provide novel solutions to current problems involving health and environmental issue in many areas such as medicine and agriculture.

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Carotenoids as Colorants 135

Bernardo Dias Ribeiro and Raquel Grando de Oliveira

Contents

1 Introduction 4018
2 Biosynthesis in Plant 4021
3 Health Benefits of Carotenoids 4023
4 Carotenoid Modification 4024
5 Analytical Methods 4026
5.1 Sample Extraction and Storage 4026
5.2 Spectrophotometry 4028
5.3 High-Performance Chromatography Methods 4029
5.4 Other Techniques 4030
6 Sources and Production Methods 4031
6.1 Supercritical Fluid Extraction 4031
6.2 Membrane Separation 4032
6.3 Enzymatic Treatment 4032
6.4 Other Methods 4033
7 Conclusion 4034
References 4034

Abstract

Carotenoids, precursors of vitamin A, are natural pigments synthesized by plants and some microorganisms. Humans and animals are not able to synthesize and need to acquire them by alimentation. Beyond the health benefits promoted by vitamin A, these compounds have antioxidant capacity which gives protection for cardiovascular and degenerative diseases. The presence of conjugated double bonds in carotenoid structure contributes for their pigmentation, absorption of ultraviolet/visible radiation, and antioxidant activity but also is the main reason

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of their chemical instability. Some factors such as heat, light, acids, organic solvent reflux, crystal melting, and iodine treatment can promote carotenoid *cis–trans* isomerization and oxidation. In the carotenoid qualitative and quantitative analysis, steps of extraction and storage are important to prevent degradation and total recovery. The extraction is generally realized using organic solvents in addiction or not a saponification step. The high-performance liquid chromatography is the most widely used technique, but others may be employed. The industrial production of carotenoids can be made by simple extraction or microbial process.

Keywords

Analysis • carotenoids • health benefits • natural pigments • plant biosynthesis • production

Abbreviations

BHT	2,6-bis(1,1-dimethylethyl)-4-methylphenol
CEC	Capillary electrochromatography
DAD	Diode array detector
DXP	1-Deoxy-D-xylulose-5-phosphate
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IPP	Isopentenyl diphosphate
MS	Mass spectrometry
MVA	Mevalonic acid
NIRS	Near-infrared reflectance spectroscopy
NMR	Nuclear magnetic resonance
OCC	Open column chromatography
TDN	1,1,6-Trimethyl-1,2-dihydronaphthalene
THF	Tetrahydrofuran
UHPLC	Ultrahigh-performance liquid chromatography

1 Introduction

Carotenoids are natural pigments synthesized by plants and some microorganisms. Humans and animals are not able to synthesize and need to acquire them by alimentation [1]. These compounds are widely distributed in fruits, flowers, roots, seaweeds, invertebrates, fishes, birds, bacteria, fungi, and yeasts [2–4]. They are responsible for the red, yellow, and orange colors, but green, purple, or blue color could be obtained when carotenoids are bound with some proteins [1]. Such molecules act as photosynthesis aid and for the photoprotection of their hosts [2–4]. Due to this, it is used in food and feed as colorant, flavoring, and nutritional supplement, being source of provitamin A.

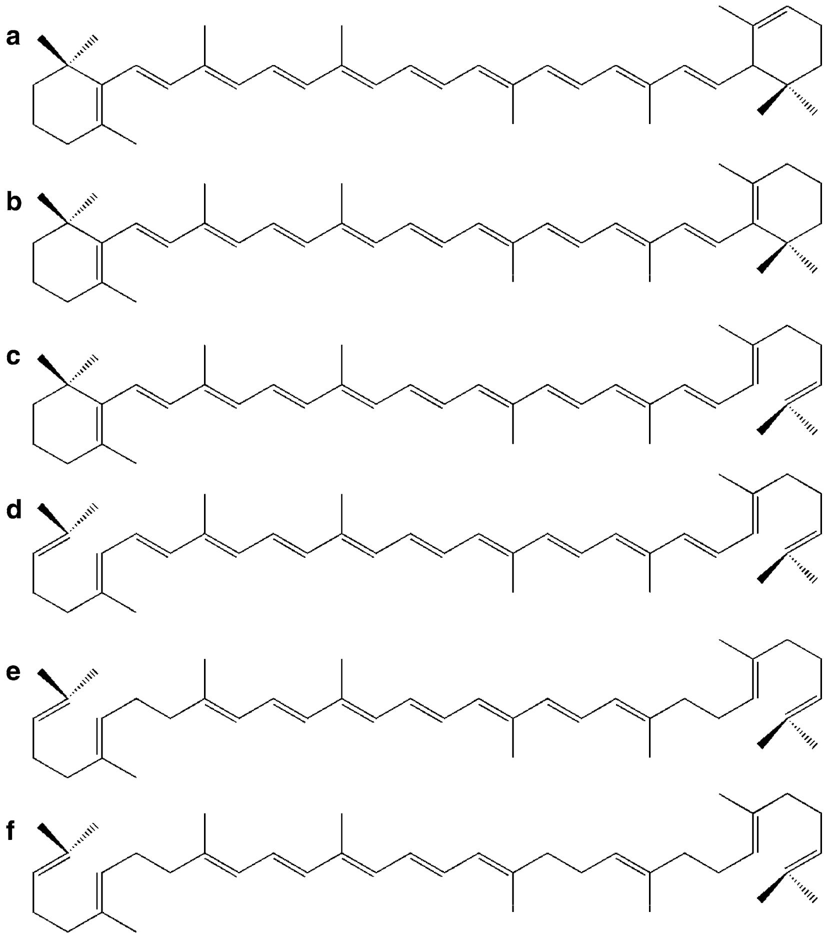

Fig. 135.1 Carotenes (**a**) α-carotene, (**b**) β-carotene, (**c**) γ-carotene, (**d**) lycopene, (**e**) ζ-carotene, (**f**) phytofluene

The structures of these compounds have numerous conjugated double-bonds and cyclic end groups; thus, carotenoids present a variety of stereoisomers with different chemical and physical proprieties [5]. Carotenoids can be divided in two groups: *carotenes*, constituted by polyunsaturated hydrocarbons C_{40} (Fig. 135.1), and *xanthophylls*; their oxygenated derivatives and main contain epoxy, carbonyl, hydroxy, methoxy, or carboxylic acid functional groups (Fig. 135.2).

Lycopene and ζ-carotene are the most common acyclic carotenes. *Lycopene* is the major pigment of many red fleshy fruits, such as tomato (*Lycopersicon esculentum*), watermelon (*Citrullus lanatus*), papaya (*Carica papaya*), guava (*Psidium guajava*), and grapefruit (*Citrus paradisi*). *ζ-carotene* is present in low quantities in many plants, but in starfruit (*Averrhoa carambola*) and passion fruit (*Passiflora alata*), this carotene becomes more significant as main pigment. Other

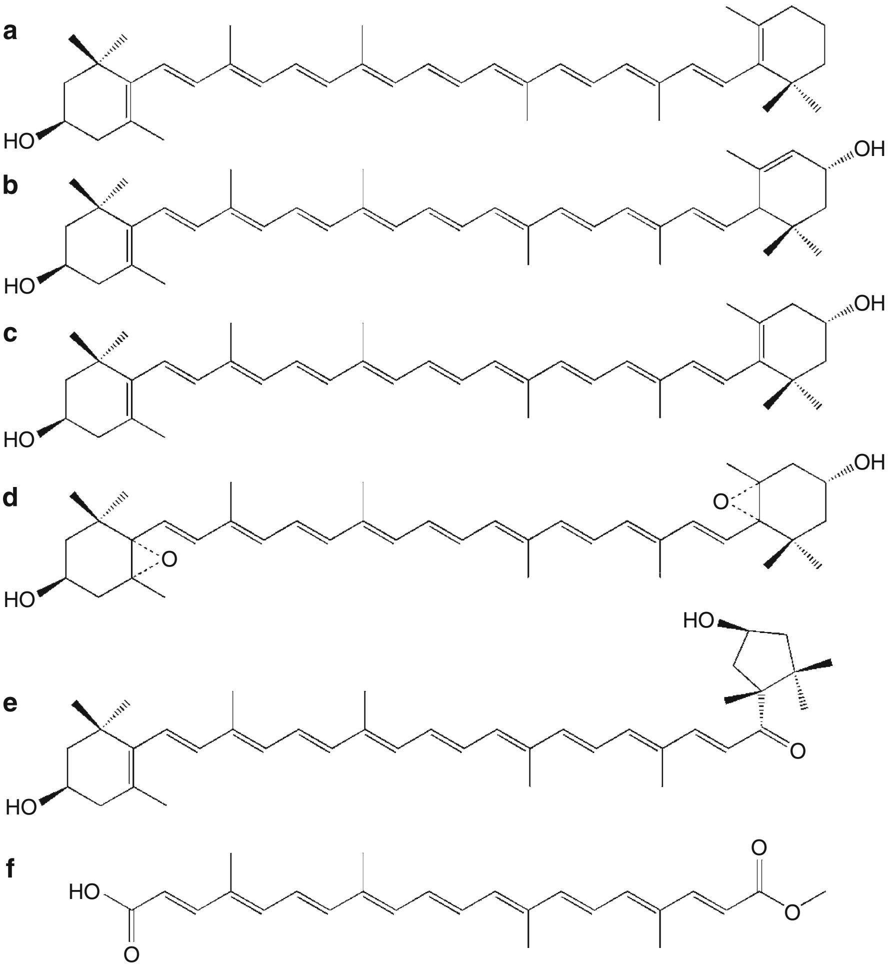

Fig. 135.2 Xanthophylls (**a**) β-cryptoxanthin, (**b**) lutein, (**c**) zeaxanthin, (**d**) violaxanthin, (**e**) capsanthin, (**f**) bixin

carotenes as phytofluene and phytoene are colorless and probably more widely distributed than the reported [2, 4]. Among cyclic carotenes, *β-carotene* is the most prominent orange pigment, being present in carrots (*Daucus carota*), mango (*Mangifera indica*), acerola (*Malpighia glabra*), apricot (*Prunus armeniaca*), medlar (*Mespilus germanica*), and Palmae/Arecaceae fruits. The *α-carotene* and *γ-carotene* are generally in minor concentration than β-carotene, which the former found in carrots and pumpkins (*Cucurbita* sp.) and the latter found in dog roses (*Rosa canina*) and Surinam cherry (*Eugenia uniflora*). *δ-Carotene* is less frequent but can be detected in tomatoes and pupunha (*Bactris gasipaes*) [6].

Among hydroxylated xanthophylls, β-cryptoxanthin, lutein, and zeaxanthin, derived from α- and β-carotenes, are the main yellow pigments. *β-cryptoxanthin* is the major pigment from orange fruits as peaches (*Prunus persica*), persimmon (*Diospyros kaki*), and hog plum (*Spondias mombin*). *Lutein* is the prevailing carotenoid in green leaves and vegetables and yellow flowers such as marigold (*Tagetes erecta L.*), and *zeaxanthin* is the main pigment in maize (*Zea mays*) and in piqua (*Caryocar villosum*). Epoxidized xanthophylls as *violaxanthin* and *antheraxanthin* are degradation products from other carotenoids, like zeaxanthin, and their presence is underestimated in many foods [6–8]. There are some uncommon xanthophylls, as *capsanthin*, present in chili pepper (*Capsicum frutescens*), *bixin*, major pigment in annatto (*Bixa orellana*), and *crocetin*, which is predominant in saffron (*Crocus sativus*) [2, 4].

In green vegetables, these compounds are located in chloroplasts with the xanthophylls non-unesterified and have the same qualitative pattern and relative proportions, with lutein, β-carotene, violaxanthin, and neoxanthin as the principal carotenoids, but the absolute concentrations differ considerably. However, there are two exceptions: lettuce, which also has lactucaxanthin as a major carotenoid and which α-carotene, α- or β-cryptoxanthin, zeaxanthin, antheraxanthin, and lutein-5,6-epoxide as minor pigments may be yet found; and kiwifruit (*Actinidia chinensis*), which remains green even when matured and the carotenoid esterification of which does not occur [7, 9]. In ripe fruits, the hydroxycarotenoids are found in chromoplasts, and carotenes are mostly esterified with fatty acids. The composition is highly complex and variable being affected by genetic and environmental factors, influenced by the cultivar, variety, maturity at harvest, climate, season, geographic site of production, part of the plant utilized, farming practices, harvesting and post-harvest handling, processing, and storage conditions [10].

Industrially, the carotenoids are used in nutrient supplementation, for pharmaceutical purposes, as food colorants and fragrances, and in animal feed [1]. The worldwide market of carotenoids is growing fast; in 2010, carotenoids were responsible for about USD 1.2 billion, mainly β-carotene and lutein, which accounts for 22 % and 19 % of this market, respectively, and was estimated to grow 2.3 % per year, reaching USD 1.4 billion in 2018. Currently, the international trade is dominated by private companies, such as Roche, BASF, Merck, Rhône-Poulenc, and DSM [11, 12].

2 Biosynthesis in Plant

Terpenoid biosynthesis is now well established in plants, which the major precursor isopentenyl diphosphate (IPP) can be synthesized in the plastids through 1-deoxy-D-xylulose-5-phosphate (DXP) pathway, generating monoterpenes, diterpenes, and carotenoids, and in the cytosol through the mevalonic acid (MVA) pathway, forming sesquiterpenes, triterpenes, and steroids [9, 13].

In the case of carotenoids, the main precursor phytoene is generated by action of phytoene synthase, strictly dependent upon Mn^{2+} enzyme, on geranylgeranyl pyrophosphate. Then, the desaturation of phytoene to lycopene involves four steps (Fig. 135.3) that are catalyzed by phytoene desaturase and ζ-carotene desaturase,

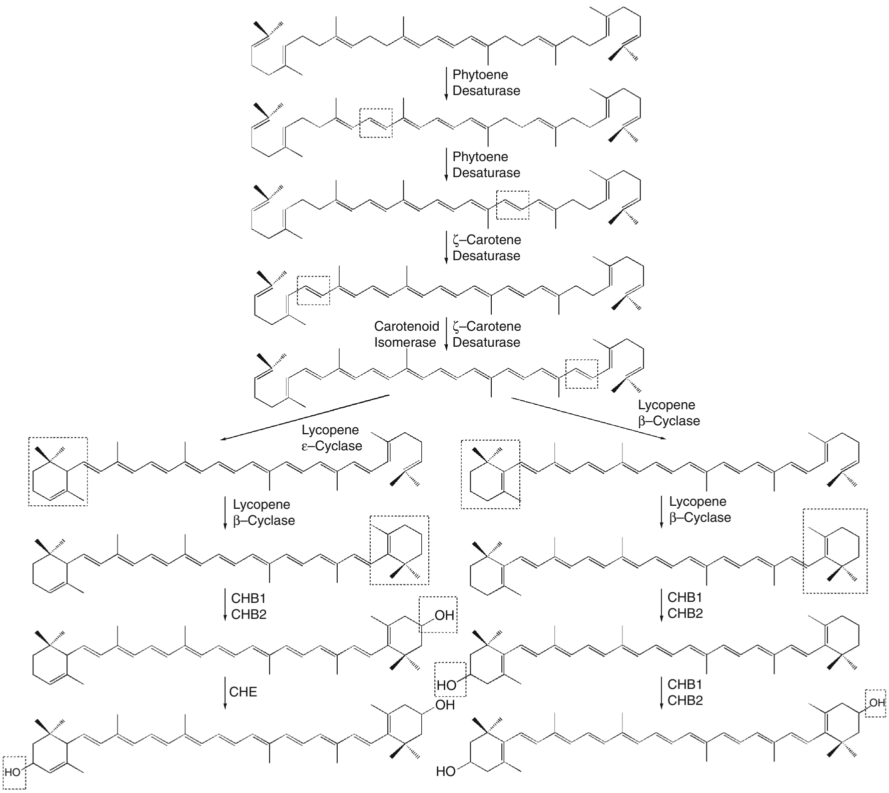
Phytoene
Desaturase
Phytoene
Desaturase
ζ–Carotene
Desaturase
Carotenoid
Isomerase
ζ–Carotene
Desaturase
Lycopene
ε–Cyclase
Lycopene
β–Cyclase
Lycopene
β–Cyclase
Lycopene
β–Cyclase
CHB1
CHB2
CHB1
CHB2
OH
HO
OH
CHE
CHB1
CHB2
OH
HO
HO

Fig. 135.3 *Carotenoid biosynthesis*. *CHB1* carotenoid β-hydroxylase nonheme di-iron monooxygenase, *CHB2* carotenoid β-hydroxylase heme-binding cytochrome P450, *CHE* carotenoid ε-hydroxylase heme-binding cytochrome P450 [9,14]

which promote the hydrogen removal alternately and thus become targets to photobleaching herbicides. Both α-carotene and β-carotene can be generated by lycopene cyclization, catalyzed by lycopene cyclases, as lycopene β-bicyclase, lycopene ε-bicyclase, lycopene β-monocyclase, lycopene ε-monocyclase, and bifunctional lycopene β, ε-bicyclase. These two carotenoids can be modified by hydroxylases, epoxydases, ketolases, and synthases forming xanthophylls as lutein and zeaxanthin (Fig. 135.2) [9,11,14].

Plants synthesize carotenoids to regulate the growth and development, which serve as accessory pigments in photosynthesis, photoprotectors, and precursors for the hormones abscisic acid and strigolactones. Carotenoid color further helps in attracting other organisms, such as pollinating insects and seed-distributing herbivores [1]. The composition of carotenoids is affected by the genetic and environmental factors. In food, the carotenoid composition is influenced by cultivar, variety, maturity at harvest, climate, season, geographic site of production, part of the plant, farming practices, harvesting and post-harvest handling, processing, and storage conditions [10].

With the knowledge of higher plants carotenoid biosynthesis and its regulation, it enabled new methods to obtain biofortified foods such as golden rice, tomato, potato, maize, and sweet potato, which can contribute significantly to reduce vitamin A deficiency in the world, since they represent cultivars widely adopted [11,15–20].

3 Health Benefits of Carotenoids

More than 700 carotenoids have been described in nature, but it is estimated that we only have access to about 40 carotenoids that can be absorbed, metabolized, and/or used in our bodies. That number is reduced to 6 if we consider the carotenoid profile that is usually detected in human blood plasma: α- and β-carotene, lycopene, β-cryptoxanthin, zeaxanthin, and lutein [21].

Nevertheless, carotenoids are known as vitamin A precursor (provitamin A), and this is its main nutritional function. Approximately 10 % of carotenoids meet the main structural requirement for acting as vitamin A precursors, that is, with at least one β-type ring, without oxygenated functional groups, along with one polyene chain containing at least 11 carbon atoms [21]. The main carotenoid with such characteristics is β-carotene, and it can be cleaved by a β-carotene-15,15′-dioxygenase, resulting in the formation of retinal [22]. The involvement of retinal (vitamin A) as visual pigments chromophore in the eyes is of paramount importance in the vision process. Hypovitaminosis A is still one of the major nutritional problems in least-developed regions of the world, presenting as frequent consequences the xerophthalmia (deficiency in the production of tears) and blindness, particularly in children, as well as premature death [23]. Vitamin A also presents important systemic functions in the growth and reproductive efficiency, besides the epithelial tissue maintenance and prevention of its keratinization. Thus, retinoids have been used in dermatological treatments, such as for the elimination of acne [4,24].

The antioxidant property of carotenoids is independent of provitamin A activity and is associated to its binding capacity with a singlet oxygen by conjugated double-bonds systems, mainly in the 5,6 and 5′,6′ double bonds in their cyclic end groups, which can undergo epoxidation with O_2, and the maximum protection is given by carotenoids with more than 9 double bonds. Due to this reason, lycopene shows higher antioxidant efficiency than β-carotene. In high oxygen partial pressure or high carotenoid concentration, however, lycopene and β-carotene can present prooxidant activity [25–28]. Due to its antioxidant capacity, β-carotene is utilized as oral sun protector to prevent skin from photoaging and sunburn, and must be consumed along several weeks to increase its content in the blood plasma and skin and increase its protection effect [25].

Carotenoids, mainly lycopene, have been used in the treatment of cardiovascular and degenerative diseases, besides prostate, stomach, and lung cancers acting on possible modulation mechanisms of carcinogens, inhibition of cell proliferation, increase of cell differentiation by retinoids, stimulation of intercellular communication, and high immunological response [11,21,28,29], whereas zeaxanthin and lutein presented high performance on prevention of age-related macular degeneration [7,11,25,28].

Lutein is an important carotenoid in the human macula. This compound is a blue filter at wavelengths around 450 nm with peak absorption at 446 nm, and these wavelengths are known to induce light-mediated damage to the retina. Damage in retina is also caused by the oxidation, reactive oxygen species have been shown to induce apoptosis of photoreceptors, and lutein has been shown to be able to block paraquat or H_2O_2-induced apoptosis of cultured retinal photoreceptors. Lutein show yet benefices in the immune response and in the inflammatory process [30].

However, only a fraction of carotenoids absorbed by the human body can effectively contribute to human health, reaches up to 10 % when a natural food is consumed [21]. A number of factors can affect the bioaccessibility and/or bioavailability, including the species of carotenoids, linkages at molecular level, amount of carotenoid, matrix, effectors, nutrient status, genetics, host-related factors, and interactions among these variables. In carrots, for example, β-carotene is located in the chromoplasts (surrounded by a double bilayer membrane) of the plant cells (surrounded by a cell membrane and a cell wall), where it is often associated with proteins and/or residual membranes. This fact results in several physical barriers that have to be broken to make β-carotene accessible for absorption.

In processed food, the maceration reduces the particle size and removes some barriers, increases the contact superficies for interaction with digestive. Effectors, such as the presence of oil, can also have an influence on the bioaccessibility and/or bioavailability of carotenoids: these compounds are lipophilic molecules, and they have to be incorporated in mixed micelles in the duodenum before they can be absorbed in the mucosa [31]. Certain structural differences may alter fat solubility and modify the efficiency of the micellization. One of them is the esterification of xanthophyll with fatty acids. Esterified xanthophylls exhibit increased fat solubility relative to their corresponding free xanthophylls and even against carotenes.

4 Carotenoid Modification

The presence of conjugated double bonds in carotenoids structure contributes for their pigmentation, absorption of ultraviolet/visible radiation, and antioxidant activity but also is the main reason of their chemical instability, since conjugated double bonds are very susceptible to oxidation and geometric isomerization. Some factors such as heat, light, acids, organic solvent reflux, crystal melting, and iodine treatment can promote carotenoids *cis–trans* isomerization (Fig. 135.4). *Trans*-carotenoid isomerization, which is the usual configuration of carotenoids in nature, to the *cis*-form enhances solubility and decreases color intensity, melting point, and provitamin A activity [4,6,7,10,32].

Oxidation, major degradation mechanism of carotenoids, can be accelerated by heat, light, unsaturated fatty acids, peroxides, iron, copper, manganese, and the presence of some enzymes, as lipoxygenases, phenoloxidases, and peroxidases [4,6,7].

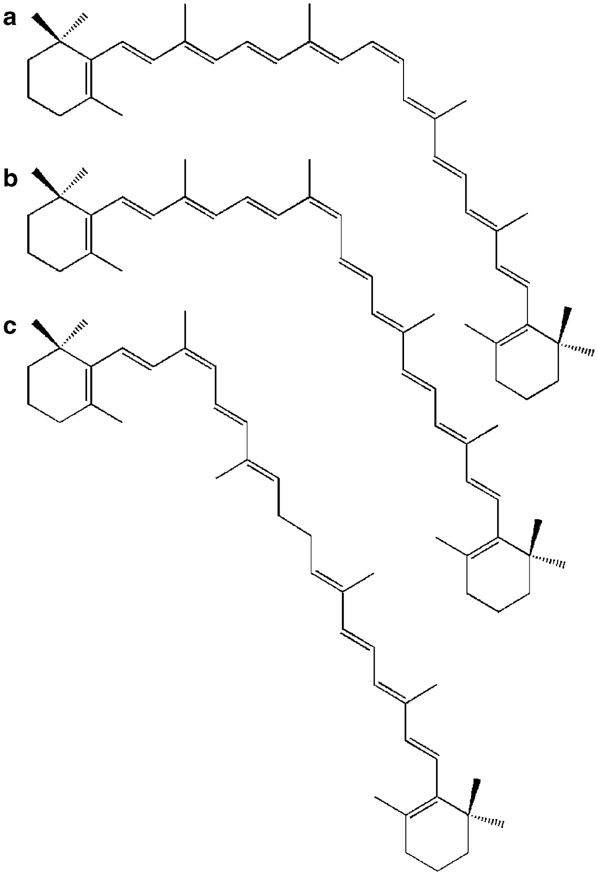

Fig. 135.4 β-*Carotene cis-isomers* (**a**) 15-*cis*-β-carotene, (**b**) 13-*cis*-β-carotene, (**c**) 9-*cis*-β-carotene

The major degradation by-products of carotenoids are epoxycarotenoids, apocarotenoids (Fig. 135.5), and volatile compounds, which, although having very low provitamin A activity, have metabolites that can be used as food dyes, in the case of apocarotenoids, or natural flavoring from volatiles compounds [33–35] These by-products are generated in many types of food processing, such as cooking, frying, pasteurization, extrusion, and dehydration, with losses around 25–35 % [33,36–38], and in vegetable oil refining, such as bleaching, deodorization, and deacification steps [39].

Norisoprenoids, responsible for the typical aroma of some grape varieties, could originate from direct degradation of carotenoid molecules such as β-carotene, lutein, neoxanthin, and violaxanthin. Some degradation carotenoid products have an important sensorial impact on wine, such as β-damascenone, β-ionone, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), and vitispirane. These aromatic molecules have very low olfactory perception thresholds [40].

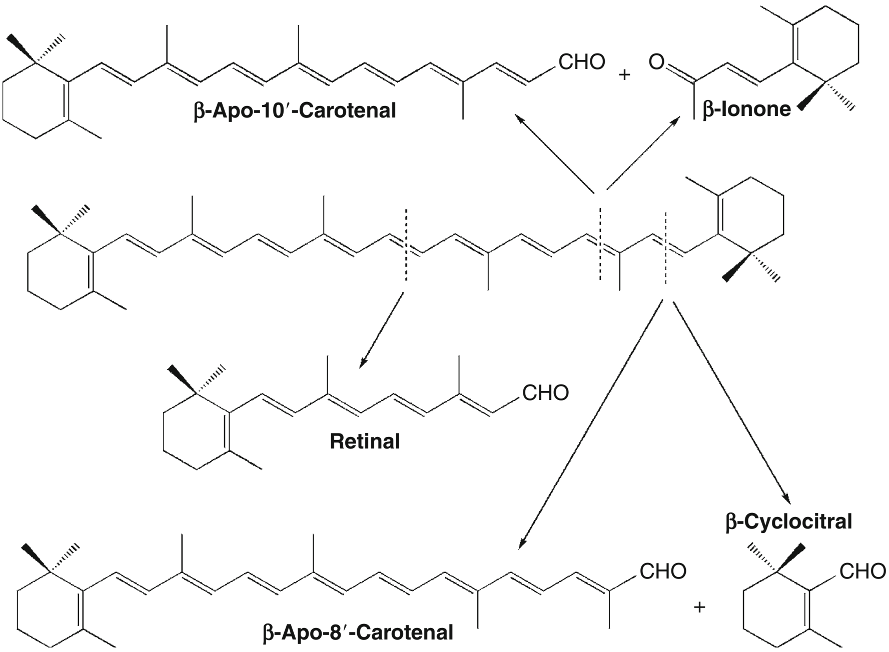

Fig. 135.5 Apocarotenoids derived from β-carotene

5 Analytical Methods

For a long time, quantification of only β-carotene or the major provitamin A carotenoids was considered sufficient. In the course of time, advances in studies have identified health benefits independent of the provitamin A activity, and this fact has encouraged the development of new techniques to quantify all-E- (*trans*) and Z-isomers (*cis*) [10].

Carotenoids have a long structure with alternating double and single bonds, constituting a conjugated system in which the π-electrons are delocalized along the entire polyene chain which gives carotenoids a unique molecular shape, chemical reactivity, and light-absorbing properties. These characteristics are the base of the analytical methods describes above [1].

An important point to emphasize is the difficulty to analyze the existence of a large number of carotenoids, concentration range, uneven distribution in the sample, varied nature of the matrix and susceptibility of carotenoids to isomerization and oxidation during analysis, and extraction and storage of samples and standards.

5.1 Sample Extraction and Storage

The procedure for the preparation of the analytical sample should be adapted to the nature of the food, analyte, and analytical method, as well as the distribution of the

Table 135.1 Carotenoids extraction solvents

Food	Extraction procedure	Detection method	References
Fruit and vegetables	50 % tetrahydrofuran, 50 % methanol/ saponification with 10 % KOH	HPLC and UV–Vis detector	[42]
Marigold	Methanol/ethyl acetate/light petroleum (1:1:1)/OCC	HPLC and MS detector	[8]
Red palm oil	Hexane	Spectrophotometry	[43]
Wine	50 % ether, 50 % hexane	HPLC and diode array detector	[40]
Grape	50 % hexane and 50 % diethyl ether	HPLC and MS detector	[44]
Pumpkin puree	Acetone/saponification with 10 % KOH	HPLC and diode array detector	[45]
Carrot roots	50 % hexane, 25 % acetone, 25 % ethanol	HPLC	[46]
Carrot puree	50 % hexane, 25 % acetone, 25 % ethanol, 0,1 % BHT	Spectrophotometry	[31]
Orange juice	50 % dichloromethane, 25 % methanol, 25 % acetone, 0.1 % BHT/saponification with 15 % KOH	HPLC and UV–Vis	[47]

analyte in the food [10]. In the cassava rot, for example, the concentration of carotenoids is higher in the part of the root closest to its attachment to the stem (proximal section) and is gradually decreasing toward the opposite end (distal section) [41].

Usually, carotenoid extraction has been carried out with acetone. This solvent penetrates the food matrix well and dissolves both carotenes and xanthophylls efficiently, and subsequent partitioning to an apolar solvent occurs more easily. Tetrahydrofuran (THF) has excellent solubility for β-carotene and lutein; however, it easily accumulates peroxides. Other solvents, such as hexane, petroleum ether, methanol, and ethanol, have also been utilized. The first two solvents readily dissolve carotenes but not the xanthophylls; on the other hand, methanol and ethanol dissolve the xanthophylls efficiently but not the carotenes. Thus, mixtures of solvents have been preferred [10]. Saponification with KOH is another procedure that can be applied to increase the extraction because some carotenes are esterified with fatty acids. Step of clean up may be made before analysis, such as open column chromatography (OCC) or solid-phase extraction (Table 135.1). The solubility of carotenoids depends on its molar mass, polarity, and solvent strength. Carotenoids are transported together with triglycerides, with large molar mass (807–885). Sometimes, a transesterification is necessary to reduce this weight, producing fatty methyl and ethyl esters [48].

The time between the storage of samples and analysis should be short. Ideally, the analysis should be planned to be realized after collection because it is difficult to avoid changes in carotenoid composition during storage, even at very low temperature. Lyophilization, an alternative method of storing used in some studies, however results in significant degradation of β-carotene and especially lycopene, and it increases sample porosity, consequently raising its exposure to oxygen during storage [10].

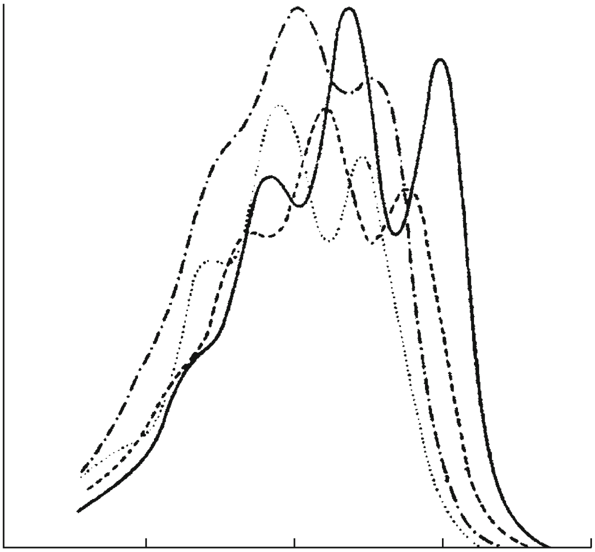

Fig. 135.6 Visible absorption spectra of lycopene (- - - -), γ-carotene (- - -), β-carotene (-.-.-.), and α-carotene (....) in petroleum ether [7]

5.2 Spectrophotometry

This method is based in the light-absorbing chromospheres and the visible absorption spectrum. The wavelength of maximum absorption (λmax) and the shape of the spectrum (spectral fine structure) are specific characteristics of each chromophore structure. In the past, most data on the amount of carotenoids found in foods were based on the total absorbance at a specified wavelength (450 nm). In some cases, a separation in an OCC was previously made followed by a spectroscopy.

Most carotenoids absorb maximally at three wavelengths, resulting in three-peak spectra (Fig. 135.6). The greater the number of conjugated double bonds, the higher the λmax values. Thus, the most unsaturated acyclic carotenoid lycopene, with 11 conjugated double bonds, is red and absorbs at the longest wavelengths (λmax at 444, 470, and 502 nm). At least 7 conjugated double bonds are needed for a carotenoid to have perceptible color. The introduction of hydroxy and methoxy substituent in the carotenoid molecule does not affect the chromophore and therefore has virtually no effect on the absorption spectrum.

Cis-isomerization of a chromophore's double bond causes a slight loss in color, small hypsochromic shift (usually 2–6 nm for mono-*cis*), and hypochromic effect, accompanied by the appearance of a *cis* peak in or near the ultraviolet region. The intensity of the *cis* band is greater as the *cis* double bond is nearer the center of the molecule.

The absorption spectra of carotenoids are markedly solvent dependent. The λmax values relative to hexane and petroleum ether are practically the same in diethyl ether, methanol, ethanol, and acetonitrile and higher by 2–6 nm in acetone, 10–20 nm in chloroform, 10–20 nm in dichloromethane, and 18–24 nm in toluene [4].

Table 135.2 Wavelength of maximum absorption and absorption coefficient $A^{1\%}{}_{1cm}$ of some carotenoids [7]

Carotenoid	Solvent	Λ_{max}, nm	$A^{1\%}{}_{1cm}$
Bixin	Petroleum ether	456	4,200
α-Carotene	Petroleum ether	444	2,800
	Hexane	445	2,710
β-Carotene	Petroleum ether	450	2,592
	Ethanol	450	2,620
	Chloroform	460	2,396
β-Cryptoxanthin	Petroleum ether	449	2,386
	Hexane	450	2,460
Lutein	Ethanol	445	2,550
	Diethyl ether	445	2,480
Lycopene	Petroleum ether	470	3,450
Violaxanthin	Ethanol	440	2,550
	Acetone	442	2,400
Zeaxanthin	Petroleum ether	449	2,348
	Ethanol	450	2,480
	Acetone	452	2,340

The absorption coefficient $A^{1\%}{}_{1cm}$ of a carotenoid (absorbance at a given wavelength of a 1 % solution in spectrophotometer cuvette with a 1-cm light path) used in the calculation of the concentration also varies pronouncedly in different solvents (Table 135.2).

5.3 High-Performance Chromatography Methods

Among the high-performance chromatographic methods available, gas chromatography (GC) is unsuitable for the analysis of carotenoids because of the inherent instability and low volatility of these molecules [1]. The high-performance liquid chromatography (HPLC) is a separation technique that coupled with some detector permits qualitative and quantitative analysis of small quantities of carotenoids in liquefied samples. Ultrahigh-performance liquid chromatography (UHPLC) has recently been developed; this is an improvement in HPLC. This technique uses narrow-bore columns packed with very small particles and mobile-phase delivery systems operating at high back pressures. UHPLC offers several advantages over conventional HPLC, such as faster analyses (shorter retention times), narrower peaks (giving increased signal-to-noise ratio), and greater sensitivity [1]. In the HPLC system, the chromatography column mostly applied in carotenoid separation was the polymeric C_{18}, but in recent years, the use of polymeric C_{30} column has grown (Table 135.3). Several solvents may be employed as mobile phase, but the mostly used are based on methanol and acetonitrile.

The detector based in the absorbance of ultraviolet–visible (UV–Vis) is currently the most common technique. Diode array detector (DAD) is a detector that can scan a variety of wavelengths, and it is widely used. A number of structurally related

Table 135.3 HPLC systems used in carotenoid analysis

Compound	Food	Column material	Detection	Mobile phase	Refernces
Lutein, zeaxanthin, β-cryptoxanthin, lycopene, α- and β-carotene	Vegetables	C_{18}	UV–Vis at 450 nm	Acetonitrile, methanol, dichloromethane, water, BHT	[42]
β-Carotene isomers	Standard	C_{30}	NMR	Acetone/D_2O	[49]
Lutein, zeaxanthin	Spinach	C_{30}	NMR	D_2O	[50]
β-Carotene, lutein	Wine	C_{18}	DAD at 270 and 550 nm	Ethyl acetate, acetonitrile, water	[40]
Violaxanthin, lutein isomers, α-, β-carotene	Grapes	C_{30}	MS	Triethylamine, methanol, water, methyl tert-butyl ether	[44]
Violaxanthin, lutein, zeaxanthin, α-, β-, and ζ-carotene	Pumpkin puree	C_{18}	DAD	Acetonitrile, triethylamine, methanol, and ethyl acetate	[45]
Trans and *cis* β-carotene	Carrot puree	C_{30}	DAD at 450 nm	MeOH, methyl t-butyl-ether, water	[31]
Violaxanthin, lutein, zeaxanthin, α-, β-, and ζ-carotene	Orange juice	C_{30}	DAD at 450 nm	Methanol, methyl tert-butyl ether and water	[47]
Lutein isomers	Marigold	C_{30}	MS	Methanol/tert-butyl methyl ether/water	[8]

molecules can coelute, and their analysis can be difficult because the spectra of many carotenoids are similar. The mass spectrometry (HPLC–MS) is another detector widely employed; this detector provides information about molecular mass and structural conformation, but does not distinguish stereoisomers. Other detectors have been used in accord with an interest in identifying carotenoids directly in the biological matrix (without preliminary sample preparation), such as near-infrared reflectance spectroscopy (HPLC–NIRS), Raman spectroscopy, and nuclear magnetic resonance (HPLC–NMR) spectroscopy; this last has the capacity to identify the geometric isomers, which are not distinguished by MS. These approaches allow a rapid overview of carotenoids while saving time and cost [1].

5.4 Other Techniques

The MS technique can be used without being coupled to HPLC; however, it requires steps of isolation and purification, such as thin layer chromatography, OCC, or preparative HPLC. The capillary electrochromatography (CEC) has been increasingly used in substitution of HPLC with good result to β-carotene, lycopene, and lutein. It is a technique that combines the versatility of HPLC and the high plate efficiency of capillary electrophoresis. The CEC use fused silica capillaries packed with a stationary phase that has fixed charges at the surface. The flow of the eluent across the column is produced and maintained by electroosmotic means by application of high electric field. The components are separated by the differences in their partitioning between the mobile and the stationary phases and the differences in their electrophoretic mobilities if they are charged [51]. Sander et al. quantified 17 carotenoids (such as *trans*-lutein, *trans*-zeaxanthin, β-cryptoxanthin, *cis*- and *trans*-β-carotene, *cis*- and *trans*-lycopene) in food by this technique using C_{30} as stationary phase and acetone/sodium borate buffer [52].

Near-infrared spectroscopy (NIRS) and resonance Raman spectroscopy are techniques with rapidity, simplicity, safety, and low operational costs and are nondestructive techniques. The results obtained were shown to have high equivalency with HPLC. Brenna and Berardo compared NIRS and HPLC in the quantification of 8 carotenoids in maize (α- and β-carotene, α- and β-cryptoxanthin, isolutein, lutein, violaxanthin, and zeaxanthin) and found good correlation between HPLC values and NIRS estimates [53].

6 Sources and Production Methods

A great part of carotenoids in the market are from synthetic processes, as, for example, β-carotene synthesis by Wittig condensation using β-ionone as precursor, but the natural carotenoid market is growing. Both synthetic and natural carotenoids have the same molecular polyenic structure; the natural compound contains several other carotenoids in low concentrations, which provides further health benefits and can be consumed in larger quantities.

Natural carotenoids can be produced by biotechnological processes using filamentous fungi, yeasts, bacteria, or microalgae or through extraction from vegetable resources [54]. For example, β-carotene and lycopene originated from vegetables are generally obtained by solvent extraction with, for example, hexane, acetone, ethyl acetate, ethanol, and ethyl lactate from carrot, palm, and tomato, which present small contents of α-carotene, γ-carotene, and some xanthophylls [55–57]. Lutein is industrially obtained from marigold petals by solvent extraction and saponification [30,58]. Additionally to those described above, several other technologies have been studied for the production, extraction, or concentration of carotenoids in vegetable matrices.

6.1 Supercritical Fluid Extraction

Carotenoid extraction with organic solvent generates a large amount of waste, and the extraction with supercritical fluids emerged as a good alternative with advantages of inertness, low toxicity, and reactivity. França and Meireles studied the extraction of carotenoids from palm oil retained in press cake using supercritical fluids [59], while França et al. reported the utilization of supercritical CO_2 in the extraction of carotenoids from buriti fruit [60]. However, in these works, although the extraction yields until 10,000 ppm, carotenoids were achieved, triglycerides and fatty acids were also obtained, and other step would be needed for their separation from carotenoids. Davarnejad et al., using supercritical CO_2 for the extraction of β-carotene from palm oil, obtained 174.1 ppm of β-carotene at a pressure of 75 bar, temperature of 120 °C, and time of extraction of 1 h, respectively. Nevertheless, temperatures above 60 °C promote degradation of β-carotene, and this probably influenced their results, which could be even better [61]. Jo et al. proposed the use of a supercritical fluid with acetone for β-carotene extraction from persimmon (*Diospyros kaki*) skin with conditions of 40–50 °C and 150–250 bar [62].

Some modifications were proposed by Chuang and Brunner in this supercritical fluid extraction process to contour the low selectivity that they have previously observed [63]. The authors utilized a process of transesterification of palm oil and subsequently after the three-step extraction obtains a product 200-fold concentrated in carotenoids but virtually absent of esters, fatty acids, and triglycerides. Nevertheless, the temperature used (60 °C) and the presence of O_2 in supercritical CO_2, conditions that lead to carotenoid degradation, require addition of antioxidants (such as butylated hydroxytoluene) to the process [64].

Other carotenoids such as lycopene from tomato and its industrial waste [65–68] and lutein esters from marigold (*Tagetes erecta*) petals [69–71] had been extracted with supercritical fluids, achieving better extraction yields when modifiers and cosolvents were used as acetone, chloroform, ethanol, and vegetable oils.

6.2 Membrane Separation

The membrane separation process was initially conducted in degumming vegetable oil and then was adapted for the recovery of carotenoids. Dense polymeric membranes are employed in this system and are very effective in the separation of xanthophylls, phospholipids, and chlorophyll, with retention of 80–100 %, producing an oil rich in carotenes [72,73]. This process, however, requires an additional step of hydrolysis or transesterification. Chiu, Coutinho, and Gonçalves examined the membrane technology as an alternative to concentrate carotenoids from crude palm oil in detriment of ethyl esters. A flat sheet polymeric membrane constituted by polyethersulfone was used and obtained a retention rate of 78.5 % [74]. Darnoko and Cheryan obtained similar results using nanofiltration with 2.76 MPa and 40 °C in red palm methyl esters [75]. Whereas Tsui and Cheryan combined ultrafiltration with nanofiltration to separate zein and xanthophylls from ethanolic corn extract [76].

6.3 Enzymatic Treatment

The enzymatic hydrolysis had also been tested as a pretreatment of chemical extraction in order to improve carotenoid recovery, and it was observed that it could replace successfully the traditional process of alkaline hydrolysis, without losses of the desired product, due to degradation or isomerization. Lietz and Henry tested the hydrolysis of palm oil using a lyophilized lipolytic preparation from *Candida rugosa*, at a dosage of 10 % (w/v) in relation to oil volume, solubilized in phosphate buffer 0.08 M. The volume ratio between oil and aqueous solution was maintained as 1/175, and the system was stirred in a N_2 atmosphere, at 35 °C, for 4 h. The authors obtained a yield of 96 % of fatty acids, without variations in carotenoid concentration [77]. Similar results were achieved by Fernandez et al., which only modified the ratio between oil and aqueous solution to 1/350, also showing in these conditions that the quantity of carotenoids remained virtually constant and equal to 330 ppm. When the authors tested an alkaline saponification, it was observed a reduction by 15 % in the carotenoid content. According to the authors, the decrease was even greater when the formed soap was removed by filtration [78].

You et al. used palm oil and immobilized lipolytic preparation from *C. rugosa* for improved carotenoid extraction, but in different conditions as cited above. In their work, the enzyme dosage used was 1 % w/v, the phosphate buffer was replaced by water, the oil/water ratio was changed to 1/1, and the temperature and the reaction time were increased to 50 °C and 24 h, respectively. The authors obtained a yield of 94 % of fatty acids and a reduction in the carotenoid content of 15 %, probably due to the temperature used [79].

Ribeiro et al. tested three different lipolytic preparations obtained from *Thermomyces lanuginosus* (immobilized), *Candida antarctica* (liquid), and *Yarrowia lipolytica* (liquid) for the hydrolysis of buriti oil, reaching values around 75 %, 35 %, and 18 % of yield of free fatty acids, respectively, without considerable losses in the total carotenoid content. After hydrolysis, carotenoids were concentrated up to 3,900 ppm by deacidification methods, such as partition using ethanol, as well as winterization, thus removing 70 % of the free fatty acids [80].

Other enzymes could be utilized if the vegetable matrix is non-oleaginous, as protease hydrolyzes corn gluten meal allowing the extraction of lutein, zeaxanthin and β-cryptoxanthin [81], and pectinases and cellulases to degrade cell wall polysaccharides from tomato skins to obtain more yields of lycopene in the extraction with hexane [82].

6.4 Other Methods

Molecular distillation process represents a type of vaporization at low pressure and low temperature. The transesterification can also be used as pre-step in molecular distillation process, like centrifuge distillatory. Batistella and Maciel studied the extraction in palm oil with 2,400 ppm of carotenoids applying a centrifugal distillator and a falling film distillator. The first process obtained a decomposition of 12 % at 155 °C and 25 % at 175 °C. The centrifugal distillator results in loss of 13 % at 190 °C and 14 % at 210 °C [83].

Other extraction methods have been used which regard a green approach besides supercritical fluid extraction, utilizing less solvent, lower extraction time, and higher efficiency and replacing thermal energy by microwave [84] or ultrasound [85, 86].

7 Conclusion

Carotenoids are an important group of natural pigments with pronounced antioxidant activity, provitamin A factor, and many health benefits. The structure with conjugated double bonds governs mainly the proprieties of color, stability, detection, and quantification. With an increase of natural carotenoid markets, more different sources will be necessary and new technologies will be developed greener and cleaner, showing how promissory and profitable industrial sector can be.

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Oleoresins from Pine: Production and Industrial Uses

136

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Contents

1 Introduction 4038
1.1 Biological Aspects of Pine Oleoresin Production 4040
1.2 Mechanical Wounding Methods and Physiological Treatments Used to Improve Pine Oleoresin Yields 4041
2 Commercial Aspects of Oleoresin Production 4045
2.1 Industrial Applications of Terpenes Obtained from Pine Oleoresin 4047
2.2 Applications of Monoterpenes 4047
2.3 Applications of Diterpenes 4050
2.4 Applications of Sesquiterpenes 4050
3 Conclusion 4051
References 4051

Abstract

Pine oleoresin is an abundant source of useful terpenes. It has two major fractions: turpentine, which is the volatile fraction, and rosin, which is the solid fraction. A key element of tree defense, oleoresin is an important non-wood forestry product because of the various conventional and potential uses of its terpenes. Oleoresin derivatives can be used by different industries, including pharmaceutical, cosmetic, and food industries, as well as by the chemical industry in the manufacturing of various products, such as paint, varnishes, adhesives, insecticides, and disinfectants. Biotic and abiotic factors that affect oleoresin production can be used to improve yields by promoting specific

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signaling and biochemical defense pathways. Oleoresin production strategies and the industrial applications of this complex blend of natural products are analyzed in this chapter.

Keywords

Pine oleoresin applications • Pine oleoresin biosynthesis • Resin tapping methods • Terpenes • Wounding defense response

Abbreviations

IPP	Isopentenyl diphosphate
MEP	2-*C*-Methyl-D-erythritol 4-phosphate
DXP	1-deoxy-D-xylulose-5-phosphate
DMPP	Dimethylallyl diphosphate
FPP	Farnesyl diphosphate
GPP	Geranyl diphosphate
GGPP	Geranyl geranyl diphosphate
TPS	Terpene synthases
RDs	Resin ducts
TRDs	Traumatic resin ducts

1 Introduction

Pine oleoresin is probably one of the oldest natural products used in large scale by humans. In ancient times, oleoresin was used for lighting, to seal, and preserve wooden ships [1] and as sealant of Greek urns in religious ceremonies [2]. Presently, pine oleoresin has been widely applied as feedstock for chemical industries to produce several types of by-products, such as cleaners, insecticides, solvents, paper sizing, paint, printer ink, pharmaceuticals, cosmetics, aroma and flavor compounds, food additives, among others [3–5].

The oleoresin synthesized by *Pinus* species represents a copious source of terpenes, the largest group of natural products with more than 30,000 known structures [6–8]. Biosynthetically derived from isoprenoid units (IPP – isopentenyl diphosphate), [4, 9] terpenes of conifers are mostly biosynthesized in the cambium zone and associated vascular tissues [10], either through the classic mevalonic acid pathway (in the cytosol) from acetyl-CoA or through the plastid or MEP (2-*C*-Methyl-D-erythritol 4-phosphate) pathway [5]. In the MEP pathway, DXP (1-deoxy-D-xylulose-5-phosphate) is formed from condensation of glyceraldehyde-3-phosphate and two carbon atoms derived from pyruvate. Then, DXP is rearranged and reduced to form MEP, which leads to the basic unit of the terpene biosynthesis (IPP). Once available, IPP and its isomer DMPP (dimethylallyl diphosphate) undergo successive additions of other hemiterpene units catalyzed by prenyltransferases [11, 12], yielding the basic acyclic precursor units of the main terpene classes: geranyl diphosphate (GPP) – monoterpenes (C_{10}), geranyl geranyl diphosphate (GGPP) – diterpenes (C_{20}) and tetraterpenes (C_{40}), mainly through the MEP pathway; farnesyl diphosphate (FPP) – sesquiterpenes (C_{15}) and triterpenes (C_{30}), mainly via the mevalonate pathway. After the formation of the basic terpene units (acyclic), there are fusion reactions and unit attachments (e.g., GGPP+GGPP in carotenoid synthesis), cyclization reactions driven by terpene synthases, additional prenylations, and other modifying reactions involved in the final steps of terpene biosynthesis, including oxidations, methylations, and binding to phenolic or lipidic compounds [6, 9, 13, 14].

The immense diversity of terpene metabolites known today is especially due to the large terpene synthase (TPS) gene family evolution and the versatility of the enzymes encoded by it. Most gymnosperm terpene synthases form a different family from those in angiosperms. However, phylogenetic analyses support the fact that all plant TPS have evolved from an ancestral diterpene synthase implicated with primary metabolism [4].

Various genes encoding for TPS of conifer species have been isolated and characterized [2]. The TPS of *Pinus* spp., and specially those of *Abies grandis* (Grand fir), are among the best known enzymes due to the ease of adaptation of the species to greenhouses and for presenting oleoresin production inducible by injury, simulating the response to the attack of bark beetles [14, 15]. The monoterpene synthases of *A. grandis* are soluble monomeric proteins, with 50–70 kDa and optimum pH for activity between 6.8 and 7.8. Monoterpene synthases of conifers require a divalent metal ion for catalysis (Mn^{2+} or Fe^{2+}) [16, 17], and their activity is stimulated by monovalent cations (mainly K^{+}). Although angiosperm monoterpene synthases require Mg^{2+} or Mn^{2+} as cofactors, magnesium is ineffective as a cofactor for conifer monoterpene synthases [14, 17, 18]. TPS may synthesize unique or multiple products. Limonene synthase, capable of producing limonene, myrcene, α- and β-pinene [2], and *Pinus taeda* abietadiene/levopimaradiene synthase, which synthesizes levopimaradiene, abietadiene, palustradiene, and neoabietadiene, are examples of multiple product TPS [19].

Pine oleoresin probably constitutes the most important ecological trait as defense against highly aggressive conifer pests, the boring bark beetles, and their associated pathogenic fungi [6, 20–27]. The volatile portion of pine oleoresins is composed of turpentine, and the nonvolatile fraction is made of rosin [6, 28, 29], both showing equivalent ecological importance in the successful evolutionary history of pines. Turpentine is a complex mixture of mono (C_{10}) and sesquiterpenes (C_{15}) responsible for the earliest emissions of chemicals/olfactory signals to discourage insect predation. Turpentine also acts as an efficient solvent that facilitates the mobilization of resin acids to injury sites. Diterpene (C_{20}) resin acids are the major components of rosin. The ecological role of rosin is to entrap and kill the undesirable visitors (bark beetles and wood borers), by sealing wounds in stems, after the volatilization of turpentine [30].

In many species of Pinaceae oleoresin biosynthesis may occur as a constitutive pattern of growth and development (primary or preformed resin), or its formation may be inducible by external factors (secondary resin), such as mechanical wounding, or biological challenge, like insect or pathogen attack [13, 14, 26, 31–34].

In general, conifer oleoresins are mixtures of mono-, sesqui-, and diterpene resin acids that accumulate in resin blisters (pockets or cysts), resin cells, or resin canals (or ducts). In pines, both preformed and induced oleoresin are produced and stored in structures named resin ducts (RDs), the most elaborate reticular system of ducts distributed throughout the wood and bark of conifers [14, 35]. RDs are arranged both horizontally (radially) and vertically, often connected at different levels [36]. RDs are formed by the separation of clusters of epithelial cells at the schizogenous level [37–39], which delimits a lumen (or extracellular storage cavity) where the resins are stored. Similar to the patterns observed for oleoresins accumulation, RDs can be primary (constitutive), or originated in response to external stimuli, commonly classified as traumatic resin ducts (TRDs). TRDs are the main features observed in secondary xylem of other genera of the Pinaceae family, such as true firs (*Abies*), and spruces (*Picea*), since constitutive RDs are absent or sparse in unwounded trees of these taxa. In contrast to the origin site of preformed resin (secretory epithelial cells), the production of induced resin is carried out by parenchyma cells surrounding the site of injury [12].

1.1 Biological Aspects of Pine Oleoresin Production

The great international demand for by-products of pine resins in industry and the copious and long-term production of this feedstock by extensive commercial plantations [40] have transformed resin tapping operations in a very profitable business. Unlike agricultural crops, conifer native forests or plantations do not require intensive investments in maintenance [5], and are relatively more tolerant to conditions such as drought and low fertility soils. On the other hand, for commercial purposes, the establishment of resin tapping operations must take into account some important aspects of pine biology and chemistry.

In spite of the fact that all pines are capable of producing resin, the quality (composition) and quantity of both rosin and turpentine can be strongly determined by genetic traits, intrinsic of each species. For instance, *P. patula* (Schiede ex Schltdl. & Cham.), an exotic species widely cultivated in Africa, does not yield sufficient resin, and the quality of its resin is poor (less than 10% of pinenes in its turpentine composition versus approximately 90% observed in *P. elliottii* var. *elliottii* [41]). These features hinder the possibility of *P. patula* use in viable commercial tapping. Similarly, *P. kesyia* is not the first choice of species to be commercially tapped (except in regions where it can be tapped throughout the year, such as the Philippines [2]), since, in spite of its natural and widespread occurrence in Thailand, the species yields very low resin [42].

Trade parameters of by-products of pine oleoresins are also crucial when choosing the species to be tapped. For example, pinenes are the most versatile and widely used compounds of turpentine purchased by the chemical industry to produce mainly pine oil and food additives [3, 43–51]. Alpha-pinene is often the most abundant component of pine turpentine, whereas β-pinene is the isomer with highest value for the chemical industry. *Pinus radiata* D. Don (Monterey pine, insignis or radiate pine) synthesizes turpentine with a superior quality (rich in α- and β-pinenes); however, it does not produce sufficient resin to become economically viable. On other hand, the occurrence of undesirable compounds in turpentine, such as the monoterpene 3-carene (comprising 50 % of the Indian turpentine), can compromise trade values of this by-product in the international market of resin derivates [42].

Historically, the same pine species have been tapped all over the world [2, 42]: *P. elliottii* Engelm., which has reasonable oleoresin yields and good composition of turpentine – Slash pine (Brazil, USA, South Africa, Zimbabwe, Kenya, China); *P. pinaster* Aiton – Maritime pine (Portugal, Spain [52]; France); *P. halepensis* Mill. – Aleppo pine (Greece); *P. massoniana* D. Don – Masson pine (China); *P. merkusii* – Merkus, Tenasserim, or Mindoro pine (Indonesia, Thailand); *P. yunnanensis* Franch. (China); *P. caribaea* Morelet – Caribbean pine (South Africa, Kenya, Brazil, China).

Patterns of heritability of resin production and some important commercial traits (e.g., viscosity) have been investigated in southern pines (*P. palustris* Mill. – longleaf pine, *P. elliottii*, and *P. taeda* L. – loblolly pine) [53–58]. Repeatability estimates for oleoresin yields of loblolly, slash, and longleaf pines proved to be higher than 55% [59].

Besides the genetic control, both constitutive and inducible resin yields may be influenced by environmental conditions [32]. Several investigations have been performed to evaluate the effects of biotic and abiotic factors on oleoresin biosynthesis, including plant age [56, 60], plant physiological status [58, 61, 62], fungal inoculation, water availability [62–68], temperature [58, 60, 69–72] season [54, 58, 60, 73, 74], irradiance [29, 61, 62, 66], and site fertilization status [22, 72, 75]. Some examples of the main results reported are briefly described below.

One-year-old plants of slash pine had increased oleoresin production after flooding [63]. Site fertilization increased constitutive flow of resin in 6-year-old loblolly pine [72], but reduced it in 11-year-old plants [22]. Rates of monoterpenes emission increased exponentially with temperature in slash pine [70] and *P. halepensis* [66]. However, variable emission responses were observed in Aleppo pine plants after exposure to different irradiance levels and limited water availability [66]. Light and water stresses reduced the constitutive level of monoterpene cyclase activity in Grand fir saplings [62]. Highest production of oleoresin in slash pine was observed in spring [54, 73] when compared to other seasons examined.

1.2 Mechanical Wounding Methods and Physiological Treatments Used to Improve Pine Oleoresin Yields

Besides genetic and environmental influences, oleoresin biosynthesis may be physiologically modulated, at least in part, by means of mechanical wounding (extraction method, frequency of bark removal) and/or chemical stimuli [76].

In Europe, the initial aggressive methods of resin exploitation (*le gemmage à mort*) have led to a severe exhaustion of entire pine forests in France. After the establishment of a well-managed reforestation program in the 1800 s, the earlier resin tapping method was replaced by another one, more sustainable and less destructive (described below), to ensure the survival of trees and extend their useful life (*le gemmage à vie*) [2, 77].

Burning the base of the trunks was the first method used in North America to promote gum exudation by trees, followed by the "boxing" method, whereupon a cavity called "box" was cut at the base of trees and above it streaks were made, both highly destructive methods for the trees [2, 78]. Nevertheless, techniques of resin stimulation used worldwide currently (French, Chinese, Mazek, and American) [79] are variations of the "boxing method" briefly described above.

In general, from the outside to the inside of a pine trunk fully grown, the following main tissues (or cell layers), through which resin ducts are distributed, can be recognized: bark (confers protection against predators, by acting as the first barrier encountered by visitors), phloem (vascular tissue that conducts photosynthates, e.g., sucrose), vascular cambium (lateral meristem involved in secondary growth, by giving rise to secondary phloem and secondary xylem – wood), and xylem (vascular tissue for water and mineral nutrient transport) [80] (Fig. 136.1). The current commercial resin tapping operations are performed on trunks presenting a well-developed secondary growth by removing the bark and further tissues beneath it, at variable depths, according to the extraction method employed (Fig. 136.2).

The French (or Hugues) method, the first technique of resin stimulation developed by Pierre Hugues in 1844, (which became popular around 1860 [77]) consists in removing long vertical bark streaks (about 10 cm wide), deep enough to reach

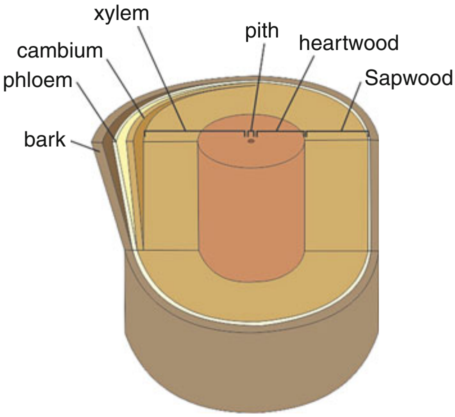

Fig. 136.1 Schematic section of a pine trunk showing the main layers that can be removed in resin tapping processes (Adapted from [185])

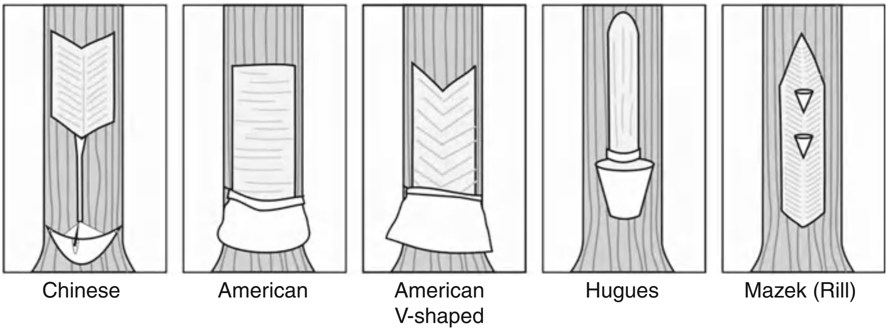

Fig. 136.2 Methods of resin tapping (Adapted from [79])

the secondary xylem of the trunks. This is the main method used in Indonesia, one of the major producers of crude gum resin today.

In the Chinese method, V-shaped streaks are daily removed in a downward direction, the starting point being about 1.2 m aboveground, to expose the secondary xylem. No chemical stimulation is used in French or Chinese methods of resin tapping operations.

The method known as "Mazek or Rill," today used in India, was adapted by Mazek Fialla in Europe (1950s) based on a US patent deposited by Steele in 1869, which describes the principle of the fish bone tapping system to stimulate and collect resin. In this system, V-shaped streaks (2–3 mm wide) are cut in variable intervals (every 24 h, 3 or 7 days) in upward direction, much like in the American method.

In the American method, horizontal (or V-shaped) strips of bark (2.5–3.0 cm wide) are removed every 14 days, from roughly one third of the tree circumference, exposing the cambium (only the bark and phloem are removed) or preferably the sapwood surface. This method is used in Brazil, Argentina, Portugal, and Spain [79]. The influence of mechanical wounding shape in resin production was tested on slash pines in southern Brazil, indicating that both horizontal and V-shaped streaks were equivalent for oleoresin yield [81].

Although mechanical wounding is well established as an efficient defense-response inducer in plants (especially in conifers) [33, 56, 61, 62, 82, 83], and this principle has been applied for centuries to obtain oleoresins, over the years numerous chemical stimulating treatments (Table 136.1) have been tested in order to enhance oleoresin yields in commercial pine plantations, including several US Patents [84–93]. For example, in the Rill method, a spray solution composed of an equal proportion mixture of hydrochloric (HCl) and sulfuric (H_2SO_4) acids is applied on the exposed "face" to improve resin yield. In the American method, a chemical stimulating paste containing sulfuric acid (20%) and CEPA (2-chloroethylphosphonic acid) (3.5–4.0%) in its formulation is placed on the sapwood or cambium surfaces [40, 73, 81]. This practice reduces the damaged area in pine trunks originally affected by the destructive traditional methods of mechanical injury.

Table 136.1 Treatments used to increase oleoresin production

Compound	Mode of action and responses observed in conifers (besides oleoresin yield enhancement)	Species tested	References
Sulfuric acid	Potential generator of free radicals; increases and prolongs gum resin yields by maximizing the effect of the wounding at the injury zone or "face"	*Pinus elliottii* Engelm., *P. palustris* Mill., *P. caribaea* var. *bahamensis, P. oocarpa, P. kesiya, P. caribaea* var. *caribaea, P. caribaea* var. *hondurensis, P. patula, P. ponderosa, P. pinaster*	[40, 41, 53, 69, 73, 76, 81, 86, 88, 89, 91, 94–99]
Ethephon, CEPA[a] or Ethrel®	Precursors of ethylene, signaling molecule involved in stress-induced responses; increases biosynthesis of mono- and diterpenes, induces radial growth, RDs formation in xylem, and alters the anatomical pattern of tracheids	*P. elliottii* Engelm. *P. palustris* Mill., *P. caribaea* var. *hondurensis, P. taeda* L., *P. resinosa*	[15, 81, 90, 93, 96, 98, 100–107]
Paraquat[b]	ROS[c] generator; increases ethylene synthesis, induces lightwood formation	*P. elliottii, P. taeda* L., *P. palustris*	[73, 90, 93, 102, 103, 108–111]
2,4-D[d] (Auxin)	Stimulates the cambial activity and RDs formation, and promotes the expression of ACC[e] synthase gene (enzyme involved in ethylene biosynthesis)	*P. elliottii, P. palustris*	[38, 73, 85, 97, 112, 113]
Salicylic acid (SA)	Mediator in pathogen-associated responses, acts to improve the defense performance of plants against their predators	*Pseudotsuga menziesii* (Douglas-fir) (Pinaceae), *Sequoiadendron giganteum* (Giant sequoia) (Taxodiaceae), *Picea abies, P. elliottii*	[73, 114–118]
Benzoic acid	Precursor of SA in pathogen-signaling pathways	*P. elliottii*	[73, 115]
Metal cofactors (Mg^{2+}, Mn^{2+}, Fe^{2+}, K^{+}, Cu) valence in Cu: Cu^{+}	Required as cofactor or stimulators of TPS activity or ethylene perception (Cu valence in Cu: Cu^{+})	*P. elliottii*	[9, 14, 16–18, 40, 119]
Yeast extract	Simulates pathogen attack	*P. elliottii, Abies grandis*	[73, 120]
Chitosan or fungal cell wall fragments[f]	Simulate pathogen attack; promote hyperoleoresinosis (transient increased accumulation of mono- and diterpenes) and increased monoterpene cyclase activity (limonene cyclase); promote formation of large lesion sizes on xylem	*P. contorta* var. *latifolia* (Lodgepole pine), *A. grandis, P. taeda, P. echinata* (Shortleaf pine), *P. palustris, P. virginiana*	[104, 120, 121]

(*continued*)

Table 136.1 (continued)

Compound	Mode of action and responses observed in conifers (besides oleoresin yield enhancement)	Species tested	References
Methyl Jasmonate (MeJA)[g]	Induces defense responses in conifers, induces the transcription of ACC oxidase gene involved in ethylene biosynthesis; increases biosynthesis of monoterpenes and diterpenes in needles and wood, promotes cell activation and TRDs formation on xylem, induces ethylene production, increases TPS transcripts	*Picea abies* L. (Karst) (Norway spruce), *Metasequoia glyptostroboides* (Dawn redwood), *Sequoiadendron giganteum*, *Sequoia sempervirens* (Coast redwood), *Cryptomeria japonica* (Japanese cedar), *A. grandis*, *Cedrus libani* (Cedar of Lebanon), *Tsuga heterophylla* (Western hemlock), *Pseudotsuga menziesii*, *Araucaria araucana* (Monkey puzzle), *Araucaria heterophylla* (Norfolk Island pine), *Podocarpus totara* (Totara), *Podocarpus lawrencei* (Mountain plum pine), *Cupressus macrocarpa* (Monterey cypress), *Picea sitchensis* (Sitka spruce)	[28, 29, 116, 122–127]
Fungal elicitors[h]	Induce hypersensitive responses in conifers; induce pronounced increase of ethylene production, monoterpene biosynthesis associated with increased ethylene production, increase systemic and traumatic resin accumulation in stems, induce expression of TPS genes (mono-, sesqui-, and diterpenes synthases), induce monoterpene cyclase activity (limonene cyclase)	*P. elliottii* Engelm. var. *elliottii, P. taeda* L., *P. nigra* Arn. (Austrian pine), *P. resinosa* Aiton (Redwood pine), *Picea sitchensis* (Sitka spruce), *P. echinata*, *P. palustris*, *P. virginiana, A. grandis*	[33, 72, 106, 107, 120, 121, 123, 128, 129]

[a] 2-chloroethylphosphonic acid
[b] *N*′,*N*′-dimethyl-4-bipyridinium dichloride
[c] reactive oxygen species
[d] 2,4-dichlorophenoxyacetic acid
[e] 1-amino-cyclopropane-1-carboxylic acid
[f,g,h] chemical stimuli or elicitors not used in large scale

2 Commercial Aspects of Oleoresin Production

In Europe, there is evidence of oleoresin exploitation predating 100 B.C. in France, albeit it is known that Greeks were the pioneers in production of resin from *P. halepensis* Mill. in ancient times. In spite of wide geographical spread of Aleppo

Table 136.2 Brazilian pine resin (and by-products) exportations and main international customers

Product	Year	Total annual (kg)	US$ per ton (FOB)	Customer
Gum resin	1989	6,653,978	496.18	India
	1999	10,420,534	420.64	Portugal
	2007	365,000	679.50	Argentina
	2008–2011	Not informed	–	–
Turpentine	1989	2,496,618	620.15	USA
	1999	2,698,705	492.05	France
	2007	2,942,720	808.89	France
	2008	1,746,080	848.85	France
	2009	2,335,760	1,016.75	France
Rosin	1989	6,333,340	542.05	USA
	1999	8,705,260	534.79	Netherlands
	2007	5,773,500	1,160.89	Netherlands
	2008	4,941,900	1,080.61	Netherlands
	2009	9,285,100	862.09	Netherlands

Pine along the Mediterranean coast, the maritime pine (*Pinus pinaster* Ait. syn. *P. maritima* Mill.) has been the main species used in France since at least the mid-1800s [2] for resin tapping commercial operations [77, 130]; today, resin tapping is practically nonexistent in this country.

In North America, the diversified industry of pine-based oleoresin, known as "Naval Stores," was the first and long-lasting industrial activity in the USA, which started roughly in the1600s, introduced by the English settlers during the Colonial period and nearly abandoned at the end of the twentieth century. The main species tapped to supply the Naval Stores industry in the 1900s were longleaf (*Pinus palustris* Mill.) and slash (*P. elliottii* Engelm.) pines [2, 78].

From 1987 to 1989, China, the former Soviet Union, and Portugal contributed, respectively, with about 55%, 12%, and 10% of the total crude resin produced in the world [42]. Currently, China, Brazil, and Indonesia are the major world producers of pine gum resins [79].

The international market of gum resin is quite variable, affecting the trade values of turpentine and rosin. Besides international standards usually required (particular chemical composition of turpentine, and specific physical properties of rosin, [76]), the prices of gum resin exported (and its by-products) may be subject to negotiation, depending on the type of customer (end user or fractionator) and the amount of tons purchased. For instance, in 2008, the average value of turpentine exported from Brazil was US$ 1,023.46/t Free on Board (FOB) however, the charged prices for France (the main customer of Brazilian turpentine, Table 136.2) and Spain (which acquired 584,600 kg in 2008) were US$ 848.85/t FOB and US$ 1,169.88/t FOB, respectively [131].

In the last 3 years, the average Brazilian total production of gum resin was more than 91,000 t. The largest Brazilian producers are the states of São Paulo (~50%)

and Rio Grande do Sul (~25%). Slash pine is the major species tapped in Brazilian plantations contributing 72% of the total oleoresin produced, followed by tropical pines (*P. caribaea* var. *caribaea, P. caribaea* var. *hondurensis*, *P. caribaea* var. *bahamensis*) [131]. Crude gum resin was initially the major feedstock purchased in large scale by the international market, but currently its by-products are preferable, with France and the Netherlands being the main customers of Brazilian turpentine and rosin, respectively (Table 136.2). In 2012, prices per ton of Brazilian resin, turpentine, and rosin reached US$ 638.50, US$ 1,658.11, and US$ 1,800.00, respectively.

2.1 Industrial Applications of Terpenes Obtained from Pine Oleoresin

Estimates indicate that terpenes represent 55% of all secondary metabolites in plants, whereas alkaloids and phenolics account for 27% and 18%, respectively [7]. Various important biological properties have been reported for plant essential oils and related terpenes [132]. A key aspect of using terpenes from plants is the fact that these represent renewable resources, yielding sustainable economic returns for longer periods of time. Moreover, terpene extraction from live trees provides a means to remove excess carbon dioxide from the atmosphere through forest plantations.

Different oleoresin terpenes have been identified in pine species (Table 136.3) [133–137]. Oleoresin obtained by tapping the bark in pine trees constitutes an important forestry product because of the chemical diversity of these secondary metabolites and their multiple possibilities of use. Oleoresin has two major fractions: rosin (diterpenes), which is the solid fraction, and turpentine (mono- and sesquiterpenes), which is the volatile fraction [6, 40].

Different types of industries can make use of oleoresins from pines: pharmaceutical and perfume industries, food additives, and other chemical industries (household cleaning products, paints, inks, varnishes, rubber, insecticides, aromatherapy). These various uses are due to their pleasant fragrance [140, 141], antimicrobial activity, among various other physical and biological properties. Examples of different terpenes obtained from pine oleoresin and their importance in distinct industrial segments are listed in Table 136.4.

2.2 Applications of Monoterpenes

Turpentine is mostly composed of monoterpenes and these can have different industrial applications. Borneol is used in cosmetics and in non-cosmetic products, such as household cleaners [145]. Carveol, linalool, myrcene, α- and β-pinene, phellandrene and terpineol are used in the food, perfume, and cosmetics industry as fragrance and to impart flavors to foods [133, 146–149]. Also, it has been reported that turpentine could be an interesting resource for the pharmaceutical industry due

Table 136.3 Examples of terpenes found in pine species

Compound[a]	References
Borneol	[136]
Camphene	[40, 133, 135–137]
Carene	[135, 136]
Carvacrol	[136]
Carveol	[135, 136]
p-cymene	[135–137]
Limonene	[135–137]
Linalool	[133, 135]
Myrcene	[40, 135, 137]
Phellandrene	[40, 133, 135, 137]
α-pinene	[40, 133–135, 137]
β-pinene	[40, 133–135, 137]
Terpineol	[135, 137]
Aromadendrene	[135]
Isopimaric acid	[138, 139]
Bisabolene	[135–137]
Germacrene	[133, 135–137]
Longifolene	[133, 135, 136]

[a] These reports might not reflect all of the terpenes present in pine species because of differences in experimental analysis and biological conditions of plant growth

to its analgesic and antioxidant activities [150]. Linalool and myrcene were shown to have considerable protective effect against genotoxicity, due to their strong antioxidant activities, both in *Escherichia coli* and human hepatoma cultured cells [151]. Pathogenesis caused by *Staphylococcus aureus* and *Salmonella enterica* draw great medical attention. Efforts have been made to understand and inhibit the formation of biofilms produced by these two important bacteria [152, 153]. Carvacrol was capable of inhibiting the formation of *S. aureus* and *S. enterica* biofilms [154]. These results highlight the important potential of antioxidant and antimicrobial effects of compounds such as linalool, myrcene, and carvacrol for the pharmaceutical industry.

In the industry of insecticides, one of the central issues is to develop products that are not harmful to the environment and to human health. It has been shown that α-pinene, camphor and limonene can be good natural repellents for insects [155]. Besides the use of geraniol in the industry of fragrances, this compound has also proven effective as a mosquito repellent [156, 157]. Terpene halides, 3-carene, and toxaphene, a chlorinated camphene, are important for agriculture because of their use as pesticides [158, 159]. However, especially for toxaphene, several restriction issues have been raised because of its pollutant capacity and carcinogenic potential in humans [159–161].

Terpineol, which has a pleasant aroma, is one of the most common ingredients of Chinese black tea [162]. Camphor and menthol are also interesting for the

Table 136.4 Classes of different terpenes and specific components present in oleoresin of pine trees and their respective industrial applications

Category	Compound	Industrial use
Monoterpenes [136]	Borneol	Cosmetics, household cleaners
	Camphene	Fragrances, pharmaceuticals, plasticizers, repellents, explosives
	D-3-carene	Repellents, manufacture of menthol, pesticides
	Carvacrol	Pharmaceutical
	Carveol	Fragrances, cosmetics, household cleaners, detergents
	p- cymene	Pharmaceuticals, repellents, disinfectants, solvents, flavorings
	Limonene	Fragrances, solvents, cleaning agents, flavorings, insect attractants, perfume industry
	Geranyl Linalool	Perfume industry, cosmetics, household cleaners, pharmaceutical
	Myrcene	Perfume industry, pharmaceutical
	Phellandrene	Perfume industry
	α-pinene	Fragrances, repellents, plasticizers, solvents, perfumery, insecticides
	β-pinene	Pharmaceuticals, fragrances, repellents, plasticizers, solvents, perfumery, insecticides
	α-terpineol	Fragrances, cosmetics, household cleaners, detergents, repellents, perfumery
	Toxaphene	Pesticides
Diterpenes [142]	Abietic acid	Paints, varnishes, lacquers, pharmaceuticals
	Dehydroabietic acid	Pharmaceuticals
	Geranylgeraniol	Potential antibacterial agent
	Isopimaric acid	Paper industry, pharmaceuticals
	Levopimaric acid	Paper industry
Sesquiterpenes [143]	Aromadendrene [144]	Potential antibacterial agent, food industry
	Bisabolene	Food additive, biofuel industry
	β-Caryopphyllene	Cosmetics, biofuels, perfume industry, food safety
	Farnesene	Insect repellent, chemical industry
	Germacrene	Food industry
	Longifolene	Chemical industry
	Isolongifolene	Fragrance, pharmaceutical

pharmaceutical industry. Camphor is an active molecule in vapor-steam products and the application of ointments that contain camphor has been effective to treat cough in children [163]. Interestingly, in Phase I trial studies, *L*-menthol was used as smooth muscle relaxant and proved to be readily metabolized and excreted in urine [164].

Esters of pinic acid derived from pinenes, which are additives to increase plasticity and fluidity of a material, are used as plasticizers [165]. *P*-Cymene and *p*-menthane are used as disinfectants, solvents, and also as flavoring ingredients [166]. Terpene phenols and terpene polymers are important in the chemical industry to make adhesives [167].

2.3 Applications of Diterpenes

There is a large-scale industry involved in producing gum rosin. In 2005, for example, China produced 700,000 t of tall oil resin (TOR) derived from tapping oleoresin of pine trees [168]. Rosin is a highly valued derivative of pine oleoresin.

Resin acids, such as abietic acid, can be converted to ester gum and used to make varnishes. Also, it is rubbed on the surface of musical instruments to make them less slippery, and used in paints [169]. Although abietic acid and its derivatives have been used in the chemical industry and as household cleaning agents, they have also shown potential as antiobesity and antioxidant molecules [170–172]. Tuberculosis is a very important disease caused by *Mycobacterium tuberculosis*, which is increasing and resurging in various parts of the world, often with the development of chemotherapy-resistant strains. It has been reported that geranylgeraniol is a potent inhibitor of *M. tuberculosis* in vitro [173]. Other diterpenes found in *P. spez*, such as isopimaric acid and dehydroabietic acid, have been identified as potent anticancer and antiviral agents [138]. Although further research is necessary to investigate the biological potential of geranylgeraniol to fight tuberculosis, the antiobesity and antioxidant effect of abietic acid and its derivatives, and of the anticancer and antiviral potential of isopimaric acid and dehydroabietic acid, these readily available compounds may be lead molecules for the pharmaceutical industry.

Levopimaric acid and isopimaric acid are very important adjuvants for cellulose pulp production and the paper industry [168]. Another relevant aspect of isopimaric acid, extracted from immature cones of *P. nigra*, is that it showed activity against a multidrug-resistant bacteria strain of *S. aureus* [139].

2.4 Applications of Sesquiterpenes

Farnesene can be used as an insect repellent [174]. Also, there are many other applications for farnesene, which can be converted into lubricants, fuels, and polymers for the biofuel industry. In Brazil, a genetically modified organism was approved by the National Committee of Biosafety (CTNBio) to produce farnesene at large scale by the chemical industry [184].

Bisabolene is a sesquiterpene used in the flavoring industry to add fruity flavor to foods [166]. In South Asia, cucurmin, which is the main compound of turmeric, is used in tradicional medicine to treat epilepsy. A detailed investigation found that bisabolene sesquiterpenes were additional molecules responsible for anticonvulsant effects [175]. Interestingly, because of its physicochemical properties, bisabolene has been investigated as an alternative biofuel for replacing D2 diesel [176, 177].

Together with the monoterpenoid terpineol, the sesquiterpenoid longifolene has been characterized as one of the main components of the aroma of Chinese black tea [162]. Isolongifolene, obtained by isomerizing longifolene, has been used in the fragrance and pharmaceutical industry [178, 179].

Beta-caryophyllene has been detected as one of the most abundant terpenes in *P. caribaea* by spectrometric methods [133]. This sesquiterpene has long been used in the perfume and cosmetics industry. Recently, using biotechnological approaches, phototrophic organisms have been genetically transformed to produce β-caryophyllene to be used by the biofuel industry [180].

Another interesting application of caryophyllene and other terpenoids found in essential oils is for enhancing aroma and safety of fresh foods, such as fresh fruit-cuts [181, 182]. Plant extracts containing germacrene and aromadendrene had a strong growth inhibition effect on food-borne microorganisms such as *Bacillus cereus* and *Salmonella thyphimurium* [183].

3 Conclusion

The plantations of commercial and renewable pine forests are sources of biomass to the continued rational exploration and extraction of oleoresin [40]. As analyzed in this chapter, there is a large array of industrial applications of different terpenes present in pine tree oleoresins. The scope of traditional and new applications of terpenes is wide and broadening. Recent advances in the management of tapped forests, by means of low-cost physiological treatments stimulating signaling cascades and terpene biosynthetic activity, may further improve oleoresin yields. Genetic selection for oleoresin production in pine forests is an important venue to pursue. The growth of different markets, with considerable social, economic, and environmental impacts, depend on future research and technology generation for oleoresin production and pine terpene products.

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Pyrethrins and Synthetic Pyrethroids: Use in Veterinary Medicine

137

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Contents

1 Introduction 4062
2 Classes of Pyrethroids 4065
3 Mode of Action 4066
3.1 Effects on Sodium Channels 4066
3.2 Effects on Chloride Channels 4067
3.3 Other Actions 4068
4 Pharmacokinetics/Toxicokinetics 4068
5 Antiparasitic Activity 4069
5.1 Ruminants 4070
5.2 Horses 4071
5.3 Pet Animals 4071
5.4 Birds 4072
6 Veterinary Applications (Uses) 4072
6.1 Uses in Ruminants 4074
6.2 Uses in Horses 4075
6.3 Uses in Swine 4076
6.4 Uses in Nonmammalian Species 4076
6.5 Uses in Pet Animals 4077
7 Toxicity 4078
7.1 Toxicity in Domestic Animals 4078
7.2 Toxicity in Aquatic Organisms 4079
8 Maximum Residue Limits and Drug Withdrawal/Withholding Periods 4080
9 Conclusion/Prospects 4083
References 4083

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Abstract

Pyrethrins and synthetic pyrethroids are used extensively as insecticides and acaricides for the treatment of a broad range of ectoparasites in large and small animals, as well as in nonmammalian species such as birds, fish, and honeybees. These compounds are used in veterinary medicine in different formulations including spot-on, sprays, ear tags, dips (immersion), soluble powders, and shampoos to control fleas, mite, lice, and ticks between other insect infestations both outside and inside the house. The synthetic pyrethroids have been classified in two classes: type I and type II; the addition of the alpha-cyano group to the 3-phenoxybenzyl alcohol group in type II has increased the insecticidal potency. The mode of action of these compounds suggests that the voltage-dependent sodium channel in the nerve membrane is the common target in insects and mammals. The pharmacokinetic/toxicokinetic properties of these compounds are also presented. This chapter also reviews the antiparasitic activities and the veterinary applications (uses) of pyrethrins and synthetic pyrethroids in several animal species. The clinical signs of poisoning in particular in the cats by permethrin are described. The chapter also provides the EU's maximum residue limits (MRLs) established for the pyrethroids as antiparasitic agents in food-producing animals; the MRLs are necessary to establish the withdrawal/withholding periods of the final veterinary drug formulations containing these chemical compounds.

Keywords

Animals • antiparasitic activity • classes • maximum residue limits • mode of action • poisonings • pyrethrins • pyrethroids • uses • withdrawal/withholding periods

Abbreviations

CNS	Central nervous system
EU	European Union
MRLs	Maximum residue limits

1 Introduction

Pyrethrins are a group of closely related, naturally occurring compounds that are the active insecticidal ingredient of pyrethrum and have been used for centuries. Pyrethrum, which has been used as a pesticide in commercial applications since the early nineteenth century, is extracted from the flowers of *Chrysanthemum cinerariaefolium* and *Chrysanthemum cineum* [1]. Most of the insecticides derived from plants traditionally have been considered safe for use on animals, and pyrethrum is an example of such material. There are six active insecticidal compounds that comprise the natural pyrethrins: pyrethrin I and II, cinerin I and II, and

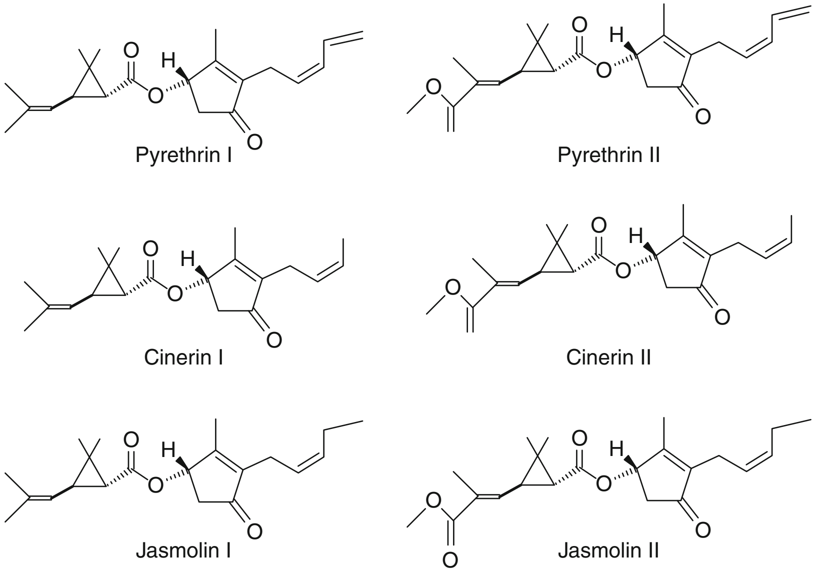

Fig. 137.1 Structure of natural pyrethrins

jasmolin I and II, the proportions being dependent on the extracts in questions. Most commercial preparations contain 20–25% pyrethrins with the ratio of pyrethrin: cinerin:jasmolin approximating 71:21:7. The structure of natural pyrethrins is shown in Fig. 137.1. Rapid knockdown of flying insects is a characteristic of the pyrethrin II compounds, but the pyrethrin I compounds possess greater insecticidal activity. Pyrethrins cause hyperexcitability with very little cytotoxicity. However, because the natural pyrethrins breakdown rapidly in the presence of moisture, air, light, and heat, they are rapidly biodegradable. The pyrethroids were developed to improve the stability of the pyrethrins, usually are biodegradable, but are sufficiently stable when exposed to air and light; the success of this chemical modification has led to widespread use of these agents as animal, agronomy, and industrial insecticides, providing excellent control of insects.

In the last century, the development of organophosphorus, carbamate, and organochloride insecticides was followed by synthetic pyrethroids. As a result, pyrethroids are now used frequently in the domestic milieu. Pyrethroid insecticides are synthetically derived from the molecular structure or sharing the same mechanism of action of natural pyrethrins that have broader spectrum of activity, more stability, and residual activity (persists longer than that of natural pyrethrins) and include the following: allethrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, fenvalerate, flumethrin, fluvalinate, tau-fluvalinate, and permethrin (see structure in Fig. 137.2). They are lipophilic compounds and generally of low acute oral toxicity to mammals but are very toxic to aquatic organisms. When synthetic pyrethroids are administered to mammals parenterally, the synthetic pyrethroids are neurotoxic.

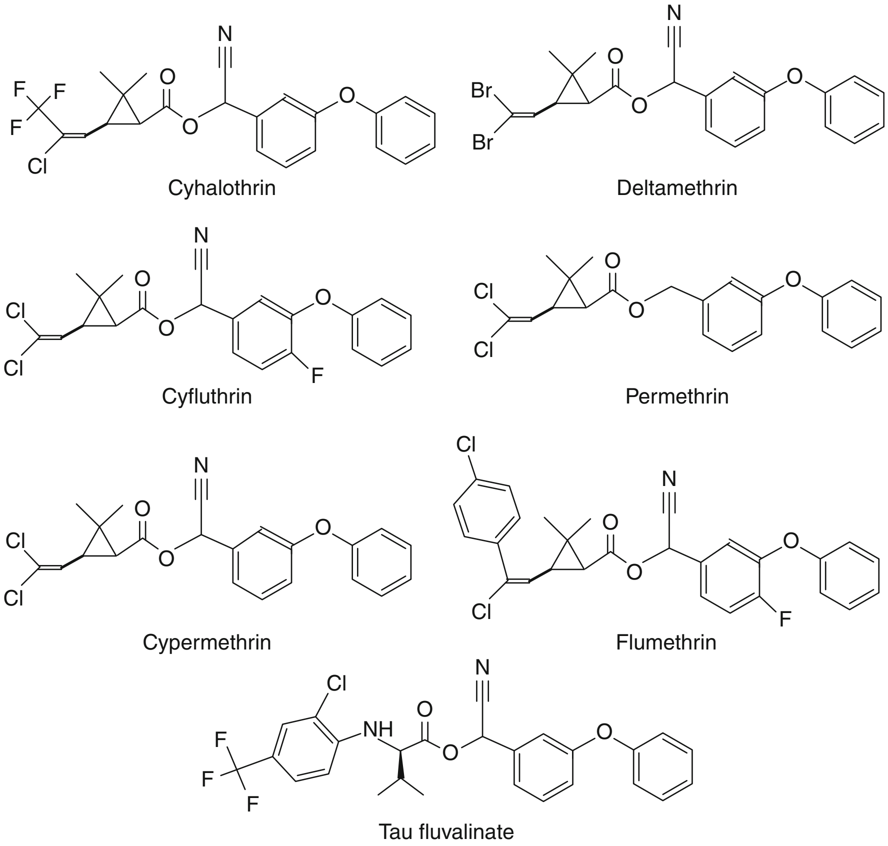

Fig. 137.2 Structure of synthetic pyrethroids

Pyrethrins and pyrethroids are used widely in numerous formulations for control of a broad spectrum of ectoparasites (i.e., fleas, lice, ticks, mites, and flies) affecting to the animals as well as in the home and garden and in agriculture to control insects for more than 45 years and account for approximately one-fourth of the worldwide insecticide market [2]. Many factors may influence the choice of product as active chemical ingredient, target parasite, efficacy and safety, regulatory status of the product, convenience, and aesthetic acceptability. In the EU and some other countries, these products are regarded as veterinary medicines, whereas elsewhere they are regulated as pesticides.

Pyrethroids have different pharmaceutical formulations and use varying solvents. Studies have shown significant differences in the efficacy of formulations with the same concentration of pyrethroids dissolved in diverse solvents since the solvent may play a role in the distribution of the active ingredient and therefore influence the efficacy of the active ingredient. Moreover, distinction between the effect of the pharmaceutical formulations in different areas of the animal body (i.e., close to and far away from the application site) could be observed.

Since pyrethrins are highly photolytic, antioxidants are often added to preparations to stabilize formulations; antioxidants adjoin include pyrocatechol, pyrogallol, hydroquinone, and 1-benzene-azo-2-naphthol. Practically, all pyrethrins and many pyrethroids are commonly combined to additives (including synergists), some formulations include additional insecticides, insect repellents, or both, and many contain hydrocarbon solvents [3] to enhance their insecticidal activity. Pyrethrin and pyrethroid sprays may also be water based or be alcohol or petroleum based, which increases the overall toxicity. It is known that concomitant use of pyrethrins and pyrethroids with synergists such as piperonyl butoxide, *N*-octyl bicycloheptene dicarboximide, sulfoxide, sesamin, sesame oil, sesamolin, isosafrole, and organophosphorus compounds or carbamates may increase toxicity by mechanisms involving inhibition of microsomal oxidation [4].

2 Classes of Pyrethroids

Pyrethroid insecticides are generally classified into one of two large groups on the basis of the central neurotoxic syndrome that they produce [5, 6]. Type I pyrethroids are esters of chrysanthemic acid and an alcohol, having a furan ring and terminal side chain moieties, and absence of a cyano moiety. Allethrin was the first pyrethroid identified in 1949. Allethrin and other pyrethroids such as phenothrin and permethrin with the basic cyclopropane carboxylic ester structure are type I pyrethroids. The insecticidal activity of these synthetic pyrethroids was enhanced further by the addition of a cyano group to give α-cyano type II pyrethroids such as deltamethrin, fenvalerate, cyfluthrin, cyhalothrin, and lambda-cyhalothrin (Fig. 137.2).

Type II pyrethroids have 3-phenoxybenzyl alcohol derivatives in the alcohol moiety and have had some of the terminal side chain moieties replaced with a dichlorovinyl or dibromovinyl substitute and aromatic rings. They are neurotoxic both for mammals and insects. In all species tested, pyrethroids show a pattern of toxic action typical of a strongly excitant effect on the nervous system. The type II pyrethroids, exerting their action on higher brain centers and interfering with the transmission of nerve impulses, act on the sodium channel of the axonal membrane; they induce a long-lasting prolongation of the membrane during excitation, leading to prolonged trains of impulses. The action on the nervous system of Type II pyrethroids is specific and tends to be reversible.

In mammals, two distinct toxic syndromes have been described: the T syndrome (characterized by tremor) induced by type I pyrethroids and the CS syndrome (characterized by choreoathetosis and salivation) induced by type II compounds [7]. Type I pyrethroids cause hyperexcitation, ataxia, convulsion, paralysis [7–9], and repetitive nerve firing [10]. In contrast, type II pyrethroid poisoning is characterized by hypersensitivity, profuse salivation, choreoathetosis, tremor, and paralysis [8, 9] but no repetitive nerve firing in sensory nerves [11]. Some pyrethroids produced tremors and salivation, classified as the intermediate TS-syndrome. In humans, the most prominent effect of the pyrethroids is paresthesia mainly in the face, and there is little evidence of any permanent effects.

3 Mode of Action

All pyrethroids share the same basic mode of action, but the different types cause somewhat different symptoms as described previously. Although the molecular aspects of pyrethroids action are not fully understood, detailed electrophysiological investigations strongly suggest that the voltage-dependent sodium channel in the nerve membrane is the common target in both insects and mammals, including humans [9]. Type II pyrethroids also depress resting chloride conductance, thereby amplifying any effects of sodium or calcium.

The mechanisms by which pyrethroids alone are toxic are complex and become more complicated when they are co-formulated with piperonyl butoxide, an organophosphorus insecticide, or both, as these compounds inhibit pyrethroid metabolism. The main effects of pyrethroids are on sodium and chloride channels. As a result, excitable (nerve and muscle) cells are the principal targets of pyrethroid toxicity, which is manifested as disordered function rather than structural damage. In that way, the major toxic effect of dermal exposure is paresthesia, supposable also due to hyperactivity of cutaneous sensory nerve fibers [12].

3.1 Effects on Sodium Channels

Pyrethroids act primarily on the nervous system. The primary mode of action of pyrethroids in both insects and mammals is disruption of voltage-sensitive sodium channel function, but chloride and calcium channels are also affected. Perturbation of sodium channel function by pyrethroids is stereospecific [13]. Using cell-attached patch-clamp electrophysiology recordings can be observed that individual voltage-gated sodium channels open for a few milliseconds and then fail to open again for an extended period due to channel inactivation. In the presence of pyrethroids (tetramethrin), the channels open with a long delay, and then often remain open for several seconds even after the termination of the depolarizing pulse. In conclusion, the pyrethroids that hold the sodium channel open the longest will cause the greatest amount of depolarization. There is marked stereospecificity of the action of pyrethroids on the sodium channel; some isomers are more toxic than others. This interaction with the sodium channel has the effect of slowing the activation and inactivation properties of the sodium channel leading to a stable hyperexcitable state in all excitable tissues. The proportion of sodium channels modified is dose-dependent, but the duration of their hyperexcitable state is determined by the structure of the pyrethroids and is not dose-dependent. The *cis*-isomers are usually more toxic than the *trans*-isomers. As an example, the 1*R* and 1*Scis*-isomers bind differently than the 1*R* and 1*Strans*-isomers that bind noncompetitively to another [14]. In mammals, the 1*R* isomers are toxic and the 1*S* isomers inactive, making the 1*S* isomers nontoxic. The stereospecificity, due to isomerism about the third carbon atom of the cyclopropane ring, accounts for the reported differences in toxicity of the pyrethroids available in isomeric mixtures. In general, type II compounds delay the inactivation of voltage-sensitive sodium channel substantially longer than type I compounds. Type I compounds prolong voltage-dependent sodium channels opening only long enough to cause repetitive firing of action potentials (repetitive discharge), whereas type II compounds hold the channels open for such long periods that the membrane potential ultimately becomes depolarized to the point making the generation of action potentials impossible (depolarization-dependent block). These differences in prolongation of channel open times are hypothesized to contribute to the differences in the CS and T syndromes of neurologic toxicity after exposure to type II and I pyrethroids, respectively. The depolarizing action results in a massive discharge of neurotransmitter at nerve terminals and hence severe disruption of synaptic transmission. Paresthesia results from the direct action of pyrethroids on sensory nerve endings, causing repetitive firing of these fibers, and they are more likely to follow exposure to type II than type I pyrethroids.

Finally, some differences have emerged between sodium channels of insects and vertebrates in terms of their intrinsic sensitivity to pyrethroids indicating that the selectivity of pyrethroids to insect is at least in part to their higher activity on insect sodium channels than vertebrate channels [15].

3.2 Effects on Chloride Channels

Pyrethroids also affect the voltage-dependent chloride channels. These channels are found in the brain, nerve, muscle, and salivary gland and are modulated by protein kinase C, and their function is to control cell excitability. There are many different functional types of chloride channels in contrast to sodium channels. Most pyrethroid-sensitive channels belong to the maxi chloride channels class [16]. Maxi channels are activated by depolarization, have high conductance, are calcium independent, and are inactivated by protein kinase C phosphorylation. Pyrethroids decrease maxi chloride channel current, which serves to increase excitability and therefore would synergize pyrethroid actions on the sodium channel. Of the pyrethroids that have been tested, only some of those producing type II poisoning syndrome (deltamethrin and fenvalerate) seem to affect maxi chloride channels. Since agents such as ivermectin and pentobarbital, which open chloride channels, antagonize pyrethroid-evoked salivation, choreoathetosis, and repetitive firing in skeletal muscle [17], it is probable that chloride channel actions contribute most to the features of poisoning with type II pyrethroids, such as salivation and myotonia.

The decrease in chloride-open channel state produced by type II pyrethroids serves to increase excitability and therefore to synergize pyrethroid actions on the sodium channel. At relatively high concentrations, type II pyrethroids have also been shown to interfere with the GABA-gated chloride channels [18] which may contribute to the seizures (the pyrethroids can act as "proconvulsants") seen in severe type II poisoning. These effects occur only at doses higher than those required to affect sodium flux. Several other reports have suggested a role for the $GABA_A$ receptor-ionophore complex in components of type II pyrethroid toxicity [11, 19]. The mechanism whereby pyrethroids interact with ion channels is not known, but type II pyrethroids (i.e., deltamethrin, fenvalerate, cypermethrin) stimulate protein kinase C-dependent protein phosphorylation at as low a concentration as 10^{-13} M in vitro by a direct mechanism [20]. Since both sodium- and chloride-ion channel activities are modulated by phosphorylation state, this could be an important mechanism of action although pyrethroids are also capable of acting directly in systems with no phosphorylation capacity, but at somewhat higher concentrations [21]. Diazepam (a GABA agonist) prevented the seizures associated with late-stage poisoning in experimental animals [22] but was ineffective in fenvalerate-poisoned humans. Thus, it appears that the undoubted potential of type II pyrethroids to act at the GABA receptor is of limited clinical significance, other than in severe poisoning [12]. Type II pyrethroids antagonize the $GABA_A$ receptor (decreased synaptic inhibition), resulting in a strychnine-like effect. This is similar to that of organochloride insecticides.

3.3 Other Actions

Other channel and receptor systems in neuronal tissues have been proposed to play a role in the generation of compound-specific clinical symptoms in mammals. The complex nature of the effects of pyrethroids on the central nervous system (CNS) has led various workers to suggest that they also act via modulation of nicotinic cholinergic transmission, reduce peripheral presynaptic adrenoceptor sensitivity [23] which leads to an enhancement of noradrenaline release [24], and affect the serotonin neurotransmission [25]. However, because neurotransmitter-specific pharmacological agents offer only poor or partial protection against poisoning, it is unlikely that any one of these effects represents an alternative primary mechanism of action of the pyrethroids.

Other possible mechanisms of pyrethroid toxicity have been investigated. Inhibition of Ca^{2+}, Mg^{2+}-ATPase, and calcium-binding protein calmodulin has been observed with some pyrethroids.

In the scientific literature, two characteristics have been mentioned which are allegedly typical of pyrethroids and responsible for the health risks involved: (a) due to their lipophilic properties, pyrethroids are supposed to be capable of accumulating in nerve tissue, and (b) there is no evidence indicating that the nervous system is reversible or irreversible [26].

4 Pharmacokinetics/Toxicokinetics

Only few studies have been conducted to analyze the toxicokinetic properties of types I and II in laboratory animals [27–29]. The lipophilicity of pyrethroids also enables rapid access to tissues, including the CNS. Non-cyano pyrethroids (permethrin) as well as cyano pyrethroids (deltamethrin and cyhalothrin) have an accumulation in the nervous tissues [25, 28, 29]. The toxicokinetic behavior of these pyrethroids revealed prolonged elimination half-lives (typically in the order of 10 h but may be larger) and high concentrations in several regions of the brain and other nervous tissues of the peripheral nervous system. For example, for lambda-cyhalothrin, the peak concentration in hypothalamus (C_{max} 24.12 μg/g) and myenteric plexus (C_{max} 25.12 μg/g) was about 1.5 times higher than in plasma (C_{max} 15.65 μg/mL) and 1.3 times higher than in liver (C_{max} 18.42 μg/mL).

The metabolic pathways for the breakdown of the pyrethrins and pyrethroids vary little between mammalian species but vary somewhat on structure. Essentially, pyrethrins and pyrethroids can be hydrolyzed in the gastrointestinal tract. Once absorbed, they are rapidly metabolized by oxidation of methyl groups and aromatic rings, hydrolysis of the ester linkage, and by conjugation reactions producing a wide range of metabolites that are excreted mainly in urine (conjugated metabolites with glucuronide, glycine, taurine, sulfate, and/or glutamate). The advantageous properties of the synthetic pyrethroids result from the fact that they are hydrolyzed relatively easily, both in the mammalian body and in the environment too; consequently, bioaccumulation in the latter does not occur, and they do not persist in soils. There is some stereospecificity in metabolism, with *trans*-isomers being hydrolyzed more rapidly than the *cis*-isomers, for which oxidation is more important metabolic pathway [30]. These metabolic reactions take place in the liver, kidneys, and other organs, as well as to a minor extent in nerve tissue. Cleavage of the ester bond results in substantial reduction in toxicity. The presence of α-cyano group will decrease the rate of hydrolysis of the ester bond. Cleavage of the α-cyano group results in rapid conversion of the cyano group to thiocyanate. As a rule, the metabolic reactions are exclusively detoxification processes, but there are exceptions. Epoxychrysanthemates are formed metabolically [31] and retain moderate insecticidal activity.

5 Antiparasitic Activity

Pyrethrins and synthetic pyrethroids are used extensively for the treatment of a broad range of ectoparasites in large and small animals, and in nonmammalian animal species (birds, fish, and honeybees). The "family member" of the pyrethroids is used in veterinary medicine to control fleas (*Ctenocephalides felis*, *Ctenocephalides canis*, *Haematobia irritans*, *Stomoxys calcitrans*, *Musca* species, *Hydrotaea irritans*, *Pulex irritants*), lice (*Trichodesctes canis*, *Damalinia bovis, Solenopotes capillatus, Linognathus vituli, Haematopinus eurysternus*, *Haematopinus suis*), ticks (*Ixodes ricinus*, *Ixodes hexagonus*, *Rhipicephalus sanguineus*, *Rhipicephalus turanicus*, *Dermacentor reticulatus*), and mites (*Otodectes cynotis*), between other infestations both outside and inside the house. Insects and acari [an order of the Class Arachnida (subphylum Chelicerata) comprising of mites and ticks] are distributed worldwide, and they are vectors of important diseases in animals and humans. Moreover, several of these insect- and acari-borne infections influence zoonotic potential and may produce serious infections in persons. The antiparasitic activity of pyrethroids has been linked to their ability to affect the sodium channel, but chloride and calcium channels are also affected. Specifically, electrophysiology studies have confirmed that pyrethroids slow the kinetics of opening and closing of sodium channels at the molecular level, thus impacting the insect's nervous system by slowing action potential decay [32]. The duration of the sodium action potential is much longer for type II pyrethroids than for type I. Type I pyrethroids result in primarily repetitive charges, and in type II, pyrethroid toxicity cell membrane depolarization is the main mechanism of action. Pyrethroids cause a phenomenon in insect called "knockdown" [10], which is caused by inhibiting the cell but not causing a lethal effect throughout ability of sodium channel to retain many of the functions such as selectivity for sodium ions and conductance after exposure to pyrethroids. The alliance of repellence and fast-kill of parasite is the most effective way of pyrethroids to reduce the risk of transmission of parasite vector and reservoir of infection.

Pyrethrins and synthetic pyrethroids have antiparasitic activities, but in general, pyrethroids have a broader spectrum than pyrethrins. The widespread and uncontrolled use of pyrethrins and pyrethroids might lead to environmental damage, insecticide and acaricide resistance in infected population, and a possible exacerbation of insect and arcari-borne population. The development of insect resistance can appear as a result of increased potency and especially increased persistence in the environment [33].

5.1 Ruminants

Insecticides and acaricides are periodically used to protect different ruminant species (cattle, sheep, and goat) from the attachment and blood sucking of various tick species which are known to be important vectors to transmit a broad spectrum of agents of diseases in particular cattle or sheep. Pyrethroid insecticides such as cyfluthrin, deltamethrin, fenvalerate, flumethrin, lambda-cyhalothrin, and permethrin had been successfully tested on their activity against a broad spectrum of insects, although their activity against ticks has been occasionally examined. Differences were found in the susceptibility of different species of insect and arcari (e.g., mites and ticks) certainly due to different long-reaching efficacy of insecticides on all treated species. One example is deltamethrin that is active against insects and ticks. The efficacy of deltamethrin pour-on against nymphs and adult ticks (*Ixodes ricinus* and *Rhipicephalus sanguineus*) in treated hair of cattle and sheep has been proven. *I. ricinus* adults and nymphs died from at least being exposed to hair taken even at 4 weeks after application (from backside to feet) of sheep; member of the species *R. sanguineus* may survive if ticks come into contact with treated hair beginning 3 weeks after treatment. While protection on the back is rather good for 3 weeks, the effects are less good if the hair of the feet comes into contact with the ticks. In cattle, the efficacy against *Rhipicephalus* is lower than in sheep. With respect to protection from insect vectors such as *Culicoides* species (vector of bluetongue disease), it was recommended to use deltamethrin at intervals of 3 weeks [34] although few insecticidal veterinary medicines have authorized claims regarding use against *Culicoides* species of insects. To control the *Culicoides*, alpha-cypermethrin has been used and applied to cattle and sheep against the biting midge *Culicoides nubeculosus* (Diptera: Ceratopogonidae). It is not known if commercial pour-on insecticides, which are most commonly applied along the dorsum of ruminant livestock, give sufficient coverage to protect animals from biting midge at potential sites of *Culicoides* feeding such as the belly and legs. Alpha-cypermethrin has shown convincing evidence that the commercial formulations tested on sheep and cattle will be at least partially effective in reducing onward transmission of bluetongue virus [35].

5.2 Horses

Culicoides biting midges can occur in large number on horses, and their bites have been linked to an immediate-type hypersensitivity reaction which causes a chronic skin disease (known as "sweet itch"). In general, *Aedes aegypti* and *Culex quinquefasciatus* appeared to be less susceptible to cypermethrin than *Culex nubeculosus*, and the attenuation of the toxic effect declined more quickly with time after treatment. There were differences in the toxicity of hair from different body regions, with hair from the back consistently inducing the highest mortality and hair from legs the lowest; this effect was more pronounced for *C. nubeculosus* than *A. aegypti* or *C. quinquefasciatus* [36].

5.3 Pet Animals

Fleas and ticks on dogs and cats usually have a widespread prevalence. For instance, ticks are known to transmit viruses, bacteria, fungi, protozoa, and nematodes, with every genus and species possessing its own specific germ flora. As blood sucking, adult fleas and all stages of ticks penetrate the skin of their hosts with their sucking mouthparts and inject saliva during the feeding process. This permit adult fleas and both immature and adult ticks to transmit disease agents throughout their saliva. One of the most important ticks in companion animals is *Dermacentor reticulatus* which achieves its main significance from being a vector of *Babesia canis* which produce the canine babesiosis. To minimize the risk of tick-transmitted diseases, a compound should ideally prevent tick attachment or should kill attached adult ticks larvae and nymphs rapidly, and permethrin has been used successfully for the prevention and control of many arthropod species.

Leishmaniasis is a group of zoonotic diseases transmitted to human and animals by the bite of phlebotomines and flies. *Leishmania infections* in dogs have great health importance as dogs are the reservoir hosts of zoonotic visceral leishmaniasis caused by *Leishmania infantum*. To control canine leishmaniasis, strategies have been proposed: cut transmission by treating domestic dogs with insecticides, lotions, or insecticide-impregnated dog collars originally developed for flea and tick control [37]. The anti-feeding and lethal effects of deltamethrin-impregnated dog collars have been tested, and this pyrethroid protects the dogs from sandfly-borne diseases when used topically [38]. For full efficacy, deltamethrin collar should be put on approximately 2 weeks before an anticipated sandfly challenge; the effects of deltamethrin on sandfly blood feeding and survival persisted for up to 8 months.

By contrast, sandfly blood feeding and survival rate of both feed and unfed flies were significantly reduced by permethrin-treated dogs after 1 week suggesting topical application of permethrin lotion can have a relatively immediate effect [38].

5.4 Birds

The northern fowl mite, *Ornithonyssus sylviarum* (Arcari: Macronyssidae), is an obligatory blood-feeding ectoparasite (hematophagous mite), which is considered to be one of the most important and common pests of poultry. This mite is sensitive to lambda-cyhalothrin, cypermethrin, fenvalerate, and permethrin. Due to short residue time, many registered compounds often need to be repeated after 2–4 weeks. Residue time of an insecticide on feather depends not only on the nature of but also on the formulation of the drug [39].

Most of the available pyrethroids applied in the northern fowl mite control are usually formulated as wettable powder or emulsifiable concentrate. For these two formulations, dilution with water before spraying is required. Since water has very low affinity to feathers, a great quantity of dilution of pyrethroids will fall on the ground, feed bin, or the cage, and some quantity will drift in the air during the process of spraying. An oil solution of pyrethroids applied has a higher affinity on the feathers of the birds and can stay on the feather for a longer time, and may provide a high efficacy and be effective for a longer, persistent period against the northern fowl mite. Lambda-cyhalothrin oil solution has a potential to become an effective and safe formulation to control northern fowl mite in breeders.

6 Veterinary Applications (Uses)

Pyrethrin and pyrethroid insecticides are effective against a variety of insect pests on companion animals and livestock, and are used on farms, in the home, and garden and have many public health implications because of the safety associated with these compounds. The great potential of pyrethroids for the control of flies, tsetse flies, fleas, and ticks is known.

Concentrations of pyrethrins range from 0.05% to 0.2% in the ready-to-use products. Concentrates are marketed for dilution as a pour-on and are not greater than 2%. A practical method of providing a pyrethrin spray for a large dog is the use of a pour-on concentrate diluted in a commercial lawn/garden pressure sprayer. Many of pyrethrin-based products incorporate the use of synthetic pyrethroids (permethrin, resmethrin, sumithrin, D-*trans*-allethrin) which are added predominantly for residual effects. Permethrin is by far the most common pyrethroid used [40] in a range from 0.2 to 71.5 [4].

Most chemotherapeutic agents registered and labeled for use as external parasiticides for large animals are pyrethroids. Pyrethroids used in veterinary medicines for domestic animals are listed in Table 137.1. For some spot-on products, particularly those intended for use on large animals and containing active ingredients

Table 137.1 Pyrethroids used in veterinary medicines for domestic animals

Pharmacologically active substance	Formulation	Animal species	Indications	Doses
Alpha-cypermethrin	Topically (spray, pour-on)	Bovine, ovine	Ectoparasites (ticks, fleas, lice, blowflies)	150 mg/animal
	Spray	Poultry	Ectoparasites	8–10 mg/bird
Cyfluthrin (8 enantiomers)	Topically (pour-on)	Bovine, caprine	Ectoparasites (horn fleas, horse flies)	100 mg/animal cattle
Cyhalothrin (contains two pairs of *cis*-isomers A and B (ratio 60/40)	Topically (pour-on, spray, dip tanks)	Bovine, pigs, and sheep (including dairy animals)	Ectoparasites (horn and face flies)	*Pour-on*: 0.2 mg/cattle, 0.1 mg/sheep or pigs
				Sprays (up to 250 mg/animal/treatment)
				Dip tanks (up to 50 g/100 L of water)
Cypermethrin (contains a mixture of 4-*cis* and 4-*trans*-isomers) (isomer ratio 40:60)	Topically	Cattle, sheep, goats, pigs, chickens including laying birds and lactating cattle, sheep, and goats	Ectoparasites (ticks, fleas, lice, blowflies)	
		Salmonidae	Treatment and control of parasites such as sea lice in *Salmonidae*	Seawater cage at a dose of 5 µg/L of seawater for a period of 1 h
Deltamethrin (90% *cis*-isomer)	Topically (dip, spray, pour-on)	Cattle, sheep, chickens	Ectoparasites (flies, including tsetse flies)	*Cattle*: 0.25–1.5 mg/kg bw as a single application or as repeated application (every 3–6 weeks)
				Sheep: 0.94–4.5 mg/kg bw as a single application, repeated 10 or 21 days later
				Chicken: 0.08 mg/kg bw
	Bath	Fin fish (Atlantic salmon, rainbow trout)	Ectoparasites treat sea lice (*Lepeophtheirus salmonis* and *Caligus elongatus*)	Bath dose 2 µg/L for 30 min in a closed container or 3 µg/L for 40 min in a particular closed sea cage

(*continued*)

Table 137.1 (continued)

Pharmacologically active substance	Formulation	Animal species	Indications	Doses
Flumethrin	Topically (pour-on, as a plunge dip)	Bovine, ovine	For control of ticks, lice, mites, and scab	*Pour-on*: 2 mg/kg bw *Plunge dip*: 1 L product in 900 L of water
		Honeybees	Diagnosis and treatment varroatosis in honeybees (3.6 mg are hung in beehives)	*Strips*: 4 strips/hive for mature colonies and 2 strips/hive for immature colonies
Permethrin (isomer ratios of *cis:trans*: 80:20, 40:60, or 25:75)	Spray (including udder spray), powders, pour-on, ear tag	Cattle, pigs, sheep, goats, poultry	Ectoparasites (horn and face fleas, mites, ticks, and lice)	4 mg/kg bw for cattle ≈6 mg/kg bw for pigs, sheep, and poultry
Tau-fluvalinate (contains two of four isomers of racemic mixture, fluvalinate)	Topically treatment	Honeybees	Parasitic mite *Varroa jacobsoni*	Polymer matrix strip (8 g in weight containing 800 mg tau-fluvalinate suspended midway for 6–8 weeks

such as synthetic pyrethroids, gloves are recommended as the quantities involved are larger than with companion animal products, and a large number of animals are likely to be treated.

6.1 Uses in Ruminants

The treatment and prophylaxis for cattle, sheep, and goat ectoparasites involves the use of ectoparasiticidal formulations containing insecticides/acaricides. There are several methods of applying these insecticides: plunge dipping (immersion) into water containing those chemical products, shower dipping, jetting races, pour-ons (actually sprays in common parlance) by application of chemical products along the vertebral column of the animals, and injection. Products available for use in sheep dips contain ectoparasiticidal solutions must to kill the larvae of green and blue bottle flies responsible for flystrike and to control sheep scab and keds. Up until 1985, lindane was the main substance used in sheep dips, but although it is less persistent in soil, lindane was banned throughout the EU, largely on environmental grounds. Dips containing synthetic pyrethroids (flumethrin and cypermethrin) have become more widely used, but the environment is targeted. Pour-ons containing pyrethroids are available and control a range of ectoparasites but not

Psoroptes ovis. None of the pour-on products provides protection against the range of ectoparasites that is achieved by plunge dipping.

In lactating dairy cows, only those veterinary medicinal products that are not excreted in the milk are approved for use in this category of animals. Fenvalerate, permethrin, and pyrethrins may be applied directly or throughout tags impregnated with permethrin or fenvalerate attached to the ears of lactating dairy cows. These pyrethroids usually need a withdrawal period when being used for human consumption, and the period may be as short as zero days or as long as several days. All parasiticides approved for lactating dairy cattle may also be used on non-lactating dairy cattle and beef cattle. The same restrictions are applied to their use on lactating dairy sheep and goats.

Sheep and goat (except when their milk is being used for human consumption) and non-lactating sheep and goats may be treated with products that contain the following: fenvalerate, permethrin, and pyrethrins. Minimum time from treatment to slaughter varies from 0 to 60 days. Fenvalerate and permethrin are marketed in spray and pour-on formulations and are used for control of the target parasites lice, mites, keds and flies, ticks, and blowflies, respectively.

In the cattle, the following pyrethroids are used:

- Cyfluthrin is approved as ear tag for use on beef cattle to control horn flies, face flies, Gulf Coast ticks, and ear ticks. The ear tags containing cyfluthrin should be removed at the end of fly season and before slaughter.
- Cyhalothrin is marketed as ear tag and pour-on formulations to control horn flies and face flies.
- Cypermethrin is a potent synthetic pyrethroid which is available as an ear tag in combination with chlorpyrifos. It is used on beef and dairy cattle to control horn and face flies, Gulf Coast ticks, and ear ticks and helps control stable flies, houseflies, and lice.
- Fenvalerate is characterized to be very photostable and potent being marketed as ear tag and pour-on formulations; the ear tags are used in dairy and beef for control of the target parasites (i.e., horn and face flies, Gulf Coast ticks, and ear ticks), and as an aid in the control of stable and houseflies, and lice. It can also be applied to livestock buildings for control of filth flies *Musca domestica* and *Fannia cannicularis*.
- Permethrin is marketed in spray (liquid or wettable powder), dust, ear tag, and pour-on formulations and is used in cattle being the target parasites horn flies, face flies, stable flies, back flies, houseflies, ticks, mites, and lice. Permethrin is available as ear tag formulation in combination with chlorpyrifos, and the target parasites are the same for permethrin.

The pyrethroids marketed as ear tags must be removed from the animals before slaughter.

6.2 Uses in Horses

Horses that are not to be used as food for human consumption may be treated with products that contain fenvalerate, permethrin, phenothrin, and pyrethrins.

Fenvalerate is marketed as spray formulation, and the target parasites are horn flies, face flies, stable flies, and houseflies. Permethrin is marketed as spray, wipe, and dust formulations, and the target parasites are horn and face flies, horseflies, stable flies, deer flies, mosquitoes, biting gnats, and ticks. Pyrethrins are marketed as spray, and the target parasites are the same than for permethrin. Ticks are capable of surviving in the environment for prolonged periods (e.g., more than 300 days without feeding), and some are not strictly species-specific and are adapted to several animal species being the control on the wet season and in some area throughout the year.

6.3 Uses in Swine

Pigs may be treated with products that contain fenvalerate, permethrin, and pyrethrins. Fenvalerate is marketed as spray and pour-on formulations and can be applied to control the target parasites lice (pour-on), mites, and flies, and permethrin is marketed in spray, paint, dip, and dust formulations, and the target parasites are lice, mites, ticks, and horn flies.

6.4 Uses in Nonmammalian Species

Fish. The only available pyrethroids intended for the treatment and control of parasites such as sea lice (*Lepeophtheirus salmonis* and *Caligus elongatus*) in *Salmonidae* are cypermethrin and deltamethrin. Usually, the diseased fish is treated with veterinary drugs either soluble or suspended in the water. The recommended doses (route and dose rate) for both pyrethroids are listed in Table 137.1.

Honeybees. Honeybee diseases are predominantly infectious and parasitic conditions accentuated by the close confinements in which they congregate, either in man-made hives or in colonies in a natural cavity. One of the most important parasitic diseases is the *Varroa* mite where pyrethroids such as flumethrin in the form of impregnated strips or tau-fluvalinate in topical application of honeybees is used. Two particularly important features of flumethrin thus formulated are the differential toxicity of the drug to the bees and their parasitic mites and the potential problem of drug residues in honey. In honey, we know there is massive variability not only between hives in the same apiary but also between frames in the same hive. Bees have a practice of moving honey around the hive as required, and this can lead to significant variations in residue concentration even across the same frame in all three dimensions. With regard to drug residues in honey, no migration of the acaricide from the plastic strip has been recorded, and levels in honey are below the limit of detection. The product may, however, be absorbed into the wax of the honeycomb [41]. If the product leaves residues in wax, it is generally assumed that honey and wax from the treated colonies are consumed in the proportions 9:1. Residues in both honey and wax need to be considered in exposure estimates (20 g honey for EU and 50 g for Codex Alimentarius, amounts assumed to be consumed daily by a 60-kg person).

6.5 Uses in Pet Animals

A wide variety of ectoparasiticides is available, and brand switching is frequent, which is an indication of the problems faced in achieving acceptable parasite control. Concentrated ectoparasiticidal dermal spot-on products are used for the control fleas and ticks and are commonly used on household pets such as dogs and cats. Synthetic pyrethroids such as permethrin and deltamethrin are extremely effective against fleas and other ectoparasiticides in the dog [42], but because of their adverse effects in cats, they are usually contraindicated for this species.

Pyrethrins are used noteworthy for rapid "knockdown," paralysis, and killing of arthropods, an effect of pyrethrin II that is further potentiated in a concentrated organic solvent such as alcohol. Unfortunately, alcohol sprays have disadvantages, particularly for use in animals with flea allergy already having dry, sensitive, and irritated skin and in cats [40]. Concern for product decay should be emphasized if solutions are left diluted for greater than 24 h. Concentrations of pyrethrins range from 0.05% to 0.2% in the ready-to-use products. Concentrates are marketed for dilution as a pour-on and are not greater than 2%. A practical method of providing a pyrethrin spray for a large dog is the use of a pour-on concentrate diluted in a commercial lawn/garden pressure sprayer. Many of pyrethrin-based products incorporate the use of synthetic pyrethroids (permethrin, resmethrin, sumithrin, D-*trans*-allethrin) which are added predominantly for residual effects. Permethrin is by far the most common pyrethroid used [40].

The following compounds are used in dogs and cats:

Pyrethrins: Some formulations contain piperonyl butoxide (synergist), *N*-octyl bicycloheptene dicarboximide (synergist), di-*n*-propyl isocinchomeronate (repellent), permethrin, carbaryl, or rotenone. The marketed formulations are spray, foam, dust, shampoo (0.2%), dip, or ear drops, and the target parasites are mainly fleas, ticks, mites, and lice (some products).

Allethrin: This first-generation pyrethroid is a mixture of several optical isomers. It is used in the control of flies, mosquitoes, ticks, and fleas in infested animal quarters.

D-trans-Allethrin: Some formulations contain piperonyl butoxide (synergist), *N*-octyl bicycloheptene dicarboximide, or sumithrin. The marketed formulation is shampoo, and the target parasites are fleas and ticks.

Permethrin: Is an extremely active insecticide with rapid "knockdown" effect against a variety of insects (fleas and ticks). Permethrin is marketed in a diversity of formulations such as collar (0.8–1.5%), topical concentrate (spot-on), spray (0.2–1%), shampoo (1%), dip, or cream rinse. Some formulations of permethrin contain furthermore piperonyl butoxide, *N*-octyl bicycloheptene dicarboximide, di-n-propyl isocinchomeronate, pyrethrins, pyriproxyfen, or butoxypolypropylene glycol (repellent).

Tetramethrin: This compound is available in a total release fogger in combination with permethrin to kill cockroaches and fleas in pet sleeping areas. Moreover, it is used in dogs as solution (at concentration of 0.6%) and as shampoo (at concentration of 0.2%).

Resmethrin: This pyrethroid shows excellent “knockdown” effect and is marketed for use in sprays and shampoo, fleas and ticks being the target parasites.

Other pyrethroids used in dogs and cats are cyfluthrin, cypermethrin (solution, 5%), deltamethrin (collar, 4–4.5%), fenvalerate (solution 6%, to dilute in water), flumethrin (collar, 4%), and phenothrin (shampoo, 0.25%) [4]. Flumethrin (4.5%) in combination with imidacloprid (10%) as a neonicotinoid has been introduced as insecticidal, acaricidal and repellent in collars for dogs (from 7 weeks of age) and cats (from 10 weeks of age); this combination is effective against fleas (*Ctenocephalides felis* and *canis*) and *Pulex irritants* of dogs and cats, ticks of dogs and cats and for treatment of biting/chewing lice (*Trichodectes canis*) infestation of dogs.

To reduce exposure, keep pets indoors during and for about 30 min after spraying. Pets that remain outdoors could be exposed to small amounts of pyrethroids, but would not be expected to experience adverse health effects from spraying. These compounds should not be applied to kittens less than 4 weeks old or the suckling puppies.

7 Toxicity

7.1 Toxicity in Domestic Animals

Dermal shampoos, spot-on type products, and collars containing insecticidal ingredients for the control of fleas and ticks are habitual on household pets. Cats are more likely than dogs to develop pyrethroid toxicosis. Cats, especially young animals less than 6 weeks of age, are particularly sensitive to the toxic effects of synthetic pyrethroids as they have low ability to conjugate these compounds through glucuronide conjugation. Most brands of permethrin spot-on products are restricted to use in dogs only. Inappropriate use of permethrin spot-on on cats can cause severe toxicity. These permethrin compounds may be obtained over the counter in grocery stores or pet stores. Permethrin toxicity usually occurs when the owner applies the dog spot-on product highly concentrated (45–65%) to the cat; however, cats which actively groom or engage in close physical contact with recently treated dogs may also be at risk of toxic exposure. The severity of permethrin toxicity varies with each individual animal. Some cats develop signs when only “one drop” is applied, while others show no clinical signs after an entire vial is used. Overall, overdosing small pets is easier than overdosing large pets due to the larger body surface to weight ratio of smaller pets [43]. Animals with small body mass and high hair density are more susceptible to poisoning due to the great surface area created by the hair. If dogs are accidentally sprayed in the mouth, the risk for poisoning increased from 60- to 150-fold. Onset of clinical signs is usually within a few minutes to hours after exposure but may be delayed up to 24 h (i.e., as a result of prolonged exposure from dermal absorption or grooming), and effects lasted in some cases for 3 days or more. The most common clinical signs of permethrin toxicity in cats are muscle tremors and seizures. Hypersalivation, depression, vomiting, anorexia, and even death may also be seen. Most cats affected by pyrethroid poisoning are presented as an emergency; the patient should be stabilized if hypothermia or hyperthermia is present [4].

In a retrospective study of 87 cases of pyrethrin and pyrethroid intoxication in cats, central neuropathies were reported to be the most common clinical signs [44]. Central neuropathies manifested primarily as hyperexcitability, tremors, or convulsions and occurred in 69% of intoxicated cats. Skeletal muscular weakness and fasciculations, which are signs of peripheral neuropathies, occurred in 28% of affected cats. Clinical signs were evident only in cats younger than 4 years of age, with more than half the intoxicated cats younger than 12 months. In a review of 286 cases reported to the Veterinary Poisons Information Service regarding inappropriate feline exposure to permethrin spot-on preparations, 96% were symptomatic. Increased muscular activity was common and occurred in 87% of cases. Death occurred in 10.5% of cases [45].

The clinical signs of pyrethroid acute toxicity are similar in dogs, cats, and large animals. The main circumstances of poisoning are excessive use of flea control sprays or dips which can lead to overdosed dermal exposure or accidental ingestion of concentrated pesticides by livestock. Clinical signs observed in pyrethroid intoxication include salivation, vomiting, hyperexcitability, hyperesthesia, tremor and seizures, dyspnea, prostration, and death. The signs begin in all animal species within minutes to hours (24–72 h) of exposure, depending on the route of exposure. Clinical signs generally last 2–3 days [4].

7.2 Toxicity in Aquatic Organisms

Pyrethrins and pyrethroid products are highly toxic for fish. It is known that the relatively high toxicity of pyrethrum and pyrethroids to fish and invertebrates is in part due to high density of specific binding sites that are much less prevalent in higher vertebrates. Moreover, toxicity of pyrethrum and pyrethroids was indirectly related to temperature (i.e., greater insecticidal effect when the temperature is lowered) and directly related to pH and water hardness [46]. However, the biological activity of several pyrethroids was not significantly altered by pH. In several countries, sheep are treated for various infestations of external parasites by immersion (plunge dipping). The practice of treating sheep in this way has been carried out for over 100 years. In EU, the number of active substances used in sheep dips has been reduced over the years. There were only two left on the market: diazinon, an organophosphorus compound, and cypermethrin. Both of these are acutely toxic to nontarget organisms in the environment particularly invertebrates and fish [47]. In general, pyrethrins and pyrethroids bind tightly to particulate organic matter, and also pyrethrins bind tightly to soil and rely, to a large extent, on photolysis for degradation. The pyrethroids in aquatic acute toxicity studies can reach LC_{50} values of less than 1 μg/L, and can cause chronic toxicity at concentration as low as 0.01 or even 0.001 μg/L. Pyrethroids dumped into dishes can kill fish and other water organisms.

8 Maximum Residue Limits and Drug Withdrawal/ Withholding Periods

A critical factor in the medication of all food-producing animals is the mandatory withdrawal/withholding period, defined as the time during which drug must not be administered prior to the slaughter of the animal for consumption. The withdrawal period is an integral part of the regulatory authorities' approval process and is designed to ensure that no significant drug residue is present in the animal at slaughter. Drug residues in food-producing animals should comply with the MRL values for their target tissues in the animal species. The withdrawal period is intended to ensure that no harmful residues remain in edible tissues (muscle, liver, kidney, skin + fat, milk, eggs, or honey) after slaughter, and it is usually established as the slaughter time when residue levels in all the edible tissues are below the MRL. Adherence to the withdrawal period provides assurance that food derived from treated animals will not exceed the MRL (termed "tolerances" in the USA) for the drug substance. Failure to keep the preslaughter withdrawal period while using animal drug is the major cause of violation of permissible drug residues in animal meat in the EU. Even if the withdrawal period involves only a few days or a few hours, the resulting residues can violate the national regulations against sale of adulterated foodstuffs which can originate distortions of competition between member states of the EU. Human food safety can have a significant impact on the withdrawal time of a drug which might make it unacceptable in the market [48].

Article 14(7) of Regulation (EC) No. 470/2009 [49] states "where it appears necessary for the protection of human health, the classification shall include conditions and restrictions for the use or application of a pharmacologically active substance used in veterinary medicinal products which is subject to a MRL, or for which no MRL has been set" (e.g., not for use in animals from which milk or eggs are produced for human consumption).

Under EU legislation (Article 14(2) of Regulation (EC) No. 470/2009) [49], the classification of pharmacologically active substances shall also establish, in relation to each such substance, and, where appropriate, specific foodstuffs or species, one of the following: (1) an MRL, (2) a provisional MRL (pending further data), (3) the absence of the need to establish an MRL, and (4) a prohibition on the administration of a substance. Those substances included in Annex I, II, or III of Council Regulation (EEC) No. 90/2377 [50] are listed in the Annex of Commission Regulation (EC) No. 37/2010 [51] (Table 137.1, allowed substances, where the pharmacologically active substance, marker residue, animal species, MRL value, target tissues, other provisions (according to Article 14(7) of Regulation (EC) No. 470/2009) [49], and therapeutic classification are listed, and Table 137.2 (prohibited substances) (where an MRL cannot be established)). This classification substitutes the four annexes of Council Regulation (EEC) No. 2377/90 [48].

The EU MRL values for pyrethroids in food-producing animals are listed in Table 137.2.

Table 137.2 EU MRLs for pyrethroids as antiparasitic agents [Regulation (EU) No 37/2010 of 22 December 2009]

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Alpha-cypermethrin	Cypermethrin (sum of isomers)	Bovine, ovine	20 µg/kg	Muscle	For milk MRL further provisions in Commission Directive 98/82/EC are to be observed
			200 µg/kg	Fat	
			20 µg/kg	Liver	
			20 µg/kg	Kidney	
			20 µg/kg	Milk	
Cyfluthrin	Cyfluthrin (sum of isomers)	Bovine, caprine	10 µg/kg	Muscle	For milk further provisions in Council Directive 94/29/EC are to be observed
			50 µg/kg	Fat	
			10 µg/kg	Liver	
			10 µg/kg	Kidney	
			20 µg/kg	Milk	
Cyhalothrin	Cyhalothrin (sum of isomers)	Bovine	500 µg/kg	Fat	For milk further provisions in Council Directive 94/29/EC are to be observed
			50 µg/kg	Kidney	
			50 µg/kg	Milk	
Cypermethrin	Cypermethrin (sum of isomers)	All ruminants	20 µg/kg	Muscle	For milk further provisions in Commission Directive 98/82/EC are to be observed
			200 µg/kg	Fat	
			20 µg/kg	Liver	
			20 µg/kg	Kidney	
			20 µg/kg	Milk	
		Salmonidae	50 µg/kg	Muscle and skin in natural proportions	

(*continued*)

Table 137.2 (continued)

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions	
Deltamethrin	Deltamethrin	All ruminants	10 μg/kg	Muscle	No entry	
			50 μg/kg	Fat		
			10 μg/kg	Liver		
			10 μg/kg	Kidney		
			20 μg/kg	Milk		
		Fin fish	10 μg/kg	Muscle and skin in natural proportions		
Flumethrin	Flumethrin (sum of trans-Z-isomers)	Bovine	10 μg/kg	Muscle	No entry	Antiparasitic agents/ agents against ectoparasites
			150 μg/kg	Fat		
			20 μg/kg	Liver		
			10 μg/kg	Kidney		
			30 μg/kg	Milk		
		Ovine	10 μg/kg	Muscle	Not for use in animals from which milk is produced for human consumption	
			150 μg/kg	Fat		
			20 μg/kg	Liver		
			10 μg/kg	Kidney		
	Not applicable	Bees	No MRL required	Not applicable	No entry	
Permethrin	Permethrin (sum of isomers)	Bovine	50 μg/kg	Muscle	For milk further provisions in Commission Directive 98/82/EC are to be observed	Antiparasitic agents/ agents against ectoparasites
			500 μg/kg	Fat		
			50 μg/kg	Liver		
			50 μg/kg	Kidney		
			50 μg/kg	Milk		
Tau-fluvalinate	Not applicable	Bees	No MRL required	Not applicable	No entry	No entry

9 Conclusion/Prospects

Pyrethrins and synthetic pyrethroids are considered as very effective insecticides and acaricides for the treatment of a broad range of ectoparasites in veterinary medicine such as fleas, flies, mite, lice, and ticks between other infestations both outside and inside the house. Insects and acari are distributed worldwide, and they are vectors of important diseases in animals and humans. Pyrethrins and synthetic pyrethroids are available in a large number of formulations, in particular spot-on, sprays, dusts, dips (immersion), shampoos, and ear tags. Pyrethrins and pyrethroids are neurotoxic as a result of their availability to bind to sodium channels in the nerve membrane and cause repetitive nervous discharges or membrane depolarization. All pyrethroids share the same basic mechanism of action, but the different types cause somewhat different symptoms. In general, pyrethroids have a broader spectrum, and their residual activity persists longer than that of the natural pyrethrins. Clinical signs associated with acute poisoning with pyrethroids are related to nervous system stimulation. These chemicals are generally of low toxicity to mammals and birds; however, they exert potent acute toxicity to arthropods and are highly toxic for fish. Fish and cats represent the most susceptible animals to pyrethroids. In fish, at the recommended doses, deltamethrin and cypermethrin can be safely used. The concomitant use of pyrethrins and pyrethroids with synergists may increase toxicity by mechanisms involving inhibition of microsomal oxidation. In addition, pyrethroids cause induction and/or inhibition of cytochrome P450 enzymes which may lead to an enhancement of the toxicity and drug interactions. Pyrethrins and pyrethroids should not be used in dogs and cats <6 weeks of age, and when both compounds are utilized as spray, formulations should be used in well-ventilated rooms because inhalation can cause serious adverse effects.

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Essential Oils in Insect Control 138

Catherine Regnault-Roger

Contents

1 Introduction 4088
2 Essential Oils: Very Complex Natural Mixes 4089
3 Insecticidal Activities of EOs 4091
3.1 Diversity of Activities on Insect Targets and Routes of Exposure 4091
3.2 Mechanisms of Action 4096
4 Trends for the Development of EOs as BCAs 4097
4.1 Benefit-Risk Ratio for Environment 4097
4.2 Benefit-Risk Ratio for Human and Animal Health 4098
4.3 Commercial Prospects and Impediments 4099
5 Conclusion 4101
References 4102

Abstract

The societal claim for a friendly environmental use of pesticides today implicates to promote alternative solutions for a better and relevant using of chemicals. Because they have a broad spectrum of uses, essential oils (EOs) have many industrial applications. They are used now in plant protection and as biocide. They occupy a significant place among insect pest biocontrol agents (BCAs) and represent a consistent part within the market of botanicals used as alternative to chemicals. After phytochemical considerations, this chapter

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presents the wide range of activities of EOs on insect. Trends and prospects including a discussion about their advantages for an ecological friendly approach but also the factors that impede their commercial development conclude.

Keywords

Benefit-risk ratio • commercialization • essential oils • insect control • mechanisms of action • monoterpenes • phytochemistry • regulation • toxicity

Abbreviations

AOPWIN	Atmospheric oxidation program for Microsoft Windows
BCAs	Biocontrol agents
CAGR	Compound annual growth rate
cAMP	Cyclic adenosine monophosphate
CSPs	Chemosensory proteins
EOs	Essential oils
GABA	Gamma-aminobutyric acid
GOBPs	General odorant binding proteins
GS-FID	Gas chromatography-flame ionization detector
GS-MS	Gas chromatography–mass spectrometry
IPM	Integrated pest management
LC_{50}	Lethal concentration 50
LD_{50}	Lethal dose 50
OBPs	Odorant binding proteins
PPP	Plant protection product
UIPP	Union des industries de la protection des plantes
USD	US dollar

1 Introduction

Because they have a broad spectrum of uses, essential oils (EOs) have many industrial applications in perfumery, cosmetics and detergents, pharmacology, and fine chemistry as well as aromatics for the food industry. They also occupy a significant place to control insect pests and in fact are presently considered to be among most efficient botanicals used as alternative to chemicals. It exists today a societal claim for a friendly environmental use of pesticides that implicates to promote alternative solutions for a better and relevant using of chemicals. EOs take place in the market of biopesticides or BCAs which is expecting to increase to $3.3 billion in 2014 for a 5-year CAGR of 15.6 % (BCC Research; www.bccresearch.com). However, progress has to be done before biopesticides will share with synthetic pesticides the PPP (plant protection products) global market which is estimated at 38,318 billions of USD for 2010 with around 25 % represented by insecticides (9,985 billion USD) [1].

EOs play in fact an important role in the protection of the plants in agricultural and nonagricultural areas (e.g., orchards, gardens). They have a wide range of activities (insecticide, antifeedant, or repellent) against bacteria, virus, and fungi, and also against insects. This chapter is focused on the uses of EOs as biopesticides (BCAs) to control pest insect, the challenges they face, and the factors that nowadays impede their development. After defining the essential oils and their main chemical components, it details their relevant uses to control insect pest insects, including a discussion about their advantages for an ecological friendly approach. Trends and prospects conclude this overview.

2 Essential Oils: Very Complex Natural Mixes

The essential oils have been used since centuries. The first distillation of these products was mentioned during the thirteenth century in Andalusia after which their pharmacological properties induced their inclusion into the very early Pharmacopoeias of several European countries [2].

EOs are biosynthesized by aromatic plants belonging to a few families. They are particularly abundant in conifers, Rutaceae, Umbelliferae, Myrtaceae, Lamiaceae, and Lauraceae. Depending on the species and families, they are localized in specialized histological structures: glandular trichomes (Lamiaceae), secretory canals (Myrtaceae), or resin ducts (Apiaceae). They could be stored in different parts of the plant such as flowers (e.g., *Citrus bergamia*), leaves (*Citronella* spp.; *Eucalyptus* spp.), wood (*Santalum* spp.), roots (*Chrysopogon zizanioides*), or seeds (*Myristica fragrans*) [3].

Essential oils appear to be very complex natural mixes. Terpenoids are major constituents of EOs and, to a lesser amount, phenylpropanoids. EO constituents belong mainly to two phytochemical groups of terpenoids: monoterpenes and sesquiterpenes of low molecular weight. They generally consist of several tens of constituents of which the great majority possess an isoprenoid skeleton. Most of the compounds have ten atoms of carbon (monoterpenes) and 15 atoms of carbon (sesquiterpenes) or more rarely 20 atoms of carbon (diterpenes). Monoterpenes present in EOs may contain terpenes that are hydrocarbons (α-pinene), alcohols (menthol, geraniol, linalool, terpinen-4-ol, p-menthane-3,8-diol), aldehydes (cinnamaldehyde, cuminaldehyde), ketones (thujone), ethers (1,8-cineole e.g., eucalyptol), and lactones (nepetalactone). As the elongation of the chain to 15 carbons increases the number of possible cyclizations, sesquiterpenes have a wide variety of structures (over 100 skeletons). Aromatic compounds are less common and are derived mainly from the shikimate pathway. Some are typical of EOs of particular species, for example, vanillin (*Vanillia* spp.) or estragol (*Artemisia dracunculus* L.). Some compounds identified in EOs result from the degradation of fatty acids (jasmonic acid) or are glycosylated volatile compounds (e.g., linalool glucoside) [3].

The majority of EOs contains a limited number of main compounds, but some of the minor compounds play an important role as vectors of fragrance and make up the richness of an extract. It is thus well established that essential oil composition is very variable depending of the species and of chemotypes within the species and also physiological parameters. As examples, the EO of eucalyptus (*Eucalyptus globulus* Labill.) is characterized by a monoterpene 1,8-cineole and that of coriander (*Coriandrum sativum* L.) by another monoterpene linalool. Thyme (*Thymus vulgaris* L.) is a species with numerous chemotypes named according to the major compound, for example, thyme with chemotype thymol or chemotype carvacrol or terpineol or linalool. A typical EO may contain 20–80 phytochemicals. The analyses of EOs of the African basil *Ocimum canum* Sims contain no less than 80 compounds identified by GS-MS and GS-FID [4].

This complexity of EOs phytochemistry led to a certain inconsistency of the chemical composition of an essential oil. In fact, several factors influence the balance of the compounds within EOs. Terpenoids and isoprenoid are synthesized through secondary metabolism of the plant. Monoterpenes are biosynthesized in plastid via two 5-carbon precursors, that is, isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which condense to give the monoterpenes (10-carbon). The sesquiterpenes (15-carbon) are formed via the mevalonate pathway in the cytosol. Phenylpropanoids are derived mainly from the shikimate pathway [5].

These metabolic pathways do not have the same importance at all stages of the plant development and in all the organs. The physiological development of the plant, its degree of maturity at the harvest, and the choice of the organ to be extracted play a role. Gershenzon et al. [6] showed that limonene and menthone are the major monoterpenes existing in the youngest leaves of peppermint, but limonene content declines rapidly with development of the plant, whereas menthone increases and then declines at later stages. Thus, menthol becomes the dominant constituent. External factors like the climatic and soil conditions or seasonal variations may also change the main compounds identified within an EO. Several studies confirmed that separated geographic areas led to observe different chemotypic races or populations [7]. Isman and Machial [8] reported that rosemary oil extracted from plants harvested in two different areas of Italy contained 1,8-cineole concentrations ranging from 7 % to 55 % and α-pinene concentrations ranging from 11 % to 36 %.

The variability of the composition of an EO is also impacted by the choice of the method of extraction of EOs. One characteristic of EOs is the volatility of their compounds which allows them to be easily extracted by water vapors, in contrast to fixed lipid oils and essences (concrete, absolute, oleoresins, and resinoids) which are extracted by solvents and alcohol. Guenther [9] distinguished three kinds of water and steam distillation methods for obtaining essential oils. These methods are far more restrictive than more recent extraction and separation methods which are mentioned in the European Pharmacopoeia [10] using supercritical fluids, steam distillation, dry distillation, or mechanical cold pressing of plants.

The diversity in the EOs phytochemical composition induces as a consequence that the production of a standardized product is a real challenge for their commercialization.

3 Insecticidal Activities of EOs

An abundant literature, more than 2,000 scientific papers, is devoted to study the EO-insect relationships. Among these, some major reviews would be mentioned [11–15].

3.1 Diversity of Activities on Insect Targets and Routes of Exposure

The insect control by EOs is the result of several kinds of modes of action and depends on the routes of the exposure. EOs develop toxicities by ingestion or contact through cuticle or inhalation for volatile compounds. Some EOs repel the insect or are deterrent or antifeedant. Others disturb oviposition or disrupt the larvae growth or modify the imago's behavior or physiology. In fact, the activities the EOs exert on an insect could impact several physiological targets at the same or at different stages of the insect development. This complexity in the way they act is illustrated by the numerous following examples.

Early studies were mainly focused to observe the activities of EOs and their volatile constituents on insects of the stored products. Twenty-two essential oils were extracted mainly from Lamiaceae and Umbelliferae families and showed a range of LC_{50} from 2 to more than 300 mg dm^{-3} air on the bruchid *Acanthoscelides obtectus* (Say) imagos [16]. Some EOs produced also ovicidal and larvicidal activities and consequently inhibited the reproduction of the insect [17]. From Lamiaceae were extracted the most efficient of those EOs, that is, thyme (*T. vulgaris*) and wild thyme (*Thymus serpyllum* L.), rosemary (*Rosmarinus officinalis* L.), summer savory (*Satureja hortensis* L.), oregano (*Origanum vulgare* L.), and sweet basil (*Ocimum basilicum* L.), which also had an antifeedant effect on larvae inside artificial seeds [18]. This beetle *A. obtectus* has been shown to be a convenient model to point out with accuracy which reproductive stage is targeted and which is the speed of the activity of essential oils. Table 138.1 shows that the fumigant toxicity of EOs on imagos and reproduction could be quite different. As an example, parsley *Petroselinum sativum* L. [Umbelliferae] did not have significant fumigant toxicity on the beetle adults but inhibited strongly its reproduction, whereas the *Satureja hortensis* presented a high toxicity on adults but inhibited poorly the bruchid reproduction. In the same way, some EOs produced fumigant toxicity and antifeedant effect with variable intensity (e.g., mint *Mentha piperita* L., bay tree *Laurus nobilis* L. or dill *Anethum graveolens* L.).

A lot of EOs protected stored grains against the damages of Coleoptera. EOs of sweet basil, of patchouli (*Pogostemon* spp.), of *Eucalyptus* spp., of thyme, and of African basil with its major component linalool were toxic to Mexican bean weevil *Zabrotes subfasciatus* Boheman, rice weevil *Sitophilus oryzae* L., lesser grain borer *Rhyzopertha dominica* Fab., drugstore beetle *Stegobium paniceum* L., bean weevil *Acanthoscelides obtectus* Say, red flour beetle *Tribolium castaneum* Herbst, and pulse beetle *Bruchus chinensis* L. [19–21]. The toxic effect of essential oils is not

Table 138.1 Toxicity of some essential oils on *Acanthoscelides obtectus Say* (Coleoptera: Bruchidae)

Essential oil species	Family of plant	Main components[a] (%)	Acute toxicity by fumigation [b]LC_{50} (mg dm^{-3}air) (NK test)	Inhibition of reproduction (emergence) by fumigation[c] (NK test)	Inhibition of larval development into artificial seeds[d] (NK test)
Origanum majorana L.	Lamiaceae	Terpinene-1-ol-4 (20.6), linalool (15.3), γ-terpinene (10.3)	1.9 (a)	+++ (a)	+++ (a)
Thymus serpyllum L.	Lamiaceae	Thymol (30.4), carvacrol (28.9), p-cymene (10), citral (4.2)	2.0 (a)	+++ (a)	+++ (a)
Cinnamomum verum Presl.	Lauraceae	Cinnamaldehyde (90), eugenol (3.6)	2.1 (a)	++ (b)	+++ (a)
Rosmarinus officianalis L.	Lamiaceae	Camphor (30.6), borneol (22.1), 1,8-cineole, β-phellandrene (10.6)	3.2 (a)	+++ (a)	+++ (a)
Ocimum basilicum L.	Umbelliferae	Linalool (50), limonene (7.5), eugenol (3.6), estragole (3.2)	4.0 (a)	+++ (a)	+++ (a)
Cuminum cyminum L.	Umbelliferae	Cuminaldehyde (42.5), β-pinene (11.8), γ-terpinene (11.4), nerol (11.4)	4.3 (a)	+++ (a)	+++ (a)
Satureja hortensis L.	Lamiaceae	Carvacrol (39.9), thymol (13.4), linalool (6.4)	5.7 (a)	++ (b)	+++ (a)
Thymus vulgaris L.	Lamiaceae	Thymol (47.5), *p*-cymene (17.3), β-caryophyllene (6.1)	7.0 (a)	+++ (a)	++ (b)
Salvia officinalis L.	Lamiaceae	α- and β-thuyone (28), linalyl acetate (21.4), β-caryophyllene, and α-humulene (5,15)	7.1 (a)	+++ (a)	+++ (a)
Origanum vulgare L.	Lamiaceae	Carvacrol (39.4), thymol (26.6), *p*-cymene (16.3)	10.0 (a)	+++ (a)	+++ (a)
Lavandula augustifolia P. Muller	Lamiaceae	Linalyl acetate (34.2), linalool (31.8), β-Caryophyllene (11.1)	12.6 (b)	++ (b)	+++ (a)

Laurus nobilis L.	Lauraceae	1.8-Cineole (48.6), sabinene (9.1), terpinyl acetate (8.2), α-pinene (7.1)	17.8 (b)	++ (b)	NS (d)
Verbena officinalis L.	Lamiaceae	Carvone (32.4), limonene (18.9), citral (17.6)	19.9 (b)	+ (c)	+++ (a)
Coriandrum sativum L.	Umbelliferae	Linalool (68.2), α-pinene (5.5), γ-terpinene (5.5)	19.9 (b)	+ (c)	+++ (a)
Mentha piperita L.	Lamiaceae	Menthone (3.5), menthol (18.8), isomenthone (12.8)	22.38 (b)	+++ (a)	NS (d)
Anethum graveolens L.	Umbelliferae	Limonene (35.9), carvone (34.8), β-myrcene (11.8)	50.0 (c)	+ (c)	+++ (a)
Cymbopogon nardus L.	Poaceae	Citronellal (33.8), geraniol (21.6), citronellol (9.2), geranyl acetate (3.4)	60.4 (c)	+++ (a)	+++ (a)
Eucalyptus globulus Labill.	*Myrtaceae*	*1.8-Cineole (86), α-pinene (3.9), p-cymene (2.4)*	*66.0* (c)	++ (b)	+ (c)
Citrus limon (L.) Burm f	Rutacae	Limonene (63.9), β-pinene (12.2)	100.0 (d)	++ (b)	++ (b)
Myristica fragrans L.	Myristicaceae	β- pinene (23.2), sabinene (22.6), myristicine (7.9)	125.8 (d)	NS (d)	+++ (a)
Apium graveolens Houtt	Umbelliferae	Limonene (73.2), sequiterpene (13)	302.0 (e)	+ (c)	+++ (a)
Petroselinum sativum L.	Umbelliferae	Apiole (43), thymol (10.2)	NST (f)	+++ (a)	+++ (a)

Newman-Keuls test: classified with the same letter (a, b, c, d, e, f) signifies no difference at the 0.05 level

NST Nonsignificant toxicity (fumigation), *NS* no difference from control

[a] Data from [16]

[b] Data from [10]

[c] 5.10^{-2} μL cm^{-3} air EO in the experimental arena (Data from [17])

[d] 0.5 % EO incorporated within *Phaseolus vulgaris* flour in artificial seeds (Data from [18])

only suitable for granary insects but also for flying insects. The Lamiaceae *Mentha* spp. and *Lavandula* spp. or *Pinus* spp. (Pinaceae) EOs were noted to be toxic against the green peach aphid *Myzus persicae* and the greenhouse white fly *Trialeurodes vaporariorum* as well as the pear bug *Stephanitis pyri* [22]. Greek aromatic plants, especially from genus *Satureja*, *Origanum*, and *Mentha* (Lamiaceae), prevented egg hatching and provoked prohibition or malformation of the puparium of the flies *Drosophila auraria* [23]. And some of these aromatic Mediterranean plant EOs tested on *A. obtectus* were also toxic to the Mediterranean fruit fly, *Ceratitis capitata*, and the cereal aphids *Rhopalosiphum padi* and *Metopolophium dirrhodum* [24]. More recent studies demonstrate that a wide range of insect taxa are affected by EOs. Park et al. [25] demonstrated the fumigant activity of EOs of *Schizonepeta tenuifolia* (Lamiaceae) against the sciarid fly *Lycoriella america*. Papachristos et al. extended the studies of the toxic effect of Mediterranean plants' EOs on *C. capitata* with this of citrus peel [26]. In the same way, Liu et al. [27] extended previous works observing the toxicity of EOs to the four major stored-product insects: *Tribolium castaneum*, *Sitophilus zeamais*, *R. dominica*, and sawtoothed grain beetle *Oryzaephilus surinamensis* [28]. They determined the fumigant toxicity of the water dropwort *Ostericum sieboldii* (Apiaceae) EOs with LC_{50} values of 27.4 mg dm^{-3}air (*T. castaneum*) and 20.9 mg dm^{-3} air (*S. zeamais*). Extracted oils from leaves and bark of Chilean laurel *Laurelia sempervirens* (Atherospermataceae) and *Drimys winteri* (Winteraceae) carried on fumigant activities of EOs against the aphid *Acyrthosiphon pisum* [29].

Besides fumigation, other routes of penetration of EOs are effective. Coleopteran insects, maize weevil *Sitophilus zeamaïs* (Motschulsky), *T. castaneum*, and larger grain borer *Prostephanus americana* (Horn) were very sensitive to topical applications of the *Citrus* spp. essential oils [30]. EOs decimated numerous agricultural pest insects and affected disease-vector insects as well. The Annonaceae *Dennettia tripetala* EOs decimated a wide range of agricultural pest insects, for example, the American cockroach *Periplaneta americana* and the grasshopper *Zonocerus variegatus* [31]. The Myrtaceae *Eucalyptus saligna* EOs killed lice *Pediculus capitis*, mosquitoes *Anopheles funestus*, bed bugs *Cimex lectularius*, and American cockroach *Periplaneta orientalis* within 2–30 min [32].

Antifeedant effects could decimate insects too, but the insects' decision to avoid feeding on a plant could be influenced by such factors as phagodeterrency of the substances, post-consumption physiological stress, or a repellent effect, and it is not always easy to discriminate them. Laboratory choice tests were conducted to evaluate the antifeedant effect of pine EOs and terpenes for the weevil *Hylobius pales* [33]. Discriminant assays were developed to observe the deterrent activity of EOs of *Minthostachys mollis* (Lamiaceae) and *Melaleuca quinquenervia* (Myrtaceae) essential oils on the flour beetle *T. castaneum* [34]. These assays were conducted to screen the antifeedant activity of Uruguayan plants that belonged to Bignoniaceae (*Clytostoma callistegioides*, *Dolichandra cynanchoides*, *Macfadyena unguis-cati)*, Sapindaceae (*Dodonaea viscosa*, *Allophylus edulis, Serjania meridionalis*), Lamiaceae (*Salvia procurrens*, *Salvia guaranitica*; Solanaceae: *Lycium cestroides*), and Phytolaccaceae (*Phytolacca dioica*) against the specialist Coccinellidae *Epilachna paenulata* and the larvae of the generalist Lepidoptera *Spodoptera littoralis* [35].

Since the last 25 years, repellent effects of EOs were also fully described with plants of all continents. The Indian plant *Adhatoda vasica* (Acanthaceae) EOs exhibited repellent activity ag*ainst S. oryzae and B. chinensis* [36]. Essential oils of Kenyan plants *Ocimum suave* (Lamiaceae) and *Lippia* spp. (Verbenaceae) repelled *S. zeamaïs* [37, 38], and *Acorus calamus* (Araceae) EOs *T. castaneum* [39]. Wang et al. [40] revisiting the traditional use of a very common weed in China *Artemisia vulgaris* to protect stored products showed the repellent activity of this EO to the Tenebrionidae *Tribolium castaneum*. In Europe, Kalemba et al. [41] demonstrated that EOs from the berries of the Cupressaceae *Juniperus communis* were a very good mosquito repellent. A review recently gathered the numerous studies published during the last 10 years on the repellent effect of EOs [42].

Nevertheless, if a lot of essential oils are repellent, some have been found to be highly attractive [10, 12]. The attractiveness of sandalwood oil, basil oil, and grapefruit oil in yellow sticky traps improved the number of trapped greenhouse whitefly *Trialeurodes vaporariorum* Westwood [43]. Cade oil, an essential oil produced by destructive distillation of juniper (*Juniperus oxycedrus L.*) twigs, synergized the attraction of alpha-ionol to tephritid fruit fly *Bactrocera latifrons* (Hendel) male [44].

Some essential oils develop a combined activity on the insects, for example, EOs of *Ocimum* spp. exhibited both a repellent and a larvicidal action [45]. *Acorus calamus* EO and its active ingredients, asarone and its analogues, were both antifeedant and potent growth inhibitors to the variegated cutworm *Peridroma saucia* (Lepidoptera: Noctuidae) [46]. It has also been shown that some essential oils exert quite opposite effects on different insect species. As an example, the tansy (*Tanacetum vulgare* L.) EOs impacted in different ways the following three beetles: it was attractive and paralyzing for *Rhizoperta dominica*, repulsive for *Tribolium confusum*, and toxic for *Sitophilus americana* [47].

A difference could be made between accurate and chronic toxicity. A short (24-h) exposure to tansy oil exerted an antifeeding activity in larvae and significantly decreased egg laying in adult females of obliquebanded leafroller (*Choristoneura rosaceana*), while the chronic (long-time exposure) of this EO mixed into the diet during 75 days decreased significantly larvae survival rate [48].

Besides the variability of the phytochemical composition of EOs mentioned above, the point is that insects vary enormously in their responses to secondary plant products. It is well known that the sensitivity of different insect species could be quite different for the same substance [11]. Oils from *Cymbopogon nardus* which killed quickly the bruchid *A. obtectus* [12] only knocked down and disabled the Angoumois grain moth *Sitotroga cereallela* [49].

From all these observations, it could be deduced that essential oils present a widespread range of activities on insects that necessitate to be sharpened by a case-by-case study before application in pest management. Because of the chemical complexity of essential oils and the variability of sensitivity of the insect species, to be significant, the comparison of the toxicity of essential oils necessitates an evaluation which must be (1) conducted with samples of an EO having a quite similar phytochemical composition at each test and (2) experimented on homogenous insect populations.

3.2 Mechanisms of Action

Although the effects of EOs for insect control are abundantly studied, a very few researches are devoted to understand the mechanisms of these observed effect. They involve both physical and chemical properties of EOs. An update was recently published on this point [15].

Because oils are lipophilic, a topical application of EOs laid a film on insect cuticle that modifies the physiology of the insect. The film changed the conditions of penetration inside the body of insect of the air and of substances because of the disruption of lipid bilayers of cell walls. A review on the biological and pharmacologic effects of EOs was recently documented [50].

The second point is linked with volatility of monoterpens. Because they are small volatile molecules, they are involved in the transmission of airborne signals from plants to insects. Detection of bouquets of fragrant and chemosensory-active compounds by insects involves different families of proteins, including OBPs and chemosensory proteins (CSPs). OBPs and CSPs are found on the periphery of the sensory receptors and function in the capture and transport of molecular stimuli [51]. In the sensilla of insects, specialized odorant binding proteins (OBPs) respond to volatile monoterpenes. For example, trichoid sensilla of the female silkworm, *Bombyx mori*, respond to linalool [52]. In moths, the OBPs include proteins that bind general odorants GOBPs (general odorant binding proteins) such as volatile compounds from plants. The protein identified in tobacco hornworm *Manduca sexta*, GOBP2, preferentially interacts with floral aromas and green plant odors such as [Z]-3-hexen-1-ol, geraniol, geranyl acetate, and limonene [53]. The different types of GOBPs serve to detect the different categories of odorants released by plants and play an important role in the response of the insect to an EO blend.

Several monoterpenoids (thymol, α-terpineol, linalool, geraniol, eugenol), which have been identified as important components of essential oils, induce a neurotoxicity. The mechanisms of this neurotoxicity have now been explored. They involve receptors of nervous system. Huignard et al. [54] described several different types of receptors which are playing a role. Thymol binds to GABA receptors associated with chloride channels located on the membrane of postsynaptic neurons and disrupts the functioning of GABA synapses [55]. Eugenol acts through the octopaminergic system by activating receptors for the neuromodulator octopamine which increases the concentration of cAMP. This AMPc increase was inhibited in the presence of a mixture of eugenol, α-terpineol, and cinnamic alcohol, but low doses of eugenol and octopamine lead to an increase in adenyl cyclase activity of cells in the nervous system of the cockroach *Periplaneta americana* [56]. Further studies on cultures of brain cells of *P. americana* and of *Drosophila melanogaster* demonstrated that eugenol mimics the action of octopamine with the consequence to increase intracellular calcium levels [57]. The cytotoxicity of EOs through the octopaminergic system was also demonstrated in cultures of epidermal cells of *Helicoverpa armigera* [58]. Thymol, carvacrol, and α-terpineol influence the production of cAMP and calcium at the cellular level in *D. melanogaster*. In this insect, tyramine receptors are involved in the recognition of monoterpenes (tyramine is a precursor of octopamine) [59].

Some EOs and their constituents also act on the transmission of the nervous impulse. Price and Berry [60] conducted electrophysiological experiments which showed that eugenol inhibits deeply neuronal activity, whereas citral and geraniol have a biphasic effect that is dose dependent. At low doses, these compounds induce an increase in spontaneous electrical activity but at high doses cause a decrease. Using a similar electrophysiological experimentation, Huignard et al. [54] observed that *O. basilicum* EOs have a complex neurotoxic activity. The neuronal electrical activity was fully inhibited by the EO which decreased the magnitude of nerve-action current and also reduced the post-hyperpolarization phase and the frequency of nerve-action current firing. The authors hypothesized that this effect could be the result of the combined action of linalool and estragole, two major components of the *O. basilicum* EO. The mere application of pure linalool in fact produced a reduction in the amplitude of nerve-action current and decreased the post-hyperpolarization while estragole specifically induced a reduction of post-hyperpolarization.

This activity of EOs on nervous system involves also another well known target for neurotoxicity, the enzyme acetylcholinesterase. Tea tree oil inhibited acetylcholinesterase [61]. Lopez and Pascual-Villalobos [62] demonstrated that several types of inhibition are involved with monoterpenes. It could be the result of a reversible competitive inhibition occupying the hydrophobic site of the enzyme's active site or of a mixed inhibition by linking to a different site from the active site where the substrate bounded.

These studies confirm that the insecticidal activity of EOs and monoterpenes, which are among the major components of EOs, is the consequence of several mechanisms that affect multiple cellular and physiological targets.

4 Trends for the Development of EOs as BCAs

One of the most attractive features of EOs is that they are, in general, low-risk products which provide a friendly environmental pest management. They develop many ecological advantages, but if the pros prevail, some unintended effects have to be mentioned.

4.1 Benefit-Risk Ratio for Environment

On an ecological point of view, EOs present several advantages that could consider them to be useful complementary or alternative method to the intense use of chemical insecticides. Because they are natural and most compounds they included are volatiles, they are biodegradable with short half-life. The AOPWIN Program for alpha-pinene, beta-pinene, camphene, and trans-pinane gave half-life in air ranging from 1.4 to 9.4 h [63]. One of the most common constituents of EOs, α-terpineol, has a short half-life of 4 h in air, 466 h in surface water, and 2 days in soil where it is degradable by soil microflora [64]. This biodegradability induces little persistence in the environment, and in contrast to some synthetic insecticides, no bioaccumulation or biomagnification has been reported to date. As a consequence, this could improve the biodegradability of insecticide treatments and therefore decrease the quantity of toxic insecticide residues not only in the environment but also for food and feed.

EOs generally affect very specific physiological and cellular target. Therefore, they increase insecticide selectivity, and most of the time, by comparison to classical pesticides, the hazards and unintended effects on nontarget species appear to be limited. Very few works, however, were devoted to study the environmental fate and pathways of EOs as well as their effects on agricultural nontarget arthropods. Requiem® is a *Chenopodium* EO-based biopesticide recently registered in the United States [65]. It is used as insecticide against thrips (*Frankliniella occidentalis*), green peach aphid, and the greenhouse whitefly (*Trialeurodes vaporariorum*) and as acaricide against the two-spotted spider mite and the European red mite *Panonychus ulmi* [66, 67]. Bostanian et al. [68] demonstrated that Requiem® is also operational against two beneficial arthropods used as BCAs, the anthocorid minute pirate bug *Orius insidiosus* and the micro-Hymenoptera *Aphidius colemani* which currently control thrips in flower and vegetable greenhouses and also the aphid *Rhopalosiphum padi*. In Africa, Huignard et al. [69] have shown that EOs of citronella and sweet basil were not only toxic to the bruchid *Callosobruchus maculatus* but also to its parasitoid *Dinarmus basalis*, thus compromising its biocontrol.

In same way but on plant species, phytotoxic and allelopathic effects have been described. EOs of wild marigold (*Tagetes minuta*) and pepper tree (*Schinus areira*) inhibited roots of maize (*Zea mays*) and thus have allelopathic effect [70]. Dudai et al. [71] observed that *Cymbopogon citratus* or *Origanum vulgare* EOs not only inhibited weed species such as amaranth (*Amaranthus palmeri*) and *Euphorbia hirta* but also the growth of crops such as wheat (*Triticum aestivum*) and tomatoes (*Lycopersicon esculentum*).

These examples demonstrate that in terms of ecotoxicology, EOs are safe to use but not without potential problems. Because of the variability of species sensitivity to EOs, unintended effects on nontarget species could be observed.

4.2 Benefit-Risk Ratio for Human and Animal Health

It is not because they are natural products that the EOs are without any toxicity when they are used in an inappropriate way. The toxicity of EOs is relatively well studied experimentally and clinically because of their use in human and veterinary medicine. This EOs toxicity may result from the mere toxicity of particular components included therein or from synergistic effects between these numerous compounds being in a same extract.

Their mammalian toxicity of EOs is generally low. Most of EOs (e.g., citronella, lavender, clove, eucalyptus, anise, marjoram, etc.) have an oral LD_{50} value ranging from 2,000 to 5,000 mg kg^{-1} in rats. Less than a dozen EOs (e.g., basil, tarragon, hyssop, oregano, savory, tea tree, and sassafras) have LD_{50} values ranging from 1,000 to 2,000 mg kg^{-1}. But a few are toxic to very toxic. EOs of Pennyroyal (*Mentha pulegium* mixed with *Hedeoma pulegiodes*) and *Thuja* spp. have LD_{50} values of 400 and 830 mg kg^{-1}. EO of Boldo (*Peumus boldus*) has LD_{50} value of 130 mg kg^{-1} but causes convulsions at a dose of 70 mg kg^{-1} in rats. The EO of rosemary is also convulsant and can cause epilepsy [2, 72].

Dermal toxicity was observed with some EOs. Tea tree oil is now described since near 15 years to cause skin allergies [73, 74]. Bergamot (*Citrus bergamia*) and angelica (*Angelica archangelica*) EOs are identified to be photosensitizing [75] and EOs of wintergreen (*Gaultheria procumbens*), eucalyptus, clove, and sage to be irritant [76].

In veterinary medicine, some EOs have demonstrated manifestations of toxicity. Dogs dermally exposed to pennyroyal oil at 2 g kg^{-1} exhibited diarrhea, hemoptysis, and epistaxis 30 h after exposure. A histopathologic examination of liver tissue showed massive hepatocellular necrosis caused by a bioactivation of the major component of this oil pulegone into its hepatotoxic metabolite menthofuran. Commercially available shampoos containing the tea tree (*Melaleuca alternifolia*) EO and the pure oil have been sold for use on dogs, cats, ferrets, and horses. Clinical signs of three cats dermally exposed to pure melaleuca EO for flea control were described. They included hypothermia, ataxia, dehydration, nervousness, trembling, and coma. Two cats recovered within 48 h following decontamination and supportive care, but one cat died within 3 days following exposure. Terpinen-4-ol, the main constituent of this EO, was detected in the urine of the cats [77].

These examples underline that some EOs need to be handled with caution despite most EOs are not particularly toxic. It is remarkable that these toxicities of some EOs do not coincide with that of the plant from which they are extracted and whose safety is generally recognized [78]. Because risk includes both hazard and exposure, the use of EOs for biocide or for plant protection effects requires that the applicators follow carefully the labeling recommendations given for each situation. EOs are most often delivered by spraying or fogging that may induce a dermal or respiratory exposure. The need of suitable equipment for handling EO products and the treated plant must be observed to avoid accident or chronic intoxication. The EO risk, whatever minimal is it, must not be ignored simply because EOs are natural products.

4.3 Commercial Prospects and Impediments

The insect control by EOs receives attention very soon. In the years 1980–1990, the patents involving essential oils showed that a majority of the inventions focused on household uses. Japanese companies understood very soon the interest to use EOs in such a way. To prevent reinfestation by Blattarias, particularly the German cockroach *Blatella germanica*, a cleaning solution including clove essential oils and pyrethrinoids [79] and adhesives containing acrylic polymers and high levels of essential oils [80] were commercialized as well as mixtures associated with essential oils and pyrethrinoids to control mosquitoes and flies [81, 82]. *Eucalyptus* spp. EOs were used as a synergistic insecticide in addition to growth inhibitors [83], and EOs of spearmint, bitter almond, and birch (*Betula lenta*) bark essential oils were incorporated into a formulation sold for acaricide, insecticide, and insect repellent properties [84]. Domestic uses prevailed. To prevent the clothes from moths and beetles, filter papers or tablets soaked with EO *Juniperus rigida* were placed in the wardrobe [85]. To protect pet dogs, a flea collar was manufactured by adding essential oils (eucalyptus, cedarwood, citronella, and peppermint) to ethylene-vinyl acetate polymer [86]. To improve resistance to insects, handicraft veneer-faced panels were impregnated with polymer layer and hiba or kinoki EOs [87, 88]. EOs also showed some usefulness for building and hand-manufactured materials. *Eucalyptus* spp. EOs were mixed with pyrethrinoids and borax in a solution to preserve wooden beams [89].

But the important sector in which EOs present applications as insect BCAs, still is the area of agriculture, the stored-product storage and feed. Mustard essential oil was used very soon into formulation containing insecticide, microbicide, and repellent substances absorbed onto silica used to prevent infestation of mites in feed [90]. In Europe, pine EOs were also incorporated in the 1990s with polymers into sheets to develop attractant adhesive films or coating materials to enhance the control of harmful insects in agriculture, livestock structures, and horticulture [91, 92].

Twenty years after, Arnason [93] underlined that essential oils are now considered to be the most important commercial application of botanical insecticides. He indicated that no less that 88 insect repellent products are actually sold in the United States market containing an essential oil as one of the active ingredients in the formulation. Eight EOs are distinguished. The most commonly used ingredient is citronella oil (45 products), followed by geranium oil-geraniol (33 products), lemongrass oil (24 products), cedar oil (22 products), peppermint oil (16 products), rosemary oil (15 products), soybean oil (15 products), and eucalyptus oil (14 products). According to this author, 57 of these 88 formulations contain a blend of two or more active ingredients. Among reasons that facilitate the use of EOs as BCAs, the need to replace methyl bromide, which is considered to deplete the ozone in the stratosphere, renews interest in essential oils as fumigants.

This enhancement of using EOs to control insect pests in orchards and to protect high-value crops is probably the result of the regulation rules in the USA. Because the procedure for regulatory approval of plant protection products is expensive and the market for BCAs appear to be presently a niche market (about 2 % of pesticides global market), industrial producers of BCAs need a simplified procedure for registration to achieve a reasonable return on investment. As indicated by Regnault-Roger et al. [15]: “A reduced regulation process for these products has existed in the United States since 1996 and it is particularly relevant to EOs. Biopesticides are subject to special procedures outlined in Title 40, Code of Federal Regulations, of FIFRA. A number of natural substances, such as EOs of mint, thyme, rosemary, and lemon grass that did not benefit from this simplified procedure, however, were classified as GRAS (generally regarded as safe). They were placed on a list (FIFRA Section 25 [b]), exempting them from the registration process [94].” Paulizt and Bélanger [95] confirmed that this simplified approval procedure has led to a large diversity of EO-based products available in the United States. Moreover, this exemption has become a marketing strategy to promote these products [96, 97]. The company EcoSmart^{TM} was a pioneer, 15 years ago, to develop a line of products on this exemption. These products now are named “EcoEXEMPT$^{®}$ Minimum-Risk & EcoPCO$^{®}$ Products” and are commercialized on a large scale by several suppliers [98].

Compared to United States, only few EO products are available in the European Union [EU] and Canada, where registration is stricter. Pest Management Regulatory Agency [PMRA, Canada] decided in 2004 to deregister citronella products due to a lack of safety data. However, since “Health Canada did not identify any imminent health risks, citronella-based personal insect repellents will remain on the market until a final decision is made” [99, 100], but strict recommendations accompany the use of these products.

In UE, the procedure for reevaluation of plant protection products (PPP) ended in 2008. To be authorized on market, all PPP derived from biological as well as chemical have to be listed in Annex 1. Because they meet the purposes of Directive 2009/128/CE to promote integrated pest management (IPM), some vegetable oils have been recently authorized but for uses that are not insecticidal [101]: tea tree oil for use as a fungicide, citronella oil as an herbicide, clove oil as a fungicide and bactericide, and spearmint oil as a plant growth regulator. The status of EOs extracted from thyme or marigold (*Tagetes* sp.) for insecticide use is presently pending and should be approved soon. Orange oil is now allowed in France for control of sweetpotato whitefly, *Bemisia tabaci*, on field pumpkin (*Cucurbita pepo*) and of greenhouse whitefly on tomato [102].

The situation for using EOs in developing countries is quite different. The tropical flora of many developing countries is remarkably diverse and can be a rich source of potent and valuable EOs. Aromatic plants are traditionally and widely used for stored-product insects or to repel harmful insects in fields. Currently, there is a move to enhance the use of steam-distilled EOs, but the lack of technologic resources leads to use crafty solution like a domestic pressure cooker for extracting EOs by steam distillation. Another point that impedes the proper development of EOs in Africa is that results most of the time are unsupported by scientific experimentation [15]. The promotion of BCAs in developing countries needs more consistent risk analyses as well as stricter regulatory systems.

5 Conclusion

The development of EOs as PPP and biocide to control insect is an alternative or a complementary approach to synthetic insecticides. They are environmentally friendly products, that is, they have natural origin and are biodegradable, and they have diverse physiological targets within insects that may delay the evolution of insect resistance. Thus, they are especially suited to organic farming as well as to integrated pest management. As a result, EOs have been embraced by the public.

Two key points impede their development as BCAs. Because the field of researches is widespread, there is a lack of data on environmental features of EOs and their components and also on plant-insect interrelationships and mechanisms. More fundamental and applied studies are needed; even the publications devoted to EOs improved the last years. The second key point is the difficulty to have a relevant registration both in developed and developing countries. Most of developing countries need to have appropriate and stronger regulation whereas many developed countries need to have adapted regulation. In some countries, it is difficult to meet the consumers demand because of registration requirements. Among the arguments used to seek reduced regulation for EOs is the fact that many active ingredients of EOs are used daily at home or in food. Therefore, it is rather logical to conclude that it would be unreasonable to request heavy and costly registration requirements for these products that have no history of adverse effects [97]. However, the safety of products which are used in a precise context sometimes led to the reality of unintended effects in a new context. As a consequence, an evaluation of the benefit-risk ratio on a case-by-case basis must be required to prevent this undesirable situation and to have a reasonable management of real risk if there is some.

Therefore, most of government policies are now seeking low-risk and alternative plant protection products. The US EPA is the only regulatory regime that has considered reduced risk seriously and, as a result, has allowed a significant number of EOs for commercial use more than 10 years ago. The results of this American position must be checked carefully to determine positive and negative inputs of using EOs in such a way. Because of the numerous advantages of EOs in controlling harmful insects, they certainly have a room in the BCAs approaches which promote sustainable development.

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Biological Activities of Selected Mono- and Sesquiterpenes: Possible Uses in Medicine

139

Gerhard Buchbauer and Anja Ilic

Contents

1 Introduction 4111
2 Hydrocarbons 4112
2.1 (+)-Limonene 4112
2.2 β-Caryophyllene 4116
2.3 α-Humulene 4119
2.4 Myrcene 4120
3 Alcohols 4121
3.1 (−)-Menthol 4121
3.2 Nerolidol 4124
3.3 Farnesol 4126
3.4 Linalool 4128
3.5 Bisabolol 4130
3.6 Carvacrol 4133
3.7 Thymol 4136
3.8 Perillyl Alcohol 4139
4 Ether 4140
4.1 1,8-Cineole 4140
5 Carbonyls 4142
5.1 Thujone 4142
5.2 Camphor 4145
5.3 Citral 4146
5.4 Pulegone 4149
References 4150

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Abstract

Reports on the biological properties of mono- and sesquiterpenes (MTs and SQTs) have been on the increase. Although MTs and SQTs are already in wide use as flavoring and antimicrobial agents in cosmetics, perfumes, household and cleansing products, and food additives, many of their pharmacological properties are yet to be discovered. Studies report on their anticancer, antiinflammatory, antinociceptive, antidiabetic, and antimicrobial activities and effects on the central nervous system that make them potential targets for development of new therapeutics and for usage for medical purposes. This chapter provides an overview of the biological activities and aromatherapeutic uses of chemical classes of MTs and SQTs, compiling the scientific achievements mainly from 2010, 2011, and the first part of 2012. Because hundreds of MTs and SQTs and their derivates exist, only some prominent representatives of MT- and SQT-hydrocarbons, -alcohols, -oxides and -carbonyls are discussed.

Keywords

Monoterpenes • sesquiterpenes • (+)-limonene • β-caryophyllene • α-humulene • myrcene • (−)-menthol • nerolidol • farnesol • linalool • bisabolol • carvacrol • thymol • perillyl alcohol • 1,8-cineol • thujone • camphor • citral • pulegone • biological activity

Abbreviations

AC	Adenylate cyclase
AUC	Area under the plasma level/time curve
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CB receptor	Cannabinoid receptor
CDK	Cyclin-dependent kinase
CNS	Central nervous system
COX	Cyclooxygenase
CREB	cAMP response element-binding
CYP	Cytochrome P450
E-BCP	(*E*)-β-Caryophyllene
EO	Essential oil
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GABA	Gamma-aminobutyric acid
GI	Gastro-intestinal
HepG2	Human hepatocellular liver carcinoma
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
IL	Interleukin
JNK	C-Jun N-terminal kinase
K_{ATP+}	ATP-dependent potassium channels
L-NAME	*N*-(ω)-nitro-L-arginine methyl ester

LPO	Lipid peroxidation
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAPK p38	Mitogen-activated protein kinase p38
MDA	Membrane lipid peroxidation
MPO	Myeloperoxidase
MT	Monoterpene
NMDA	*N*-methyl-D-aspartate
NFkB	Nuclear factor "kappa-light-chain-enhancer" of activated B-cells
NO	Nitric oxide
NOS	Nitric oxide synthase
PG	Prostaglandin
ROS	Reactive oxygen species
SOD	Superoxide-dismutase
SQT	Sesquiterpene
TNF-α	Tumor necrosis factor alpha
TRP	Transient receptor potential
TRPA1	TRP Ankyrin 1
TRPM8	TRP Melastatin 8
TRPV1	TRP Vanilloid 1

1 Introduction

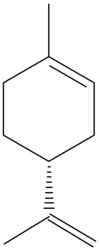
Monoterpenes and sesquiterpenes are small molecules compared with the majority of drugs used in classical pharmacotherapy. N-containing molecules cannot be found among them, which is one reason why terpenic medicines are not usually encountered, except for MT-ic alcohol menthol, which is used as a spasmolytic drug to treat bile problems, along with a few other examples. Nearly all naturally occurring MTs and SQTs are volatile and thus fragrant, rendering them suspicious to the majority of pharmacologists and physicians. Another fact hinders the entrance of these natural compounds into the pool of established medicaments, namely, that they occur as multi-component mixtures in EOs and are therefore not compatible with the so-called "-one-molecule-one-target-" dogma of classical pharmacotherapy (Hannelore Daniel, Oral presentation, entitled: Genetic and Nutrient Determination of the Metabolic Syndrome (Nutrigenomics), 59th Intern. Congress and Annual Meeting of the Society for Medical Plants and Natural Product Research, Antalya, Turkey, 4th–8th September 2011. See also S. Frantz, Nature (2005), 437:942). Thus, the medicinal uses of these terpenes remain in the domain of complementary and alternative medicinal therapies. But the question is offered: "Why should these fragrant, small molecules do not possess other biological, namely therapeutically usable properties?"

As already mentioned, MTs and SQTs are the major components – besides some phenylpropanes and small alkene derivates, the latter often the catabolic products from unsaturated fatty acids – in the EOs that are produced by plants mainly as protection against herbivores, insects, mites, fungi and bacteria, for use as pollinators, or for release when under attack to warn neighboring plants and/or to "cry for help" in order to lure the enemies of the attacking aggressors [1, 2]. The biological properties of EOs and thus also of their constituents have already been covered in reviews by one of the authors [3–7] and other colleagues [8, 9]. Therefore, to avoid a repetition of already discussed material, the present overview focuses on biological activities and aromatherapeutic uses of chemical classes of MTs and SQTs, specifically hydrocarbons, alcohols, oxides, and carbonyls. Because hundreds of MTs and SQTs and their derivates exist, only a few prominent representatives of each class will be discussed. Finally, the term *biological* must be defined, as has already been done in [4]. Therefore, in this treatise the following are not discussed: plant care, inter-plant communication (see also [1, 2]), pheromones (discussed in detail in [10]), veterinary therapeutics, cosmetic uses, perfumes, household and cleaning products, and flavors for food and drinks. Other activities will be partially discussed; they are also covered in [7]. The "penetration enhancement properties" of MTs and SQTs have been excluded here as they are discussed in ▶ Chapter 124, "Terpenes and Improvement of Transdermal Drug Delivery".

2 Hydrocarbons

2.1 (+)-Limonene

(Former: d-limonene, ᴅ-limonene)



One of the most prominent MT-hydrocarbons is (+)-limonene, which occurs in nearly every EO of the citrus oils and is a major compound (up to 97 % [11]) in sweet orange oil (from the peel of *Citrus sinensis* (L.) Osbeck, syn. *Citrus aurantium* var. *sinensis*). Its odor is reminiscent of the typical sweet orange flavor, whereas its antipode (–)-limonene possesses an odor that recalls turpentine [12]. Sweet orange oil achieves its main importance in the flavor and food industry because it is easily obtainable (yield: ~5 % [11]) and due to its pleasant odor, which is accepted by everyone and is caused by the "character impact compound" [13] (+)-limonene. In the past, the use of (+)-limonene has experienced a great expansion. Besides its use in the food industry, it is used as flavor and fragrance additive in cosmetics, soaps, and perfumes, and also in medicine to mask the bitter taste of alkaloids in pharmaceutical products. (+)-Limonene is consumed by people mostly as a natural ingredient in common foods such as oranges and other citrus fruits, juices, vegetables, coffee, meat, and spices [14]. (+)-Limonene is also realizing increasing use in cleaning and disinfection products in both industrial and household products.

There has been increasing interest in the use of EOs as plant-based antimicrobials in the food industry. They are an alternative to synthetic antimicrobials, attractive because of the growing resistance of foodborne microorganisms to synthetic chemicals, and also because these plant-based antimicrobials are less expensive and more environmentally friendly. Singh et al. [15] confirmed the use of (+)-limonene as a plant-based antimicrobial and, due to its antioxidative effects, a food preservative. They investigated the antifungal, antiaflatoxigenic, and antioxidant activity of EOs of the leaves of *Citrus maxima* Burm. and of the peels of *Citrus sinensis (L.) Osbeck*, and the 1:1 combination of them. The major components, analyzed by GC-MS, in the oil of in *C. maxima* were 31.8 % DL-limonene and 17.7 % *E*-citral; in the oil of *C. sinensis*, DL-limonene represented 90.7 % followed by 2.8 % linalyl acetate. The 1:1 combination contained 69.8 % of DL-limonene. An antifungal assay was first performed due because fungi are among the major destroyers of food that is being stored. The results showed, according to ANOVA and Tukey's comparison test, that the EOs were in all concentrations effective compared with a control. A broad fungitoxic spectrum was established. Furthermore, the efficacy of suppression of aflatoxin production was investigated and at 500 ppm the EOs of *C. maxima, C. sinensis*, and their combination, showed a complete inhibition of AFB1 production and AFB1.

The antioxidant activity was observed by DPPH radical scavenging assay on TLC, which proved the strong antioxidative effect. Tests on mice showed a high value for LD50, which confirmed the safety of oral consumption. (+)-Limonene is generally recognized as safe (GRAS) by the US FDA. By oral consumption it has a relative small toxicity, although when applied in high concentrations it may cause dermal irritation [16].

Because limonene possesses such strong antioxidant activity, it could potentially provide protection from diseases caused by oxidant damage, for example, cancer.

Previous studies in rats and mice showed that limonene prevented the growth of tumors in chemical-induced carcinogenesis models.

Roberto et al. [14] analyzed the effect of limonene on proliferation of normal lymphocytes and its connection to the H_2O_2 level and its effect on the cell antioxidant enzymes (catalase, peroxidase, and superoxide dismutase). H_2O_2 has a large impact on the process of growth and death of cells. In small concentrations it stimulates cell proliferation, whereas in higher concentrations proliferation is decreased. H_2O_2 also is connected with damaging the DNA and genetic mutations. The results showed that, at low concentrations, limonene decreased H_2O_2, whereas in higher concentrations the level increased.

The enzymes peroxidase and catalase reduce the concentration of organic hydroperoxides and hydrogen peroxides, whereas superoxide dismutase generates H_2O_2. Limonene presented its activity relative to the applied concentrations.

Limonene applied in low concentrations leads to an increase of catalase and peroxidase, which then leads to decrease of H_2O_2 and vice versa. In addition, limonene can stimulate cell proliferation through decreasing the level of H_2O_2 by increasing the activity of the enzymes catalase and peroxidase. Limonene also protected the cells from oxidative damage when H_2O_2 was exogenously added.

Chaudhary et al. [17] investigated the exact mechanism of how limonene provides its antitumor effects. The chemopreventive and chemotherapeutic effects of D-limonene were tested against chemically induced tumors in female Swiss albino mice. The development of the tumors was initiated by DMBA (7,12-dimethylbenz-[a]anthracene) and promoted by TPA (12-*O*-tetradecanoylphorbol-13-acetate). DMBA and TPA activate a few carcinogenesis pathways. One way is by triggering the Ras-ERK pathway; another is the genetic mutagenesis made by ROS that are generated by TPA. As mentioned in the study above, the activity of antioxidative enzymes is reduced and there is an up-regulation of proinflammatory genes such as COX-2. Limonene showed significant results: by reducing the edemas and hyperplasias that were chemically induced, it reduced COX-2 expression, the activity of ornithine decarboxylase while the level of antioxidant enzymes was increased, and the amount of [3H] thymidine incorporated in the genetic material reduced. A topical treatment with d-limonene, prior to TPA alleviated the TPA-induced increase of COX-2 enzymes, which implies that COX-2 might be a potential target for d-limonene.

A significant inhibition of the Ras/Raf/ERK signaling pathway can be confirmed, which is also connected to a suppression of the induced down-regulation of Bax and up-regulation of Bcl-2. Namely, when ERK is activated, it has activating effects on proteins such as transcription factors and other protein kinases. By its inhibition, it affects the expression of apoptotic proteins such as Bim, Bax, and Bcl-2, leading to an apoptosis. By attenuating the inflammatory process, oxidative stress, and Ras-cascade, limonene provided its chemopreventive effect and the induced skin tumorigenesis could be delayed.

One study showed that d-limonene alleviates insulin resistance and liver injuries induced by oxidative stress. Victor Antony Santiago et al. [18] performed their investigations on young, male Wistar rats that were previously fed a high-fat diet together with L-NAME for 8 weeks and subsequently with 2 % (+)-limonene in the last 4 weeks. They examined the effect of (+)-limonene against biochemical and histological alterations of the liver in a high-fat diet and L-NAME-induced metabolic syndrome. Dietary d-limonene supplementation improved the biochemical changes in the liver induced by HDF and L-NAME, especially the hepatic lipid accumulation, liver function indicators, circulatory antioxidants, hepatic histology, and insulin resistance. (+)-Limonene restored the pathological changes of liver and pancreas. These findings indicate the potential therapeutic efficacy of (+)-limonene against the development of NAFLD (nonalcoholic fatty liver disease), especially as a promising complementary treatment. NAFLD is most probably the hepatic manifestation of the metabolic syndrome (linked to obesity, insulin resistance, diabetes type 2, and hyperlipidemia).

> d-Limonene is known to inhibit lipid peroxidation, arrest the free radical-induced damage and prevent physical stress, psychological stress, stress-induced hypertension, and stress responses in stroke-prone spontaneously hypertensive rats. d-Limonene is also known to regulate the development of pulmonary hypertension, induce glutathione (phase II detoxification) and inhibit 3-hydroxy-3-methylglutaryl coenzyme A(HMG-CoA) reductase activity. In addition, d-limonene is reported to exert potent biological activities, such as antioxidant properties, chemopreventive or chemotherapeutic properties against many types of cancers, antiinflammatory properties, hepatoprotective activities and immunomodulatory effects. [18]

Limonene also shows other effects on the cellular metabolism. Park et al. [19] came, in their study, to the conclusion that limonene binds directly to the adenosine A2a receptor. This leads to the activation of receptor-mediated signaling pathways – the increase of the cytosolic cAMP concentration and activation of protein kinase A – and further to the phosphorylation of the CREB transcription factor. Through binding on the adenosine A2a receptor, limonene also increased the intracellular calcium level. Both these effects are typical for agonists of the receptor, which leads to the conclusion that limonene also acts as an agonist on the A2a receptor. The ligands of A2a receptors have, in general, an impact on the inflammation process through modulating the release of the pro- and anti-inflammatory cytokines, so they act as a potential protection of tissue injuries. Therapeutically, they can be used as potential sleep inducers due to their effects in sleep regulation. This holds implications for the possible sedative effects of limonene. The activation of the receptor has also influence on the cardiovascular system.

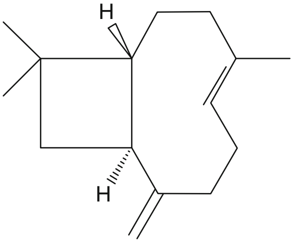
Fletcher [20] investigated the effects of (+)-limonene and its metabolites perillyl alcohol, perillaldehyde and perillic acid on the membrane lipid bilayer. The effect was assessed by bilayer-spanning gramicidin (gA) channels using two methods. The first was a fluorescence assay, which showed that at micromolar concentrations (+)-limonene decreased the gA channel activity and all its metabolites (except perillic acid, which had no effect) increased the activity. The second method, using single-channel electrophysiology, showed, however, that each terpene increased the lifetime and occurrence of the gA channel. Thus the disagreements appeared between (+)-limonene and perillic acid using these two methods; nevertheless, these terpens have been confirmed to have significant bilayer-modifying potential.

Limonene also has an effect on the central nervous system. Further studies have shown the relaxant properties and anxiolytic effect of EO of *Citrus sinensis*, suggesting a possible depressant activity of these constituents [21]. De Almeida et al. [22] analyzed the effects of (+)-limonene epoxide on the CNS of male Swiss mice.

(+)-Limonene epoxide is synthesized from (+)-limonene and is a mix of cis and trans-isomers that are found in many plants. It has been shown to have antitumor and antinociceptive activities. In the study, the acute toxicity of (+)-limonene epoxide in mice was examined, showing, dose-dependence and relatively good safety, though it fell into the group of slightly toxic substances. Furthermore, the anxiolytic, sedative, and motor coordination effects were investigated using diazepam as a positive control. (+)-Limonene epoxide was able to significantly decrease the number of crossings, grooming, and rearing. It increased the percentage of open arms entries and the time spent in those arms. At higher doses, it produced an inhibition of motor coordination, presented through a muscle relaxation effect. Flumazenil reversed the diazepam and (+)-limonene epoxide effect, suggesting that its mechanism might be involved in an action on the $\mathrm{GABA_A}$ receptor complex. These findings suggest (+)-limonene epoxide as a therapeutic approach in the treatment of anxiety inasmuch as it is relatively safe. The results also indicated that (+)-limonene epoxide might be responsible for the effects of limonene on the CNS.

2.2 β-Caryophyllene

Synonym: (−)-trans-Caryophyllene



Another prominent representative of the ST-hydrocarbons with significant scientifically proven biological activities is (*E*)-β-caryophyllene. It occurs in large amounts as a major plant volatile in the EOs of spice and food plants like *Origanum vulgare* L. (oregano), *Cinnamomum spp*. (cinnamon), and *Piper nigrum L.* (black pepper). *E*-BCP is in nature found together with small amounts of its isomers (*Z*)-β-caryophyllene (*Z*-BCP) and α-humulene (former name α-caryophyllene) or with its oxidation product β-caryophyllene oxide [23]. β-caryophyllene possesses a woody, spicy aroma, so traditionally it is used in the fragrance and cosmetic industry. Because its antibiotic, anesthetic, anti-inflammatory, antioxidant, and other effects have been established by scientific studies, there is great interest in the use of this natural product as a starting point for the development of new drugs. At the present time, β-caryophyllene is being isolated by various methods of purification from oleoresins extracted from huge amounts of plant materials. To avoid this wasteful means of production, Reinsvold et al. [24] engineered phototropic microorganisms with sesquiterpene-synthase genes. The β-caryophyllene synthase gene from *Artemisia annua* was inserted into the genome of the cyanobacterium *Synechocystis ssp.*

The experiment was successful and the synthesis of β-caryophyllene was confirmed in the transgenic strain using GC-FID and GC-MS analysis. This was an important step in developing alternative ways of synthesizing relevant terpenoids, both for pharmaceutical research and for biofuels.

(*E*)-BCP is a selective agonist of the cannabinoid receptor type 2. Investigations were performed in 2008 by Gertsch et al. [23] in the EO of *Cannabis sativa* L., which contains (*E*)-BCP up to 35 %. It was the first *Cannabis*-derived CB receptor ligand with a basically different structure than the one of typical cannabinoids. Traditional cannabinoids are agonists of CB_1- and CB_2-receptors, so, despite their potential therapeutic effect by activating the CB_1-receptor, they cannot be taken for a pharmacological development because of their central CB_1-receptor activity. In this study, a CB_2-receptor-selective agonist was discovered that provided all the potential therapeutic effects of a CB_2-receptor activator but without the psychoactive effects associated with the CB_1-receptor activation. This makes (*E*)-BCP an excellent candidate for the development of new drugs for treatment of inflammation and pain, atherosclerosis, and osteoporosis. (*E*)-BCP binding of the CB_2 receptor initiates a complete stimulation program:

- Inhibition of the adenylate cyclase, which leads to calcium transiency in the cell
- Weak activation of the mitogen-activated kinases ERK1/2 and p38 in primary human monocytes. Three major MAPK pathways are known, ERK1/2, JNK, and p38, which further on leads to the phosphorylation of cytoplasmic and nuclear targets. ERK is mostly activated by mitogenic factors, whereas JNK and p38 are usually activated by stress-inducing stimuli such as UV light. MAPKs have, in general, an important role in cell proliferation [25].
- Inhibition of LPS-induced proinflammatory cytokine expression in peripheral blood
- Alleviation of LPS-stimulated ERK1/2 and JNK1/2 phosphorylation in monocytes, because these pathways are critical for expression of IL-1 and TNF-α (both cytokines involved in inflammation processes in the body). The experiment confirmed that (*E*)-BCP also provides its effect in vivo.

After this discovery, interest in further investigations of (*E*)-BCP was awoken. Horváth et al. [26] investigated the possible therapeutic effects of BCP in a cisplatin-induced murine nephropathy model. Cisplatin is a chemotherapeutical agent often used in cancer therapy but with nephrotoxicity as a side effect. This side effect is probably caused by oxidative and nutritive stress and inflammation, so a solution for preventing or reducing these complications is in great demand. β-Caryophyllene attenuated cisplatin-induced kidney dysfunction and morphological damage, inflammatory response in the kidney, the increased oxidative and nutritive stress, and the enhanced cell death. All of these effects were provided in a CB_2-receptor-dependent manner, which was proven by the fact that the protective effect of BCP was absent in CB_2-knock-out mice.

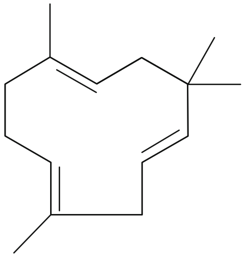
CB_2-receptors also exist, in low levels, in cells of the gastrointestinal and cardiovascular system, bone and neuronal cells, liver tissue, and other cell types [26]. CB_2 is up-regulated in inflamed colonic tissue of colitis patients. It is believed that the CB_2-receptors are in close interaction with the PPARγ-receptor, and both are considered targets for treatment of inflammatory bowel diseases. That was the motivation for Bento et al. [27] to investigate the effect of oral BCP in DSS (dextran sulfate sodium)-induced colitis experimental models. The results showed that BCP inhibited the influx of inflammatory cells, decreased damage to the colon, and reduced the production of inflammatory mediators and cytokine release from LPS-stimulated macrophages. It also inhibited the activation of transcription factors NFkB, CREB, and ERK ½ and activation of colonic caspase-3 but not claudin-4. The effects of BCP could be reversed by CB_2 and PPARγ selective antagonists. That leads to the conclusion that BCP activates the CB_2 receptor and reduces the inflammation of the colon by directly or indirectly interacting with the PPARγ-receptor. The examination showed, with small significant differences, however, that a preventive treatment was more effective than the therapeutic treatment, so BCP exhibits both preventive and therapeutic effects in DSS-induced colitis models. A preventive treatment with BCP also improved oxazolone-induced colitis by reducing weight loss and increasing the survival rate. Investigations also showed that applications of BCP in high concentrations induced an antiedematogenic effect in CB_2 knockout mice, which could suggest that BCP does not act on the CB_2 receptor exclusively.

β-Caryophyllene also showed antispasmodic activity. Leonhardt et al. [28] examined the effect of BCP as the main constituent of the EO of *Pterodon polygalaeflorus* F. on the isolated ileum from rats. Both BCP and the EO of *P. polygalaeflorus* F. showed a dose-dependent relaxant effect on the ileum and were able to inhibit the acetylcholine and KCl-induced contractions of the ileum and alleviate $CaCl_2$-induced contractions. This effect on muscle contractility is provided by an intracellular mechanism and is myogenic. This leads to the conclusion that BCP plays a crucial role in the relaxation and antispasmodic effect that the EO of *P. polygalaeflorus* F. provides in the ileum.

β-Caryophyllene shows a potential anti-cancer effect. Previous studies have demonstrated that BCP possesses strong antimutagenic activity against 2-nitrofluorene mutagene [29] and that it has a potentiating effect in the anticancer activity of α-humulene, isocaryophyllene, and paclitaxel against tumor cell lines [30]. To investigate whether BCP provides an antitumor effect and its possible mechanisms, di Sotto et al. [31] studied the in vitro effects of BCP at the chromosomal level by using human lymphocytes. The cultured lymphocytes were exposed to the genotoxic effects of two different mutagens: the alkylating agent EMS (ethyl methanesulfonate) and the aneugenic agent COL (demethylcolchicine). The treatment with BCP was performed three times: a pre-treatment before the treatment with the mutagenes (to examine the capability to prevent the damage), a co-treatment (to see whether BCP can directly interfere with the mutagene) and after the damage was made by mutagens, and a post-treatment (to see whether BCP is able to repair the genotoxic damage). The results showed that, compared with the control, BCP by itself did not provoke any cytotoxic or genotoxic effects. BCP's provision of its anticlastogenicity potential exclusively in the pre- and co-treatment with EMS was significant but not dose-dependent. The post-treatment could not assert any antimutagenc effect of BCP, which means that it could not promote a reversion of the damage made on the DNA. In addition, testing in the presence of COL could not confirm a protective effect. This could lead to the conclusion that BCP acts as a des-mutagen and is an active pre- or co-treatment antimutagen, which means that it deactivates mutagens before they attack the DNA. The exact mechanism is not yet clear. The anticlastogenic activity may be involved in the antioxidant effect that BCP provides, or a chemical interaction with the mutagens is possible; another hypothesis is that is has a destabilizing effect on the cellular membrane. Nevertheless, due to the lack of genotoxic effects and the anticlastogenic activity, BCP provides valid reasons for further investigations and interest as a potential chemoprotective agent.

2.3 α-Humulene

Synonym: Humulene, α-Caryophyllene



α-Humulene is a naturally occurring monocyclic sesquiterpene, with a structure built of three isoprene units. The name α-humulene is derived from *Humulus lupulus*, in whose EO it is found. Humulene and its oxidation products play an important role in the hoppy flavor of beer [32]. In plants it is often found together with its isomer β-caryophyllene, like in the EO of *Cannabis sativa* L., where they are the major sesquiterpenes [33], and it contributes to the characteristic odor of this plant.

α-Humulene is also an important constituent of *Cordia verbenacea*, which is used in folk medicine for its antirheumatic, anti-inflammatory, analgesic, and healing properties. It is believed that the oral-inflammatory actions that *C. verbenacea* provides are related to the presence of the SQTs α-humulene and β-caryophyllene.

α-Humulene showed rapid and relatively good absorption by oral and topical administration, which plays an important role in the topical and systemic anti-inflammatory and antinociceptive effects it provides. Chaves et al. [34] reported that the oral anti-inflammatory effects of α-humulene and β-caryophyllene, isolated from *C. verbenacea*, could be compared to the effects observed in animals treated with dexamethasone. They examined the inhibitory effects of these two compounds in different inflammatory models in mice and rats.

Fernandes et al. [35] reported that these SQTs were able to inhibit the activation and/or release of inflammatory mediators like bradykinin, platelet activating factor, histamine, IL, IL-1β, TNFα, and PGE_2. They also inhibited the up-regulation of the enzymes COX-2 and iNOS (inducible nitric oxide synthase). α-Humulene stood out in the study as it was the only compound that could, in a systematic treatment, reduce the histamine-induced mouse paw edema and largely prevent both TNFα and IL-1β generation in carrageenan-injected rats, whereas β-caryophyllene reduced only TNFα release.

Based on these findings, Rogerio et al. [36] investigated the anti-inflammatory properties of α-humulene, in order to identify potential targets that could prevent or treat inflammatory diseases like allergies or asthma. α-Humulene was applied preventively and therapeutically in a allergic airway inflammation murine model. The examination results revealed that SQT reduced the eosinophilic migration into the bronchoalveolar lavage fluid and lung tissue, similar to that reported with corticosteroids.

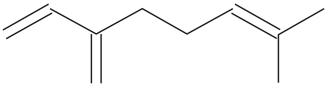
It is believed that the mechanism is related to a reduction of inflammatory mediators, adhesion molecule expression, and activation of transcription factors. Namely, by modulation of the Th1/Th2 (T-helper1/T-helper2) balance, reduced production of mucus, and inhibition of IL-5, CCL11 (chemokine (C-C motif) ligand 11 (eotaxin)) and LTB4 (leukotriene B4) levels and P-selectin expression, probably by inhibiting the NF-κB and AP-1 pathways. An interesting fact was that the animals treated with α-humulene gained weight similar to animals from the control, while dexamethasone-treated animals suffered weight loss. This implies a minor collateral effect of this compound. α-Humulene was successful, by oral or aerosol treatment, though application via aerosol was more effective.

It is reported that α-humulene also provides an antitumor effect. In the study, α-humulene inhibited the growth of MCF-7 breast cancer cells by about 50 %. This effect can be potentiated by β-caryophyllene by increasing the inhibition up to 75 % [30].

El Hadri et al. [37] investigated the cytotoxic effect of both α-humulene and β-caryophyllene from *Salvia officinalis* on breast cancer MCF-7, colon cancer HCT-116, and murine macrophage RAW264.7 cellular lines by the MTT assay (a colorimetric assay to assess viability and cell proliferation and also the cytotoxicity of substances). The results showed that the subfraction of *S. officinalis* EO, containing α-humulene, provided the highest activity at the RAW264.7 and HCT-116 cell line, whereas the subfraction with β-caryophyllene showed less activity on the same cell lines. This suggested that both SQTs were able to inhibit the growth of tumor cells.

2.4 Myrcene

Synonym: β-myrcene



Myrcene is an unsaturated acyclic MT, which exists in two isomeric forms: β-form, which can be found in nature, and the α-isomer, which does not occur naturally but can be easily synthesized. The EO has a pleasant odor of geranium, but in pure form it is not used as a flavor; a solution with at most 5 % would be recommended for smelling. Myrcene tends to polymerize, which is why it is unsustainable in the air [38]. It has a reactive diene structure, which makes it an eclectic starting material for flavoring agents and fragrances. It also is used in cosmetics, soaps, detergents, vitamins, and pharmaceuticals and as a flavoring agent in food and beverages. It is the main constituent of hop and bay oils, which are used in the production of alcoholic drinks [38, 39]. Myrcene can be found in *Humulus lupulus*, *Pimenta racemosa*, *Rosmarinus officinalis*, and *Salvia officinalis*.

Behr and Johnen reported [38] that myrcene is an important starting point for the synthesis of menthol, nerol/geraniol, and linalool. Further derivates are citral, citronellal, and citronellol, which are used because of their lemon-like smell. Based on the diene structural component, it can be used for Diels-Alder reactions with unsaturated structures, which leads to synthesis of amberlike flavors and anticancer therapeutics. By a C-C linkage, geranylacetone and β springene can be obtained, derivates which can be used to synthesize side chains of vitamin E.

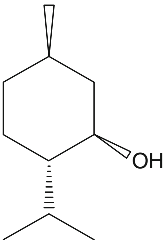
Another use of myrcene is the synthesis of pheromones that can be used as traps for insects. Myrcene can be found in many plants, but its extraction would not be economical, so industrially it is obtained by pyrolysis of β-pinene, which is contained in turpentine.

The National Institute of Environmental Health Sciences [39] studied myrcene for its cancerogenic activity, because it is heavily produced and shows structural connections to limonene, which induces tumors in male rat kidneys. The study was performed on male and female rats and mice by force-feeding with myrcene for either 3 months or 2 years. The results of the 2-year studies showed that myrcene possesses a carcinogenic effect based on increased incidence of renal tubule neoplasms in male rats and renal tubule adenomas in female rats. Increased incidence of hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma was seen in male mice and marginally increased incidences hepatocellular adenoma and carcinoma were observed in female mice.

3 Alcohols

3.1 (−)-Menthol

Synonym: *Levomenthol*



Menthol belongs to the group of monocyclic terpenes, which can be found as a major compound in the EO of leaves of mentha species like *Mentha piperita* and *Mentha arvensis*.

Because of the presence of three asymmetric C-atoms in its structure, menthol occurs in four pairs of optical isomers, (−)- and (+)-menthol, (+)- and (−)-neomenthol, (+)- and (−)-isomenthol, and (+)- and (−)-neoisomenthol. (−)-Menthol is the isomer that mostly occurs in nature and, besides its characteristic odor, it possesses a cooling effect on the skin and mucosa. It can be obtained synthetically or from peppermint or other mint oils, or from essential oils such as citronella oil, eucalyptus oil, and Indian turpentine oil. Due to its minty smell and flavor, it is used in pharmaceuticals, soaps, hygiene products like toothpaste, cosmetics, chewing gum, teas, sweets, and tobacco products [40, 41].

Because of its antispasmodic, carminative, choleretic, and cholagogic effects it is traditionally used for treating gastrointestinal disorders and also in mucus-dissolving and broncholytic preparations. In pharmaceuticals it is a compound in antipruritic, antiseptic, and cooling preparations [40].

The cooling effect and tingling sensation of menthol by topical application is related to its stimulation of cold-receptors. This stimulation is caused by inhibiting Ca^{++}-currents of neuronal membranes, since Ca^{++}-channel blockers are connected to painkilling properties [40]. Both (+)- and (−)-menthol show equiactive local anesthetic activity, but only (−)-menthol also elicits an analgesic effect [40].

Kahner et al. [41] reported on the effects of menthol in tobacco-products. Approximately a quarter of worldwide use of menthol is in tobacco products. According to the tobacco industry, it gives the tobacco a more intense and pleasant taste. The addition of menthol actually has strong pharmacological effects, such as easing inhalation and increasing the addictive potential, which can further lead to numerous chronic diseases and death. The authors detail the mechanism by which menthol interacts in the body. Menthol interacts with channels responsible for perception of heat, cold and pain, the so-called TRP ion channels, particularly with TRPM8, which reacts with cold. Menthol inhibits TRPA1, responsible for pain perception, so it works as an analgesic and anesthetic. With long and recurrent consumption it causes a desensitization of the mouth, which affects the perception of irritant substances such as nicotine. Menthol also increases the transdermal and transbuccal absorption of substances and prolongs the time the breath can be held and suppresses the need to cough. Other studies reported that menthol inhibits the oxidation of nicotine to cotinine, so nicotine stays longer in the body,

Subsequently, there have been reports of menthol pyrolysis-products containing the cancerogenic agent benzopyrene and menthol inducing the absorption of benzopyrene.

In conclusion, menthol in tobacco products is not simply a flavor, increasing the tobacco taste; it affects sensoric perception, smoking habits, and addiction potential.

The mechanism of menthol interaction in the body, as mentioned above, is confirmed by other studies. Willis et al. [42] concluded that menthol in mentholated cigarettes acts as a counterirritant that diminishes the chemosensory responses of inhaled irritants. Irritations were elicited in mice by irritants that occur in cigarette smoke (acrolein, acetic acid, cyclohexanone) and menthol abolished the irritation responses caused by these irritants, which are agonists of the TRP channel family. Menthol effects could be reversed by a TRPM8 antagonist.

The effect of menthol in mentholated cigarettes on nicotine pharmacokinetics is an important topic for investigation for the tobacco industry. Abobo et al. [43] performed their examination by exposing rats to the smoke of mentholated and nonmentholated cigarettes, then collected blood samples and analyzed the nicotine and cotinine concentrations. The results showed that mentholated cigarettes decreased the maximum concentration (Cmax) of nicotine in plasma and the plasma AUC compared with nonmentholated cigarettes. The values for cotinine were reduced by menthol as well. These results showed that menthol in mentholated cigarettes decreased the absorption and increased the clearance of nicotine. Kreslake and Yerger [44] reported that menthol, besides its use as a flavor and to ease inhalation, also reduces the irritation from inhaling smoke and modulates the subjective effects, like smoke harshness and increased smoothness.

In addition to its use in the tobacco industry, menthol provides many biological activities, which make it extremely attractive in pharmacy and medicine. It has been reported that menthol showed anticancer effects by being effective in treating prostate cancer in vitro. Menthol induced cell death in prostate cells through TRPM8 activation and the resulting increase in Ca^{++} [45].

Bhadania et al. [46] reported that menthol had a protective effect on β-amyloid peptide–induced cognitive deficits in mice. Menthol's ability to interfere with cognitive actions opened another area of medical research on the effects of this MT. Young and aged mice were examined, using interceptive and exteroceptive memory models (modified elevated plus-maze test and Morris water maze test, which are used in behavioral neuroscience to study spatial learning and memory processes in rodents) and various biochemical parameters were assigned (brain glutamate, glycine, glutathione, and thiobarbituric acid reactive substances). The nootropic effect of menthol on learning and memory was evaluated with piracetam as a control. Menthol maintains the glutamate concentration in the mouse brain throughout its antioxidant activity. It triggers the glutamate release by acting directly on the presynaptic Ca^{++} stores of sensory neurons to release Ca^{++}. It is believed that the glutamate concentration plays an important role in cognitive functions. The results showed a significant enhancement in learning and memory. Menthol did not modify the level of glycine in the brain, but it increased the glutamate level, which leads to the conclusion that the effect is most probably based on the glutamatergic neuronal effect.

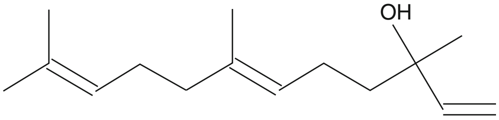
Menthol has been recognized for many years as a treatment for gastrointestinal disorders because it relaxes the GI smooth muscles. Thus, the use of menthol as an antispasmodic agent before GI endoscopy has been examined. The application of the EO in form of a spray on the gastric mucosa inhibited gastric peristalsis, having the advantage of being connected with fewer undesired drug effects than the substances typically used. Hik et al. [47] investigated the mechanism and pharmacokinetics of menthol used for GI endoscopy. Menthol showed fast absorption and was excreted mainly through the urine in form of menthol-glucuronide. It had good safety and only a few adverse effects, whose relation to the GI treatment cannot be excluded. The C_{max} and AUC of menthol increased dose-dependently, but the elimination half life showed it to be dose-independent. The study showed that the C_{max} can be obtained faster if sprayed on the gastric mucosa (0.17–1.00 h) rather than taken orally.

Menthol is traditionally used to relieve pain caused by exercise because of its cooling and relaxing effect. Topp et al. [48] examined the mechanism by testing the effect of menthol on blood flow and arterial diameter. The investigation was performed on a small group of eight males and eight females, and with two doses of menthol (3.5 % and 10 %), assessing the blood flow and arterial diameter before and after MVMC (maximum voluntary muscular contraction) were performed on the quadriceps and hamstrings. Exercise with high intensity and short duration increases the blood flow of the surrounding tissue and menthol acts by stimulating the thermoreceptors, leading to a vasoconstriction and localized cooling. The results showed that application of both doses decreased the local as well as the generalized blood flow after a MVMC. This effect may be attributed to an inhibition of local NOS and NO and also to an increase in systemic a_{2C} adrenergic tone.

The effects of ice and a menthol gel (3.5 % menthol) on blood flow and muscle strength of the lower arm were compared in another study [49]. The results suggested that menthol provides a fast-acting but short-lived reduction of the blood flow, while with topical application of ice a similar vasoconstrictive effect can be achieved only by a longer application of ice.

3.2 Nerolidol

Synonym: Peruviol



Nerolidol is a natural occurring aliphatic SQT-alcohol that possesses one chiral center in its structure; it prevails as a mixture of its cis and trans-form. It is an isomer of farnesol, from which it is distinguishable by a different position of one double bond and the hydroxyl-group. Nerolidol is a major component of EO extracted from many plants [50–52]. It has a woody aroma reminiscent of tree bark. It is used to enhance flavor and aroma and is also used as a fragrance in perfumes, cosmetics, shampoos, toilet soaps and, household products. [53].

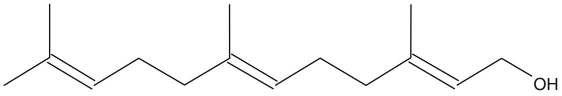
It has been reported that this long-chain SQT is an enhancer for the transdermal delivery of therapeutic drugs and for substances that permeate the human skin membranes in general [54]. It reinforces the bilayers, possibly by orientating alongside the lipids of stratum corneum [55]. Nerolidol also exhibits antineoplastic activity, probably by having an impact on protein prenylation or affecting the mevalonate pathway [56]. The antibacterial effect of nerolidol was confirmed in several studies, for example, on *Staphylococcus aureus*, reporting that the mechanism of this action is probably the damaging of the cell membrane [57]. Other studies accounted the antifungal effect against *Microsporum gypseum* [58] and its anti-Leishmania effect by inhibiting the growth of *Leishmania amazonensis, L. braziliensis*, and *L. chagasi promastigotes* and *L. amazonensis* amastigotes [59]. Nerolidol also displays an anti-ulcer activity. As a main constituent from the essential oil of *Baccharis dracunculifolia* DC, it inhibited the formation of ethanol-, indomethacin-, and stress-induced ulcer models in rats [60]. Nerolidol and farnesol were classified in 1986 as active ingredients in biochemical pesticides [61].

Ferreira et al. [62] investigated the effect of nerolidol in mitochondria and also correlated the results with its cytotoxic effect on HepG2 cells. The mitochondria might be a target for compounds of EO, provoking changes, possibly leading further on to enzyme inhibition or cell death. And nerolidol can, as a hydrophobic compound, cross the plasma membrane easily and interact with cellular proteins and intraorganelle sites. Nerolidol was shown to increase respiratory chain activity and decrease phosphorylative efficiency. The inhibitory effect in the phosphorylative system is related to a reduced concentration of ATP in cells, through inhibition in the ATP-ase enzyme activity. Nerolidol also was shown, dose dependently, to induce a decrease of the mitochondrial transmembrane electric potential. The permeability transition in the mitochondria was delayed, most probably as a result of a Ca^{2+}-uniporter reduced activity. The decrease of the calcium-induced permeability transition susceptibility can be a consequence of the decreased membrane potential and modifications of the mitochondrial membrane fluidity. In connection to the HepG2 cell line, nerolidol presented hepatic cell cytotoxicity. It induced cell death and inhibited cell growth.

Nerolidol is classified (as is farnesol) in “Toxicity Category IV” for acute oral toxicity, “Toxicity Category III” for acute dermal toxicity, primary eye irritation and primary dermal irritation, and “Toxicity Category II” for acute inhalation toxicity, according to the US Environmental Protection Agency (EPA) [61]. Considering this and the above-mentioned cytotoxicity, nerolidol may present a possible risk in use as a therapeutic agent, or as a flavor enhancer at high doses; therefore, differentiation between the therapeutic and toxicological effects is important.

Pículo et al. [63] assessed the in vivo gentoxicity of nerolidol. The authors investigated whether a single treatment with this compound was able to induce DNA damage in peripheral blood and liver cells of mice and micronuclei in polychromatic erythrocytes of their bone marrow cells. The comet assay was used to assess the genotoxicity, and *N*-nitroso-*N*-ethylurea was a positive control for the comet and micronucleus assay. In both peripheral blood and liver cells, nerolidol induced weak, dose-dependent DNA damage compared with the control. Nerolidol also induced a clastogenic effect on bone marrow cells of mice by enhancing the average number of micronucleated cells in high doses tested. In conclusion, the study pointed out the clastogenic and weak genotoxic effects of nerolidol.

3.3 Farnesol



Farnesol is an acyclic SQT-alcohol that possesses a floral odor; some people report it reminds them of *Convallaria majalis*. It is often used to enhance the odor and flavor of sweet floral perfumes and as an antibacterial compound in cosmetics. Farnesol has been shown to decrease biofilms of *Staphylococcus epidermidis*, which often causes infections and is resistant to antimicrobial agents. Thus, farnesol has been shown to be a potential therapeutic for clinical *S. epidermidis* biofilm infections [64]. There has been an increase in interest in the use of this compound as an antifungal agent. In *Candida albicans*, it caused a down-regulation of the expression of some aspartyl proteinase genes, provoking morphological changes [65]. Farnesol is believed to be endogenously produced by dephosphorylation of farnesyl-PP, a metabolite of the cholesterol biosynthetic pathway [66].

Hyuck Joo and Jetten [67] reported on the anti-cancer and chemoprotective effects of farnesol and summarized the mechanisms of its apoptosis-inducing activities. In vitro, farnesol was shown to inhibit cell proliferation and induce apoptosis in different types of malignant cells. It was notable that tumor cells were more sensitive to the growth inhibition induced by this compound than normal cells. Cells treated with farnesol were shown to have a G_O/G_1 cell cycle arrest, reduction in in CDK2 activity, and an increased generation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ with cyclin E/CDK2 complexes. The inhibitory effect of this terpene is suggested to be dependent on these CDK inhibitors ($p21^{Cip1}$ and $p27^{Kip1}$), because their down-regulation provided protection from the proliferation-inhibitory effect. Farnesol also proved its anti-tumor effects in vivo. Liver of farnesol-treated rats had an increased number of phase I and phase II enzymes, which metabolize drugs and carcinogens, so farnesol might interfere in the metabolism, toxicity, or carcinogenesis of drugs. A farnesol has inhibitory effects on HMG-CoA reductase can be related to its anti-cancer effect. Tumor cells need an increased cholesterol biosynthesis, so by inhibiting it, farnesol might provide its growth-suppressing activity. A farnesol-induced endoplasmatic reticulum stress is a major factor leading to cell death. It can activate ERK1/2 and MAPK p38, and by activating this MEK-ERK-pathway, the ER-stress is most probably induced. The authors also reported that farnesol inhibited phosphatidylcholine synthesis by changing the subcellular localization and activity of CCTα (CTP: phosphocholine cytidylyltransferase α), which catalyzes its biosynthesis. Phosphatidylcholine is important in maintaining the structure of membranes and it is a precursor of a few second messengers, which control several cellular processes, including proliferation and cell death. Namely, under treatment with farnesol, CCTα translocates to the inner nuclear envelope, following a further export to the cytoplasm and causing an inhibition of the phosphatidylcholine synthesis.

Apoptotic stimuli can lead to an assembly of an apoptosome, a protein complex that also includes caspase. Farnesol activates caspases 3, 6, 7, and 9, but not caspase 8, which leads to the conclusion that the apoptosis is mediated by the intrinsic, mitochondrial-dependent pathway and not the extrinsic pathway. A higher level of expression of the pro-apoptotic protein Bak and lower level expression of anti-apoptotic proteins BCL2 and BCL-X are also related to a farnesol-induced apoptosis. Farnesol activates the NF-κB signaling pathway and expression of inflammatory genes and increases the level of ROS. Studies in vivo demonstrated that this substance can reduce oxidative stress, inflammations, and injuries in rat lungs exposed to intratracheal installation of cigarette smoke extract. In conclusion, the anti-tumor effects probably involve a few mechanisms, and farnesol can act at the initiation phase (reducing the DNA strand breaks and formations of DNA adducts) or at the progression phase of tumor development.

Qamar et al. [68] investigated the chemopreventive effects of farnesol on rats what were intratracheally exposed to the cancerogene benzo(a)pyrene. Pretreatment with farnesol alleviated the inflammation, edema, surfactant dysfunction, and injuries caused by this cancerogene. Farnesol was shown to have an effect on the benzo(a)pyrene metabolizing enzymes (NADPH-cytochrome P450 reductase, microsomal epoxide hydrolase (mEH), and glutathione S-transferase (GST)) and it was able to normalize the reduced levels of the lung surfactants.

Farnesol is an interesting and promising compound due to its antioxidant, anti-inflammatory, and chemopreventive properties. Khan and Sultana [69] explored its anticipatory effect against DMH (1,2-dimethylhydrazine)-induced oxidative stress, inflammatory response, and apoptotic tissue damage in the colon of Wistar rats. The study showed that a prophylactic treatment with farnesol increased the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, and quinone reductase and the cellular antioxidant-reduced glutathione. Farnesol was shown to have a protective effect against DMH-induced lipid peroxidation in colonic tissue. The pretreatment may also down-regulate caspase-3-activity, which was up-regulated by DMH, a colon-specific cancerogene. Farnesol was shown to suppress the initial stages of colon cancerogenesis, and the mechanism is, according to these findings, probably the amelioration of the oxidative damage, inflammatory processes, and apoptotic responses.

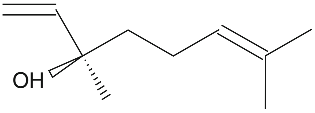
Because farnesol proved its antioxidant effect and and antioxidative agent can have a protective effect against neurotoxicity, de Oliveira Júnior et al. [70] investigated the antinociceptive effect of farnesol and its effect on the brain of adult mice. Mice were treated with doses of 50, 100, and 200 mg/kg, injected intraperitoneally. In the group treated with the highest dose, 16 % of the mice had a brain injury that affected 12 % of the hippocampus, but no lesions were found on mice treated with doses of 50 and 100 mg/kg. This leads to the conclusion that farnesol provides an antinociceptive effect, with no significant neurotoxicity.

A number of studies have previously shown that farnesol has an impact on the metabolism of lipids and can regulate serum lipid concentrations. This effect is a consequence of farnesol up-regulating the PPARα and genes of fatty acid oxidation, as well as down-regulating the synthesis of fatty acid in liver cells, which results from a decreased mRNA and protein level and activity of fatty acid synthase. Farnesol lowered serum triglyceride levels, and is potentially a protective factor to hypertriglyceridemia [71].

Goto et al. [72] made further investigations on farnesol being a ligand of PPARs and its effect on metabolic abnormalities. PPARs control energy homeostasis. Farnesol was shown to improve metabolic abnormalities by decreasing plasma glucose concentration, glucosuria, and the hepatic triglycerides. The study confirmed the previously mentioned mechanism of action, observing that farnesol could not up-regulate the mRNA expression of PPARγ target genes in adipose tissues. This showed that an up-regulation of fatty acid oxidation genes requires the function of PPARα, but farnesol was shown to act on two types of receptors: PPARα and FXR (farnesoid X receptor). FXR regulates genes important for bile acid homeostasis and lipid and glucose metabolism [65]. The decrease of the hepatic triglycerides is probably related to activation of both receptors, with FXR presenting a PPARα-independent way of acting.

3.4 Linalool

Synonym: β-linalool



Linalool is a MT-alcohol that possesses one chiralic C-atom, so it occurs naturally in the form of two enantiomeres: (–)-linalool and (+)-linalool. This compound is widespread in plants; the most commonly used *Lavandula* species are *L. angustifolia, L. latifolia, L. stoechas.* and *L. x intermedia* [73]. Other prominent linalool-producing species would be *Citrus bergamia* R., *Melissa officinalis* L., *Rosmarinus officinalis* L., *Cymbopogon citratu*s DC, and *Mentha piperita* L. [74]. Linalool is one of the best-examined terpenes. In ancient times it was used as a compound of these plants, providing sedative, analgetic, and anxiolytic effects, which has been later on proved in scientific studies. Studies also reported strong anti-oxidative, antibacterial, antifungal, anticonvulsive, and antihypercholesterinemic effects. Because of its pleasant scent, it is used as a flavor and fragrance; incorporated in soaps, cosmetics, and hygiene products; used in aromatherapy; and is a common compound of herbal essential oils and teas.

Linck et al. [74] investigated the effect of inhaled linalool on anxiety, aggressive behavior, and social interactions in mice. The results showed that inhaled 3 % linalool extended anxiolytic effects on mice, due to the fact that it increased the time spent in the lit area in the light/dark test. A step-down inhibitory avoidance test was performed, showing that linalool possesses amnesic effects. All results were compared with diazepam as a control. Linalool decreased aggressive behavior and increased social interactions at a concentration of 1 %. Three percent inhaled linalool showed a lack of effect in the social interaction test, most probably related to the fact that linalool acts as an antagonist on NMDA receptors, which is a common effect of NMDA antagonists in general. The same team of authors showed in a previous study that inhaled linalool can bolster pentobarbital-induced sleep and decrease body temperature and locomotion. These results can be taken as further proof of the psychopharmacological effects of inhaled linalool and EO containing this compound.

Takahashi et al. [75] compared the EO from six *Lavandula* species, investigating how the interspecies differences affect the expression of their anxiolytic activity. The result showed a qualitative as well as a quantitative compositional variance between the EOs, leading to significant differences in the provided anxiolytic effect. The authors also investigated the influence of the major constituents of the EOs of these species, suggesting that linalyl-acetate acts synergistically with linalool, and that the presence of both compounds is required for the anxiolytic effect of the inhaled EOs.

Linalool is, as already mentioned, a competitive antagonist of the NMDA receptor, and is believed to have an important role in the building of memory. Coelhoa et al. [76] evaluated the effect of (−)-linalool on the acquisition of long- and short-term memories using three types of behavioral models: recognition task, inhibitory avoidance test, and habituation in a new environment. With an open field test, the effect on motivation, locomotion, and exploration level was investigated. The test was performed on more than 200 male Wistar rats, using a glutamate antagonist as a positive control. (−)-Linalool showed different effects in the three types of tests. In the object recognition task, (−)-linalool impaired the formation of long-term memory without impacting short-term memory. The building of both STM (short-term memory) and LTM (long-term memory) was impaired in the inhibitory avoidance test, while in the habituation test, the LTM was impaired. In the open field test, the tested rats showed no difference in the crossing and locomotion, but higher concentrations of (−)-linalool decreased rearing behavior. The effect was different in each assay, but the compound still impaired memory acquisition in every assay. This suggests that (−)-linalool, probably due to its antagonistic effect on the NMDA receptor, affects the memory, like other antagonists of this receptor also do.

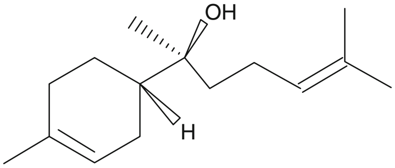
The anxiolytic and anticonvulsant effect of linalool is related to its mechanism of action in the CNS, blocking glutamatergic NMDA receptors, stimulating GABA receptors, and blocking voltage-dependent ion channels. Sampaio et al. [77] investigated the inhibition of adenylate cyclase by rosewood oil (*Aniba rosaeodora* Ducke), due to the fact that an increased cAMP concentration plays an important role in development of seizures in epilepsy, so inhibitors of the AC could be potential anticonvusant therapeutics. Rosewood oil, (−)-linalool, and the racemate (±)-linalool were tested against the increase of cAMP concentration, also involving the effect on adenosine receptors (adenosine decreases cAMP concentration through binding to the A1 receptor). Chick retinas were used as a CNS model and a phosphodiesterase inhibitor and an adenosine receptor antagonist as controls to determine the involvement of these receptors in the resulting effect. Rosewood oil and the linalool isomers inhibited the accumulation of cAMP but only when the AC was activated by a forskolin stimulus, suggesting that they act on the forskolin binding site of the AC. The effect was provided even when the adenosine receptor were blocked, showing that the antagonist did not interfere in the effect of the EO in the AC activity.

De Sousa et al. [78] investigated the difference in the anticonvulsant activity between the two linalool enantiomeres and the racemate. The results showed that a pretreatment with all types of linalool could increase the latency of convulsions, but rac-linalool was more effective, providing the effect at lower doses than the enantiomeres. In addition, all types of linalool could inhibit convulsive actions, with effects comparable to diazepam. (−)-Linalool was, in general, more potent than (+)-linalool, but still less potent than rac-linalool. When it comes to preventing tonic convulsions, both enantiomeres were equipotent and rac-linalool was even more effective than phenytoin. The study reported the presence of a chiral influence, observing that the two enantiomeres have similar anticonvulsant effects but different potencies.

Cho et al. [79] investigated the hypocholesterolemic effect of linalool in high-fat fed mice and in HepG2 cells. A treatment with linalool on high-fat fed mice reduced the total- and LDL-cholesterol level, with an accompanying reduction in the hepatic lipid concentration; the levels of HDL cholesterol increased. In hepatocytes, linalool extended a dose-dependent reduction of cholesterol and triglyceride concentration. Linalool decreased cholesterol by decreasing the expression of the sterol regulatory element binding protein-2 and an accompanying decrease of HMG-CoA reductase protein expression through transcriptional and posttranscriptional mechanisms. The reduction of expression of HMG-CoA reductase is a result of the reduced binding of SREBP-2 (sterol regulatory element binding protein-2) to its promoter and the induction of a ubiquitin-dependent proteolysis of HMG-CoA reductase.

Nevertheless, the antitumor effect of linalool should be mentioned. Gu et al. [80] investigated the antitumor effect of linalool on different hematopoietic tumor cell lines along with the effect on healthy blood cells. Linalool inhibited proliferation and induced rapid apoptosis on different human leukemia cells, but it spared normal blood cells. The effect is associated with an activation of the tumor suppressor gene p53 and cyclin-dependent kinase inhibitors.

3.5 Bisabolol



The name bisabolol includes both α- und β-isomeres of this compound, with each existing in two enantiomeric forms. In nature, the most common form is

(−)-α-bisabolol. (−)-α-Bisabolol is an unsaturated, optically active sesquiterpene alcohol that possesses a delightful floral odor [81]. It is part of the EOs of a variety of plants, the most commonly utilized source being *Chamomilla recutita* L, but it is also found in *Salvia runcinata*, *Plinia cerrocampanensis* [82], and *Vanillosmopsis erythropappa* [81]. (−)-α-Bisabolol is used in different formulations, and due to its antiseptic effect often in cosmetics, aftershave lotions, moisturizers, and creams for sensitive skin. Previous studies have reported on the anti-inflammatory, antibiotic, anti-ulcerative, anti-oxidative, anti-tumor, and other effects of this compound [82]; a few of them will be discussed here.

Rocha et al. [81] were among the first teams to examine the anti-nociceptive and anti-inflammatory potential of (−)-α-bisabolol as an isolated drug and not just as a plant containing this compound. The examinations were performed on male Swiss mice and male rats in classic models of pain and inflammation. The study showed that (−)-α-bisabolol reduced carageenan- and dextran-induced paw edemas, and at higher doses also reduced edemas produced by direct application of 5-HT. This suggests that the substance does provide anti-inflammatory effects. Bisabolol has been shown to be a peripheral anti-nociceptive and anti-inflammatory drug. This finding is supported by the fact that the anti-nociceptive test on hot-plate response did not suggest central analgesic activity and the formalin test also supported this conclusion. The formalin test includes two phases: the first phase confirmed the peripheric mechanism of action and the second phase proved the anti-nociceptive activity of the substance by influencing inflammatory mediators (histamine, serotonin, prostaglandins, and bradykinin). Pre-treatment with (−)-α-bisabolol decreased leukocyte migration, protein concentration, and myeloperoxidase activity in rats with peritonitis. It could also decrease TNFα in the peritoneal fluid of rats with carrageenan-induced peritonitis. The effects of (−)-α-bisabolol might be related to the effect on TNFα, but the effect of other inflammatory mediators cannot be excluded. The study proves the substance exhibits anti-inflammatory effects but without ulcerative potential like the commonly used analgetics and inflammation therapeutics (diclofenac, indomethacin). In contrast, (−)-α-bisabolol provides gastroprotective effects.

Moura Rocha et al. [83] investigated the gastroprotective effect of isolated (−)-α-bisabolol in ethanol and indomethacin-induced ulcers in mice. The substance showed anti-ulcerative activity in both ulcer models. The authors assessed the possible mechanisms involved in this action. (−)-α-Bisabolol was able to protect the gastric mucosa from lesions caused by NSAIDS, similar to ranitidine. To examine the role of prostaglandins in the effect of this substance in ethanol-induced ulcer models, mice were pretreated with indomethacine, but this did not prevent the effect of (−)-α-bisabolol. Therefore, an increased prostaglandin synthesis is not the mode of action. The involvement of K_{ATP+} channels and (−)-α-bisabolol in gastric functions was investigated, but it showed not to be related in the mechanism of action due to the fact that there was no difference in the gastroprotective effect of (−)-α-bisabolol in animals pre-treated with glibenclamide (glibenclamide closes the ATP-dependent potassium channels) or not. Also, the nitric oxide pathway is not involved, because the anti-ulcerative effects could not be reversed by L-NAME, an inhibitor of nitric oxide synthase. Finally, the effect was shown to probably be related to a decreased reduction of non-protein sulfhydryl groups, which leads to an increase of their occurrence and strengthening of their protective effects on gastric tissue, leading to a reduction of gastric oxidative injuries induced by ethanol and indomethacin. Namely, ethanol is able to diminish the levels of non-protein sulfhydryl groups, such as reduced glutathione in gastric tissue, which provides its gastroprotective effects by scavenging free radicals and preventing the gastric damage made by free-radicals' accumulation.

In a previous study, (−)-α-bisabolol was shown to have the ability to reduce gastric ulcer in response to absolute alcohol, but the way of acting was not clear, although a few mechanisms could be excluded. Moura Rocha et al. [84] have done further experiments, evaluating the gastroprotective effect in ethanol-induced lesions on the gastric mucosa. The methods they used were histopathological determination, measuring the membrane lipid peroxidation, myeloperoxidase, superoxide-dismutase, and catalase activity and the nitrite level. Ethanol produces characteristic necrotic gastric lesions, but the damage is also related to a massive production of free radicals. That is why the authors investigated the connection between the capability of (−)-α-bisabolol to reduce oxidative stress and inflammation, and the anti-ulcerative effect on ethanol-induced lesions.

The study showed that (−)-α-bisabolol prevented the ethanol-induced increase of MDA, showing its antioxidant activity. The substance increased the SOD activity and the dismutation of superoxide anion and it prevented the reduction in CAT activity. (−)-α-Bisabolol also reduced the influx of neutrophils in the gastric lesions. In agreement with the findings mentioned in the previous study, the pathway of nitric oxide is not related to the effect, because the substance did not significantly modify the nitrite levels.

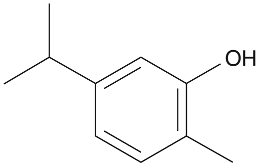
Seki et al. [82] reported that (−)-α-bisabolol is, in vitro, capable of suppressing cell proliferation which leads to death in pancreatic cell lines. The substance was effective and did not cause significant side effects. The mechanism of action includes the inhibition of Akt activation (one of the most often activated serine/threonine kinases in pancreatic cancer) and an up-regulation of the expression of the tumor suppressor early growth response-1 (EGR1). The authors did not exclude that other mechanisms, next to those two mentioned, are involved in the activity. They report that (−)-α-bisabolol might be a potential therapeutic in treatment of pancreatic cancer.

Cavalieri et al. [85] first gave evidence of α-bisabolol being a pro-apoptotic substance for primary human acute leukemia cells. The cells used in the study were Philadelphia-negative and -positive B acute lymphoid leukemias (Ph-/Ph + B-ALL), acute myeloid leukemias (AML), normal leukocytes, and bone marrow stem cells. α-Bisabolol was shown to be effective in ALL cells at all concentrations and duration of the treatment, and it spared normal leukocytes and bone marrow cells. At a bit higher concentration, it also acted as apoptotic against primary AML cells. The apoptotic activity was present even in imatinib mesylate-resistant Ph + B-ALL. The mechanism of action might involve the disruption of the mitochondrial membrane potential, which goes along with a decrease of oxygen consumption in the presence of glutamate/malate and the unpersuaded respiration levels in the presence of succinate/glycerol-3-phosphate.

De Siqueira et al. [86] investigated the pharmacological effect of (–)-α-bisabolol in various smooth muscle preparations of rats. The substance was shown to be biologically active in smooth muscle, but it had different effects depending on the tissue and applied concentration. For example, in preparations that were electromechanically or pharmacologically pre-contracted, (–)-α-bisabolol had a relaxing effect. At concentrations of 30–300 μmol/L, (–)-α-bisabolol relaxed duodenal strips; it contracted endothelium-intact aortic rings and urinary bladder strips, but relaxed the same tissues at higher concentrations (600–1,000 μmol/L). On tracheal or colonic tissue the effect was relaxing but with less potency than in mesenteric vessels. In vivo, (–)-α-bisabolol alleviated the increase of carbachol in tracheas of ovalbumin-sensitized rats challenged with ovalbumin, but it did not interfere with the decreasing responsiveness of urinary bladder strips in ifosfamide-treated mice. The authors suggested that a possible mechanism of action is the inhibition of voltage dependent Ca^{++} channels.

Alvesa et al. [87] studied the pharmacological effect of (–)-α-bisabolol on the peripheral nervous system of mice. The examination was performed ex vivo, observing the effect on the compound action potential characteristics, using a modified single sucrose-gap method. (–)-α-Bisabolol was dose-dependently able to decrease neuronal excitability. The effect was similar to lidocaine but not to 4-aminopyridine; both are known as inhibitors for sodium and potassium voltage-gated channels. In contrast to lidocaine, the (−)-α-bisabolol action showed an irreversible and non-use-dependent pathway. Based on this finding, the effect might be provided through irreversible inhibition of voltage-dependent sodium channels.

3.6 Carvacrol



Carvacrol is a member of MT phenols that occur in many EOs of the family *Labiatae*, including *Origanum, Satureja*, *Thymbra, Thymus*, and *Coridothymu*s species. This alcohol is generally recognized as safe, so it is commonly used as a flavoring substance. Carvacrol is described as having a pungent and warm scent similar to oregano. The EO from *Origanum vulgare* contains carvacrol at the highest naturally occurring concentration (up to 80 %) [88]. Carvacrol, in rat models, is metabolized and excreted very quickly. After 24 h, only small amounts could be found in urine, suggesting the excretion is almost complete in 1 day.

The substance is excreted mostly unchanged, but an oxidation of the methyl and isopropyl group can also occur, leading to benzyl alcohol and 2-phenylpropanol and their carboxylic acids [89].

Carvacrol was shown to strongly activate and sensitize TRPV3 channels, which are warm-sensitive Ca^{++}-permeable channels, often occurring in skin and neural tissues, causing the sensation of warmth. Carvacrol also activates and desensitizes TRPA1, a pain receptor, giving a possible explanation for the pungent taste of oregano [90]. Years after this finding, Parnas et al. [91] found carvacrol to inhibit the non-thermoTRPs, TRPL and TRPM7 channels. TRPM7 channels are mediators of anoxic neuronal death, so by inhibiting the expression, protection from ischemic cell death is provided.

Hotta et al. [92] reported on carvacrol acting on other receptors. They found that this substance, as a major part of the EO of thyme, is an activator of PPARα and γ, leading to an inhibition of COX-2 expression. This finding is a strong indicator for the anti-inflammatory effect of carvacrol since COX-2 is known to play important roles in inflammation processes and circulatory homeostasis.

Liu Y et al. [93] examined the in vitro anti-inflammatory effect of seven plant extracts, including carvacrol, on alveolar macrophages collected from pigs. Carvacrol was shown to significantly suppress TNFα and decrease IL-1β secretion from LPS-treated macrophages. Carvacrol also suppressed TGF-β from macrophages with LPS stimulation. An even more detailed report about the effect of this substance was given by Guimarães et al. [94]. The authors evaluated the effect of carvacrol on inflammatory hypernociception and inflammation on different mice models, and also on in vitro–stimulated murine macrophages. The inflammations were induced by carrageenan, TNFα, PGE2, and dopamine. The effect on leukocyte-accumulation and production of TNFα in carrageenan-induced pleurisy, as well as the effect on the NO building in murine macrophages, were also examined. A cavacrol-pretreatment was successful in reducing hypernociception and edema induced by carrageenan and TNFα, but there was no effect when induced by PGE2 and dopamine. In agreement with the study mentioned before, the TNFα concentration was decreased and an accumulation of leukocytes could be inhibited. Carvacrol inhibited LPS-induced nitrite production. The authors suggest that the suppression of TNFα production and NO release play the most important roles in the anti-inflammatory effect of carvacrol.

Carvacrol elicits an inhibitory effect on histamine receptors, as Boskabady et al. [95] report. They examined the effect of an aqueous-ethanolic extract of *Zataria multiflora* Boiss (Labiatae) and its constituent carvacrol on H1 (histamine 1) receptors in tracheal chains of guinea pigs. The results confirmed the inhibitory effect of both extract and carvacrol on H1 receptors with no significant difference when different concentrations of extract and carvacrol where applied.

Aristatile et al. [96] investigated the effect of carvacrol on mitochondrial enzymes, oxidative stress, and DNA damage in hepatic tissue in a model of D:-galactosamine (D:-GalN)-induced hepatotoxicity. The studies were performed on male Wistar rats and silymarin was used as a control drug. Carvacrol normalized the changes that were induced, providing antioxidant and defensive effects against mitochondrial enzymes and DNA damage. Carvacrol was able to bring the hepatic mitochondrial enzymes isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, NADPH dehydrogenase, and cytochrome C oxidase, after they were decreased by D:-GalN, to normal levels again. In addition, the increased concentration of thiobarbituric acid reactive substances was also decreased. Carvacrol was able to modulate the enzymatic antioxidants SOD and glutathione peroxidase and the non-enzymatic antioxidants vitamin C and vitamin E, and it and reduced glutathione back to higher concentrations. Carvacrol decreased DNA damage, probably due to the scavenging of free radicals before they caused the damage.

The effect of carvacrol on the mitochondrial pathways plays an important role in its anti-hepatocarcinogenic activity, as the study of Yin et al. [97] found. The study was performed on HepG2 cells showing that carvacrol was able to induce apoptosis and suppress further growth of cancer cells. The apoptosis mechanism involved an activation of caspase-3, PARP cleavage (a marker for apoptosis in tissue sections), and reduction of Bcl-2-gen expression. An important mechanism in the antitumor activity might be the influence in the mitogen-activated protein kinase pathway, by reducing phosphorylation of ERK1/2 and activating the p38 phosphorylation, but not interfering with JNK MAPK.

The effect of carvacrol on hepatocellular carcinoma has also been the subject of Jayakumar et al. [88]. The authors examined the preventive effect of the substance against carcinoma induced by diethylnitrosamine in rats. The main topic of research was the strong antioxidant effect and free radical elimination as an anti-cancer mechanism. Carvacrol modulated LPO levels and enhanced the endogenous antioxidant defense in cancerogenesis and decreased the high levels of serum markers.

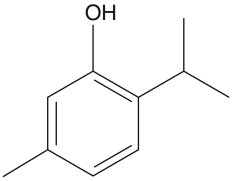
An interesting fact for the application of carvacrol and carvacrol-containing plants is that this substance exerts inhibitory properties on UGTs (UDP-glucuronosyltransferases). UGTs are responsible for metabolizing about 35 % of all drugs metabolized by phase II enzymes. Its inhibition could result in serious drug-drug interactions and cause metabolic disorders. Dong et al. [98] investigated the inhibition of main isoforms of UGT using a nonspecific probe substrate 4-methylumbelliferone and recombinant UGT enzymes as enzyme resources. Carvacrol was able to inhibit UGT1A9, one of the most important UGT isoforms, with an irrelevant effect on other UGT isoforms.

Yu et al. [99] investigated the neuroprotective potential of carvacrol against cerebral ischemia and/or reperfusion damage in mice using the middle cerebral artery occlusion model. The study showed the protective effect of carvacrol by decreasing the infarct volume and the level of neuronal cell death. Noticeably, a post-treatment was also able to provide protection. The authors' suggestion is that the PI3K/Akt pathway is related to the protective mechanisms of carvacrol on cerebral I/R damage. With intracerebroventricular treatment after cerebral I/R damage, carvacrol was shown to have a wide therapeutic window by still providing its protection even when applied 6 h after reperfusion. The therapeutic window was shortened when carvacrol was intraperitoneally applied, so this method might affect its protective efficiency. The authors suggest the use of carvacrol as a therapeutic drug, better yet as nanoformulations, which would make it even more efficient and easier to apply for an infarct treatment.

Carvacrol also improves cognitive activity. Azizi et al. [100] examined the effect of carvacrol and thymol in two rat models of dementia: deficits caused by amyloid β and by scopolamine. The method they used was the Morris water maze test and they also assessed the acute toxicity of both carvacrol and thymol. The result showed that both substances could reverse and alleviate the induced cognitive impairments, for example, the escape latency and reduction in target quadrant entries. Both substances also were shown to be relative safe, with LD50s of thymol (565.7 mg/kg) and carvacrol (471.2 mg/kg) that were significantly higher than the therapeutic concentration. The authors also suggest that the antioxidative, anti-inflammatory, and anti-cholinesterase activity could be involved in these activities.

Carvacrol possesses antibacterial, antifungal, and anti-insecticidal effects that are worth noting. The antibacterial potential of this substance has been ascribed to its effect on the structural and functional integrity of the cytoplasmic membrane. Due to this effect, carvacrol is used to extend the time before food becomes spoiled by bacteria [101]. For example, carvacrol inhibited, in sub-lethal concentrations, the virulence of *Salmonella typhimurium* by reducing the motility and invasion in porcine epithelial cells, which is an important finding inasmuch as carvacrol is commonly used in sub-lethal concentrations [102].

3.7 Thymol



Thymol is a MT-ic phenol derivate of cymene, which can be found in EOs of thyme, *Thymus vulgaris* or *Thymus zygis* L. *var. gracilis* Boissir. Thymol constitutes up to 80 % of the major compound of thyme EO, but it can be found in various citrus plants as well [103]. Thymol possesses a well-known antimicrobial and antiseptic activity, and because of its pleasant taste it has been used in mouthwashes and toothpastes for many years [104].

Thymol is a ligand of odorant receptors that are expressed in the intestinal mucosa, and by binding to those receptors, serotonin secretion is stimulated. These receptors belong to the group of chemical receptors that play an important sensoric role and can modulate functions of the GI system. Due to the fact that the ion transport, as a result of the binding, had not been previously evaluated, Kaji et al. [105] investigated the effect of thymol on ion transport in human and rat colonic epithelial cells by using a Ussing chamber (used to measure the short-circuit current to determine the ion transport taking place across an epithelium). The results showed that thymol can interfere in the permeability and anion secretion in colon cells. The mucosal application of thymol induced dose dependently an anion secretion that is probably related to an activation of TRPA1 channel. The authors came to this conclusion because the anion secretion could be reversed either under Ca^{++}-free conditions or application with a blocker of TRPA1.

Thymus vulgaris L. and/or *Thymus zygis* L. extracts from leaves and flowers have been traditionally used for diseases of the respiratory tract due to their broncholytic, secretomotoric, and anti-spasmodic effects. Thymol is known to relax the trachea and binds with α1-, α2, and β-receptors of smooth muscles, so this effect is believed to be related to the activity of thymol and carvacrol, the main phenolic compounds in the extract. To get evidence of this hypothesis, Engelbertza et al. [106] investigated the spasmodic effect of thymol-deprived thyme extracts and determined which compounds are responsible for the actual effect. The thyme extract was split into fractions, the compounds were isolated from them and the anti-spasmodic effect was determined on smooth muscle trachea model of rats with papaverin as a control. The results showed that thymol possesses an anti-spasmodic effect, but for the complete effect it was not responsible by itself alone but probably in synergistic effect with the flavone luteolin.

The pro-apoptotic and anti-cancer effects of thymol are reported in several studies. Xuan et al. [107] investigated the effect of thymol on the immune response, by examining the effect, survival, and function of dendritic cells. Dendritic cells are important for inducing an immune reaction against pathogens, but also to prevent unnecessary immune reactions against harmless antigens, and it plays important roles in the intestine. The study was performed on dendritic cells either from wild-type mice or from mice lacking acid sphingomyelinase, treated and untreated for 24 h with thymol. The treatment with thymol was shown to stimulate sphingomyelinase and formation of ceramide, down-regulation of Bcl-2 and Bcl-xL expression, activation of caspase-3 and -8, and suicidal death of the cells in the end. This finding is interesting because thymol was shown to protect from suicidal cell death in erythrocytes, which is also triggered by sphingomyelinase stimulation and ceramide formation. So, there is an opposite effect on erythrocytes and dendritic cells. The authors suggest, besides the fact that the thymol-induced apoptosis could induce anti-inflammatory actions, caution is necessary with use in infectious diseases because there is a possibility it might induce the pathway of an infectious disease.

Hsu et al. [108] examined the effect of thymol on Ca^{++} and the viability in human astrocytes, using glioblastoma cells for a model. The study showed that thymol induced a rise of the Ca^{++} concentration and cell death in the glioblastoma cells. The Ca^{++} rise was thymol-dose-dependent and realized through releasing Ca^{++} from the intracellular stores and inducing Ca^{++} entry from extracellular medium via non-store operated Ca^{++} channels. The mechanism is, according to the authors, related to the phospholipase C- and protein kinase C-dependent release from stores from the ER. Thymol induced cell death that was not triggered by the rise of Ca^{++} concentration. The cell death most probably involves apoptosis and necrosis.

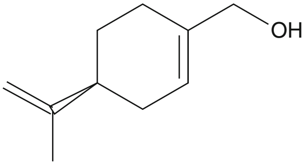
Chang et al. [109] investigated the same topic, using MG63 human osteosarcoma cells. The results were partially similar. Thymol provoked a Ca^{++} rise by triggering the phospholipase C-dependent release from the stores in the ER and the Ca^{++} entry through kinase C- dependent store-operated Ca^{++} channels. Thymol was also able to induce cell death, probably related to apoptosis via mitochondrial pathways.

Satooka and Kubo [110] investigated the inhibitory effect of thymol on the formation of melanin. They came to the conclusion that this effect is due to the radical scavenging activity of thymol. Thymol inhibits the redox reaction between dopaquinone and leukodopachrome without any interaction with tyrosinase, although, tyrosinase is the key enzyme in melanin synthesis. This finding is important due to the fact that a high melanogenesis produces free radicals and can be further on related with development of diabetes mellitus, cardiovascular diseases, or cancer. One year later, the same authors investigated [104], on basis of the previous findings, the effect of thymol on B16-F10 murine melanoma cells. They wanted to examine whether thymol is capable of inhibiting melanogenesis in cultured melanocytes, but without interfering the cell growth. Thymol exhibited moderate cytotoxity but not an antimelanogenic activity. With vitamin C and D, the moderate cytotoxic activity could be inhibited and cell viability enhanced. Actually, B16 melanoma cells cultured with thymol showed a significant increase of oxidative stress. In conclusion, at high concentrations, thymol acts as a pro-oxidant rather than an antioxidant, so the authors suggest rather caution when using it as a food additive.

The anti-cancer effect of thymol is related to its ability to scavenge free radicals, but besides these antioxidant properties, other effects exist. That is why Deb et al. [111] investigated the anti-cancer effect of thymol on acute promyelotic leukemia HL-60 cells. Thymol exerted a cytotoxic effect on HL-60 cells but not on normal human peripheral blood mononuclear cells. The authors suggest that the different effect on normal and cancer cells can be related to a different gene expression or an activity-modulation of thymol, and, in general, the case that a plant extract is pro-oxidative in vivo on one tissue and is antioxidative on another tissue is already known. The cytotoxic effect of thymol is related to an increase of ROS production, mitochondrial H_2O_2 generation, depolarization of the mitochondrial membrane potential, decrease in Bcl-2 protein and increase in Bax protein expression and activation of caspase. So, thymol was able to induce the cell death in HL-60 cells both caspase dependently and independently.

Archana et al. [103] investigated the role of thymol against radiation-induced DNA damage under in vitro conditions, determined by micronuclei and comet assay in Chinese hamster lung fibroblast cells. Radiation is commonly used for cancer treatment, but the DNA damage it causes can also affect normal tissue. That is one of the reasons the researchers were interested in finding new, effective substances as a protection against damage of healthy tissues while exposed to radiation. Thymol protected cells against genotoxicity and apoptosis induced by radiation. This effect is most probably related again to its antioxidant and radical scavenging properties. On basis of this in vitro information, the same team investigated the in vivo radioprotective effect of thymol in Swiss albino mice [112]. Besides the radioprotective effect, the anticlastogenic effect of thymol was investigated against a whole-body gamma radiation. The results showed that with a pre-treatment with thymol on gamma radiation-sensitized mice, a decrease in LPO levels and increase of the antigenotoxic, anticlastogenic, and radioprotective effects and an increase in viability of the animals could be caused. This effect is again due to the antioxidative and free radical scavenging activity, but the existence of other mechanism cannot be excluded.

3.8 Perillyl Alcohol



Perillyl alcohol is a MT-ic alcohol that can be found in the EOs of lemon, lavender, mint, ginger, and some vegetables. Perillyl alcohol is best known for its anti-cancer activities and several studies report on its mechanism of action.

For example, Garcia et al. [113] reported on the inhibitory effect of this substance on the Na/K-ATPase from guinea pig kidney and brain tissues, and from A172 human glioblastoma cells. They suggest that the anti-cancer activity could be related to its Na/K-ATPase binding properties, thus showing a non-competitive inhibition of perillyl alcohol to Na^+ and K^+ and an un-competitive inhibition towards ATP. The authors also suggest that the drug probably acts in the initial phase of the catalytic cycle of the enzyme, different from the standard inhibitor ouabain (binding and inhibiting on the plasma membrane Na^+/K^+-ATPase).

The results of many recently published studies point out the anti-cancer effect on glioblastoma cells. Those discoveries are important because glioblastoma multiforme is the most deadly primary brain tumor in humans. Da Fonseca et al. [114] investigated the efficacy of perillyl alcohol, when intranasally administered, on the survival rate of patients with recurrent malignant glioma. The study was performed in comparison to historical untreated patients. The intranasal administration was chosen due to the evidence that the substance takes its route through perineural and/or perivascular channels along the olfactory and the trigeminal nerves. The study showed that perillyl alcohol can increase the overall survival of recurrent glioblastoma patients, compared with the historical control group. The effects were outstanding in patients with secondary glioblastoma multiforme and patients who had tumors in deep areas of the brain. It is important to note that there were almost no side effects, even in patients who were treated for 4 years. The mechanism of action of perillyl alcohol is believed to be related to the inhibition of Ras/Raf/ERK pathways, NFkB, and also the isoprenylation of the Ras small GTPase superfamily of proteins that induce tumor-related angiogenesis. In the primary glioblastoma multiforme, the EGFR and its mutant EGFRvIII are overexpressed, so by alterations of the EGF/EGFR pathway, perillyl alcohol likely influences the development of the disease.

The previous study followed up on earlier findings of the same group. In 2008, their study proved that perillyl alcohol was able to extend average life by more than 8 months in recurrent glioblastoma patients, to decrease the tumor growth, and to reduce tumor size.

However, after 7 months, tumors became resistant to perillyl alcohol and continued to grow [115]. Based on these findings, de Saldanha da Gama Fischer et al. [116] investigated the molecular changes that finally lead to the resistance of the tumor to perillyl alcohol. For the purposes of the study, a new glioblastoma cell culture was generated heretofore as A172r, which was able to tolerate doses of perillyl alcohol from which the standard cell line would die. The result was a list of protein markers that are representative in the resistant or the non-resistant cell line. The proteins are related to cellular growth, negative regulation of apoptosis, ras-pathway, and other functions of the cell.

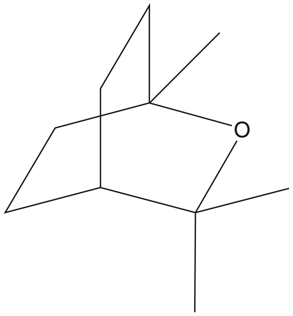
Malignant gliomas are related to alterations in EGF/EGFR signaling. Thus, da Silveira et al. [117] examined the influence of an EGF + 61A > G gene polymorphism on the development of the disease and the different responses to an intranasal administered perillyl alcohol therapy. The study showed that patients who had lower EGF levels survived longer after the perillyl alcohol treatment, probably due to the fact that high levels of EGF can be related to larger tumor sizes and greater degree of malignancy. Thus, EGF level in the serum may be predictive of treatment response. The authors also suggest that perillyl alcohol, as a lipophilic substance, can cross the blood-brain barrier and induce cell death even in cells with low rates of EGF/EGFR signaling.

Khan et al. [118] reported on perillyl alcohol exerting antioxidant effects and modulating TNFα release and NFκ-B activation. With these activities, which are related to inflammation processes and damaging of cells, perillyl alcohol showed protective effects in models of ethanol-induced liver injuries in Wistar rats. The study demonstrated that perillyl alcohol has the capability to prevent liver toxicity by boosting the endogenous antioxidant system, inhibiting lipid peroxidation, and suppressing the inflammatory cytokines and NFκ-B activation. The authors suggest this monoterpene has a possible role in the prevention of liver toxicities.

4 Ether

4.1 1,8-Cineole

Synonym: Cineole, Eucalyptol



The 1,8-cineole MT compound can be found abundantly in nature. It is the major compound of the *Eucalyptus* EO, with up to 80 %, but it can be also found in *Rosmarinus off., Salvia off., Mentha* ssp. and in other plants as well. It is used in different cosmetic products, like toothpaste, soaps, and creams, and also in household products like air fresheners and cleaning products [119]. The substance is a colorless liquid, which possesses a fresh, camphor-like odor. It is reported that with appropriate handling of 1,8-cineole, no toxic effects can be expected, but after admission of high concentrations, systematic effects such as blood pressure drop-down, CNS disturbance, and somnolence can be caused [120].

The pharmacological effects of 1,8-cineole, which have been documented in the past, are mostly focused on the therapeutic activity of this substance on the respiratory tract and inflammation in general. A long-term systematic treatment can have therapeutic, mucolitic effects in asthma, sinusitis, and COPD and in diseases of the lower and upper airways in general. The normalizing effect of the mucus hypersecretion by 1,8-cineole is related to its ability to inhibit the arachidonic acid metabolism and generation of cytokines in human monocytes. So, the substance exhibited a steroid-saving effect on steroid-dependent asthma [121]. 1,8-Cineole is also an inhibitor of TNFα and IL-1β [122].

Worth et al. [123] investigated whether 1,8-cineole can, due to its mucolytic, bronchodilating, and anti-inflammatory activity, reduce the exacerbation rate and improve the health status when applied as a concomitant therapy in COPD patients. The substance possesses positive effects on the beat frequency of the cilia in the mucus. 1,8-Cineole reduced the exacerbation rate and improved lung function by improving the airflow obstruction and reducing severity of dyspnoea. Due to its positive effect on the health status, lack of side effects, and relative low cost, concomitant therapy can be recommended with therapy of the rather costly COPD, in the opinion of the authors.

The antioxidant property of the EO as well as of methanolic extracts of *Eucalyptus loxophleba* Benth. ssp. were evaluated by Rahimi-Nasrabadi et al. [124], where 1,8-cineole presents, with 39.4 %, the major compound. For the examination, the DPPH, β-carotene/linoleic acid, and reducing power assays were used. The results showed that the methanolic extracts are very effective antioxidants in in vitro assays and that the compounds of *E. loxophleba* exhibit antioxidant activities.

It is recognized that EOs with MT compounds such as 1,8-cineole can be used to evoke epileptic seizures. For this reason, Ćulić et al. [125] investigated the effect of this compound in the camphor EO using wavelet and fractal analysis to quantify the electrocortical changes. Animals were intraperitoneally treated with camphor EO or 1,8-cineole, and then by wavelet analysis the frequency bands in pre-ictal, ictal, and inter-ictal stages were examined. The properties of the acute, epileptic-like seizures, caused by either the EO or 1,8-cineole, could be described through frequency bands in wavelet analysis. δ frequency bands dominated brain activity, with ≈45 % mean relative wavelet energy (MRWE) in the control group (no treatment) increasing up to ≈76 % MRWE after drug application. The effect seems to be concentration-dependent.

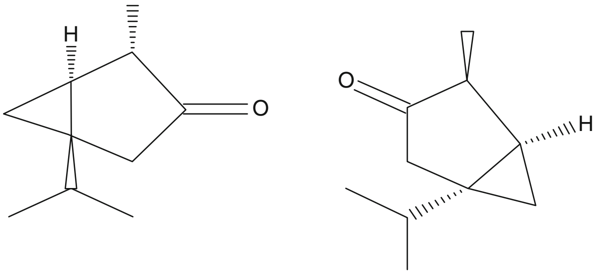
Kirscha et al. [119] investigated the flavor changes in breast milk after the oral intake of a preparation that contains 1,8-cineole. The background of this investigation was the fact that odorants in breast milk can potentially affect the breastfed child. Newborns are extremely sensitive to olfactory stimuli, and this influence is believed to affect food preferences in the years to come and may be a potential preventive of nutrition-related diseases. The study showed that after ingestion of a preparation containing 100 mg 1,8-cineole, the substance was transferred into the milk in a time-dependent manner. The change of the flavor of the milk could lead to rejection of the milk, and over the long term, might have an effect on food preferences later in life.

Yoshimura et al. [126] examined the influence of 1,8-cineole on proliferation and elongation in plant cells by using BY-2 suspension-cultured tobacco (*Nicotiana tabacum*) cells. 1,8-Cineole inhibited cell elongation more efficiently than cell proliferation. The authors suggest that the inhibitory effect of this substance is not specific, but it seemed to affect several cellular activities in an almost nonspecific way by direct contact with the cells.

Tomsheck et al. [127] found that 1,8-cineole can be produced in *Hypoxylon* sp., an endophyte of *Persea indica.* This novel source is easier to use and apply in medicine, industry, and even as a fuel additive, due to the fact that it is a derivate of octane.

5 Carbonyls

5.1 Thujone



Thujone belongs to the group of bicyclic MT ketones and it occurs in two stereoisomeric forms, the α-thujone and β-thujone. It is an ingredient of the EOs of *Salvia* spp.*, Thuja* spp.*, Artemisia* spp., and some others [128]. Thujone is a compound in aromatic plants that are used to flavor food and beverages. The substance is often associated with absinthe, the spirit flavored with *Artemisia absinthium* L. Much discussion has taken place regarding the possible role of thujone in causing adverse psychoactive effects in the “absinthism” syndrome. But the symptoms may have been wrongly attributed to thujone, and may rather be caused by ethanol or other toxic adulterants. Today, thujone-containing plants can be used in food without restrictions, but in pure form, it is still forbidden to directly add thujone to food. That decision was made in 2008 by the European Union Regulation on flavorings [129]. Thujone is believed to be neurotoxic, by providing a convulsant effect, with α-thujone being even more toxic than *β*-thujone. The EO of *Salvia off*., which is rich in thujone (35–50 %, mainly α-thujone) is known to be an abortifacient and an emmenagogue agent [130].

In 2010, Lachenmeier and Uebelacker [129] re-evaluated the toxicological evidence for thujone by making a new risk assessment using the benchmark dose (BMD) approach. The current limits set for thujone in food products are based on short-term animal studies from the 1960s, and they estimated the acceptable daily intake (ADI). As mentioned above, the restrictions for food were lowered in 2008, but the opposite happened for use in medicine when in 2009 the European Medicines Agency (EMA) introduced limits for the substance. *Artemisia absinthium* L. and *Salvia offinicinalis* L. are often used in medicine in various preparations. Due to the lack of toxicological data for thujone, it was not possible to set an accurate value for ADI for thujone, so the ADI for *A. absinthum* has been determined as 3.0 mg/person for maximum 2 weeks use and 5.0 mg/person for *S. officinalis*. The results of the evaluation were similar to previous short-term studies, thus, the authors proposed an ADI of 0.11 mg/kg bw/day, which would not be possible to reach even when consuming high levels of food containing thujone – between 2 and 20 cups of wormwood or sage tea would be required to reach this ADI.

In 2011, the National Toxicology Program [131] published the results of their study on the effect of α,β-thujone on male and female rats and mice. The aim was to determine the potential toxic and cancer-related activity of thujone. The rats receiving 50 mg/kg died; all other rats, which received 25 mg/kg, had seizures. Similar results were obtained dose dependently with mice. Male rats showed high frequency of preputial gland cancer and a slight increase of pheochromocytomas in the adrenal gland, but no increases in cancer were shown in female rats or male and female mice.

Abass et al. [132] described the metabolism of α-thujone using human hepatic preparations in vitro. Their aim also to determine the relevance of cytochrome P450 and the possible interference of other enzymes in the metabolization of α-thujone. The substance was shown to have two major metabolites (7- and 4-hydroxy-thujone) and two minor metabolites (2-hydroxy-thujone and carvacrol), and glutathione and cystein conjugates were also detected. CYP2A6 was responsible for 70–80 % of the metabolism, followed by CYP3A4 and CYP2B6.

To address the controversy regarding the psychoactive effects of thujone in absinthe, the thujone content in this alcoholic drink is too low to produce such effects. However, modern studies report that at high concentrations, thujone can indeed induce seizures, which is one of the symptoms of absinthism. Because this effect can be attenuated by benzodiazepines, an interaction with GABA_A receptors has been suggested. Because the effect of thujone on GABAergic synaptic transmission and also the mechanism of GABA_A modulation was unknown, Szczot et al. [133] investigated this effect. They used cultured hippocampal neurons and compared the effect of thujone and dihydroumbellulone on GABAergic miniature inhibitory postsynaptic currents and on responses caused by rapid exogenous GABA applications. α-Thujone reduced the frequency and amplitude of miniature inhibitory postsynaptic currents and also modulated their kinetics, suggesting both pre- and postsynaptic mechanisms. The current response on exogenous GABA showed reduced amplitude, modulated onset, desensitization, and deactivation, which indicates receptor gating. Dihydroumbellulone was ineffective or showed much smaller effects. α-Thujone was confirmed to exhibit a specific action on GABAergic activity, indicating the existence of a MT-recognition site on $GABA_A$ receptors. The authors suggest further systematical investigations.

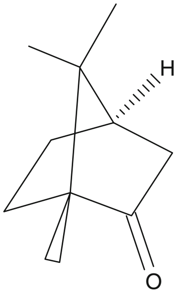
Thujone is a major compound in several plants that are believed to have antidiabetic properties. For this reason, Alkhateeb and Bonen [128] examined the use of thujone per se in the therapy of insulin resistance. In the study, insulin resistance was rapidly induced with high concentrations of palmitate in the skeletal muscle and the restoration of insulin sensitivity with thujone was assessed. The study showed that this substance was able to completely recover insulin sensitivity, even when palmitate was continuously present. This effect is related to the complete restoration of AS160 phosphorylation and palmitate oxidation. Thujone showed improvement in insulin-stimulated glucose transport and GLUT4 (glucose transporter type 4) translocation (an insulin-regulated glucose transporter), but those two effects were not totally parallel to each other. The authors suggested a possible improvement of the intrinsic activity of GLUT4 as a secondary mechanism, next to the modulation of GLUT4 translocaton. Thujone was able to activate AMP-activated proteinkinase (which usually stimulates fatty acid oxidation via inhibition of acetyl-CoA carboxylase activity), but not every restorative effect was related to this activation, such as the oxidation of palmitate, for example. The insulin-stimulated AS160 phosphorylation and glucose transport was shown, however, to be related to AMP-activated proteinkinases. The finding that thujone can improve insulin sensitivity in skeletal muscle suggests it as a, relatively cheap potential therapy, although the exact mechanisms and its safety should further examined.

Although, as already mentioned, thujone has been shown to induce cancer when applied in high doses, it seems that, in the opposite direction, it can also provide anti-cancer effects. The ethanolic extract of *Thuja occidentalis* is commonly used, in form of a mother tincture (TOΦ), in homeopathy and also in traditional medicine to treat moles and tumors. The EO of fresh leaves from this plant contains approximately 65 % thujone, related to the MT fraction. Biswas et al. [134] investigated the anti-cancer effect of the mother tincture and a thujone-rich fraction (TRF), which was separated from it, on the cancer melanoma cell line A375. TOΦ had four fractions, chromatographically separated, of which the TRF was shown to have the best anti-cancer and pro-apoptotic effect. The TRF might actually be the key-component for this effect in general. The anti-cancer activity was provided by inducing a pro-apoptotic pathway via activation of Bax, caspase-3, and cytochrome 3. Both TOΦ and TRF also caused a reduction in cell viability, induced DNA fragmentation, mitochondrial transmembrane potential collapse, and higher ROS production.

The study of Siveen and Kuttan [135] provided further proof for the anti-cancer and, more precisely, antimetastatic effect of thujone. The examination was performed on mice, where the metastasis was induced by injecting highly metastatic B16F-10 melanoma cells through the lateral tail vein. Administration of thujone, either prophylactic or parallel to tumor induction, suppressed the tumor nodule formation in lungs and increased the surviving rate. The parameters that thujone influenced where extensively discussed. The treatment led to an inhibition of pro-inflammatory cytokines (TNFα, IL-1β, IL6, GCSF), down-regulation of the matrix metalloproteinase 2 and 9, tissue inhibitor of metalloproteinase 1 and 2, VEGF, ERK-1, and ERK-2 in the lung of the animals. The invasion of the melanoma cells across the collagen matrix in a Boyden chamber was suppressed by thujone treatment, as well as the adhesion of the cancer cells to collagen-coated microtitre plate wells and the migration of the melanoma cells across a polycarbonate filter.

The possible antioxidative effect should be mentioned. Laciar et al. [136] tested the EO of *Artemisia echegarayi* for it antioxidant activity. It inhibited, with one exception, the growth of Gram-positive and -negative bacteria. It had the lowest minimal inhibitory concentration against *Listeria monocytogenes* and *Bacillus cereus*. Thujone and camphor are believed to be responsible for the antibacterial activity.

5.2 Camphor



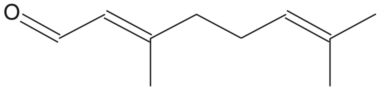
Camphor is naturally occurring in the camphor laurel tree (*Cinnamomum camphora*), but it can be obtained synthetically from turpentine oil. It possesses a cyclic turpentine structure, so it is very lipophilic, which is the reason why it is so well distributed in the body and can make crossings through mucus membranes and probably attract myelinated axons. Camphor is used in medicine for its local anesthetic, antipruritic, and antiseptic activities and as a expectorant in pharmaceutical preparations [137]. Camphor has been cherished for its medical uses for ages in Asia; it remains less known in other parts of the world. Camphor vapor is not irritating to the eyes, so it is used in cosmeticproducts and also in room fresheners or in food as a disinfectant [138].

In the eighteenth century, Leopold Auenbrugger reported on the use of camphor in the treatment of psychosis by inducing epileptic seizures. Camphor was considered to be similar to opium in pain treatment or quinine in treatment of malaria fever [139]. Indeed, camphor can cause seizures; camphor poisoning can also result in apnoea, renal insufficiency, high hepatic enzyme levels, and vomiting, which can end in pneumonitis due to the aspiration or even death. Many everyday products contain this substance. For example, several cases of camphor poisoning by ingestion of camphor mothballs for persistant headaches are known [140]. The most common symptoms in serious poisoning are neurological, such as irritability, hyperreflexia, tonic muscle, contraction, confusion, and coma. The only care is supportive help since no antidotes are available. During acute camphor toxicity, changes occur in axonal excitability. There is an excessive response to hyperpolarizing currents in the threshold electrotonus and the current-threshold relationship, which leads to a decrease in the conductance that was initiated by the hyperpolarization [137].

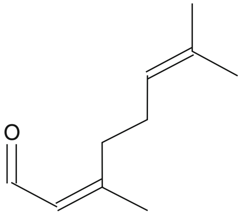
Camphor has been shown to act on two members of the TRP family, TRP vanilloid subtype 1 and subtype 3. That is the reason for the modulating sensation of warmth in humans by this substance [141]. Marsakova et al. [142] investigated the molecular mechanism of this action and the possible interaction site on TRPV1. The results showed that camphor acts on the channel by affecting the gating equilibrium of the outer pore helix domain of the channel. Camphor might also induce changes in the spatial distribution of phosphatidylinositol-4,5-bisphosphate on the inner leaflet of the plasma membrane, since it is known that the substance can decrease fluidity of the plasma membrane.

Worth mentioning is the finding of Nikolic et al. [143] that camphor (eucalyptol and thujone as well) can stimulate error-free DNA repair processes and act as a bioantimutagen. The studies were performed on prokaryotic and eukaryotic cells. The results showed the antimutagenic potential of these MT, although at higher concentrations these substances induced DNA strand breaks.

5.3 Citral



Geranial



Neral

Citral is a naturally occurring aliphatic aldehyde MT. The name citral stands for the mixture of the cis and trans-isomer called geranial and neral. With approximately 80 %, it is the major component of lemongrass oil (*Cymbopogon citratus*), but it can be found in all other citrus fruits as well. Citral possesses a fresh, intense lemony scent, which is why it is extensively used in food, cosmetics, and household products. *Cymbopogon citratus*, which is the most prominent source of this MT, is an evergreen plant growing widely in Asia and traditionally used in oriental households. Citral is believed to be non-toxic and does not induce cancer in animal models [144]. Reports on the pharmacological activities of citral are made continuously, so a few recent discoveries will be mentioned within the next lines.

C. citratus is traditionally used against GI disorders, and citral is believed to be responsible for most activities of this plant. For this reason, Devi et al. [145] investigated the extract of different parts of *C. citratus* (leaves, stems, and roots) and citral on the visceral smooth muscle in the rabbit ileum. The aim of the study was to examine the spasmolytic activity of citral, so the effect was tested on acetylcholine (ACh)- and KCl-induced contractions. The study showed that citral and the leaf extract (LE) were capable of inhibiting spontaneous contraction in a dose-dependent manner, while the extracts of the root and stem did not show results worth noticing. Citral and LE were also able to reduce the contractions induced by ACh, which was similar, although less strongly, to atropine, an antagonist of the muscarinic receptor, suggesting the possible mechanism of action. Citral was also able to inhibit contractions induced by high concentrations of K^+, very similar to verapamil (80 % inhibition with citral compared with 90 % by verapamil). The inhibitory effect of citral on the visceral smooth muscle was the strongest on spontaneous contractions, followed by KCl- and ACh-induced contractions. The mechanism of relaxation is probably by interfering in the NO-pathway and inhibiting calcium channels, due to the fact that the spasmolytic effect of citral could be reduced by L-NAME, an inhibitor of the nitric oxide synthase. Another possible mechanism is the blockage of muscarinic receptors, as mentioned above, and also inhibition of IP3, resulting in a relaxation of the smooth muscle.

It is believed that citral provides anti-inflammatory and analgesic activities, which was examined and proved in several studies. Katsukawa et al. [146] evaluated this effect using established assays for COX-2 and PPAR. Citral was shown to be a dual activator of PPARα and γ and also a PPARγ-dependent inhibitor of COX-2 expression. This finding was assessed by the fact that the NF-κB site of the COX-2 gene was involved in the inhibition of LPS-induced COX-2 promoter activity by 15d-PGJ2, a natural PPARγ ligand, as well as by dexamethasone. In U937, human macrophage-like cells, citral was able to inhibit both LPS-induced COX-2 mRNA and protein expression dose dependently. Citral is known to act on the TRP channels, particularly TRPM8 and TRPA1. The TRP channels are believed to be related to inflammation processes and even cancer, so this mechanism cannot be excluded. The authors suggest that this finding of citral is useful for consumption as a compound in the daily diet but not as a pharmacological drug, because the activity is, after all, lower compared with standard synthetic drugs.

Macrophages present an important source of inflammatory cytokines and have the potential to control an overproduction of these products, thus protecting against development of immunopathologies. Bachiega and Sforcin [147] investigated the effect of lemongrass and citral on the production of the cytokines IL-6, IL-1β, and IL-10 by peritoneal macrophages in vitro. The effect was determined before and after macrophages where incubated with LPS. The study proved the anti-inflammatory effects of citral, suggesting that the possible mechanism is involved with the inhibition of the transcription factor NF-κB. Citral inhibited the release of IL-1β, both before and after the LPS challenge; the same effect was seen with IL-6 and IL-10. Lemongrass, in comparison to citral, was not so effective; it could only inhibit LPS action after the macrophage challenge with LPS.

The synergistic action of a non-steroidal anti-inflammatory drug with plants that provide the same effects can increase the anti-nociceptive activity, with even lower rates of side effects and using lower doses. Ortiz et al. [148] investigated the anti-inflammatory effects and the gastric damage of the application of citral, naproxen, and their combination in rat models. The substances were orally administered and the effect was assessed on carrageenan-induced paw edemas and gastric damages, while the interaction type was assessed by isobolographic analysis. Naproxen, citral, and their combination exhibited anti-inflammatory effects. The advantage of their combination was that the gastric damage that naproxen significantly produced when administered by itself was not obtained when applied with citral, suggesting a potential of minimizing gastric damage in therapeutic use. Naproxen and citral were shown to have a synergistic interaction, which the isobolographic analysis demonstrated. This interaction is most probably provided by citral acting on TRP channels and inhibiting NO production, while naproxen, on the other side, suppresses the prostaglandin production.

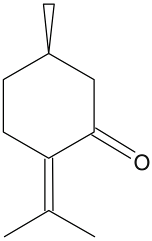
Another interesting pharmacological effect of citral is its antiadipogenic and antidiabetic activity, recently investigated by Modak and Mukhopadhaya [144]. This finding was based on the fact that citral acts as a competetive inhibitor of retinaldehyde dehydrogenase, leading to higher levels of retinaldehyde in adipocytes. Retinaldehyde is known to suppress adipogenesis and increase the metabolic rates and also influences the glucose tolerance. The study showed that citral was able to decrease the body weight gain and abdominal fat mass in rats, which were held on a high-energy diet. The effect was dose-dependent. The food intake of the rats has not changed while citral was administered suggesting that the lower weight gain is related to a lower fat absorption or higher energy expenditure. An increased metabolism is most probably the involved mechanism because an increased metabolic rate, temperature, and respiratory quotient were determined. Citral also was shown to affect insulin by decreasing its levels. which is related to an improvement in glucose tolerance and lower fasting plasma glucose levels. Taking these findings together, the authors suggest that citral has a possible role in alleviating lifestyle diseases like obesity or diabetes.

Chaimovitsh et al. [149] reported on the effect of citral on mitotic microtubules in models of tobacco BY2 cells and wheat roots. Citral disrupted mitotic microtubules and suppressed the cell cycle and also increased the occurrence of asymmetric cell plates in those cells. The effect seemed to be dose-dependent. The authors propose that at lower concentrations, citral influences cell division by disruption of the mitotic microtubules and cell plates; at higher concentrations it suppresses the cell elongation by disrupting cortical microtubules.

The antibacterial activity of citral should be briefly mentioned. Citral inhibits swarming and virulence factor expression of *Proteus mirabilis*, which can cause urinary tract infections, thus it is used to prevent development of these infections

[150]. Citral was also shown to be effective against four pathogene stains of isolated bovine mastitis, including *Staphylococcus aureus, Streptococcus agalactiae, Bacillus cereus*, and *Escherichia coli* and was also effective in destroying *S. aureus* biofilms [151]. The inactivation of *E. coli* cells is related to inducing damage in the cell envelope [152]. In addition, citral has been shown to be the responsible compound in the anti-Leishmania activity of *C. citratus*, by providing a significant inhibition of *Leishmania infantum, Leishmani tropica*, and *Leishmania major* [153].

5.4 Pulegone



Pulegone is a MT ketone that can be found as a compound in pennyroyal EO (*Mentha pulegium*). It is used in low doses as a flavoring agent in food, beverages, and hygienic products. In high doses, it is reported to cause gastritis, seizures, hepatic and renal damage, toxicity to the CNS, and coma. It has been used to induce menstruation and abortion. [154]. In 2011, the National Toxicology Program published the results of the toxicology and carcinogenesis gavage study of pulegone. The study was performed on rats and mice receiving pulegone, and data assessment was performed after 2 weeks, 3 months, and 2 years. Genetic toxicology studies have been performed on *S. typyhmurium, E. coli*, and mouse erythrocytes. The toxic effects of pulegone on rats and mice increased dose dependently. Even in the 2-week treatment group, several cases of animal death occurred when pulegone was applied in high concentrations. In the group of rats and mice treated for 3 months, besides changes in blood parameters, most damage or death was attributed to liver toxicity. The rats and mice treated for 2 years with pulegone showed symptoms like thinness, lethargy, and ruffled fur. In comparison to vehicle controls there was an increase in pathological developments in the liver (oval cell hyperplasia, bile duct hyperplasia, hypertrophy, hepatocyte necrosis, portal fibrosis), kidneys and urinary tract (hyaline glomerulopathy, nephropathy), osteoma and osteosarcoma, degeneration of the olfactory epithelium and inflammations, hyperplasia, and ulcerations of the forestomach [155].

Based on the findings of the above study, da Rocha et al. [154] investigated the mechanism of action of pulegone on the urinary bladder of female rats. It was concluded that female rats showed an increase of urinary bladder neoplasms, whereas male rats did not show an increased incidence of neoplasms of that type. The metabolism of pulegone includes hydroxylaton, reduction or conjugation with glutathione, and the metabolites identified are piperitone, piperitenone, menthofuran, and menthone. The results of the study were in agreement with those from the previous study, with rats losing body weight, bloody nasal mucus, and alopecia in the mouth and urogenital area. Scanning electron microscopy showed damage on the surface of the bladder induced by pulegone. The authors suggest that the tumors are induced due to chronic exposure to high doses of pulegone, its metabolism, excretion, and concentration and its toxic metabolites, especially piperitenone in urine, urothelial cytotoxicity, cell proliferation, and ultimately development of tumors.

De Sousa et al. [156] reported on the pharmacological effects of (R)-(+)-pulegone on the CNS. Pulegone was shown to have a central depressant effect, increased the latency of convulsions, and to inhibit both chemical and thermal models of nociception. The authors suggest, therefore, that pulegone is a psychoactive substance with activities of analgesic drugs.

De Cerqueira et al. [157] investigated the ionotropic effects of R(+)-pulegone in mammalian myocardium. They examined the effect of pulegone on L-type Ca^{++} channels, due to the assumption that it might decrease the Ca^{++} influx and thus change heart contractility. The results showed that pulegone was able to decrease myocardial contractility and reduce the intracellular Ca^{++} transient and L-type Ca^{++} current. The negative inotropic effect is similar to nifedipine, an L-type Ca^{++-}channel inhibitor, which indicates that this is the mechanism of action, but the authors do not exclude other mechanisms from possibly being involved. The effects of pulegone were almost reversible, so possible myocardial damage was unlikely.

Umezu [158] examined whether dopamine is involved in a pulegone-induced ambulation in ICR mice. The results indicate a possible involvement of dopamine and pulegone. A co-administration of pulegon and bupropion (a dopamine agonist) was shown to increase the effect on ambulation-promoting actions, and antagonists of dopamine (chlorpromazine, fluphenazine, haloperidol, and spiperone) were able to alleviate the effects of pulegone. A pretreatment with reserpine (a dopamine depletor) eliminated the sensitivity to the effect of pulegone, which implies that pulegone may not be a direct dopamine receptor agonist.

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Index

A
1A9, A431 and A549 cell lines, 3478
12α,13α-Aziridinyl epothilone derivatives, 990
Aaptos ciliata, 1292
AATs. *See* Anthocyanin acyltransferases (AATs)
Aβ (1-42), 2287
ABA biosynthesis, 1736
Aβ aggregation, 2286
Abdominal troubles, 3477
Aberrant behavior, 1375
Aberrant estrous cycles, 2421
Abies alba, 2980
Abietadiene synthase, 2725
Abiotic and biotic elicitors, 2783
Abiotic factor, 1697
Abiotic stresses, 2930
Aβ neuropathology, 2285
Aβ oligomerization, 2285
Aβ oligomers, 2283
Aβ peptides, 1308, 2285
Aβ polymerization, 2287
Abrin, 296
Abscisic acid (ABA), 2860, 3591
Absolute configuration, 930, 3320
Absolute configuration of monosaccharides, 3319
Absorbance (A), 3334, 3380
Absorbance spectrum, 3929, 3933, 3934
Absorption, 1230, 2321, 2470–2476, 2478–2481, 3134, 3377, 3616, 4024
  coefficient, 3380
  and metabolism, 1428
  wavelength, 4028
Abusive correlations, 2410
Acacetin, 1830
Acacia, 865
a_{2C} adrenergic, 4124
Acanthaceae, 801
Acanthoscelides obtectus, 4091
Acanthostrongylophora, 1293
Acari, 4070, 4083
Acaricides, 4070, 4076, 4083
Accelerated solvent extraction (ASE), 1017, 2017, 2021–2023, 2032, 2037, 2111
π-Acceptors, 692
Accretion, 2405
Accumulation, 2321
Acetaldehyde (ATL), 2902
Acetaldehyde acid, 1783
Acetate, 50, 59
Acetate-mevalonate (Ac-MEV), 2943
Acetate pathway, 2314
Acetic acid (ACE), 851, 1608, 2884
Acetogenic bacteria, 2443
Acetone, 3371
Acetonitrile, 691, 1170, 1175
3-Acetylaconitine, 1508
Acetyl-ACP, 963
Acetyl-and butyrylcholinesterase inhibitors, 3488
Acetylcholine (ACh), 1241, 1306, 1333, 1526, 1527, 1529, 1530, 3710
Acetylcholine esterase, 51, 52
Acetylcholine esterase inhibitor activity, 2906
Acetylcholinesterase (AChE), 41, 1246, 1308, 1334, 1527, 1529–1533, 1535, 4097
Acetyl-CoA, 61, 584
Acetyl-CoA carboxylase (ACC), 1658, 1825
3-Acetyldeoxynivalenol, 3127, 3145
15-Acetyl deoxynivalenol (15ADON), 3127, 3145
Acetylerucifoline, 1061
Acetylgaertneroside, 3054
6′-*O*-Acetylgeniposide, 3058
8-*O*-Acetylharpagide, 3055
Acetylintermedine, 1061
Acetyllycopsamine, 1061

K.G. Ramawat, J.M. Mérillon (eds.), *Handbook of Natural Products*,
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Acetyl/malonyl conjugates, 2391
Acetyl NIV, 3125, 3134, 3136, 3144, 3149
10-Acetylpatrinoside, 3025
N-Acetylphenylethylamine (*N*-ACPEA), 28, 1208
13-*O*-Acetylplumieride, 3026
Acetyltransferase, 3131
N-Acetyltyramine (*N*-ACTYR), 28, 1208
Acevaltrate, 3049
ACh. *See* Acetylcholine (ACh)
AChE. *See* Acetylcholinesterase (AChE)
Achillea millefolium, 2988
Achnatherum inebrians, 165
α-Acid, 2343
Acid anhydrides, 1077
Acid-butanol assay, 2074
Acidic potassium permanganate, 1087
Acidity, 2109, 2111
Acidocalcisome, 1277
Acids (“necic acids”), 361
Acids (AC), 2886, 2904, 2906
Ac-MEV. *See* Acetate-mevalonate (Ac-MEV)
Acnistin, 3470, 3471
Acnistin ramiflorus, 3470
Acnistus, 3467
Acnistus arborescens, 3478
Aconine, 1517
Aconite, 1504
Aconitine, 1170, 1188, 1506, 1508
Aconitine-induced arrhythmia, 1511
Aconitine type, 933
Aconitorientaline, 935
Aconitum, 1188, 1505
  A. carmichaelii, 1505
  A. columbianum, 1505
  A. ferox, 1505
  A. karakolicum, 1512
  A. lycoctonum, 1505
  A. napellus, 1505
Acorus calamus, 4095
Acquired resistance, 3976
Acridone, 717
Acronychia, 723
Acrosiphonia centralis (Acrosiphonia arcta), 2901
Acrylic acid (ACR), 2884, 2906
ACT. *See* Artemisinin-based combination therapy (ACT)
Actinidine, 930
Actinomadura madurae, 986, 988
Activated charcoal, 3329
Activities of daily living (ADL), 1253
Acute pain, 1379
Acute paw oedema in mice, 3217
Acute rheumatism, 1507
Acutissimin A, 1999
Acutissimins, 1993
Acyclic, 3369
Acyclic bisbibenzyls, 1921
Acyl carrier protein (ACP), 50, 61
Acyl-cholesterol *O*-acyltransferase 2 (ACAT-2), 3446
Acyl halides, 1077
Acyrthosiphon pisum, 4094
AD. *See* Alzheimer’s disease (AD)
Adaptive tolerance, 1373
Adaptogen, 3503
Adaptogenic plants, 3648
ADAS–Cog. *See* Alzheimer’s disease Assessment Scale–Cognitive Section (ADAS–Cog)
AD clinical trials, 1257
Addiction, 1207, 1375
Additive, 3983, 3984
A-delta fibers, 1371
Adenocarcinoma, 2421
Adenosine A2A receptor, 2682, 4115
Adenosine monophosphate-activated protein kinase (AMPK), 1304
Adenosine receptor, 946
Adenosine triphosphate (ATP), 3706
Adenylation protein, 51
Adenylyl cyclase, 3327
Adhatoda, 802
Adhatoda vasica, 4095
ADHD. *See* Attention deficit and hyperactivity disorder (ADHD)
Adipocytes, 3812
Adipose tissues, 2420
Adiposity, 1444
ADL. *See* Activities of daily living (ADL)
Adlercreutzia, 2444
A-domain, 703
Adrenal hormones, 1374
Adrenergic, 3709
Adrenergic receptors, 686
β-Adrenergic receptors, 1226
α2-Adrenoceptor, 599
β Adrenoceptors, 3709
α-Adrenoreceptors, 1516
β-Adrenoreceptors, 1516
ADS. *See* Amorphadiene synthase (ADS)
Adsorbents, 1699, 3329, 3376
Adsorption, 3331
AD1-8-tax, 2803
Adulteration, 294, 1084

Advantages/drawbacks, 2959
Adventitious root cultures, 188
Adverse effects, 2410, 2417
Advertizing campaign, 2423
Advise, 2416
Aedes aegypti, 4071
Aegle, 718
Aeration, 1702, 2776
Aeromonas salmonesida, 980
Aescin, 2955
Aesculus hippocastanum, 2951
Affinity, 2388, 2413, 2417
Aflatoxin, 4113
AFP, 2417
Afraegle, 732
Agaricus silvaticus, 979
Agastache rugosa, 2982
Agathosma, 753
Aged garlic, AGE, 3666–3668, 3671, 3675, 3676, 3679, 3681, 3684
Ageladine, 270
Agelastatin, 268
Ageliferins, 268
Agents, 978, 982, 984, 990, 994
Ageotropism, 2950
Age-related macular degeneration (AMD), 3937–3941, 3943–3948
Age-related macular disease, 3933, 3940, 3941
Age-related maculopathy (ARM), 3939, 3941, 3942, 3946, 3947
Age-related maculopathy disease (ARMD), 3930, 3939–3943, 3948
Aging, 1471, 1485, 1490–1491, 2266, 2267, 2349, 3477, 3703, 3707, 3711, 3712, 3722, 3727
Aglycones, 2473, 2475, 2478, 2479, 2481, 3227
Agonist, 2404
Agriculture, 3978
Agrobacterium
  A. rhizogenes, 189, 231, 918, 2774, 2948, 2955
  A. tumefaciens, 2774
Agroclavine synthase, 701
Agrocybe, 526
Agronomic factors, 2645
118A > G SNP, 1377
Ahnfeltia
  A. paradoxa, 29
  A. plicata, 2887
Ahnfeltiaceae, 2887
Ahnfeltiales, 2887
AhR. *See* Aryl hydrocarbon receptor (AhR)
Ailanthone, 3778
Airlift bioreactors, 2778
Ajmalicine, 88, 94, 96, 98, 103–110, 576–578, 584, 587–588, 591–594, 596, 1126, 2774
Ajmaline, 94, 108
Ajoene, 3668, 3678, 3680, 3681, 3683
Ajugol, 3017
Ajulemic acid, 3429
Akt, 2187
Akt/NF-kB/Bcl-2 pathway, 3485
Akt phosphorylation, 2191
Alanine aminotransferase, 1387
Alariaceae, 2898
Alaria crassifolia, 2898
Albertisia papuana, 1287
Albomitomycin A, 985
Alcaline phosphatase, 2405
$AlCl_3$ toxicity, 3653
Alcohols (ALC), 2884, 2886, 4065, 4077
Aldehydes (AL), 2884, 2886, 2904, 2907
Alectra, 3585
Alectrol, 3585
Algae, 3252–3254, 3257, 3258, 3264, 3265
Alkaline phosphate, 536
Alkaloids, 27, 41, 87–89, 92, 100, 103, 106, 109, 123, 149–167, 215, 216, 218–220, 224, 225, 228–233, 235–240, 242, 243, 245, 416, 462–476, 977–1002, 1072, 1279, 1289, 1527, 1529, 1530, 1532, 1533, 1535
  content, 323, 331, 527
  fingerprint, 386
  production, 215, 228–231, 236, 238, 242, 243, 245
  profiles, 202
Alkannin, 1552
Alkylaryquinolin, 834
Alkylboronates, 1057
Alkylphosphocholine, 1275
Allamancin, 3027
Allelochemical character, 1830
Allelochemicals, 2925, 3533
Allelopathic, 965, 3509
Allelopathic effects, 4098
Allelopathy, 151, 154, 166, 1566, 3533
Allergens, 3295, 4008
Allergic reactions, 3686
Allethrin, 4063, 4065, 4077
Alliaceae, 979
Allicin, 3666–3668, 3670, 3671, 3674, 3675, 3677, 3681, 3683, 3684

Alliinase, 3666, 3677, 3684
Alliosterol, 3233
Allium
  A. cepa, 979
  A. sativum, 3664, 3665, 3678
Allodynia, 1374
Allograft rejection, 1834
Allomelanins, 1564
Allophylus edulis, 4094
All-*trans*-β-carotene, 3588
Almazolone (ALM), 29
ALM *E*, 29
Almeidea, 731
ALM *Z*, 29
Alnus, 1553
Aloe-emodin, 1554
Alpha-cypermethrin, 4070
Alpha-fetoprotein, 2208
Alpha-ionol, 4095
Alpha-pinene, 4098
Alpha-secretase, 3714
Alteromonas, 788
Alumina, 3331, 3332
Aluminium, 3645
Aluminium oxide, 692
Alyosia triphylla, 2982
Alzheimer's disease (AD), 315, 332, 335, 337, 338, 510, 589, 848, 1244, 1307, 1333, 1471, 1475–1480, 1485, 1486, 1526, 1527, 1529, 1531, 2283, 2313, 2619, 2621, 2622, 3219, 3488, 3627, 3646, 3713, 3967
Alzheimer's disease Assessment Scale–Cognitive Section (ADAS–Cog), 1256
AM. *See* Arbuscular mycorrhizal (AM)
Amanita, 526
Amanita muscaria, 1343
Amaryllidaceae, 1335
Amaryllidoideae, 481
Amaryllis, 480
Amastigotes, 845, 1266
Amastigote-specific, 1291
Ambrosia maritima, 2782
Amenorrhea, 1374
American method, 4043
AMF. *See* Arbuscular mycorrhizal fungi (AMF)
AMH. *See* Anti-Mullerian Hormone (AMH)
Amide aziridine–2-carboxylic acid, 991
Amides (AD), 2884
Amidinium groups, 1274
Amidinotransferase, 61
Amination, 416
Amine (AM), 2884, 2886, 2904
Amino, 412
Amino acids, 884, 979, 3665
Aminoalcohols ("necines"), 361
(E)-(S)-4-Amino-7-guanidino-hept-2-enoic acid, 986, 987
4-Amino-2*H* benzo[h]chromen-2-one (ABO), 3566
(3*S*,4*S*)-4-Amino-3-hydroxy-6-methylheptanoic acid, 986, 987
4-Amino-hydroxyvaline, 988
Aminoisobutyric acid, 840
4-Amino-3-oxo-guanidinoheptane, 61
4-Aminopyridine, 64
8-Aminoquinoline, 1277
4-Amino-7,8,9,10-tetrahydro-2*H*-benzo[h] chromen 2-one analogues (ATBO), 3566
Ammonium sulfate, 3333
Amnesic, 129
Amniotic fluid, 2417
Amorphadiene, 3108
Amorphadiene synthase (ADS), 2832, 3082, 3083
AMPA/kainite receptors, 41
AMPc, 4096
Ampelopsin, 2281
Amphiroa anceps, 2889
Amphotericin B (AmB), 1274, 1289
AMPK. *See* Adenosine monophosphate-activated protein kinase (AMPK)
Amycolatopsis sulphurea, 988
α-Amylase, 1780
Amyloid, 2622, 2626
Amyloid beta (Aβ), 2283, 3712–3716, 3220
Amyloid plaques, 2283
Amyloid precursor protein (APP), 3714, 3715
Amyotrophic lateral sclerosis-Parkinsonism dementia, 41
Amyris, 722
Anabaena, 47, 58
  A. circinalis, 59
  A. crassa, 52
  A. flos-aquae, 42, 52
  A. lemmermannii, 52
Anabaseine, 1347
Anaerobic conditions, 1609
Anaerobic cultivation, 2443
Analgesic, 1389, 4119, 4122, 4131, 4150
  and anesthetic activities, 1508
  effect, 4122

Analipus japonicus, 2900
Analytical methods, 3125, 3144–3146, 3312, 3320, 4026
Anandamide, 3424
Anarchic offer, 2411
Anatoxin-a(s), 41–45, 49, 51, 52, 59
Anchor peptide, 1659
Ancistrocladus
  A. congolensis, 1286
  A. tanzaniensis, 1286
Andreadoxa, 731
Androgen receptor (AR), 2195, 2404
Anesthetic, 4116, 4122, 4145
Anesthetic activity, 4122
Anethum graveolens, 2982, 4091
Angelica archangelica, 4099
Angeloside, 3017
Angiogenesis, 2203, 2466, 2799, 3506, 3526, 3864, 3965
Angiogenic, 566
Angiogenic growth factors, 1455
Angiopoietins, 1443
Angiotensin-converting enzyme (ACE), 3675
Angostura, 767
Angustifoline, 385, 1177
Anhydrolycodoline, 1248
Anhydrovinblastine, 1123, 1129
Animal disease models, 1593
Anionic quinonoidal base, 2468
p-Anisaldehyde, 1548
Anisaldehyde-sulfuric acid reagent, 3328, 3334, 3335, 3336
Annonaceae, 850
Annona foetida, 1284
Annotine, 1248
Annotinine, 1248
Anopheles, 846
Anopheles funestus, 4094
Anorexia, 2906, 4079
Anorgasmia, 599
Antagonism, 3541
Antagonistic, 3984
Antagonist of δ-opioid and NMDA, 1212
Antagonists, 1369, 2404, 3642
Antarafacial 1,3-allylic rearrangement, 2705
Anthelminthic/anthelmintic, 308, 2906, 3792
Anthelminthic/anthelmintic activity, 337, 985
Anthocyanidin, 1854, 2119, 2260
  reductase, 1656
  synthase, 1655–1656
Anthocyanin(s), 1567, 1607, 1621, 1653, 1719, 1729, 1804, 1837, 2128, 2163, 2269, 2367, 2437, 2465–2470, 2472–2482, 2570, 2584, 2588, 2589, 2591, 2600, 2602
  anthocyanidins, 2467, 2470, 2478
  biosynthetic genes, 1631
  color, 2467, 2468, 2470, 2480
  extracts, 2466
Anthocyanin acyltransferases (AATs), 1809
Anthocyanin-flavanol, 1786
Anthocyanin-flavan-3-ol copolymerization, 2269
Anthocyanin-tannin reactions, 2262
Anthocyano-ellagitannin, 1998
Anthocyanogens, 2335
Anthonomus grandis, 1570
Anthranilic, 416
Anthranilic acid, 827
Anthraquinones, 1554, 2160
Anthriscifolcine F, 927
Anthriscifolmine A, 928
Antiangiogenesis, 2401
Antiangiogenic, 600
Antiangiogenic effects, 3216
Antianxiety, 3656
Anti-aromatase, 2401
Antiarrhythmic, 3684
Antiarrhythmic action, 1511
Antiatherogenic, 3681
Antiatherosclerotic, 694, 3681
Antibacterial, 978, 980, 985, 986, 988, 989, 1002, 1211, 2906
Antibacterial activity, 3429, 4007
Antibiotic FR-900482, 981
Antibiotic resistance, 1628
Antibiotics, 982–985, 987, 2440, 3182, 4116, 4131
Antibodies, 2803
Anticancer, 457, 978, 985–987, 990, 999, 1685, 1812, 2177, 3346, 3467, 3477, 3485, 3489, 3963
  activity, 1511, 1835, 2673, 3626, 3653
  properties, 1737
Anticarcinogenic, 2466, 4007
Anticarcinogenic effects, 2295
Anticholinergic effect, 1515
Anticonvulsive, 123
Antidepressant, 129, 317, 3219
  activity, 3645
  effect, 1206
Antidiabetic, 1937, 3450, 3964
Antidiabetic activity, 3624

Antidiarrheal activity, 1408
Antidiarrheic, 2000
Anti-DNA Topoisomerase II, 2422
Anti-doping tests, 287
Antidote, 63
Antiepilepsirine, 1352
Antiepileptic activity, 1515
Antiestrogenic, 2408
  action, 2400
  effects, 2412
Antifeedant, 3488
Antifeedant effect, 4091
Antifertility agents, 2388, 2417
Antiflogistic, 2906
Anti-forskolin monoclonal antibody, 3330
Antifungal, 994, 1211, 2388, 4113, 4125, 4126, 4128, 4136
Antifungal activity, 1212
Antiglycative, 3674
Antihepatotoxic activity, 876
Anti-herpes simplex virus–1, 2677
Anti-HIV, 3346
Antihyperglycemic, 2678, 3200
Antihyperlipidemic, 3669
Antihypertensive, 1378, 2961, 3200, 3674
Anti-inflammation, 1938, 2466
Anti-inflammatory, 123, 849, 1211, 1422, 1590, 2176, 2181, 2406, 3089, 3488, 3621, 3642, 3963, 4115, 4116, 4119, 4134, 4136, 4137, 4141, 4147–4148
  activity, 1413, 1510, 1834, 3217
  agents, 463, 475, 3449
Anti-initiation activity, 2296
Antileishmanial, 1279, 3488
Antileukemic, 981
Anti-LH, 2419
Anti-lithogenic influence, 1420
Antillatoxin, 41
Antimalarial, 455, 2776
Antimalarial activity of quinine, 608
Antimetastatic, 1451
Antimetastatic activity, 1211
Antimicrobial(s), 816, 978, 981, 984, 988, 994, 1002, 1813, 1937, 2388, 3488, 3626, 4113, 4126, 4136
  activities, 397, 1212, 2002
  agents, 3977, 3984
  components, 3981
  efficacy, 3982, 3983
  property, 1608
  therapy, 3984
Antimitotic, 3187
Anti-Mullerian hormone (AMH), 2418
Antimutagenic, 135, 848, 912, 1812, 3542, 4007
  action, 1410
  blocking agent, 2213
Antineoplastic, 2785, 2961
Antineovascularization, 3562
Antinociceptive, 1211, 4127, 4131, 4148
  activities, 945, 1508, 4115
  effects, 4119
Antinutritional, 1755
Antinutritional factors, 2391
Antioxidant(s), 123, 911, 1212, 1717, 1736, 1755, 1822, 1860, 1934, 2063, 2158–2161, 2401, 2466, 2473, 2506, 2510, 2511, 2515, 2564, 2591–2595, 2599–2605, 2619, 2626, 2627, 2785, 2960, 3618, 3670, 3701, 3712, 3713, 3727, 3925, 3938, 3942–3944, 3947, 3948, 3963, 4007, 4023, 4113–4116, 4123, 4127, 4132, 4134, 4135, 4138, 4140, 4141, 4145
  activities, 1211, 1590, 2298, 2651
  capacity, 2644
  detection, 2123
  effect, 4119
  property, 1421
  response element, 2626
  status, 2176
Antioxidative, 1730
Antiparasitic activity, 1515, 4069
Anti-photo-oxidative activity, 1212
Antiplasmodial, 3346
Antiplatelet, 848, 1936
  activity, 3567
  aggregation, 3622
Antiproliferative, 1447, 1960, 2400
  activity, 3216
  in ERβ-positive cells, 2408
Anti-promotion activity, 2296
Anti-protein kinase C (PKC), 2401
Anti-protein tyrosine kinase (PTK), 2400
  inhibition, 2401
  pathway, 2404
Antiprotozoal, 626, 816
  activity, 626
Antipruritic, 4122, 4145
Antipsychotic, 129
Antipyretic, 130, 397, 1389
Antirheumatic, 4119
Antirrinoside, 3018
Antisense, 232, 238
Antisense suppression, 1838

Antiseptic, 2906, 4122, 4131, 4136, 4145
Antispasmodic, 4118, 4123, 4137
Antispasmodic activity, 4118
Antistress, 2785, 3711, 3720
Antithrombotic, 3677
Antitumor, 318, 337, 563, 566, 978, 981–983, 988, 991–996, 999, 1002, 1812, 1932, 3467, 3477, 3485, 3488, 4114, 4120, 4126, 4127, 4130, 4131, 4135
  activity, 1210, 1514, 3200
  agents, 510
Antitumoral, 88, 2466
Antitumoral activities, 2002
Antitussive, 1378
Antiulcer activity, 1424
Antiviral, 123, 1813, 3627
Antiviral activity, 3853
Anxiety, 1307, 3711, 3720
Anxiolytic, 129, 3220, 4115, 4128, 4129
  activity, 3968
  anti-convulsive effects, 1621
  effect, 4115
Aortic fatty streak, 2298
Aortic rings, 2369
Aphanizomenon, 47
Aphanizomenon flos-aquae, 53, 58
Aphantoxin, 53
Aphidius colemani, 4098
Aphids, 157, 158, 160, 165
Aphylline, 158
Apiaceae, 1551, 3071, 3073, 3084–3086, 3090, 3093
Apigenin, 1658, 1830
Apigeninidin, 1569
Apios americana, 1852
Apocynaceae, 89, 111, 775
Apomorphine, 1349
Apoptopic, 49
Apoptosis, 1211, 1286, 1314, 1450, 1870, 1872, 1875, 1880, 1884, 2328, 2404, 2406, 2413, 2628, 2630, 3142, 3478, 3485, 3522, 3523, 3527, 3557, 3700, 3704, 3712, 3713, 3715, 3716, 3723, 3748–3750, 3790, 3853, 3963, 4114, 4126, 4127, 4130, 4135, 4137, 4138, 4140
Aporphine, 412
Appetizer, 3477
Apple, 1729
Aquaculture, 1736
Aquaporine-1, 1273
Aqueous two-phase system (ATPS), 1102
Arabidopsis, 1722
Arabidopsis thaliana, 1636, 3002, 3584
Arabinose, 2467
Arabinoxylans, 2346
Arachidonate epoxygenase, 994
Arachidonic acid (AA), 3679
2-Arachidonyl glycerol, 3424
Arachis, 1553
Araliaceae, 749
Aralia saponins V, 2952
Araliopsis, 730
Arbuscular mycorrhizal (AM), 3584
  fungal inoculation, 2650
  symbionts, 2653
Arbuscular mycorrhizal fungi (AMF), 2645, 2646
Arbuscules, 3595
Arbutin, 1548
Architecture, 2413
Arctiid moth, 155, 156
Arctiids, 155–157, 162
Area under the curve (AUC), 2323, 3336
Areca catechu, 1344
Arecoline, 1344
Areschougiaceae, 2890
Arginine, 59, 365
Arginine decarboxylase, 182
Argyrodendron peralatum, 865
Aricine, 611
A-ring modifcations, 3352
Arizona, 2150, 2161
Arnica montana, 3532
Aroma compounds, 3981
Aromatic oils, 2946
Aromatization, 2418
Aromatized, 2385
Arrhythmia, 946, 1306, 3683
Arrhythmogenic effect, 1510
Artemisia, 2947
  A. absinthium, 2983, 2999
  A. annua, 2776, 2959, 3531, 4116
  A. dracunculus L, 4089
  A. pallens, 2988
  A. vulgaris, 4095
Artemisin, 3082
Artemisinic acid, 1628, 3103, 3105, 3108
Artemisinin, 629, 1279, 2680, 2695, 2736, 2764, 2776, 2827, 2831, 2955, 3100–3115, 3531
Artemisinin-based combination therapy (ACT), 3101, 3107, 3108, 3114
Arterolane, 1279
Arthritic conditions, 3477

Arthritis, 1964
Arthrobacter, 798
Arthrogryposis, 900
Arthropods, 4077
Artichoke, 2650
Artificial biosynthetic pathway, 1657
Artificial enzyme design, 1633
Artificial organelle, 1672
Artificial polyploidization, 197
4-Arylcoumarins, 1555
Aryl hydrocarbon receptor (AhR), 2189
Ascending mode in CPC, 2150
Ascites, 1386
Ascophyllum nodosum, 2896
Ascorbate (ASA), 1588, 1732
ASE. *See* Accelerated solvent extraction (ASE)
Ashwagandha, 413, 3476, 3477
Ashwagandhanolides, 3482
Asian women, 2408, 2417
Asiatic acid (AA), 3961
Asiaticoside (AS), 3961
Asparagales, 979
Asparagopsis
  A. armata, 2888
  A. taxiformis, 2888
Aspartame, 3199
Aspartate aminotransferase, 1387
Aspartyl protease, 987, 988
Aspergillus niger, 106, 1564
Aspergillus species, 839, 1554
Asperuloside, 3024
Asperulosidic acid, 3057
Aspidosperma, 577, 590
Aspidosperma ramiflorum, 1288
Asplenium spp, 1553
Assacharobacter, 2444
Association, 3789
Association for Official Analytical Methods (AOAC), 57
Association of Poison Control Centers, 295
Associative, 1373
Astaxanthin, 3265, 3273, 3274
Asteraceae, 769, 979, 1385, 1551, 3071, 3073, 3081–3086, 3094, 3194, 3532, 3540, 3590
Asterolasia, 735
Asthma, 4120, 4141
Astragalus sinicus, 3586
Astringency, 1755, 1787, 2258, 2266, 2267, 2313, 2342
Astringency intensity, 2267
Astrocytes, 2624, 2625
Astroglial cells, 2406
Asymmetric carbons, 1787, 2398
Atalantia, 752
Ataxia, 3725, 4065
Atelopus zeteki, 54
Atherogenesis, 1811, 3623
Atheroprotective effect, 3819
Atherosclerosis, 1305, 1444, 2298, 2326, 2961, 3217, 3681, 4117
Athletes, 289
Atisane, 3180
Atisine-denudatine type, 937
Atmospheric pressure chemical ionization (APCI), 1126, 3398
ATP. *See* Adenosine triphosphate (ATP)
ATPase, 3982
ATP-binding cassette (ABC) transporters, 3446, 3451, 3590
ATPS. *See* Aqueous two-phase system (ATPS)
Atropa belladonna, 291, 1182, 1185
Atropine, 175, 291, 348, 1014, 1165, 1182, 1188
Atroxima, 3206
Atroxima liberica, 3213
Attention deficit and hyperactivity disorder (ADHD), 1207, 1471, 1474, 1482–1492
Attention task, 2414
AUC. *See* Area under the curve (AUC)
Aucubin (aucuboside, rhinanthin), 3018
AuNPs, 1106
Aurachins, 829
Aureusidin synthase (AmAS1), 1889
Aurones, 1621, 1887, 1889
Australian ewes, 2417
Autolyzate, 978–979
Autooxidation, 2370
Autopsy, 2409
Autumnaline, 461–476
Auxins, 192, 1830, 2774, 3198, 3591, 4044
Avenalumin I, 1569
Avena sativa, 1569
Avicenniaceae, 1552
Axinellamines, 268
Axinohydantoin, 268
Axonal membrane, 4065
Axone regeneration, 3219
Ayahuasca, 125, 1452
Ayurvedic, 1278, 3960
  formulations, 3327, 3328
  medications, 1399
  medicinal system, 3476

1-Azabicyclo[3.1.0]hexane moiety, 980
Azacyclo-propane, 978–979
8-*O*-Azeloyl-14-benzoylaconine, 1512
Azetidine, 978
Azicemicin A, B, 988, 989
Azimexon, 991
Azinomycin A, B, 987–988
Aziran, 978–979
Aziridine, 977–1002
Aziridine-2-carboxylic acid, 979, 991
Aziridine-containing peptides, 979
(2*R*,3*R*)-Aziridine-2,3-dicarboxylic acid, 986, 987
(2*S*,3*S*)-Aziridine-2,3-dicarboxylic acid, 980
Aziridine-2,3-dicarboxylic acid, 980, 987
Aziridine ring, 978, 986, 1000
4-(1-Aziridinyl)-3-buten-2-one, 979
N-Aziridinylspermidine, 991
Azirinomycin, 979
Azulene, 2893

B
Babesiacanis, 4071
Babies, 2422
Baccata, 2801
Baccatin III, 2784, 2801
Baccharis dracunculifolia DC, 4125
Bacillus, 806, 984
Bacillus subtilis, 979, 1627
Background electrolyte (BGE), 1103, 1155
Bacopa monnieri extract (BME), 3643
Bacoside A, 3647
Bacoside B, 3648
Bacteria, 1705, 1707, 3976
Bacteria-host interactions, 2451
Bacterial diarrhea, 1307
Bacterial flora, 2399
Bacterial microcompartment, 1672
Bacterial non-heme dioxygenases, 1670
Bacterial/viral infections, 374
Bacteriostatic action, 1835
Bacteroides, 2452, 2479
Bacteroidetes, 2441
Bactrocera latifrons, 4095
Baeocystin, 527
Baicalein, 1390
Baker's yeast, 978
Balance, 2405, 2417, 2422
BALB/c mice, 1283
Balfourodendron, 729
Balloon bioreactor, 2786
Bangiaceae, 2886–2887
Bangiales, 2886–2887
Banisteriopsis caapi C. V. Morton, 1452
Baogongteng A, 1345
bapt, 2784
Barlerin, 3055
Barrier, 3758, 3759, 3762
Basic C5 units, 2683
Basic fibroblast growth factor, 1448
Basidiomycetes, 979
Bathochromic shift, 1999
Bathochromism, 1781
Bauerella, 737
Bauhinol A, 1933
Bayogenin, 3207, 3214
Bayogenin derivatives, 3217
BBB. *See* Blood-brain barrier (BBB)
BChE. *See* Butyrylcholinesterase (BuChE/BChE)
Bcl-2, 3559
Bcl-2 family, 1313, 3524
Bean second internode bioassay, 3858
Beef, 4075
Beer-Lambert law, 3380
Bees, 2935
Behavioral sensitivity for taste, 1208
Belladonna, 291
Belotecan, 653, 665
Bemisia tabaci, 4101
Beneficial effect, 2411
Benefits, 2327
Benign breast hyperplasia, 2412
Bentonite, 2342
Benzaldehyde (BE), 2884
Benzatropine, 1345
1-Benzene-azo-2-naphthol, 4065
Benzo*[c]*phenanthridine, 1282
Benzoic, 1953, 1956
Benzoic acid (BZ), 2885, 4044
Benzothiazole (BZT), 2885, 2905
Benzoxazinones, 154, 161
Benzoyl, 3210
6-Benzoylheteratisine, 1511
Benzyl alcohol, 2887
Benzylisoquinoline alkaloids (BIA), 3–21, 160, 220, 1349
Benzyl mercaptan, 1765
4-Benzyltropanone, 352
Berberine, 6, 11, 12, 444, 1169, 1170, 1303, 1446, 1534, 2766
Berberine bridge enzyme (BBE), 11
Bergaptene, 1551

Berry, 2588
Beta-caryophyllene, 4051
Betacyanins, 2159, 2161
Beta-D-glucosidases, 2479
Beta-pinene, 4098
Betula lenta, 4100
BGE. *See* Background electrolyte (BGE)
Bi and triflavonoids, 1555
Bibenzyls, 1914
Bichalcone, 1555
Bicyclic sesquiterpenoids, 2782
Bicyclogermacrene, 2976
Bifidobacteria, 2452
Bifidobacterium animalis, 2565
Biflavonoids, 1621
Bifunctional terpene cyclases, 2725
Bilayers, 4115, 4124
Bile secretion, 1385
Bilitranslocase, 2478
  organic anion carrier, 2478
Bilobalide, 3701, 3726
Binary vectors, 2953
Bioaccumulation, 4098
Bioactive, 3664
Bioactive indole, 545
Bioactivity, 1583, 2324, 2471–2473, 2479
Bioassay-guided fractionation, 2147, 2151, 2160, 2161
Bioassays, 3858
Bioavailability, 1317, 1593, 1611, 1663, 2319, 2402, 2411, 2466, 2470, 2472, 2473, 2476, 2478–2480, 2482, 2590, 2591, 2593, 2619, 2622, 3505, 3726, 4024
  enhancer, 1402
  of micronutrients, 1407
  of resveratrol, 2294
Biochanin A, 1662
Biochemical, 3541
Biodegradability, 4098
Biodegradation, 51, 3505
Biogenesis, 830
Biogenic amines, 1732
Bio Ginkgo, LI-1370, 3708
Bioinformatics, 2513
Biokinetics, 2470, 2471, 2476, 2477, 2481
Biological achievements, 3206
Biological activity, 510, 540, 1809, 2766, 3243, 3519, 3540, 3978
  of purine alkaloids in humans, 965
  of saxitoxin and tetrodotoxin, 970
Biological and therapeutic property, 3541
Biological efficiency, 3977
Biological membrane, 2471
  membrane fluidity, 2471
Biological weapon, 296
Bioluminescence, 3485
Biomagnification, 4098
Biomphalaria, 846
Biopesticides, 4088
Biopharmaceuticals, 2942
Biopharming, 1736
Bioprocesses, 215, 243
Bioprospecting, 1672
Biopterin transporter (BT1), 1271
Bioreactors, 107, 111, 919, 1693, 1700–1703, 1706, 1707, 1862, 2775, 2950, 2958, 2959, 3198
Biosynthesis, 49, 50, 138, 215, 219, 220, 232, 233, 235–240, 242, 243, 253, 318, 365, 504, 536, 775, 959, 963, 984, 1221, 1762, 1808, 1925, 2584, 2585, 2600, 3125, 3129–3131, 3133, 3166, 3242, 3243, 3571, 3609, 3836, 3837, 3980, 4021
  de novo, 202
  genes, 61
  of long-chain PUFA, 1738
Biosynthetic efficacy, 1633
Biosynthetic gene cluster, 686
Biosynthetic genes, 1723
Biosynthetic networks, 1628
Biosynthetic pathways, 88, 89, 94, 103, 104, 108, 111, 215, 219, 220, 223, 230–233, 235, 237, 239, 240, 243, 488, 2860, 2861, 2869, 2875, 3103–3105, 3107, 3108, 3522
Biosynthetic precursors, 2770
Biotechnological, 1686, 1691, 1700, 1706, 1814, 1816, 2765
  approach, 88, 89, 101, 544
  interventions, 1728
  processes, 3575
Biotechnology, 193
Bioterrorism, 296
Biotic and abiotic stresses, 3853
Biotransformation, 215, 232, 238, 239, 1223, 1231, 1625, 2471–2473, 2479, 3108
Biphasic, 2148, 2150, 2162, 2163, 2165
Biphenyl dioxygenase, 1670
Birch reduction, 2704
Birds, 4069
Bisabolene, 2985, 4050
Bisabolol, 4130–4133
α-Bisabolol, 2985, 2997
Bisbibenzyls, 1917

Bisdesmoside, 3227
3,5-Bis(1-aziridinylmethyl)-2,6-dimethyl-pyridine, 992
3,3′-Bis(4,6-dibromo-3-methylthio) indole, 33
Bis(trifluoroacetoxy)iodo(III) pentafluorobenzene, 262
Bis-[*O*-(14-benzoylaconine-8-yl)]esters, 1513
Bis-[*O*-(14-benzoylaconine-8-yl)]-suberate, 1513
Biting gnats, 4076
Bitter, 3780
  lupins, 157, 161
  orange, 290
Bitterness, 2258, 2334, 2342, 2346
Blackberry, 1719
Black pepper, 311, 317, 318, 337
Bladder cancer, 2214
Blades, 2777
Blatella germanica, 4100
Bleaching agent, 984
Bleekeria, 590
Blocking of neuronal channels, 897
Blood, 1077
Blood-brain barrier (BBB), 52, 1251, 2478, 2482, 2619
Blood diseases, 3477
Blood flow, 4124
Blood pressure, 1737, 3668, 3675–3677, 3681, 3682, 3684, 3720, 3810
Blood purifier, 3477
Blood vessels, 1442
Blowflies, 4075
Blueberry, 1719
Blueing phenomenon, 536
Blue light, 3925, 3927, 3929, 3933, 3936, 3937, 3945–3948
Blumea balsamifera, 2984
B-lymphocyte, 991
BMC. *See* Bone Mineral Content (BMC)
BMD. *See* Bone mineral density (BMD)
B-16 melanoma, 988
BN-52021, Ginkgolide B, 3710, 3721, 3726
β-*N*-methylamino-L-alanine (BMAA), 41
Boenninghausenia, 732
Bog rhubarb, 979
Boiling, 2335, 2351
Boiling kettle, 2347
Bombyx mori, 4096
Bone disorders, 2449
Bone fracture, 2413
Bone fracture risk, 2405
Bone mineral content (BMC), 2405
Bone mineral density (BMD), 2405, 2414, 2448
Bone preservation, 2423
Bone turn-over markers (BTM), 2405, 2414
Bonistein®, 2415
Bonnemaisonia
  B. asparagopsis, 2889
  B. hamifera, 2889
  B. nootkana, 2889
Bonnemaisoniaceae, 2888
Bonnemaisoniales, 2888–2889
Borage, 1738
Boraginaceae, 1552
Borate buffers, 1169
Borneol, 2735, 4047
Bornyl angelate, 2976
Boronella, 730
Boronia, 723
Boronic acid, 3859
Bortezomib, 2221
Boschnaloside, 3047
Bosistoa, 761
Bostrychia
  B. calliptera, 2891
  B. radicans, 2892
  B. tenella, 2892
Bottleneck, 1633
Botulinum toxin, 296
Bouchardatia, 732
Bovine serum albumin (BSA), 1780
Bower Manuscript, 3664
Bowman-Birk inhibitor, 2416
Box fermentation, 1609
Brachycerine, 138
Brahmi, 3641
Brahmoside, 3962
Brain, 2592, 2593, 2598, 2599, 4068
Brain protection, 3966
Branch point, 701
Brassicaceae, 176, 851
Brassica napus, 3592
Brassinolide, 3852
Brassinosteroid (BR), 3591, 3852
Brassinosteroid-insensitive 1 (BRI1), 3856
Brassinosteroid-insensitive 2 (BIN2), 3856
Br2C:CHCHO carbonyldiiodide, 2888
BrCH2CH2I, 2889
BR-derived boronates, 3860
Breast, 1316
Breast cancer, 2212, 2448, 4120
Breast cancer cell, 3964
Breast carcinoma, 1836
Breast carcinoma (MDA-MB-231, MCF-7), 3479
Breast-feeding, 3686

Breeding, 2854, 2855, 2861–2863, 2866, 2871, 2873–2875
Breeding aim, 1603
BRI. *See* Bromoindole (BRI)
BRI1-associated receptor kinase 1 (BAK1), 3856
β-ring carotenoids, 3369
Bristol-Myers Squibb, 2784
British pharmacopeia (BP), 3664
Broken nibs, 1603
Brombya, 737
Brominated 3,4-dihydroxybenzaldehyde, 2893
1-Bromo-2-chloro(1-octene-3-ones), 2889
(Z)-1-Bromo-1-chlorovinyl group, 980
Bromocresol (BC), 2884
Bromocryptine, 707
Bromodeoxyuridine, 1445
1-Bromo-2,3-dichloro(1-octene-3-ones), 2889
Bromo-4-hydroxybenzaldehyde (BH), 2884
Bromoindole (BRI), 32, 33, 1212
Bromophenol (BP), 2884, 2905
2-Bromophenol (2-BP), 2905
4-Bromophenol (4-BP), 2905
Bronchial, 1964
  asthma, 3477
  muscle, 3991
Bronchitis, 3477
Bronchodilator, 1225
Broncho-vasodilatory activity, 3652
Broomrapes, 3585
BR signaling pathway, 3866
Brucea, 3780
Bruceantin, 3778, 3784
Bruceins, 3778
Bruceolide, 3778
Bruch's membrane, 3927, 3928, 3939–3941
Bruchus chinensis, 4091
Brucine, 590, 1451
Brusatol, 3778
Bryophytes, 1914
Bryopsidales, 2903
BSA. *See* Bovine serum albumin (BSA)
BTM. *See* Bone turn-over markers (BTM)
B-type and one A-type linkage, 1759
Bubble column, 2777
  bioreactor, 188, 197
  V-shaped, 1701
BuChE. *See* Butyrylcholinesterase (BuChE/BChE)
Budd–Chiari syndrome, 1387
Bufalin, 3747, 3748
Buffers, 2118
Bulbs, 3664, 3665, 3667
Bulbus Lycoridis, 1188
Bulleyaconitine, 1507
Burning sensation, 1417
Burundanga, 292
2,3-Butanediol, 2896
t-Butanol, 3333
Butoxypolypropylene glycol (repellent), 4077
Butylated hydroxyanisole (BHA), 3670
Butylated hydroxytoluene (BHT), 3389, 3670
6-Butylcyclo-1,4-heptadiene, 2894
3-Butyl-4-vinylcyclopentene, 2894
Butyric acid (BU), 2884
Butyrylcholinesterase (BuChE/BChE), 1247, 1527, 1529
Byproducts, 2600, 2602, 2603

C

C8 phase, 1086
C18 phase, 1086, 3385
C-18, C-19, C-20, C-22, and C-25 (Quassinoids), 3347
C30 phase, 3385
Cabernet Sauvignon and Merlot wines, 2265
C7α-C6β γ-lactone ring, 3090
Cachinol, 3051
4-Cadainene, 2895
Cadaverine, 389
Cadelene, 2893
Cadinene, 2895, 2907
β-Cadinene, 2986
1-Cadinol, 2894
α-Cadinol, 2887
Caespitane, 2892
Caespitol, 2892
Caffeic, 1549, 1954, 1960
Caffeic acid, 1629, 1657, 2164
  aspartate, 1605
  ester of arabinosyl 5-*O*-apigeninidin, 1569
  phenyl ester, 2443
Caffeine, 151, 153, 154, 160, 955, 1178, 1181, 1353–1355, 2567
  content, 955
  synthase, 959
Caffeine (1,3,7-trimethylxanthine), 954
Caffeine-producing transgenic, 971
Caffeoylhistamine, 876
Ca^{2+} influx, 3994
Cajaflavanones, 2162, 2164
CAL A. *See* Callophycin A (CAL A)

Calanolide B, 1551
Calcitonin gene-related peptide (CGRP), 1371
Calcium, 2364, 2405, 2414
  balance, 2406
  channels, 3996, 4069
  handling, 3995
  signal, 2367
Calibration, 3380
Calibration curves, 3315, 3336
Caligus elongatus, 4076
Callilepis laureola (Impila), 1384
Callophycin A (CAL A), 31
Callophycus
  C. oppositifolius, 31
  C. tridentifer, 2890
Callosobruchus maculatus, 4098
Callus cultures, 186
Calmodulin, 2364, 4068
Caloglossa leprieurii, 31
Calonectrin, 3131
Calorie restriction, 2300
Calystegine A-group, 350
Calystegine B-group, 350
Calystegine C-group, 350
Calystegines, 177, 184, 350, 1169
Cambodia, 533
Camellia
  C. assamica, 955
  C. ptilophylla (cocoa tea), 956
  C. sinensis, 955
Ca^{2+} Mg^{2+}-ATPase, 4068
cAMP, 3327, 4115, 4129, 4130
Camphene, 4098
Camphor, 2734, 2735, 2984, 2998, 4141, 4145, 4146
Camptotheca acuminata, 647, 1178
Camptothecin (CPT), 667, 670, 671
Camptothecins, 644, 645, 650, 651, 654, 659, 661, 667, 1179
  biological activity, 661
  bioproduction, 671
  biosynthesis, 659
  chemistry, 650
  conjugates, 670
  delivery, 667
  formulation, 645
  history, 644
  isolation, 647
  occurence, 647
  other activity, 670
  prodrugs, 667
  reviews, 647
  SAR, 654, 659
  total synthesis, 651
Can act as LXR ligands, 3446
Cancer(s), 588, 847, 1311, 1444, 1460, 2326, 2439, 2583, 2591, 2592, 2594, 2596, 2598, 2605, 2959, 3188, 3485, 3488, 3489, 3506, 3525, 3626, 3752, 3783, 3875–3910, 3995
  cell lines, 1447
  chemoprevention, 3477
  chemopreventive activity, 2296, 2302
  pain, 1380
  prevention, 2401, 2643
  progression, 2409
Cancerogenic, 4121, 4122
Cancerostatic, 991
Cancer stem cell (CSC), 2206, 2297
Candida albicans, 2906, 4008, 4126
Candida spp., 1310
Cannabichromanone, 3423
Cannabichromene (CBC), 3420
Cannabicoumaronone, 3423
Cannabicyclol (CBL), 3422
Cannabidiol (CBD), 3417, 3420, 3425, 3428, 3430
Cannabielsoin (CBE), 3421
Cannabifuran (CBF), 3421
Cannabigerol (CBG), 3417, 3429, 3430
Cannabigerolic acid (CBGA), 3418
Cannabimovone (CBM), 3420
Cannabinaceous, 413
Cannabinodiol, 3420
Cannabinoid (CB), 4117, 4118
Cannabinoid (CB) receptors, 3417, 3424, 4117
Cannabioxepane (CBX), 3422
Cannabiripsol, 3422
Cannabisol, 3422
Cannabis sativa L., 2986, 4117, 4119
Cannabitetrol, 3422
Cannabitriol, 3422
Cannabivarins, 3419, 3426
Canola, 1735, 1737
Canola oil, 1736
Canteyoside, 3047
Canteyoside dimethyl acetal, 3047
Canthaxanthin, 3369
Capacity factors, 2112
Capacity to modulate cytokine production, 3449
Capillaries, 1102
Capillary electrochromatography (CEC), 1181
Capillary electrophoresis (CE), 180, 1032, 1064, 1074, 1128, 4031

Capillary electrophoresis–mass spectrometry (CE-MS), 1033, 1182
Capillary gas chromatography, 3144
Capillary-like structures, 1446
Capillary zone electrophoresis (CZE), 1033, 1104, 1148, 1157
Caproic acid (CA), 2885
Caprylic acid (CAP), 2885
Capsaicin, 1456
Capsella bursa-pastoris, 864
Capsiate, 1458
Capsicum
  C. annuum L., 1457
  C. frutescens L., 1457
Carbamates, 63, 4065
Carbamate toxins, 54
Carbamoyl side-chain, 54, 59
O-Carbamoyltransferase, 61
Carbamylcholine chloride, 3656
Carbaryl, 4077
Carbinol, 2468, 2475
  carbinol base, 2468
Carbocations, 3081, 3085
Carbohydrate, 3665
β-Carboline, 554–563, 565–569
Carbolines, 1170
Carbon assimilation pathways, 1633
Carbon dioxide, 230
Carbon fluxes, 1570, 1634
Carbonium ions, 369
8-Carbon polyketoacid, 896
Carbon source, 103
N-Carboxyisoserine, 991
Carboxylic acid, 979, 988
Carcinogenesis, 2320, 3149
Cardiac action, 944
Cardiac fibroblasts, 3570
Cardiac ion channels, 63
Cardiac myocytes, 3569
Cardioprotective, 1941, 2591, 2594
Cardioprotective activity, 3967
Cardiopulmonary bypass, 1516
Cardiopulmonary failure, 63
Cardiovascular diseases (CVD), 1811, 2362, 2372, 2373, 2449, 2466, 3667
Cardiovascular health, 2298
Cardiovascular problems, 3477
Cardiovascular protection, 3622
δ-3-Carene, 2983
Carinatumin A, 1248
Carinatumin B, 1248
Carlactone, 3588
Carmagerol, 3420
α-Carotene, 3256, 3258, 3263–3265, 3369, 4020, 4022
β-Carotene, 1736, 2680, 2830, 2855, 2857, 2858, 2860–2866, 2868–2870, 2872, 2874, 2875, 3369, 4020–4025, 4028, 4031
δ-carotene, 3390
ζ-Carotene, 4019
Carotenes, 1730, 2787, 4019, 4027
Carotenogenesis, 2861, 2869
Carotenoid biosynthesis pathway, 1731
Carotenoid cleavage dioxygenases (CCD)7, 3588, 3596
Carotenoid cleavage dioxygenases (CCD)8, 3588, 3596
Carotenoid 3-hydroxyisorenieratene, 1546
Carotenoids, 1717, 1719, 1729, 2469, 2567, 2680, 2734, 2737, 2744, 2763, 2787, 2815, 2824, 2826, 2836, 2855, 2856, 2860–2862, 2867–2870, 2873–2875, 3925, 3928–3930, 3933–3935, 3937, 3940, 3942, 3948
Carpolobia, 3206
Carpolobia alba, 3211
Carrageenan, 3655
Carrier-mediated, 2471
  antiport, 2471
  symport, 2471
  uniport, 2471
Carteramine, 268
Carum carvi, 2978
Carvacrol, 2976, 2977, 2980, 2997, 4097, 4133–4137, 4143
Carveol, 4047
Carvone (CAR), 2887, 2901, 2902, 2978, 3079, 3081
Caryomene olivasans, 1287
Caryophyllene, 2925, 3521, 4116, 4118, 4119
β-Caryophyllene, 2986, 2989, 2997, 3427
Carzinophilin/Carzinophyllin, 988
Case-control study, 1835
Casimidine, 864
Casimiroa, 718
Casimiroa edulis, 864
Caspase-3, 1449, 3429, 3557
Caspases, 1313, 3523, 4118, 4127, 4135, 4137, 4138, 4144
Cassia, 1554
Castela, 3780
Castilleja indivisa, 158
Castor beans, 296
Catalase, 699, 2184, 3713, 4113, 4114, 4127, 4132

Catalpol, 3057, 3058
Catalytic domains, 61
Cataract, 1964
Catechin, 2314, 2335, 2342, 2452, 2568
(+)-Catechin, 1605
Catechinpyrylium, 1784
Catechins, 1178, 2285, 2334
Catechol, 1548
Catecholamines, 598, 967
Catechol melanins, 1564
Catechol-*O*-methyl transferase, 2475
β-Catenin, 2194
Catha edulis, 1221
Catharanthine, 88, 90, 94, 97–99, 102–110, 579, 584, 587, 588, 1123
Catharanthus, 577, 578
Catharanthus roseus, 101, 1454
  anticancer, 2094
  anti-diabetic, 2094
  antioxidant activity, 2104, 2105
  benzoic acids, 2098, 2105
  flavonoids, 2100, 2103–2105
    anthocyanins, 2101, 2104
    isorhamnetin glycosides, 2101
    kaempferol glycosides, 2101
    quercetin glycosides, 2101
  hydroxycinnamic acids, 2098, 2104, 2105
Cathepsin B, 987, 988
Cathepsin L, 987, 988
Cathinone, 913
Cation radical, 260
Cats, 4071, 4077, 4078
Cattle, 4070
Caucasian, 2409
Caulerpa
  C. ashmeadii, 31
  C. cactoides, 2904
  C. lamourouxii, 31
  C. microphysa, 31
  C. paspaloides, 31
  C. peltata, 31
  C. prolifera, 31
  C. racemosa, 31
  C. racemosa var. *clavifera*, 31
  C. racemosa var. *laetevirens*, 31
  C. racemosa var. *macrophysa*, 31
  C. scalpelliformis, 31
  C. serrulata, 31
  C. sertularioides, 31
  C. taxifolia, 31
Caulerpaceae, 2904
Caulerpales, 2904
Caulerpin (CLP), 31, 1210
Caulersin (CLS), 31
Cauliflower mosaic virus 35S promoter, 192
Caveolin 1, 2364
CB. *See* Cannabinoid (CB)
CB1-receptor, 4117
CBD. *See* Cannabidiol (CBD)
CBG. *See* Cannabigerol (CBG)
CB_2-receptor, 4117
CCa. *See* Colon cancer (CCa)
CCC. *See* Countercurrent chromatography (CCC)
C6-C3-C6, 2108
CCR7 breast cancer cell, 3485
CDF-difluorinated curcumin, 2206
C_{18}-diterpenoid alkaloid, 1507
C_{20}-diterpenoid alkaloids, 1505
C_{19} diterpenoid group, 1506
CDKI. *See* CDK inhibitors (CKI)
CDK inhibitors (CKI), 2186
CD95L, 2199
cDNA library, 3572
C-domain, 703
CDs. *See* Cyclodextrins (CDs)
CE. *See* Capillary electrophoresis (CE)
CEC. *See* Capillary electrochromatography (CEC)
Cecal ligation and puncture (CLP), 3966
β-Cedrene, 2988
Celastraloids, 3477
β-Cell, 1304
Cell and organ cultures, 2765
Cell apoptosis, 2401
Cell-based assay, 2675
Cell cultures, 92, 98, 101–110, 1685, 1686, 1688, 1690–1700, 1702, 1704, 1706, 1959
Cell cycle, 1448, 2186
Cell death, 1313, 3995
Cell differentiation, 102, 188
Cell differentiation inducers, 3481
Cell differentiation induction, 3477, 3481
Cell division, 3862
Cell growth, 1210
Cell immobilization, 101
Cell inoculum densities, 103
Cell lines, 2770, 3478
Cell proliferation, 1313, 2302, 2466, 3527, 4113, 4114, 4117, 4120, 4126, 4142, 4150
Cell signaling cascades, 2328
Cell signaling pathways, 2176
Cells suspension culture, 3110–3115
Cell suspensions, 186

Cell, tissue cultures, 2800
Cell-to-cell communication, 3525
Cellular adherence, 2327
Cellular cycle, 2960
Cellular systems, 215, 232, 245
Cellular toxicity, 1670
Cell walls, 1691, 1694, 1695
CE-MS. *See* Capillary electrophoresis–mass spectrometry (CE-MS)
Centaurium erythraea, 2956
Centella, 2764
Centellosides, 2766
Central depressants, 1209
Centrally acting analgesic agents, 1515
Central muscle-weakening action, 944
Central nervous system (CNS), 64, 317, 332, 334, 338, 967, 3642, 4115, 4116, 4129, 4141, 4149, 4150
Centrifugal partition chromatography (CPC), 2145, 2147, 2148, 2150–2153, 2155–2166
Centrilobular congestion, 373
Cephalomannine, 2803
Cephalotaxus
  C. fortunei Hook F, 1453
  C. harringtonia K. Koch var. *harringtonia*, 1453
Ceramiaceae, 2891
Ceramiales, 2891
Ceramium rubrum, 28, 2891, 2904
Ceratitis capitata, 4094
Cerberidol, 3030
Cereals, 1134, 1135, 1138–1140, 1149
Cerebral blood flow, 3703, 3706, 3721
Cerebral embolism, 3703
Cerebral flow, 2301
Cerebral insufficiency, 3705, 3708, 3720–3723
Cerebral ischemia, 1307
Cerebral ischemic injury, 3721–3723
Cerebral oedema, 3720
Cerebrovascular resistance, 3706
C-fibers, 1371
C-6G. *See* Codeine-6-glucuronide (C-6G)
C-glucosidic ellagitannins, 1979, 1983, 1986, 2002, 2003
cGMP, 2365
CGRP. *See* Calcitonin gene-related peptide (CGRP)
$C_{15}H_{24}$, 3519
Chaetomium, 815
Chaetomorpha basiretorsa, 33
Chagas' disease, 816, 2682
Chalcone isomerase (CHI), 1654, 1825, 1877, 1878
Chalcones, 1653, 1828, 1868, 1869, 1871–1875, 1877, 1878, 1883, 1884, 1887, 1889, 1890, 2468, 2475
Chalcone synthase (CHS), 1654, 1688, 1825, 1869, 1884
Chamaelirosides, 3236, 3242
Chamazulene, 2988, 2991
β-Chamigrene, 2988
Chapparinone, 3785
Characteristic features, 890
Characterization, 2318
Charaka-Samhita, 3664
Charales, 3598
Charcoal column chromatography, 3329
Charophyceae, 1545
Chavicine, 311, 337
CH_2Br_2, 2894, 2896, 2897, 2899
$CHBr_3$, 2888, 2889, 2893, 2894, 2897, 2899, 2905
$CHBr_2Cl$, 2888, 2894, 2896, 2899
$CHBrCl_2$, 2888, 2894, 2896, 2899, 2905
CH_2Br_2Cl, 2897
$CH_3CH_2CH_2I$, 2894, 2896, 2899
CH_3CHICH_3, 2894, 2896, 2899
CH_2CII, 2896, 2899
$C_3H_6Cl_2$, 2902
ChE. *See* Cholinesterase (ChE)
Chebuloyl, 1978
Cheese effect, 1208
Cheilosporum sagittatum, 2889
Chelerythrine, 1167, 1170
Chelidonium majus, 1166
Chemical(s), 3289
  classification, 218
  defence theory, 965
  ionization, 3397
  and physical characterization, 216
  preservatives, 3978
  structures, 361, 891, 2385, 2441
  substances, 1505
  synthesis, 57
  warfare agent, 57
Chemical penetration enhancers (CPE), 3758, 3762
Chemiluminescence, 1086
Chemistry of *Cinchona* alkaloids, 615
Chemistry of stilbenoids, 2278
Chemoattractant, 336
Chemo-induced tumors, 2402, 2403
Chemokine, 1811

Chemoprevention, 2177, 2179
Chemopreventive, 2598, 2601, 4114, 4115, 4127
  action, 2643
  effects, 1412
  potential, 1422
Chemo-profiles, 2960
Chemoprotective, 3483, 4119, 4126
Chemoreceptor trigger zone, 1374
Chemorepellent, 336, 337
Chemo-sensitizing, 2176
Chemotactic activity, 336
Chemotaxonomic marker, 3221
Chemotaxonomic studies, 940
Chemotaxonomy, 3174, 3540
Chemotherapeutic, 3489, 4114, 4115, 4117
  agent, 978, 997
  drugs, 990, 1316
Chemotherapy, 1279, 1445, 3525
Chemotypes, 2976, 3540, 4090
Chenopodium ambrosioides, 2982, 2984
(1-, 2-) C_3H_7I, 2897
CH_2I_2, 2894, 2897, 2899, 2905
C_2H_5I, 2897
CH_3I, 2888, 2893, 2894, 2899
C_4H_9I, 2897
Chick embryo, 1455
Childhood, 2408
Children, 2416
Children (neonates/infants), 374
Chilling injury, 1565, 2788
Chimeric cytochrome P450 enzymes, 1632
Chinese, 2409, 2422
  herbal medicine, 1303
  medicine, 1399
  method, 4043
  traditional medicine, 1243
  women, 2409
Chinotoxine, 615
CHIP-2 regulations, 3541
Chiral, 2109
Chiral selectors, 607
Chitosan, 1694, 1703
Chlamydia trachomatis, 1310
Chlorflavononin, 1824
Chloride channels, 4067, 4069, 4096
Chloride conductance, 4066
Chloroacetic acid, 2896
Chloroacetic acid ethyl ester, 2896
Chlorodesmis major, 2903
Chloroform, 3371, 3385
Chlorogenic, 1954
Chlorogenic acid, 1549, 2150
Chloromethylated polystyrene matrix, 3332, 3333
2-(4-Chlorophenyl-thio) triethylamine, 2787
Chlorophyll A, 3176
Chlorophylls, 1385, 2695, 2763, 3373
Chlorophyta, 2901–2904
Chloroplasts, 63, 972, 1732, 2856, 2858
Chloroplast system, 984
Chloroquine, 629
Chlorpyrifos, 4075
Chocolate production, 1610
Choisya, 729
Cholesterol, 1305, 1812, 2446, 2471, 2668, 3819
Cholesterol biosynthesis, 3668
Choline, 1732
Cholinergic, 1333, 2406, 3709, 3715
  effects, 1246
  hypothesis, 1526
  receptors, 1343
  symptoms, 1249
  synapses, 41
Cholinesterase (ChE), 1334, 1335
  inhibitions, 1527
  inhibitors, 1527, 1529–1531, 1535
Chondria
  C. armata, 31
  C. crassicaulis, 2892
  C. succulenta, 2892
Chondrus
  C. crispus, 2890
Chordaceae, 2898
Chordariaceae, 2896
Chorda tomentosa (Halosiphon tomentosus), 2898
Choreoathetosis, 4065, 4067
Choriocapillaris, 3927, 3928, 3940, 3941
Chorismate mutase/prephenate dehydrogenase (CM/PDH), 14–15
Chorismic acid, 536
Choristoneura rosaceana, 4095
Choroidopathy, 1450
Chromano (benzopyrano) moiety, 1619
Chromatic aberration, 3933, 3936
Chromatographic, 1967, 3353
  partitioning, 1177
  separation, 2014, 2034–2035, 3313
  techniques, 3370, 3520
Chromatography, 535, 2802, 4029
Chromatography in column, 1086

Chromodacryorrhea, 51
Chromones, 1551
Chromophores, 3472
Chromophoric features, 693
Chromoplasts, 1732, 2788, 2856, 2860, 2861
Chronic anal fissures, 65
Chronic gastric ulcers, 3652
Chronic illnesses, 3477
Chronic inflammatory disorders, 1460
Chronic pain, 1379
Chronic studies, 3139
Chrysanthemum parthenium, 2984
Chrysin, 1658
Chrysophanol, 1554
Chrysopogon zizanioides, 4089
Cicer arietinum, 1852
Cichorium intybus, 1549
CID-MS/MS. *See* Collision-induced dissociation tandem mass spectrometry (CID-MS/MS)
Cigarette smoke, 3643
Cimex lectularius, 4094
Cinchona, 121
  alkaloids, 609, 615, 626
    biological activity, 626
    biotechnological production, 632
    biotransformation, 633
    ecological role, 632
    metabolism and toxicology, 633
    in stereoselective synthesis, 607
  bark, 608, 610
Cinchonamine, 611
Cinchonaminone, 631
Cinchonidine, 611
Cinchonine, 611, 630
Cinchophyllamine, 611, 631
Cinchophylline. *See* Cinchophyllamine
Cinchotoxine, 607
1,8-Cineol, 2905, 2906, 2977, 2982
Cineol (CI), 2885
1,8-Cineole, 2682, 2906, 2977, 2982, 4089, 4140, 4141
Cinerin I, 4062
Cinerin II, 4062
Cinnabar moth, 156
Cinnamaldehyde, 4089
Cinnamate 4-hydroxylase (C4H), 1654, 1688, 1825
Cinnamic, 1953
  acid, 1629, 1809
  alcohol, 4096
Cinnamomum camphora, 2984
Cinnamomum spp., 4116
Cinnamon oil, 2676
Cirrhosis, 1385
9-*Cis*-β-apo-10′-carotenal, 3588
9-*Cis*-β-carotene, 3588
Cis-3-butyl-4-vinylcyclopentene, 2895
Cis conformation, 1756
Cis-isomers, 4066, 4069
cis-Nepetalactone, 3029
C20 isoprenoid precursor, 1730
Cisplatin, 2215
Cis-zeatin, 958, 963
Citral, 2979, 4097, 4113, 4121, 4146–4148
β-Citraurin, 3369
Citrifolinoside A, 3026
Citronellal (CIT), 2885, 2978
Citronella spp., 4089
Citronellol, 2979, 2999
Citrus, 730, 1228–1230, 1877, 1879, 1880
  C. aurantifolia, 3542
  C. aurantium, 2979, 4112
  C. aurantium var. *amara*, 290
  C. bergamia R., 4089, 4099, 4128
  C. limon, 2979
  C. maxima, 4113
  C. paradisi, 2982
  C. reticulata, 2981
  C. sinensis, 2979, 2982, 4112, 4115
c-Jun-NH_2-kinase mediated apoptosis, 3485
Cladantholide, 3079, 3082
Cladobotryum, 806
Cladophoraceae, 2903
Cladophorales, 2903
Cladophora rupestris, 2903
Cladophoropsis herpestica (Cladophora herpestica), 2903
Cladostephus spongious, 2900
Claisen, 796
Claisen-condensation, 59, 61
ε-Class squalene synthases, 2715
Classes of pyrethroids, 4065
Classes of withanolides, 3467
Classifications, 484, 488, 1266, 1806, 2051
Class II aminotransferase, 61
Class II terpene cyclases, 2724
Class I terpene synthases, 2719
Clausena, 744
Claviceps purpurea, 164, 1134, 1148, 1349
Clavine-type alkaloids, 685
Cleaning up, 1101
Cleavage, 4069
Clerodane, 3182
Climate, 2588
Climate factors, 2410

Clinical, 2583, 2591, 2596, 2599
complementary medicine, 1391
effect, 63
outcomes, 2328
studies, 2301, 2372
symptoms, 1516
toxicology, 1391
trials, 1232, 1253, 1319, 2218, 2323, 3165, 3166
uses, 1225
Cloned enzyme donor immunoassays, 1035
Cloverleaf structure, 2703
Cloves, 3664, 3666, 3671, 3673
CLP. *See* Caulerpin (CLP)
[^{14}C]-L-tryptophan, 697
Cluster headaches, 543
Clytostoma callistegioides, 4094
CM/PDH. *See* Chorismate mutase/prephenate dehydrogenase (CM/PDH)
^{13}C-NMR, 332, 3476
CNS. *See* Central nervous system (CNS)
Coactivators, 1839
Cocaine, 175, 351, 1012
Cocaine antigen–protein conjugates, 1035
Cockroaches, 4077
Coclaurine-*N*-methyltransferase (CNMT), 8–11, 21
Cocoa butter, 1603
Cocoa flavanols, 2509
Cocoa genotypes, 1601
Co-contaminants, 3144
Coculture/Coculturing, 1628, 1659
Codeine, 6, 11, 429, 430, 434, 437, 1073, 1169, 1170, 1172, 1181, 1369, 1370, 1376–1380
Codeine-6-glucuronide (C-6G), 1377
Codeinone, 430
Codex Elsers, 3664
Codiaceae, 2903
Codiales, 2903
Codium
C. decorticatum, 31
C. fragile, 2903
C. galeatum, 2903
C. lucasii, 2904
Codonopsis lanceolata, 2955
Coevolution theory, 1568
Coexpression, 1632, 2832
Cofactor, 374, 1633
Coffea arabica, 1353, 1639
Coffee, 1353, 1354, 1959
Cognition, 1244
Cognitive, 3707–3709, 3717, 3720, 3727, 4123, 4136
activity, 4136
disorder, 3707
effects, 3644
function, 2301, 2620, 2630
improvement, 3219
Cohort study, 1835
Colchicine, 198, 461–476
Colchicum, 463, 465, 466, 470
Coleonol, 3327
Coleoptera, 985
Coleus
C. aromaticus, 2981
C. forskohlii, 293, 3326
Collagen-induced arthritis (CIA), 3963
Collagen synthesis, 3965
Collars for dogs, 4078
Colletofragarones, 3477
Collision-induced dissociation (CID), 1060, 3398, 3592
Collision-induced dissociation tandem mass spectrometry (CID-MS/MS), 3474
Colloidal instability, 2335
Colloidal stability, 2342
Colon, 4118, 4120, 4127, 4133, 4136
Colon adenocarcinoma, 1836
Colon cancer (CCa), 2403, 3485
incidence, 2409
mortality, 2409
Colon cancer cell, 3485
Colon tissues, 2295
Color, 1755, 1807, 2343, 2350
Color-developing agents, 3314
Colorectal cancer, 2302
Colpomenia
C. bullosa, 2900
C. sinuosa, 31, 2900
Column chromatography, 3197, 3328, 3376, 3647
Column temperature, 1075
Combinatorial, 3347
Combinatorial biosynthesis, 1641, 1659
Combined effect, 1698
Combretastatin A-1, 1553
Combretastatin A-4, 1932
Combretastatins, 1903
Combretum caffrum, 1553
Comfrey, 1053, 1061, 1385
Commercial essential oils, 3984
Companion animals, 4072
Comparison, 2419

Compartmentalization, 220, 233, 243
Compensated, 2419
Competetive binding assay, 1667
Complex tannins, 1979, 1986, 2003
Compositae, 1869, 1871, 1877, 1887
Compositae family, 3531
Compounds, 2054, 2595
  MS/MS, 2054
Comprehensive 2-year study, 2421
Compressenolide, 3079
Comptonella, 728
Computational algorithms, 1671
Concentrations, 1766, 2293, 2417, 2421, 2588, 2589, 2593, 2595, 2599, 2602, 2603
Concentrations anthocyanin, 2265
Concordant, 2412
μ-Concotoxins, 64
Condensation, 2258
Condensed tannins, 1559, 1755
Cone photoreceptor, 3927
Cones, 2335, 2350, 3926, 3931, 3940
Conformation, 43, 1780
Confounding factors, 2409, 2410, 2418
Confusion, 2410
Conhydrine, 891
γ-Coniceine, 891
Conifers, 4089
Coniferyl alcohol, 1561
Coniine, 884
Conium maculatum, 294
Conjugate, 4078
Conjugated ketone, 43
Conjugation reactions, 4069
Conocybe, 527
Constipation, 1374
Constituents, 3664, 3665, 3667, 3668, 3684–3686
Constitutive, 1735
Constitutive defense, 2931
Construction test, 2414
Consumer goods, 3984
Consumers, 2496, 2497, 2501, 3978
Consumption of wine, 2278
Contamination, 3978
Contents, 1806
Contraception, 2420
Contraceptives, 2751
Controlled release, 354
Controversy, 2415
Convallaria majalis, 4126
Convolvidine, 354
Convolvulaceae, 176
Convulsant effects, 1207
Convulsions, 136, 4065, 4079
Copaifera
  C. langsdorfii, 3002
  C. officinalis, 3001
Copelandia, 527
Copenatus, 788
Co-pigmentation, 1781, 1787, 1805
Copigments, 1781, 1837
Copper, 374
Copper-induced pathophysiological conditions, 3477
Coptidis rhizoma, 1179, 1303
Coptis
  C. chinensis, 1181
  C. chinensis Franch var. *chinensis*, 1446
Coptisine, 445
Corallina
  C. berteri, 2889
  C. officinalis, 2889
  C. rubens (Jania rubens), 2890
Corallinaceae, 2889
Corallinales, 2889–2890
Cordia verbenacea, 4119
Core microbiome, 2441
Coriandrum sativum, 2979, 4090
Coriobacteriaceae, 2444
Corn oil, 1736
Coronary arterial calcification (CAC), 3683
Coronary flow-velocity reserve, 2300
Coronary intervention, 2799
Coronatine, 2773
Corticosteroids, 4120
Corticosterone, 3707, 3711, 3719
Cortinarius, 789
Cortisol, 1374
Corydalis spp., 1175, 1183
Corynanthe, 577
Corynanthe pachyceras, 1288
Corynanthe-type, 596
Corynascus, 807
Corynin, 596
Corytuberine, 11, 20
Corytuberine synthase (CYP80G2), 11
Cosmetic products, 3289
Cosmetics, 4049
Cosmetic treatments, 65
Costariaceae, 2898
Costaria costata, 2898
Cost-effective, 2770
Costunolide, 3081, 3083
Cotton bollworm, 160
Coulometric detector, 2120
Coumarate-CoA ligase, 1654, 1688

Coumaric, 1549, 1592, 1629, 1705, 1706, 1958, 2346, 2348
Coumarins, 1549, 2346
Coumaroylbarlerin, 3055
4-Coumaroyl-CoA, 1762
6′-*O*-*trans*-*p*-Coumaroylgeniposide, 3058
8-*p*-Coumaroylharpagide, 3044
p-Coumaroylhistamine, 876
p-Coumaryl alcohol, 1561
Coumestrol, 1830, 1850, 2385
Countercurrent chromatography (CCC), 1055, 1062, 2147, 2165
COX-1. *See* Cyclooxygenase-1 (COX-1)
COX-2. *See* Cyclooxygenase-2 (COX-2)
C-6 oxidation, 3854
CPC. *See* Centrifugal partition chromatography (CPC)
CPT. *See* Camptothecin (CPT)
CR. *See* *p*-Cresol (CR)
Cratoxylum pruniflorum, 1553
C-reactive protein (CRP), 3683
p-Cresol (CR), 2887, 2892, 2897, 2899, 2901–2903, 2907
Crinitol, 2896
Criollo, 1600
Critical fluid extraction, 1123
Crooked calf disease, 395
Crop diseases, 3976
Crop yield, 3865
Cross-linking of DNA, 370
Cross-talk, 2399
Cruciferous vegetables, 2676
Crude forskolin, 3330–3332
Cryptadine B, 1248
Cryptochrome 2, 1736
Cryptopleurine, 1455
Cryptorchidism, 2418
Cryptotanshinone, 2783
α-Cryptoxanthin, 3378
β-Cryptoxanthin, 3369, 4020
Crystallization, 3330, 3370
CS and T syndromes, 4067
CSC. *See* Cancer stem cell (CSC)
CSEI, 1104
CS syndrome, 4065
C-toxins, 55
(-)-cubenol, 2895
Cucumis sativus, 3587
Cucurbitacin R, 2679
Cucurbita pepo, 4101
Culex
  C. nubeculosus, 4071
  C. quinquefasciatus, 4071
Culicoides spp., 4070
Cultivar type (phenolic compounds), 1719
Cultivation conditions, 226, 228
Cultivation site (phenolic compounds), 1719
Culture conditions, 101, 103, 107, 229
Culture medium, 1692–1696, 1698, 1705
Cuminaldehyde, 4089
Cupreidine, 619
Cupreine, 619
Cupressaceae, 4095
Cupressus lusitanica, 2781
Curare, 294
Curare-like activity, 947
Curcuma
  C. aromatica, 2985
  C. longa, 2985
Curcumin, 2225
Curcuminoids, 1654
Curcuminoid synthase, 1654
Curvularins, 3477
Cutaneous leishmaniasis (CL), 1265
Cutoff values, 1083
Cut test, 1609
CY. *See* *p*-Cymene (CY)
Cyanidin, 1664, 2467, 2469, 2475, 2476, 2478
Cyanobacteria, 41, 54, 59, 63, 1292, 3252–3254, 3257–3259, 3263, 3265, 3266, 3273
Cyclases, 42, 4041
Cyclic AMP-phosphodiesterase, 967
Cyclic peptides, 41
Cyclin D1, 2186
Cycloartane, 3237
Cycloartenol, 445, 3237, 3243
Cyclobutane, 978
Cyclocerberidol, 3032
β-Cyclocitral, 2891, 2892
Cyclodextrins (CDs), 106, 107, 1103, 1172, 1178, 1188, 1695, 1698, 1699, 1703, 1706, 1707
β-Cyclohomocitral, 2891, 2892
Cyclooxygenase (COX), 2366, 4006
Cyclooxygenase-1 (COX-1), 3482
Cyclooxygenase-2 (COX-2), 1306, 1590, 1938, 2182, 2679, 3477, 3482, 4114, 4119, 4134, 4147
Cyclophosphamide, 2216
Cyclopropylcarbinyl cation, 2715
Cyfluthrin, 4063, 4065, 4070, 4075, 4078
Cyhalothrin, 4063, 4065, 4068, 4075
Cylindrospermopsis raciborskii, 58
Cylindrospermum, 47

Cymbopogon, 2979
  C. citratus, 2979, 4098, 4128
  C. nardus, 2979, 2998, 4095
  C. sennarensis, 2981
p-Cymene (CY), 2887, 2892, 2897, 2899, 2901–2903, 2907, 2976
Cynara cardunculus L. subsp *scolymus* (L.) Hayek, 1566
Cynometra, 867
CYP711A1, 3596
CYP71AV1, 3082
CYP80B, 8, 11, 17
CYP719B1, 13
CYP71BL1, 3083
CYP71BL2, 3083, 3085
CYP2D6, 11
Cypermethrin, 4063, 4068, 4072, 4074, 4076, 4078, 4079
Cyp80F1, 184
Cysteine proteases, 980, 987, 988, 1270
Cystocloniaceae, 2890
Cystoclonium purpureum, 28, 2890
Cystophora
  C. intermedia, 2897
  C. moniformis, 2897
Cystoseira
  C. barbata, 2896
  C. crinita, 2896
  C. stricta var. *amentacea*, 2896
  C. stricta var. *spicata (Cystoseira amantacea* var. *spicata)*, 2896
  C. trinodis (Sirophysalis trinodis), 2896
Cystoseiraceae, 2896
Cytisine, 1347, 1350
Cytochrome c, 1313
Cytochrome P450, 1252, 1659, 2179, 2803, 2820, 2821
  enzymes, 2683, 4083
  inhibitor, 1638
  reductase, 1659
Cytochrome P450, family 11, subfamily A (CYP11A), 2744
Cytochrome P450 monooxygenase (CYP), 702, 3129, 3588
Cytokines, 1443, 2405, 3704
Cytokinins (CKs), 956, 963, 3198, 3591
Cytomegalovirus, 1310
Cytomegaly, 1386
Cytoprotective effect of piperine, 1411
Cytotoxic, 123, 599, 848, 980–983, 990, 991, 993, 994, 998, 999, 1455, 2326, 3243, 3244, 3467, 3478, 3480, 3485, 3863, 3994, 4009, 4118, 4125, 4138, 4150
  activity, 1210, 1212, 3236
  effect, 1512, 4120
Cytotoxicity, 695, 945, 980, 981, 983, 990, 998, 1285, 1446, 3144, 3477, 3479, 3517, 3522, 3542, 3787, 3963
CZE. *See* Capillary zone electrophoresis (CZE)

D

Dahlia spp, 1553
Daidzein, 1655, 1830, 1851, 2388, 2437
Daidzin, 1655, 1851, 2447
Dairy cattle, 4075
Daldinia, 1564
Damalinia bovis, 4069
Damascenone, 2989
Damascone, 2989
Dammarane, 3647
Danaines, 157, 162
1D and 2D NMR, 935
DAS. *See* Diallyl sulfide (DAS)
DAT. *See* Deacetylvindoline-4-*O*-acetyltransferase (DAT)
Date rape drug, 292
Datura, 3467, 3471
  D. alba, 160
  D. met, 3480
  D. metel, 3476
  D. stramonium, 291
Daucol, 2988
Daucus carota, 2988
Davana oil, 2988
Davanone, 2988
2,4-DBA, 2893, 2902
3,5-DBC, 2893
3,5-DBH, 2893
DBP. *See* Diastolic blood pressure (DBP)
2,4-DBP, 2886–2893, 2895–2904
2,6-DBP, 2886–2891, 2895–2898, 2900–2905
DDXXD motif (biosynthesis of terpenoids), 2710, 2712
DDXX(D,E) motif (biosynthesis of terpenoids), 2719
Deacetyl asperulosidic acid, 3057
10-Deacetylbaccatin III, 2957
7-Deacetylforskolin, 3332
Deacetylvindoline-4-*O*-acetyltransferase (DAT), 584
4-*O*-Deacetylvinflunine, 1129
4-*O*-Deacetylvinorelbine, 1129
Debittering processes, 398
Debromoaxinohydantoin, 268
Decadienal, 2090, 2887, 2891, 2892

(2*E*,4*E*)-Decadienal, 2887
(2*E*,4*Z*)-Decadienal, 2887
Decaffeinated coffee beans, 970
Decaloside, 3017
Decanoic acid, 2887
1-Decanol, 2891
2-Decanone, 2891, 2892
Decarboxylation, 49, 2347, 2348
2,4,7-Decatrienal, 2902
(E)–2-Decenal, 2891, 2892
Dedifferentiation, 190
De-epoxy NIV, 3134
Deer flies, 4076
De-esterification, 1517
N-Deethylaconine, 934
De-etiolated 1 (DET1), 2835
Defense, 3181
Defense system, 3522
Deglucosylation, 3506
Deglycosylation, 2442
Degradation, 3611
  byproducts, 4025
  of caffeine, 961
  kinetics, 2805
  products, 51
Degree of polymerization (dp), 1787, 2264
Deguelin, 2191, 2204
Dehydration to the dehydropyrrolizidine alkaloids, 368
6-Dehydrochamaelirosides, 3236
Dehydrocostuslactone, 3592
Dehydrodicatechin A, 2343
Dehydroepiandrosterone (DHEA), 2740
Dehydroevodiamine, 1532
Dehydrogeissoschizine, 92, 94, 96
Dehydrogeosmin, 2989
Dehydrohexahydroxydiphenoyl (DHHDP) unit, 1978, 1989
2,3-Dehydropilosinine, 869
Dehydropiperidine, 320
Dehydroxylation, 2442
Delaumonones, 3348
Delesseriaceae, 2891
Delesseria sanguinea, 28, 2891
Deleterious effects of β-amyloid, 2406
Delisea pulchra, 2889
Delphinidin, 1831, 2467
Delphinium
  D. anthriscifolium, 927
  D. yunnanense, 928
Δ^9-Tetrahydrocannabinol (Δ^9-THC), 3417, 3421, 3425, 3428
Deltamethrin, 4063, 4065, 4067, 4068, 4070, 4071, 4076, 4077
Dementia, 697, 1333, 1334, 1338, 1353, 2278, 2621, 2623, 2624, 3707, 3708, 3716
9α-Demethyl mitomycin A, 984
Dendritic spine density, 2406
Dendritic spine growth, 2406
Dennettia tripetala, 4094
Densitometric analysis, 3334
Denticin (DTC), 1212
3-Deoxyanthocyanidins, 1569
5-Deoxyantirrinoside, 3017
6-Deoxycatalpol, 3019
Deoxycholate, 1274
3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS), 14, 15
1-Deoxy-D-xylulose-5-phosphate (DXP), 90, 2698, 2741
1-Deoxy-D-xylulose-5-phosphate (DXP) pathway, 2671, 3102, 3103
7-Deoxy-8-epi-loganic acid, 3022
5-Deoxylamiol, 3021
Deoxypumiloside, 659
5-Deoxystrigol, 3586
5-deoxystrigol, 3588
Deoxyxylulose, 3175
Deoxyxylulose phosphate (DXP), 2991
Deoxyxylulose phosphate/methylerythritol phosphate pathway, 3418
Deoxyxylulose phosphate pathway, 2696, 2698
Dephosphorylation, 533
Depolarization, 47, 4083
Depolarizing neuromuscular blocking agent, 42
Deprea, 3467
Deprea orinocensis, 3471
Depression, 1307, 4079
Depsipeptides, 41
Derivatization, 44, 1073, 1074, 1105, 1185, 2083
Derivatized BR (brassinosteroids), 3859
Dermacentor reticulatus, 4071
Dermal toxicity, 4099
Dermatitis, 3087, 3094
Desacetoxyvindoline-4-hydroxylase (D4H), 587
15-Desacetylundulatone, 3785
Descending mode (CPC), 2150, 2163, 2164
Desmarestia
  D. aculeata, 28, 2893
  D. anceps, 2894
  D. menziesii, 2894
  D. viridis, 28, 2894
Desmarestiaceae, 2893–2894

Desmarestiales, 2893–2894
O-Desmethyl-angolensin, 2398, 2446
Desorption electrospray ionisation (DESI), 181
Desorption electrospray ionization (DESI), 1038
DET1. *See* De-etiolated 1 (DET1)
Detection, 1967
  program, 2409
  window, 1082
Detoxification, 2395
Detrusor smooth muscle, 3992
Devirilization, 2418
Dexamethasone, 4119, 4120, 4147
Dexterity, 2414
DH. *See* Double haploid (DH)
D4H. *See* Desacetoxyvindoline-4-hydroxylase (D4H)
Datura, 3476
DHEA. *See* Dehydroepiandrosterone (DHEA)
DHS34 gene, 1272
DHS-like gene (DHSL20), 1272
DHT, 2413
Diabetes, 1304, 1813, 3198, 3200, 3477, 3488, 3507, 3625, 4148
Diabetes mellitus, 1304, 4138
Diabetes type 2, 4114
Diabetic neuropathy, 3656
Diabetic retinopathy, 1444, 3723, 3724
Diagnostic fragment ions, 3317
Diallyl disulfide (DADS), 3666
Diallyl sulfide (DAS), 3666–3668
Diallyl trisulfide (DTS), 3666, 3667, 3677
Diarrhea, 3795
Diastolic blood pressure (DBP), 3675, 3676
Diazepam, 4068, 4116, 4129, 4130
Diazinon, 4079
Diazocin, 412
Dibrominated *S*-antazirine, 980
Dibromoacrolein, 2889
Dibromoanisole (DBA), 2885
1,2-Dibromo-3-chloro(1-octene-3-ones), 2889
2,3-Dibromo-1-chloro(1-octene-3-ones), 2889
Dibromocresol (DBC), 2885
1,3-Dibromo-2-heptanone, 2889
3,5-Dibromo-4-hydroxybenzaldehyde, 2893
Dibromo-4-hydroxybenzaldehyde (DBH), 2885
3,3-Dibromo-1-iodo-2-heptanone, 2889
3,3-Dibromo-*n*-butylacrylic acid, 2889
3,3-Dibromo-*n*-hexylacrylic acid, 2889
Dibromophakellin, 270
Dibromophakellstatin, 270
Dibromophenol (DBP), 2885
2,6-Dibromophenol (2,6-DBP), 2886–2891, 2893, 2895–2898, 2900–2905
S,S-2,3-Dicarboxyaziridine, 980
4,4′-Dichloro-5,5′-dibromo-7,7′-dimethoxy-3,3′-bis-1 *H*-indole, 33
Dichloromethane (DCM), 2885, 2902, 3371
Dichlorophenoxyacetic acid, 2788
Dichroa febrifuga L., 1458
Dictamnus, 724
Dictyol C, 2895
Dictyol D, 2895
Dictyol H, 2895
Dictyoloma, 753
Dictyophora, 806
Dictyoprolene, 2894
Dictyoprolenol, 2895
Dictyopterene A, 2894, 2895, 2900, 2901
Dictyopterene B, 2894, 2895, 2900, 2901
Dictyopterene C′, 2894, 2895, 2900
Dictyopterene D′, 2894, 2895
Dictyopteris
  D. australis, 2894
  D. divaricata, 2894
  D. latiscula, 2894
  D. membranacea (Dictyopteris polypodioides), 2894
  D. plagiogramma, 2894
  D. prolifera, 2895
  D. undulata, 2895
  D. zonarioides, 2895
Dictyota
  D. cervicornis (Canistrocarpus cervicornis), 2895
  D. dentata (Dictyota mertensii), 2895
  D. dichotoma, 2895
  D. dichotoma var. *implexa (Dictyota implexa)*, 2895
  D. divaricata (Dictyota implexa), 2895
Dictyotaceae, 2894
Dictyotales, 2894–2896
Didehydro-orobanchol, 3586
1,9-Dideoxyforskolin, 3332
Didrovaltrate acetoxy hydrin, 3049
Dielectric constant, 1175
Diels-Alder, 805
Diester-diterpene alkaloids, 1170
Diesters, 362
Diet, 2583, 2589–2592, 2594, 2595, 2600, 2601
Dietary intake, 3440
Dietary intervention, 1740
Dietary supplement, 1245, 2564
Dietary surveys, 2407

Diethyl ether, 3373
Diferuloylmethane, 2225
Differentiated cultures, 231
Differentiation, 2403
Diflomotecan, 666
20′-Difluorovinblastine, 1129
Digallic, 1963
Digenea simplex, 2892
Digestive stimulant action, 1404, 1426
Digitalis, 3752
Digitalis purpurea, 295
Digitalis spp., 1554
Digitoxin, 3747, 3750–3752
Digoxin, 3745, 3749, 3750, 3752
Dihydroactinidiolide, 2887
3,4-Dihydro-4-arylcoumarins, 1555
(8*S*)-7,8-Dihydroaucubin, 3019
2,3-Dihydro-3*β*-methoxyixocarpalactone A, 3484
2,3-Dihydro-3*β*-methoxyixocarpalactone B, 3484
Dihydrocapsiate, 1458
Dihydrochalcones, 1883–1885
2,3-Dihydrocitral, 2891, 2892
Dihydrocorynantheol, 631
Dihydrodaidzein, 2444
Dihydrodiols, 1670
Dihydroergotamine, 707
Dihydroergotoxine, 707
Dihydroflavonol reductase, 1655
Dihydroflavonols, 1815
(2*R*, 3*R*)-Dihydroflavonols (DHFs), 1825
Dihydrofolate reductase (DHFR), 1270
Dihydroindole, 587
2,3-Dihydroixocarpalactone-B, 3484
Dihydrokaempferol, 1762
Dihydroresveratrol, 2294
15,16-Dihydrotanshinone I, 3567
Dihydrowithanolide D, 3481
3,4-Dihydroxybenzylol, 2888
7,8-Dihydroxycalonectrin, 3133
11-Dihydroxydihydroquinine, 633
6,7-Dihydroxygeraniol, 2999
1,8-Dihydroxynaphthalene, 1564
3,4-Dihydroxyphenylacetaldehyde (3,4-DHPAA), 8
3,4-Dihydroxyphenyllactic acid, 2163
Dihydroxy pigment (DHP) carotenoids, 3376
3α,6β-, 3β,6β-Dihydroxytropane, 348
3α,7β-, 3β,7β-Dihydroxytropane, 348
Dill ether, 2982
Dimeric phenanthrenes, 1924
Dimeric tropane alkaloids, 354
3,4-Dimethoxycinnamoyl, 3207
Dimethylallyl pyrophosphate (DMAPP), 2696, 2698, 2672, 2705, 2741, 2744, 3943, 3175, 3571, 4090
4-Dimethylallyltransferase (4-DMATS), 697
Dimethylamine (DMA), 2885
p-Dimethylaminobenzaldehyde, 1139
Dimethylamylamine, 290
7,12-Dimethylbenz[a]anthracene (DMBA), 1315
3,4-Dimethylcyclohexanol, 2891, 2892
Dimethyl disulfide (DMS), 2885, 2893, 2896, 2899, 2901–2903
Dimethylformamide, 2896, 3373
Dimethyltryptamine (DMT), 535, 540
Dinarmus basalis, 4098
Dinoflagellates, 53, 59, 63
Di-*n*-propyl isocinchomeronate, 4077
Diode array detection, 2118
Diode-array detector, 3387
Dioscin, 3228–3230
Diosgenin, 3227–3230
Dioxygenase, 61
Dip formulation, 4076
Diphasia, 741
1,2-Diphenylethane, 1914
2,2-Diphenyl-1-picrylhydrazyl (DPPH), 4141
Dips (immersion), 4083
Dipterocarpaceae, 1906
Direct
  analysis in real time, 1038
  defense, 2931
Directed evolution, 1666
Dirigent protein model, 1562
Discopodium, 3467
Discorhabdin A, 261, 262
Discorhabdin B, 264
Discorhabdin C, 261
Discorhabdin W, 264
Discorolides A, 3480
Discorolides B, 3480
Discrepancies, 2388, 2415
Disease prevention, 2423
Diseases, 1738, 2406, 2583, 2592, 2594, 2596–2599, 2604
Disorders, 2406
Dispacamides, 266
Dispersive liquid-liquid microextraction (DLLME), 1101
Displacement mode, 2150, 2165
Disposable pipette extraction (DPX), 1084, 1085
Disposition, 2471, 2473, 2479, 2481
Disruption, 2406
Distillate, 979

Distillation, 3977
Distribution, 1230, 1232, 2321, 2471, 2473, 2474, 2478, 2481, 3125, 3134, 3147
Distribution coefficient, 2147, 2148
3,6-and 3,7-Disubstituted-4-benzyltropanes, 352
3,6-and 3,7-Disubstituted tropanes, 348
Diterpene, 412
Diterpene alkaloid, 2948
Diterpenes, 2763
Diterpenoid(s), 2737, 2915, 2955, 3553
  alkaloids, 931
  tanshinones, 2946, 2954
2,4-Di-tert-butylphenol, 2891, 2892
2,3-Dithiomethyl-4,6-dibromo-1 *H*-indole, 32
2,3-Dithio-methyl-4,5,6-tribromo-1 *H*-indole, 33
Dithionite, 1054
Diuretic, 3477, 3488
DLD-1 4Ub-Luc cells, 3485
d-LIM, 2899, 2902
DLLME. *See* Dispersive liquid-liquid microextraction (DLLME)
DMAPP. *See* Dimethylallyl pyrophosphate (DMAPP)
DMT. *See* Dimethyltryptamine (DMT)
DNA, 528, 1319, 2771, 3704, 4113, 4118, 4119, 4125, 4127, 4134, 4135, 4138, 4144, 4146
  activation tagging, 2953
  damage, 2466
  fragmentation, 3542
  synthesis, 1457, 3144, 3790
2D-NMR experiments, 3207
Docetaxel, 2215, 2674, 2737, 3167, 3168, 3170
Docking configurations, 1669
Docking studies, 3488
Docosahexaenoic acid, 1737
Dodecanal, 2891, 2892
Dodecanoic acid, 2894
Dodecanol, 2892, 2900
Dodonaea viscosa, 4094
Dogs, 4071, 4072, 4077, 4078
Dolastane, 2895
Dolichandra cynanchoides, 4094
Dolichothele sphaerica, 865, 872–874, 876
Dolichotheline, 865, 872, 874, 876
ζ-Domains prenyltransferases, 2713
Donepezil, 1244, 1256
DOP. *See* Dopamine (DOP)
L-DOPA decarboxylase (DODC), 17, 21
Dopamine (DOP), 6, 8, 10, 11, 14–17, 19, 21, 29, 63, 541, 598, 686, 967, 1209, 1348, 3710
Dopaminergic, 1348, 1349, 1351, 1355, 3709, 3710, 3718, 3719
Dopaminergic neurotransmission, 1208
Dorstenia zenkeri, 1555
Dose-dependent (bioconversion of flavonoids), 1631
Doses, 2324, 2470, 2476, 2477, 2481, 3441
Double-blind, 3669, 3673, 3676, 3678, 3682
Double haploid (DH), 472–473
Downstream processing, 2771
Doxorubicin (DOX), 3684
DOXP. *See* 1-Deoxy-D-xylulose-5-phosphate (DXP)
DPPH. *See* 2,2-Diphenyl-1-picrylhydrazyl (DPPH)
DPPH radical scavenging assay, 4113
DPX. *See* Disposable pipette extraction (DPX)
Draught tube, 2777
Drimys winteri, 4094
Droseraceae, 1552
Drosophila
  D. auraria, 4094
  D. melanogaster, 4096
Drought resistance, 1602
Drought stress, 2927
Drug(s), 978, 986, 990, 1002
  abuse, 332, 334, 338, 1030
  bioavailability, 1400
  development, 1640
  resistant, 2214
  susceptibility, 1295
Drug-herb interactions, 3969
Drummondita, 718
Dry distillation, 4090
Dryobalanops aromatica, 2984
DTC. *See* Denticin (DTC)
DTC A, 31
DTC B, 31
DTC C, 31
D-*trans*-allethrin, 4072, 4077
DU-45 human tumor cell line, 3478
Dual-mode (solid support- free liquid–liquid chromatography), 2150
Duguetia furfuracea, 1285
Dumontia
  D. incrassata, 28
  D. incrassata (Dumontia contorta), 2890
Dumontiaceae, 2890
Dunalis, 3467
Dusts (formulation), 4076, 4083
Dutaillyea, 734

Dutching (treatment of cocoa powder), 1610
DXDD, 2725
DXDD motif, 2717
DXP. *See* 1-Deoxy-D-xylulose-5-phosphate (DXP)
DXP reductoisomerase (DXR), 2699, 3571
DXP synthase (DXS), 3571
Dynamic extraction, 1017
Dynamic pH junction, 1191
Dynorphin, 1371, 1373
Dysentery, 3795
Dysidea fragilis, 980
Dyslipidemia, 3809
Dyspnea, 4079

E

Ear ache, 3477
Early C-22 oxidation, 3854
Ear tags, 4075, 4083
Earth worms, 2906
Ear ticks, 4075
Eau de cologne, 3291
Eau de parfum, 3291
Eau de toilette, 3291
Ebenaceae, 1552
17β-E_2 bioavailability, 2398, 2417
E-β-ocimene, 2979
6-[(1E)-butenyl]-cyclohepta-1,4-diene, 2894, 2895
ECD. *See* Electron-capture detection (ECD)
Ecdysteroids, 3828–3831, 3837, 3839–3842, 3844, 3845
Ecgonine, 351
Echimidine, 1061
Echinocereus
  E. blanckii, 864
  E. triglochidiatus, 864
Echium, 1051, 1054, 1061, 1062
Ecklonia
  E. cava, 2900
  E. radiata, 2900
ECL. *See* Electrochemiluminescence (ECL)
Ecological, 1719
Economy, 1103
Ecstasy, 1077
Ectocarpales, 2896
Ectoparasites, 4069
Ectoparasiticides, 4077
Ectopic expression, 1732
ED_{50}. *See* Effective dose fifty percent ($ED_{50)}$
EDCs. *See* Endocrine disrupting compounds (EDCs)
Edemas, 3969, 4114, 4119, 4127, 4131, 4134, 4148
EDHF. *See* Endothelium-derived hyperpolarizing factor (EDHF)
Edysteroid receptor, 3503
2-[(2′E,6′E,14′E)–10′,11′-dihydroxy,-3′,7′,11′,15′-tetramethylhexadeca-2′,6′,14′-trien]-1,4-dihydroxy-6-methylbenzene, 28
Effective dose fifty percent ($ED_{50)}$, 1508
Effective therapeutic dose, 1256
Efficacy, 3716, 3724–3727
Efficiency, 370, 2416
Efficient microbial factories, 1631
Efflux, 1663
Efflux pump, 1672
EFSA. *See* European Food Safety Authority (EFSA)
EGb. *See Ginkgo biloba* extract (EGb)
EGb-761, 3701
  standard *G. biloba* extract, 3700, 3701, 3703–3721, 3723–3726, 3728
EGCG. *See* Epigallocatechin gallate (EGCG)
EGFR phosphorylation, 2197
Eggerthella, 2444
Eggs, 4080
4-[(1E)-hexenyl]-cyclopentene, 2895
Ehrlich, 535
Ehrlich carcinoma, 988
EIC. *See* Extracted ion chromatogram (EIC)
Eicosapentaenoic acid, 1737
Eicosa-5,8,11,14,17-pentamethylenoate, 2894
Eicosa-5,8,11,14-tetramethylenoate, 2894
EI-MS. *See* Electron impact mass spectra (EI-MS)
Eisenia arborea, 1560
Elaeocarpaceae, 787
Elaeocarpusoyl, 1978
Elderly women, 2449
Electrical treatment, 2773
Electrochemical biosensor, 52
Electrochemical detectors, 2116, 2119
Electrochemiluminescence (ECL), 1168, 1187, 1188
Electrolytes, 1112
Electromembrane extraction (EME), 1102
Electron-capture detection (ECD), 1075, 3144
Electronic noses, 3294
Electron impact, 3397

Electron impact mass spectra (EI-MS), 3473
Electron shuttling, 1660
Electron spray ionization–mass spectrometry (ESI-MS), 1182
Electron-transport, 1272
Electroosmotic flow, 1156
Electrophilic attack, 60
Electrophoresis-mass spectrometry, 1128
Electrophoretic mobility, 1155
Electrospray, 2123
Electrospray ionization (ESI), 58, 1060, 1061, 1105, 2080, 3195, 3398, 3592
Electrospray ionization ion trap multiple-stage mass spectrometry (ESI-ITMS), 3207
β-Elemene, 2894, 2900, 2901
Elettaria cardamomum, 2980
Elicitation, 101, 103, 105, 107, 109, 111, 1686, 1690, 1691, 1693, 1695, 1696, 1698, 1703, 1706, 2958
Elicitors, 90, 92, 106, 107, 226, 230, 235, 471, 476, 1690, 1691, 1693–1695, 1698–1700, 1706, 2770, 2962
Elimination, 1230, 2471, 2476, 2477
ELISA. *See* Enzyme-linked immunosorbent assay (ELISA)
Ellagic acid, 1549, 2163, 2211, 2213
Ellagitannins, 1559
Ellipticine, 590, 599
ELSD. *See* Evaporative light scattering detection (ELSD)
Eluant, 3376
Elution, 3329, 3330
Elution mode, 2150, 2162, 2163
Elymoclavine oxidase, 702
Embryo germination, 2952
Embryonic lung (MRC-5), 3479
EME. *See* Electromembrane extraction (EME)
Emetic effect, 125
Emetine, 125, 1351
Emodin, 1554
EMT. *See* Epithelial–mesenchymal transition (EMT)
Enantiomeric, 1249
Enantiomers, 43, 47, 57, 1172, 1174, 2447, 2977
Enantioselective GC analysis, 3242
Enantioseparation, 1032
Enantiospecific, 2446
Endarachne binghamiae (Petalonia binghamiae), 2900
Endocannabinoids (EC), 3417, 3424
Endocrine disrupting compounds (EDCs), 2751
Endocrine disruptions, 2417
Endocrine disruptors, 2386
Endocrine system, 1374
Endocytosis, 2471
Endogenous factors, 3540
Endogenous growth hormones, 2768
Endometrial disruptions, 2419
Endometriosis, 2420
Endophytes, 163–166
Endophytic fungi, 1245
Endoplasmic reticulum (ER), 92, 97, 98, 1659
Endothelial, 3677, 3682
  cells, 1442, 2366, 3969
  dysfunction, 2371, 2373
  function, 2362, 2371, 2372
  growth factor receptor subtype 2, 1449
Endothelial NOS (eNOS), 2364
Endothelin-1, 2299
Endothelium, 2362, 3993
Endothelium-dependent dilatation, 2372
Endothelium-dependent relaxations, 2369
Endothelium-derived hyperpolarizing factor (EDHF), 2363, 2365
Enediyne chromophore, 988
Enediyne polyketide synthase, 990
Enfleurage, 3290
Enhance intestinal absorption of micronutrients, 1427
Enlargement of spleen, 3477
Entamoeba histolytica, 1310
Ent-5-deoxystrigol, 3587, 3589
Ent-2′-*epi*-5-deoxystrigol, 3587, 3589
Ent-2′-*epi*-orobanchol, 3587
Ent-2′-*epi*-orobanchyl acetate, 3587
Enterobacteriaceae, 2451
Enterocyte barrier, 2395
Enterohepatic circulation, 2440
Enterolactone, 2442
Enterolignans, 2443, 2450
Enteromorpha spp., 2901
  E. clathrata (Ulva clathrata), 290
  E. compressa (Ulva compressa), 2902
  E. intestinalis, 27
  E. intestinalis (Ulva intestinalis), 2902
  E. linza (Ulva linza), 2902
Enterorhabdus, 2444
Enterotypes, 2456
Ent-kaurenes, 942
Environment, 2873–2875, 4098
Environmental, 1719, 2410
  analysis, 1099, 1100

effects, 391
fate, 4098
Environmental protection agency (EPA), 3542
Enzymatic desymmetrization, 275
Enzymatic hydrolysis, 1085
Enzymatic proteins, 3981
Enzymatic treatment, 4032
Enzyme-linked immunosorbent assay (ELISA), 1063, 1064, 1137, 1139, 1141, 1142, 1148, 1150, 2803, 3215, 3330, 3339, 3340
Enzymes, 731, 979, 980, 2395, 3333
Enzyme-substrate interaction, 1666
Eolutionary model, 2719
Ephedra, 910, 1172, 1174, 1191, 1218, 1220, 1221, 1223, 1227–1230, 1233
alkaloids, 1172, 1174
E. equisetina, 916
E. intermedia, 916
E. procera, 916
E. przewalskii, 916
Ephedrine, 290, 418, 910, 1174, 1179, 1217–1233
9-*Epi*–6α–methoxy geniposidic acid, 3057
Epiaphylline, 158
6-*Epi*-aucubin, 3019
Epibatidine, 1347, 1351
4-*Epi*-borreriagenin, 3030
24-Epibrassinolide, 3857
Epicatechin, 2314, 2335
(–)-Epicatechin, 1605, 2256
Epicatechin thresholds, 2342
Epichloë festucae, 164, 689
4-*Epi*-cubebol, 2895
Epidemiological, 2583, 2595
evidence, 1835, 2325
studies, 2277
Epidemiology studies, 2278
10-*Epi*-8-deoxycumambrin B, 3094
2′-*Epi*-5-deoxystrigol, 3589
Epidermal growth factor, 1457
Epidermal growth factor receptor, 1449
Epididymal sperm, 2421
Epigallocatechin gallate (EGCG), 2223, 2320, 2439, 2568
Epigenetic, 2404, 2834
Epigenetic effects, 2401
Epiisopiloturine, 877
8-*Epi*-kingiside, 3029, 3041
Epilachna paenulata, 4095
Epilepsy, 877, 3644, 3966
Epileptic seizures, 1208
8-*Epi*-loganic acid, 3022, 3047
Epiloganin, 136
Epimerization, 1135, 1137, 1139, 1145, 1147, 1149, 2805
Epimers, 54
Epimino fatty acids, 994
24*(RS)*,25-Epiminolanosterol, 994
9-*Epi*-mitomycin B, 984
Epinephrine, 912
Epiphytic, 2802
Epithelial–mesenchymal transition (EMT), 2201
Epithelial regeneration, 2404
Epithelisation, 3965
8-*Epi*-valerosidate, 3025
Epothilone A, 990
Epothilones, 990
Epoxides, 1670
Epoxychrysanthemates, 4069
Epoxygaertneroside, 3054
E8 promoter, 1729
Eptastigmine, 1341
Equol, 1671, 2435
Equol producers, 2444
Eranthemoside, 3020
ERβ. *See* Estradiol receptor β (ERβ)
ERCC1. *See* Excision repair cross-complementary 1 (ERCC1)
Eremanthus erythropappus, 2986
E-resveratrol bioavailability, 2292
Ergoamides, 685
Ergocornine, 1135, 1140, 1142, 1144, 1149
Ergocristine, 1135, 1140, 1142, 1144, 1147–1149
Ergocryptine, 1135, 1140, 1144, 1147, 1149, 1349
Ergoline ring, 685
Ergometrine, 1135, 1144, 1146, 1148
Ergonovine, 1140
Ergopeptines, 685
Ergosine, 1135, 1143, 1148, 1149
Ergostane, 3467
Ergosta-5,7,24(28)-trien-3*β*-ol, 1268
Ergosterol, 1274
Ergot, 1349, 1355
Ergot alkaloids, 163, 164
Ergotamine, 1135, 1136, 1140, 1142, 1144, 1148
Ergotismus convulsivus, 686
Ergotismus gangraenosus, 686
Erianin, 1933
Eriostemon, 738
ERK. *See* Extracellular signal-regulated kinase (ERK)

ERK1/2, 1309
Erlich's reagent, 1056, 1063
Erlotinib, 2217
ER-negative tumor cells, 2402
ER-positive tumor cells, 2408
ERR. *See* Estrogen-related receptors (ERR)
Ertela, 739
Erucifoline, 1061
Erwinia uredovora, 1735
Erycibe obtusifolia, 1345
Erythema, 3964
Erythroleukemia cell lines, 3561
Erythrose-4-phosphate (E4P), 14, 15
Erythroxylaceae, 176, 344, 351
Escherichia coli, 111, 240, 242, 243, 980, 988, 989, 1627, 1706, 2828, 2906
E-screen test, 2400
Esculentin, 1551
Esenbeckia, 738
ESI. *See* Electrospray ionization (ESI)
ESI-ITMS. *See* Electrospray ionization ion trap multiple-stage mass spectrometry (ESI-ITMS)
ESI-MS. *See* Electron spray ionization–mass spectrometry (ESI-MS)
ESI-MS detection, 1179
ESI-QqTOF-MS/MS, 3474
Esophageal cancer, 1836
Essential fatty acids, 1719
Essential oil (EO), 2668, 2822, 2920, 2956, 2975, 3976–3978, 3981–3983, 3990, 4088
Estafiatin, 3079
Esterases, 2346
Ester hydrolysis, 370
Esters, 361, 3371, 3373
Estradiol, 2385, 2448
Estradiol receptor β (ERβ), 2413
Estradiol receptors (ERs), 2385, 2413
Estragole, 2976
Estrogen, 1374, 1829, 3752
Estrogen-dependant human breast cancer, 3478
Estrogenic, 2419, 2447
  activity, 2291, 2466
  anti-estrogenic, 2652
  effect, 2412
  mechanism of toxicity, 2421
Estrogen receptor positive (ER+), 3564
Estrogen receptors (ER), 2447
Estrogen-related receptors (ERR), 2400
Estrogen supplementation, 2423
Ethanoic acid side-chain, 54
Ethanol, 1170, 3371
Ethanolamine phosphate, 1270
Ethephon, 1699, 4044
Ethical committee, 2412
Ethnic origin, 2399
Ethnobotanic studies, 1295
Ethnopharmacology, 2667
Ethyl acetate, 691, 3371
Ethyl alcohol-flavan-3-ol adduct, 2269
Ethylamine (EA), 2885
Ethyl-apovincaminate, 580
Ethyl-bridged flavan-3-ols, 2264
Ethylene (ET), 1690, 1695–1697, 1699, 1706, 3591
Ethylenimine, 978, 979
2-Ethylhexanol, 2902, 2905
Ethyl-linked structures, 2269
2-Ethyl-*1*-oleoyl-aziridine, 994
p-Ethylphenol, 2398
8-Ethyl-10-phenyllobelionol, 329
8-Ethyl-10-phenyl-norlobelionol, 329
$\beta-1-O-$Ethylvescalagin, 1995–1997
EU. *See* Eugenol (EU); European Union (EU)
Eucalyptol, 4089
Eucalyptus spp., 2979, 4089
  E. camaldulensis, 3002
  E. globulus, 2982, 4090
  E. oleosa, 2988
  E. perriniana, 1640
  E. saligna, 4094
Eucheuma muricatum, 31
γ-Eudesmol, 2988
Eugenia aromatica, 1552
Eugenin, 1552
Eugenol (EU), 2885, 2905, 2907, 4002, 4096
Euglena gracilis, 984
Eumelanins, 1564
Euodia, 720
Eupatorium perfoliatum, 3528
Eupatundin, 3075, 3077
Euphorbiaceae, 176, 756
Euphorbia hirta, 4098
Euphorbinoyl unit, 1983
European Food Safety Authority (EFSA), 1135, 1136, 1144, 2446, 2495, 2505–2509
European Pharmacopeia (Ph Eur), 3664
Eusiderin, 1558
Eutrophication, 2929
Euxylophora, 720
Evaporative light scattering detection (ELSD), 3207, 3238, 3315, 3337
Evaporative light scattering detector, 1024
Evening primrose, 1738
Evodiamine, 1451

Evodia rutaecarpa, 1451
Evolution, 1545
Exatecan, 665
Excelside A, 3052
Excelside B, 3052
Excelside G13, 3052
Excelside G15, 3052
Excision repair cross-complementary 1 (ERCC1), 2217
Excitability, 3996
Excitation-contraction coupling, 3991
Excitotoxicity, 3711, 3716, 3721, 3722
Excretion, 370, 1231, 3134
Exodeconus, 3467
Expectorant, 2906
Experimental animals, 1508
Experimental design, 1085
Exposure, 4091, 4099
Exposure assessment, 3145
Expressed sequence tags, 63
Expression, 3290, 3978
Extensive metabolizers, 1378
Extracellular matrix, 1458
Extracellular signal-regulated kinase (ERK), 2192, 3532, 4114, 4117, 4118, 4126, 4135, 4139, 4145
Extract, 2591, 2595, 2596, 2598, 2600–2602, 2604, 2605
Extracted ion chromatogram (EIC), 1129
Extraction, 322, 1139, 1140, 2013–2037, 2063, 2064, 2068–2072, 2076, 2111, 3354, 3612, 3978, 4031
Extraction conditions, 3313
Extrahepatic toxicity, 1385
Extraradical hyphae, 2647
Extrinsic pathway, 1314, 2199
Ezetimibe, 3444

F
FAB. *See* Fast atom bombardment (FAB)
Fabaceae, 749, 1551, 3586, 3590
Fabacol, 3587
Fabacyl acetate, 3587
Face flies, 4075, 4076
F-actin, 3485
FADD-like ICE (FLICE), 2200
Fagaceae, 797
Fagara, 763
Fagocytosis, 2471
FAK. *See* Focal adhesion kinase (FAK)
Falkenbergia rufolanosa, 2889
Fangchinoline, 1179
Fannia cannicularis, 4075
FAO/WHO, 3145, 3149
Farmaceuticals, 2566
Farming practices, 2390
Farnesene, 2985, 4050
α-Farnesene, 3000
Farnesol, 2999, 3520, 3529, 4124–4128
Farnesyl cation, 2723
Farnesyl diphosphate (FPP), 2673, 2708, 2736, 2737, 2744, 2748, 2817, 3081, 3082, 3129, 3519
Farnesyl synthases, 3081
Farvorskii ring, 3079
Fas, 1313
Fas-associated death domain (FADD), 3524
Fasciculations, 51, 4079
FASI (Field-amplified sample injection), 1103
FasL, 1313
Fas receptor, 3524
FASS. *See* Field-amplified sample stacking (FASS)
Fast atom bombardment (FAB), 3397–3398
Fasting blood glucose level, 3488
Fat absorption, 1418
Fat digestion and absorption, 1406
Fatty acid amide hydrolase (FAAH), 3425
Fatty acids, 981, 994, 3665
Fatty acid synthase, 1737
Fatty acid synthesis, 1633
Fawcettimine, 1242
F-box protein, 3596
FDA, 2495, 2505, 2506
Feathers, 4072
Febrifuge, 3477
Febrifugine, 1458
Fédération Internationale de Football Association (FIFA), 290
Feed, 1135, 1137–1139, 1142, 1144, 1146, 1149, 1150
Feedback inhibition, 1651, 1671
  allosteric, 1671
Feeding, 101, 104, 105, 111
Feeding stimulants, 1570
Fenchone, 2976, 2984
Fenchyl acetate, 2976
Fenvalerate, 4063, 4065, 4067, 4068, 4070, 4072, 4075, 4076
Fermentation, 2347, 2348, 2351
Fermented preparation, 2402
Fermented-soy-extract, 2415
Fermy’s salt, 258
Ferredoxin, 61
Ferric ion, 986

Ferruginol, 2783
Fertilizer, 2390
Ferulates, 1964
Ferulenol, 3533
Ferulic acids, 1549, 1592, 1629, 1958, 1961, 2346, 2348
Ferulin, 3071
Feruloylhistamine, 866, 876
4Fe-4S cluster, 2701, 2703
Festuca arundinacea, 164
Festucalvine synthase, 701
Fetus, 374, 1319
Fevers, 3795
F-gitogenin, 3666
FHB. *See* Fusarium Head Blight (FHB)
Fib04 (fibroblast culture), 3478
Fibers, 2451, 3665
Fibrinolytic, 3677, 3678
Fibroblast, 3965
Fibroids, 2420
Fibrosarcoma, 988, 991, 3483
Fibrosis, 373
Ficellomycin, 980
Fick's law, 2471
FID. *See* Flame-ionization detection (FID)
Field-amplified sample stacking (FASS), 1189
Fine emery, dry and grippy (astringency descriptors), 2266
Fine/flavor cocoas, 1601
Fine fragrances, 3291
Fingerprinting, 1022
Firmicutes, 2441, 2479
First-pass metabolism, 2322
Fish, 4069, 4079
Flame-ionization detection (FID), 1057, 1075, 3144
Flame photometric (FPD), 1075
Flash chromatography, 3329
Flash column chromatography, 3384
Flavan-3-en-3-ol, 1764
Flavano-ellagitannins, 1992, 1994, 1996
Flavanol-anthocyanin, 1786
Flavanol pool, 1611
Flavanols, 1653, 1719, 1755, 2342
Flavan-3-ols, 1764, 1782, 2266, 2312, 2343, 2588, 2589, 2591, 2592
Flavanone 3β-hydroxylase (F3H), 1655, 1825
Flavanones, 1653, 1828, 1876–1879, 1881–1883, 1889, 1890, 2119, 2151, 2343
Flavin mononucleotide (FMN), 2707
Flavogallonyl unit, 1983
Flavones, 1653, 1719, 1822, 1854, 2116, 2151, 2159
Flavone synthases (FNS), 1655, 1826
Flavonoid(s), 4, 1555, 1587, 1619, 1719, 1822, 1850, 2147, 2155, 2159, 2161, 2163, 2164, 2166, 2334, 2342, 2343, 2438, 2466, 2467, 2472, 2473, 2475, 2478, 2480, 2481, 2528–2531, 2533, 2534, 2538–2540, 2542–2545, 2548, 2549, 2551, 2552, 2556, 2557, 2583, 2584, 2595, 2597, 2599, 2605, 2642, 3194, 3501, 3666, 3961
  aglycones, 2162
  biosynthetic, 1729
  biosynthetic pathway, 1622, 1723
  flavan nucleus, 2467
  flavylium, 2467, 2468, 2475, 2480
  glycosides, 2163
  oligomers, 2335
Flavonoid 3′-hydroxylase, 1762
Flavonoid 3′,5′-hydroxylase, 1762
Flavonol(s), 1653, 1719, 1822, 1854, 2115, 2152, 2155, 2156, 2158, 2161, 2439, 2588, 2591
  glycosides, 1604, 2151, 2157, 2158, 2163, 2346
  synthase, 1655
Flavonone, 1719
Flavor, 2347, 3983, 4112, 4120–4125, 4141, 4142, 4149
Flavoring agents, 3983
Flavour, 2262
Flax, 1728
Flaxseeds, 1736, 2438
FLD. *See* Fluorescence detection (FLD)
Fleas, 4064, 4069, 4071, 4072, 4077–4079, 4083
FLICE. *See* FADD-like ICE (FLICE)
Flies, 4064, 4071, 4072, 4075, 4077, 4083
Flindersia, 728
Florosenine, 1385
Flow-mediated dilatation (FMD), 2372
Fluconazole, 1276
Flumazenil, 4116
Flumethrin, 4063, 4070, 4074, 4076
Fluorescence detection (FLD), 692, 1086, 1137, 1139, 1143, 1145, 2121
Fluorescence detector, 3387
Fluorescence/mass spectrometric detection, 3144
Fluorescence spectroscopy, 58
α-Fluoromethylhistidine, 876
5-Fluorouracil, 2217
Fluvalinate, 4063

FMD. *See* Flow-mediated dilatation (FMD)
FMN. *See* Flavin mononucleotide (FMN)
FNS. *See* Flavone synthases (FNS)
Foam, 2342, 2776
Focal adhesion kinase (FAK), 2203
Foeniculum vulgare, 2976, 2984
Folate, 1732
Folic acid, 1270
Folk medicine, 3214, 3220
Follicle-stimulating hormone, 1374
Food, 1959, 2391, 3978, 3983, 4112
  additives, 337, 3199
  colorants, 1816
  drugs, 2423
  extracts, 1765
  industry, 4049
  matrix, 2472
  poisoning, 286
  preservatives, 3976
  processing, 2467
  sources, 1967, 2527–2557
  supplements, 2391
  transit time, 1408
Food and Drug Administration (FDA), 3686
Food-borne, 3977
Food-borne microorganisms, 4113
Food-producing animals, 4080
Forastero(Cocoa), 1601
Forced swimming test, 3219
Forensic botanists, 296
Forensic Sciences, 1504
Formic acid (FO), 2885
Formononetin, 1852
Forskolin, 3183, 3327
Forsythide, 3024, 3041
FOSHU (Foods for Other Specific Health Use), 2494, 2496
fos proteins, 3527
Fovea, 3928, 3930, 3931, 3933, 3937
Foxglove, 295
FOXO3a, 2196
FPD. *See* Flame photometric (FPD)
FPP. *See* Farnesyl diphosphate (FPP); Farnesyl pyrophosphate (FPP)
Fraganol, 2999
Fraganyl acetate, 2999
Fragaria grandiflora, 3587
Fragilamide (FRG), 29, 1211
Fragmentation, 329
Fragmentation mechanism and rule, 3317
Fragment ions, 325, 3316
Fragrances, 3183, 3289, 3519, 4049, 4112, 4116, 4121, 4124, 4128
Frankliniella occidentalis, 4098
Free radicals, 3668, 3670, 3682
Free radical scavengers, 2319
French (or Hugues) method (resin stimulation), 4042
French paradox, 2277, 2324
Frequent urination, 899
Freudenberg (tannin classification), 1561
FRG. *See* Fragilamide (FRG)
Friend retrovirus, 1315
Frosty pod, 1602
Fructooligosaccharides, 2454
Fructose, 3813
Fructose-6-phosphate, 1670
Frugivores, 1720
Fruit(s), 1730, 1806, 2582–2584, 2595–2597, 2605
  pulp, 1601
  ripens, 1766, 2470
Fruit-specific, 1735
Fucaceae, 2896
Fucales, 2896
Fucoxanthin, 3254, 3256, 3264
Fucus
  F. distichus, 2896, 2905
  F. serratus, 2896
  F. spiralis, 2897
  F. vesiculosus, 2897
Full scan mass data, 3316
Fully characterized extracts, 2411
Fumarase, 980
Fumaria officinalis, 1175, 1183
Fumiquinazolines, 839
Functional foods, 1582, 1736, 1738, 2423, 2454, 2493, 2495, 2496, 2504, 2565, 2583, 2600, 2601, 2603–2605, 2642, 2651, 2652
Functionally expressed, 1626
Functional metagenomic, 2455
Functional properties, 3981
Functional redundancy, 2441
Functional value, 2855, 2862, 2871, 2874
Fungal, 1688, 1690, 1694, 1695, 1699
  cell membrane, 3981
  disease, 3976
  elicitors, 2781, 4045
  pathogens, 2937
Fungi, 1693, 1694, 3584, 3976, 4113
Furanocoumarins, 886, 1551
Furfural (FU), 2885, 2907
Furfuryl alcohol (FUR), 2885
Furoacridones, 850
Furoquinolines, 731, 1284

Furostanol glycosides, 3232
Furostanols, 3228, 3230, 3241
Furostanol saponins, 3227, 3229, 3230, 3232, 3239, 3244
Fusarenon X, 3125, 3139, 3145
Fusarium, 806, 3124–3126, 3129–3133, 3144, 3145
  F. asiaticum, 3126–3128
  F. culmorum, 1728
  F. graminearum, 3126–3129, 3133, 3134, 3149
    species complex, 3126
  F. oxysporum, 1728
  F. sporotrichioides, 3130–3133
Fusarium head blight (FHB), 3126
Fused-silica capillaries, 1128

G
$GABA_A$ receptor, 4006
$GABA_A$ receptor-ionophore complex, 4067
GABAergic transmission, 1208
GABA-gated chloride channels, 4067
Gaertneric acid, 3026
Gaertneroside, 3054
Gain-of-function mutations, 2953
Galactorrhea, 1374
Galactose, 2467
Galactose-inducible promoter, 1659
Galangin, 1658, 1835
Galantamine, 1335, 1337
Galanthamine, 1188, 1244, 1335, 1337, 1529, 1530, 1532
Galanthus woronowii, 1335
Galaxaura
  G. marginata, 2887
  G. obtusata, 2887
Galaxauraceae, 2887
Galbulimima, 1345
Galerina, 530
Galipea, 738
Galipea longiflora, 1283
Gallate, 2335
GALL genes, 2950
Gallic acid (GA), 1954, 2346, 2568
Gallic *p*-hydroxybenzoic, 1548
Gallocatechins, 1758, 2335
Galphimia glauca, 2786
Galphimine-B, 2786
γ-amino butyric acid-A (GABA-A)-receptor, 1621
Gamma-aminobutyric acid (GABA), 1375, 3711, 3722
  receptors, 4068, 4096
  synapses, 4096
Gandira, 3327
Gangrene, 3664
Ganstigmine, 1341
Garcinia lucida, 1282
Gardenosidic acid, 3023
Garlic, 3664, 3667, 3670, 3673, 3676, 3677, 3681, 3683–3686
  oil, 3666, 3668, 3673, 3678, 3685
  paste, 3668
  powder, 3666–3667, 3669, 3673, 3675, 3677, 3678, 3681–3683, 3685
Gas chromatography (GC), 1028, 1054, 1057, 1058, 1062, 1064, 1073, 1124, 2076, 3337
Gas chromatography-mass spectrometry (GC-MS), 44, 178, 399, 1137, 1143, 3144, 3145, 3398, 3517, 3592, 3859
Gas-phase fragmentation, 3474
Gastointestinal cancers, 2207
Gastric carcinoma cell lines, 3561
Gastric irritation, 3686
Gastric ulcers, 1307, 3967
Gastrointestinal, 1374, 1378, 2435, 4117, 4122, 4123
  disorders, 3144, 3477
  parasites, 985
  protective role, 1408
Gaultheria procumbens, 4099
Gaultheria spp., 1548
G-B. *See* Ginkgolide B (G-B)
GC. *See* Gas chromatography (GC)
G-C. *See* Ginkgolide C (G-C)
GC-EIMS, 1029
GC-MS. *See* Gas chromatography-mass spectrometry (GC-MS)
GEIR-1 (Iridoid), 3029
Geissoschizine, 94
Gelatinase, 3560
Geleznowia, 739
Gelidiaceae, 2887
Gelidiales, 2887–2888
Gelidium spp., 2887
  G. amansii, 2887
  G. crinale, 28, 2888, 2904
Gelsemiol, 3058
Gemcitabine, 2215
Gender-selective, 293
Gene, 2407

clusters, 3130, 3133, 3257, 3270, 3273
deletion, 1664, 1672
expression, 3526
General odorant binding proteins (GOBPs), 4096
Genetic, 2390
engineering, 215, 226, 237, 1622, 1718, 2856, 2862, 2867–2869, 2871, 2875, 3575
markers, 51
mobilization, 42
selection, 4051
stability, 198, 2776, 2943
transformation, 108
Genetically modified (GM) plants, 970
Genexol®, 2784
Genipin, 3033, 3050, 3056
Genipin diglycoside, 3053
Geniposide, 135
Geniposidic acid, 3023
Genisteae, 382
Genistein, 1655, 1828, 2387, 2444
Genistin, 1655, 1850
Genome organization, 203
Genome sequence, 1641
Genome shuffling, 2805
Genomic, 1686, 1690
Genomic approach, 232
Genotoxic, 49, 360, 3542, 4118, 4119, 4138
activity, 2652
effects, 4118
Genotoxicity, 3141
Genotypes, 1691, 1694, 1697
Gensenosides, 2675
Gentianaceae, 925, 1553
Gentianine, 931
Gentisic, 1960
Gentoxicity, 4125
Genus *Aesculus*, 2947
Genus *Salvia*, 2961
Geosmin, 2989
Geranial, 2979
Geraniol (GE), 90, 105, 2885, 2906, 2977, 2979, 2997, 2999, 4089, 4096
Geraniol acetate, 2888
Geraniol formate, 2888
Geranium oil, 290
Geranyl acetate, 2979, 4096
(E)-Geranylacetone, 2891, 2892
Geranyl cation, 2720
Geranyl diphosphate (GPP), 2673, 2708, 2735, 2744, 2817, 3418
Geranylgeraniol, 2896
Geranylgeranyl diphosphate (GGPP), 941, 1730, 2673, 2708, 2744, 2748, 2803, 2817
Germacrene A, 3082
Germacrene A synthases, 3082
Germacrene B, 3000, 3085
Germacrene B synthase, 3085
Germacrene D, 2986, 2990, 2997
(-)-Germacrene D, 2895
German cacao, 1607
Germfree, 2440
Germination, 392
Germination stimulant, 3584
Gerronema, 530
GFR. *See* Growth factor receptors (GFR)
GPP. *See* Geranyl pyrophosphate (GPP)
GGPP. *See* Geranylgeranyl diphosphate (GGPP); Geranylgeranyl pyrophosphate (GGPP)
Giant butterbur, 979
Giardia lamblia, 1310
Gibberellic acid, 3109
Gibberellins, 3181, 3591
Gigartinaceae, 2890
Gigartinales, 2890
Gigartina stellata, 29, 2890
Gigspora margarita, 3591
Gimatecan, 653, 666
Gingeberaceae, 3532
Ginger, 3532
Ginkbilobin, 3700
Ginkgo biloba, 2678, 3530, 3700, 3701, 3703–3706, 3708, 3709, 3711, 3712, 3715–3718, 3720–3722, 3724, 3725, 3727
Ginkgo biloba extract (EGb), 3707, 3708, 3710, 3716, 3720, 3726, 3727
Ginkgo leaf extract, 3727
Ginkgolide A (G-A), 3701, 3704, 3711, 3713, 3715, 3716, 3720, 3726
Ginkgolide B (G-B), 3701, 3703, 3704, 3710, 3713, 3715, 3716, 3719, 3721–3723, 3725, 3726
Ginkgolide C (G-C), 3701
Ginkgolide J (G-J), 3701, 3713, 3715, 3716
Ginkgolide K (G-K), 3701, 3721
Ginkgolide L (G-L), 3701
Ginkgolide M (G-M), 3701
Ginkgolides, 2737, 3185, 3700, 3701, 3703, 3707, 3709, 3711, 3726, 3728
Ginkgo seeds, 3727
Ginkgotoxin, 3700

Ginsan, 3501
Ginseng, 3498
Ginseng adventitious roots, 2786
Ginsenosides, 2766, 2785, 2947, 3501
G-J. *See* Ginkgolide J (G-J)
G-K. *See* Ginkgolide K (G-K)
G-L. *See* Ginkgolide L (G-L)
Glandular trichomes, 2822, 3521
Glaucarubolone, 3785
Glaucine, 446
Glaucoma, 877
Glial cells, 2619, 2624, 2625
Glioma, 1316
Glioma cells, 3561
Glochidicine, 866
Glochidine, 866
Glochidion philippicum, 865
Glomeromycota, 2645
Glomus
  G. intraradices, 2648–2650, 2652
  G. mosseae, 2648, 2650
Glucocorticoids, 3808
Gluconeogenesis, 3816
Gluconeogenic enzymes, 3810
Glucose, 2467, 2478, 3703, 3706, 3721
  tolerance, 3200
  transporter
    GLUT2, 2478
    SGLT1, 2478
Glucose peptone with soy flour medium (GPS), 691
Glucose-6-phosphatase (G6Pase), 3809
Glucose-1-phosphate, 1664
Glucose-6-phosphate, 1664
Glucose-1-phosphate uridylyltransferase, 1664
Glucosinolates, 2150, 2567, 2643
3-*O*-Glucosyltransferase, 1664
β-Glucuronidase, 192
Glucuronidated anthocyanin, 2475
Glucuronidation, 2293, 2397
Glucuronide, 2111, 2324, 4069
Glucuronide conjugation, 4078
Glucuronide metabolite(s), 1099
GLUT 4, 3819
Glutamate, 1249, 1371, 3710, 4069, 4123, 4129, 4133
Glutamate receptor mobility, 2406
Glutamatergic, 3710, 3711
δ-Glutamyl *S*-allyl-L-cysteines, 3666, 3667
γ-Glutamyltransferase, 2190
Glutathione (GSH), 1588, 2321, 3670, 3672, 3674, 3713, 4115, 4123, 4127, 4132, 4135, 4143, 4150
Glutathione depletion, 1385
Glutathione peroxidase, 4127
Glutathione *S*-transferase (GSTs), 2181
β−1−*S*−Glutathionyl vescalagin, 1997
Glutinoside, 3020
Glutamate, 49
GLVs. *See* Green leaf volatiles (GLVs)
Glyceollin II, 1569
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1268
Glychirriza echinata, 1852
Glycine, 4069
Glycine B, 3711
Glycitein, 2388
Glycocalyx, 1275
Glycogenolysis, 3816
Glycolysis, 1268, 1272, 3816
β-Glycosidase, 2475
Glycosides, 2319, 2346, 2350, 2395, 2467, 2473, 2475, 2478, 2481, 3195, 3200
Glycosmis, 719
Glycosomes, 1273
Glycosylated bibenzyls, 1916
Glycosylated form, 2391
Glycosylated resveratrol, 1587
Glycosylation, 1663, 1664, 1669
  deglycosylation, 1669
  microbial, 1663
  regioselective, 1664
Glycosylphosphatidylinositol (GPI), 1269
Glycyrrhiza echinata, 1852
Glyoxalase-I, 1271
Glyoxalase-II, 1271
Glyoxylate cycle, 1273
Glyoxylic acid, 1783
G-M. *See* Ginkgolide M (G-M)
GNAT-acetyl transferase, 61
Gnetaceae, 1906
Gnetum, 1912
Gnotobiotic, 2440
Goat, 4070
Golden rice, 1718
Goldenseal, 1303
Gonadal hormones, 1374
Gonorrhea, 3477
Gonyaulacales, 59
Gonyautoxins, 54
Gossypetin, 1828
Gossypium
  G. barbadense, 2984
  G. hirsutum, 3000, 3585
Gossypol, 2957, 3521
G6Pase. *See* Glucose-6-phosphatase (G6Pase)

G1 phase arrest, 1457
G6P isomerase, 1670
GPP. *See* Geranyl diphosphate (GPP); Geranyl pyrophosphate (GPP)
GPP pool, 2831
G-protein, 1371
G-protein coupled ER, 2400
GR5 (strigolactone analogs), 3588
GR7 (strigolactone analogs), 3588
GR24 (strigolactone analogs), 3588
Gracilariaceae, 2888
Gracilariales, 2888
Gracilaria spp., 2888
  G. bursa-pastoris, 28, 2888
  G. edulis, 2888
  G. secundata, 2888
  G. verrucosa, 27
Gracillin, 3229, 3244
Gradient, 2150, 2152, 2164, 2165, 2471
Gradient elution, 1086, 3337, 3339, 3378
Grahamine, 355
Gramine, 151, 153, 160, 161, 163
Gram-negative, 2906
Gram-negative bacteria, 979, 981, 988
Gram-positive, 2906
Gram-positive bacteria, 979, 980, 988, 994
Grandifloric acid, 3020
Grape berry, 2257
Grape-derived products, 2369
Grape extracts, 2369
Grape juice, 2369
Grape polyphenol fractions, 2264
Grapevine, 1685, 1687, 1688, 1690, 1692–1699, 1701, 1702, 1706
Great burdock, 3528
Greater affinity (Estradiol receptors), 2398
Green algae, 29
Green filamentous bacteria, 3257, 3258
Greenhouse, 330, 331
Green leaf volatiles (GLVs), 2934
Green pepper, 311
Green sulfur bacteria, 3254, 3257, 3258, 3262, 3271, 3272
Grooming, 4078
Groupe Polyphénols, 1546
Growth factor receptors (GFR), 2197
Growth inhibition effect, 1512
Growth rate, 2906
GSH. *See* Glutathione (GSH)
GSIR-1 (Iridoid), 3031
GSTs. *See* Glutathione *S*-transferase (GSTs)
Guaianolide, 3070–3073, 3075, 3076, 3079–3081, 3083–3090, 3093, 3094
Guaiazulene, 3530
Guaiene, 3085
Guanidine methyl phosphate ester, 42, 52
Guanidinium, 1669
Guanidinium group, 54
Guanylyl cyclase, 2364
Guarana, 955
Guatteria foliosa, 1284
Guettardine, 614
Gulf coast ticks, 4075
Gum resin, 4046
Guttiferae, 1553
Guy-Lussac, J., 1369
Gymnodiniales, 59
Gymnodinium catenatum, 54, 59
Gymnopilus, 527
Gynostemma pentaphyllum, 2947
Gynura segetum, 1179

H
Haemangiomas, 1444
Haematobia irritans, 4069
Haematological maligances, 1313
Haematopinus
  H. eurysternus, 4069
  H. suis, 4069
Haemorrhage, 3727
Hair, 1082, 4070
  care, 3476
  remedy, 3477
Hairy root(s), 2943, 2956, 2958, 2959, 2961, 2962, 3110–3115
  cultures, 189, 231, 236, 237, 239, 2774, 2954
  lines, 2957
  phenotype, 2950
  segments, 2951
  syndrome, 2774
Hairy root–derived calluses, 2952
Halfordia, 732
Haliclona, 789
Halidrys siliquosa, 2897
Halimeda
  H. cuneata, 2903
  H. discoidea, 2903
  H. incrassate, 31
  H. opuntia, 2903
Halimedaceae, 2903
Haliptilon
  H. roseum, 2890
Hallucination, 542

Hallucinogenic, 534, 695
Hallucinogenic effects, 292
Hallucinogens, 542, 3184
Halofuginone, 1458
Halogenated alcohol (HALC), 2885
Halogenated compounds (HC), 2885, 2886, 2904, 2905, 2907
Halogenated indole (HLI) alkaloids, 31, 1212
ω-Halogenated long-chain 2*H*-azirines, 980
Halogenated organic solvents, 691
Halopithys incurva, 2892
Halopteris
  H. paniculata (Stypocaulon paniculatum), 2901
  H. platycena, 2901
Halymeniaceae, 2891
Halymenia floresii, 28, 2891, 2904
Haploid germ cells, 2421
Haplophyllum, 719
Haraldiophyllum spp., 29
Harmala alkaloids, 554, 564, 568
Harmaline, 1352
Harman, 1170, 1352
Harmane, 1170
Harmine, 553–569, 1352, 1452
Harpagide, 3018
Harpagoside, 3018, 3055
HATX. *See* Homoanatoxin-a (HATX)
Hazardous pathogens, 3977, 3983, 3984
Haze, 2335, 2342
HCC. *See* Hepatocellular carcinoma/cancer cells (HCC)
HCT-8 (tumor cells), 3478
HCT-116, 5W-480 (tumor cells), 3485
HDL. *See* High-density lipoprotein (HDL)
Headache, 3477
Head-space solid-phase microextraction (HS-SPME), 1084
Head-to-head condensation, 2715, 2717
Head to tail, 2150
Health, 1816, 2422
  benefits, 1961, 2312, 4023
  claims, 2505–2507
Health-promoting activity, 2653
Health properties, 2343, 2345, 2346, 2351
Health-protecting effects, 1628
Healthy subjects, 2320
Hearing loss, 1373
Heat shock granules (HSGs), 3863
Heat shock protein 90 (Hsp90), 3485
Hedge hog (Hh), 2195
HeLa cells, 3964
Helenalin, 3088, 3089, 3532
Helianthus annuus, 3592
Helicobacter pylori, 1310
Helicoverpa armigera, 4097
Helietta, 732
Helietta apiculata, 1284
Heliobacteria, 3254, 3257, 3258
Heliotrine, 1062
Heliotropium, 1388
Helix structures, 2385
Hellinoyl group, 1980
Heloside A, 3240
Helosides, 3236, 3242
Heme biomineralization, 3791
Heme oxygenase 1, 2190
Hemiacetal, 2480
Hemiaminal formation, 942
Hemiparasites, 3585
Hemiterpenes, 2670, 2974
Hemiterpenoid, 2915
Hemlock, 294
Hencikosane, 2899, 2902
Henderson–Hasselbalch, 1165
Hepa 1c1c7 cells, 3484
Hepatic encephalopathy, 1386
Hepatocellular adenomas, 1386
Hepatocellular carcinoma (Hep G2, Hep 3B), 3479
Hepatocellular carcinoma/cancer cells (HCC), 2208, 3561
Hepatocytes, 3668, 3670
Hepatoma, 1316
Hepatoma cells, 3964
Hepatomegaly and recurrent ascites, 373
Hepatoprotective activity, 1835, 1940
Hepatotoxic, 41, 360
Heptacyclic system, 938
(8Z,11*Z*)-Heptadecadienal, 2887, 2902, 2904
Heptadecane, 2891, 2892, 2894
(8Z,11Z,14*Z*)-Heptadecatrienal, 2902
(8*Z*)-Heptadecenal, 2887, 2902, 2904
1-Heptadecene, 2891, 2892
8-Heptadecene, 2892
Heptafluorobutyric anhydride, 1058
Heptanal, 2902
2-Heptenal, 2894
Herbal drug, 3983
Herbal ingredients, 289
Herbal plant, 3983
Herbal tea, 1388
Herbicides, 2780
Herbivores, 1720
Herbivory, 138, 2917

hERG potassium channels, 63
HER-2/neu transgenic mice, 2212
Heroin, 1082
Herpes simplex virus, 1310
Hesperidin, 2570
Heterexenous, 1266
Heterochordaria abietina (Analipus japonicus), 2901
Heterochromatic flicker photometry (HFP), 3931, 3932, 3934, 3935
Heterocyclic compounds, 978
Heterodimers, 2388
Heterologous, 232, 234, 237, 240, 242, 1626
Heterologous production of different antibiotics, 1638
$\alpha_2\delta_2$-Heterotetramer, 2711
2,2′,5,5′,6,6′-Hexabromo-3,3′-bis-1*H*-indole, 33
Hexachlorobutadiene, 2896
(7Z,10Z)-hexadecadienal, 2887
Hexadecanal, 2891, 2982
Hexadecane, 2891, 2892
Hexadecanoic acid, 2894
Hexadecanol, 2891, 2892
(7Z)-hexadecenal, 2887
Hexaflavonoids, 1621
Hexahydroxydiphenoyl (HHDP), 1978, 1988
Hexanal, 2900, 2902, 2906
n-Hexane, 3330, 3371
Hexanol, 2896
(Z)-3-Hexen-1-ol, 2902
trans-1-[(1Z)-Hexenyl]–2-vinylcyclopropane, 2889
Hexose-6-phosphate dehydrogenase (H6PDH), 3809
3-Hexyl-4,5-dithiacycloheptanone, 2894
HIF-1α. *See* Hypoxia-inducible factor-1–subunit alpha (HIF–1α)
High activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), 2697, 3198
High-density lipoprotein (HDL), 2591, 2596
High density lipoprotein cholesterol (HDL-C), 3668
High doses, 2414, 2416, 2417, 2420
Highest prevalence, 2422
High-fat-fed rats, 3811
High light intensity, 2645
High-mobility group box 1 (HMGB1), 3569
High performance liquid chromatography (HPLC), 44, 58, 535, 915, 1055, 1058, 1060, 1073, 1074, 1124, 1139, 1143, 1766, 2076, 3144, 3145, 3195, 3207, 3314, 3333, 3336, 3340, 3355, 3376, 3592, 3859
High-performance thin-layer chromatography (HPTLC), 1021, 3336, 3340
High-producing *Vitis* cell lines, 1691
High soy consumption, 2419–2420, 2423
High speed counter current chromatography (HSCCC), 2077, 2147, 2148, 2150–2161, 2163–2165
High-sucrose-fed rats, 3811, 3814
High-throughput separations, 1023
High voltages (capillary electrophoresis), 1102
High-yield cell lines, 111
High-yielding stable cell line, 228
HILIC. *See* Hydrophilic interaction chromatography (HILIC)
Himbacine, 1345
Hippocampus, 2406, 3710, 3712, 3713, 3715, 3716
Histamine, 1374, 3652
Histidine, 416, 861–865, 868, 871–873, 875, 876
L-Histidine, 872, 876
Histidine decarboxylase, 864
Histopathological, 1384
HIV. *See* Human immunodeficiency virus (HIV)
Hizikia fusiformis (Sargassum fusiforme), 2897
HMBPP. *See (E)*-4-Hydroxy-3-methylbut-2-enyl diphosphate (HMBPP)
HMBPP epoxide, 2701
HMGB1. *See* High-mobility group box 1 (HMGB1)
HMG-CoA. *See* 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)
HMG-CoA reductase, 2678, 4115, 4126, 4130
HMGR. *See* 3-Hydroxy–3-methyl-glutaryl-CoA reductase (HMGR)
Hemiterpenoids, 2720
^{1}H-NMR, 332, 916, 3476
Hodgkins lymphoma, 588
Holarrhena curtisii, 1289
Holoparasites, 3585
HOMA-IR. *See* Homeostasis model assessment of insulin resistance (HOMA-IR)
Homeopathic medicine, 1507
Homeostasis, 3670
Homeostasis model assessment of insulin resistance (HOMA-IR), 3810
Homoanatoxin-a (HATX), 41, 44, 45, 49

28-homoBL. *See* 28-Homobrassinolide (28-homoBL)
28-Homobrassinolide (28-homoBL), 3862
Homocamptothecin, 652, 657, 666
Homocysteine (Hcy), 3681–3683
Homoeostrichus sinclairii, 2895
Homoharringtonine, 1453
Homoisoflavonoids, 2163
Homologous genes, 686
Homomeric prenyltransferases, 2709
Homospermidine, 365
Homospermidine synthase (HSS), 365
Homoterpenes, 2915
Homotropane alkaloid, 43
Homovanillic, 2476
Honey, 361, 1051, 1053–1056, 1058, 1064, 4076, 4080
Honeybees, 4069
Honeydew, 704
Hop, 2334, 2335, 2343, 2346, 2347, 2350
Hop conditioning, 2335
Hopeaphenol, 2279, 2282
Hopein, 2343, 2345
HORD. *See* Hordenine (HORD)
Hordenine (HORD), 153, 159–161, 1209
Horizontal gene transfer, 63, 689
Hormetic compounds, 2572
Hormone-dependant human prostate cancer, 3478
Hormone replacement, 1667
Hormone-sensitive cell lines, 3863
Hormophysa cuneiformis, 2897
Hormosira banksii, 2897
Hormosiraceae, 2897
(-)-Hormosirene, 2900
Horn flies, 4075, 4076
Horses, 4075
Hortia, 732
Hot flushes, 2407
Houseflies, 4075, 4076
Household products, 3289, 4113, 4124, 4141, 4147
Houttuynia cordata, 3587
H6PDH. *See* Hexose–6-phosphate dehydrogenase (H6PDH)
HPLC. *See* High performance liquid chromatography (HPLC)
HPLC-DAD, 324
HPLC-MS, 3315, 3320
HPTLC. *See* High-performance thin-layer chromatography (HPTLC)
hPTP1B. *See* Human protein tyrosine phosphatase 1B (hPTP1B)
HSCCC. *See* High speed countercurrent chromatography (HSCCC)
Hsp90. *See* Heat shock protein 90 (Hsp90)
Hsp90 client proteins, 3485
Hsp90-Hsp70 chaperone complex, 3485
HSS. *See* Homospermidine synthase (HSS)
HS-SPME. *See* Head-space solid-phase microextraction (HS-SPME)
5-HT$_{1A}$(serotonergic receptors), 525
5-HT$_{2A}$, 525
5-HT$_{2C}$, 525
5-HT$_{1D}$, 525
Human, 2419
  breast cancer cells, 3557
  breast milk, 1099
  cancer cell lines, 3863
  cancer cells, 2296
  cytochrome, 3969
  diseases, 3984
  endothelial cells, 3562
  estrogen receptors, 1650, 1667
  fibroblast cultures, 3478
  health, 965, 1593
  liver carcinoma Hep G2 cell lines, 3480
  lung adenocarcinoma, 3560
  lung cancer, 3478
  models, 1740
  skin, 3759
  skin barrier, 3758
  tumor cell lines, 1512, 3478
Human cervical cancer (HeLa) cells, 3561
Human colon carcinoma (CRC), 3561
Human colorectal adenocarcinoma cells (DLD–1), 3480
Human head and neck squamous cell carcinoma (JMAR and MDA–1986), 3480
Human immunodeficiency virus (HIV), 1310, 2677
Human lung carcinoma cells (A549), 3480
Human myeloma cells (Human 8226), 991
Human protein tyrosine phosphatase 1B (hPTP1B), 1211
Human tissue kallikrein (KLK), 3749
Human umbilical vein endothelial cells (HUVEC), 2204, 3216
Humulene, 4116, 4118–4120
α-Humulene, 2986
Humulus lupulus, 2986, 4119, 4121
Huntington's diseases, 589, 3488
Huperzia serrata, 1241, 1245, 1338
Huperzines A, 1244, 1338, 1340, 1530–1532
Huperzines B, 1248, 1338

Huprines, 1339
HUVEC. *See* Human umbilical vein endothelial cells (HUVEC)
Hybrid ryes, 706
Hydramarosides A, 3042
Hydramarosides B, 3042
Hydrangeaceae, 801
Hydrastis canadensis L., 1169, 1170, 1446
1,2-Hydride shift, 60
Hydrocarbon (HDC), 2885, 2886, 2894, 2904, 3981
Hydrocarbon carotenoids, 3369
Hydrodistillation, 3290
Hydrodynamic, 2147, 2148, 2163
Hydrogen peroxide (H_2O_2), 3712
Hydrogen sulphide, 3677
Hydroindole alkaloids, 1168
α/β-Hydrolase, 3596
Hydrolysable tannins, 1559
Hydrolysate of Polygalasaponins, 3219
Hydrolysis, 1085, 2805, 4069
Hydromorphone, 1376
Hydrophilic interaction, 1181
Hydrophilic interaction chromatography (HILIC), 58, 1023, 1086
Hydrophobicity, 2115
Hydroquinone, 4065
Hydrostatic, 2147
Hydrostatic pressure, 1698, 1699
Hydrotaea irritans, 4069
Hydrotropes, 3330
Hydrotropic extraction, 3330
Hydrotropic solubilization, 3331
Hydroxamic acid, 986
3-(Hydroxyacetyl)indole (HAI), 29
4-Hydroxybenzaldehyde, 1548, 2902
Hydroxybenzoate side-chain, 54
Hydroxybenzoic, 1953
3-Hydroxybenzoic, 1962
4-Hydroxybenzoic acid, 1953, 2902
Hydroxybenzoic acids, 2346
4-Hydroxybenzyl alcohol, 2888
6-Hydroxycaespitol, 2892
Hydroxycinnamates, 1549
Hydroxycinnamic acid amides, 1604
Hydroxycinnamic acids, 1953, 2346, 2589
Hydroxycitronellal, 2999
5-Hydroxydidrovaltrate, 3049
3*β*-Hydroxy-2,3-dihydrowithanolide F, 3472
6-Hydroxydopamine (6-OHDA), 3710, 3718
1β-Hydroxy-4-epigardendiol, 3047
Hydroxygeraniol, 90, 105
4-Hydroxyindole, 535, 537
2-Hydroxyisoflavanone dehydratases, 1655
6-Hydroxykynurenic acid (HKA), 3711
Hydroxylation, 1805, 2397
Hydroxylation pattern, 1756
Hydroxyl-group is introduced adjacent to the nitrogen-atom, 368
Hydroxyl groups of polyphenols, 2263
3-Hydroxyl-3-methylglutaryl group, 3320
Hydroxyl radical (OH•), 3673
4″-Hydroxy-maysin, 1831
(E)-4-Hydroxy-3-methylbut-2-enyl diphosphate (HMBPP), 2701
1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP), 2744
3-Hydroxy-3methylglutarul-co-enzyme A (HMG-CoA), 3447, 3668
3-Hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), 2782, 2832
3*β*-Hydroxymethylsubtrifloralactone E, 3484
3′-Hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT), 6, 8–11, 21
7-Hydroxyorobanchol, 3587
4-Hydroxyphenylacetaldehyde (4-HPAA), 6, 8, 17
p-Hydroxyphenylacetic acid, 1549
o,m-Hydroxyphenylcyclopentenon (HPCP), 1987, 2000
4-Hydroxyphenyllactic acid, 2902
3-Hydroxy-3-phenylpropanoic allosedaminate, 330
Hydroxyproline, 3966
Hydroxyquinine, 633
7α-Hydroxy semperoside aglucone, 3058
11β-Hydroxysteroid dehydrogenase (11β-HSD), 3808
5-Hydroxytryptamine (5-HT), 3570
4-Hydroxytryptophan, 537
27-Hydroxywithanolide D, 3481
18-Hydroxywithanolide D, 3484
Hygrocybe, 530
Hygrophiloside, 3020
Hylobius pales, 4094
Hymenialdisine, 266
Hymenidin, 266
Hymenin, 3092
Hymenolepis microstoma, 985
Hyoscine, 1345
Hyoscyamine, 175, 1013
(S)(-)-Hyoscyamine, 347
Hyoscyamine 6β-hydroxylase (H6H), 185
Hyoscyamus, 3467
Hyoscyamus niger, 291

Hypaconitine, 1170
Hyperalgesia, 1374, 1376
Hypercholesterolemic, 3669, 3675, 3678
Hyperchromism, 1781
Hyperexcitability, 4079
Hyperexcitation, 4065
Hyperglycemia, 3200, 3488, 3721
Hypericum spp., 1553
Hyperinsulinemic state, 3813
Hyperlipidemia, 1305, 3668, 4114
Hyperplasias, 4114, 4149
Hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels, 4004
Hyperpolarizations, 2365, 2370
Hypersalivation, 4079
Hypersensitivity, 4065
Hypersensitization, 1374
Hyperspectral imaging, 1138
Hypertension, 63, 1306, 2371, 3674, 3964, 4115
Hyperthermia, 4079
Hypertriglyceridemia, 3809
Hypervalent iodine, 257
Hypervalent iodine (III) reagent, 261
Hyphae, 2936
Hyphal branching, 3591
Hyphenated techniques, 1038
Hypholoma, 528
Hypnea concornis, 31
Hypochlorous acid (HOCl), 1832, 3670
Hypocholesterolemic agent, 1418
Hypocholesterolemic effect, 3441
Hypoglycemic, 397, 3624
Hypolipidemic, 3218, 3668, 3669
Hypolipidemic effects, 1417
Hypospadias, 2418
Hypothermia, 4079
Hypothyroidism, 1471, 1473, 1485–1489, 2423
Hypothyroidism goiters, 2422
Hypoxia, 3706, 3707, 3712, 3722
Hypoxia-inducible factor-1alpha, 1446
Hypoxia-inducible factor-1–subunit alpha (HIF–1α), 1446, 2204
Hypoxic, 1455
Hypusine, 1272
Hyrtios, 727

I

IAP. *See* Inhibitor of apoptosis (IAP)
Iboga, 577
Ibogaine, 1349
ICH_2CH_2OH, 2889
Ichthyotoxins, 41
Icilin, 3993
Identification, 1967, 3369, 3614
Identification tests, 890
IDI. *See* Isopentenyl diphosphate-dimethylallyl diphosphate-isomerase (IDI)
Idioblasts, 1604
IgA nephropathy, 3142
IGF-binding proteins (IGFBPs), 2198
IgGa, 3215
Ileal smooth muscle, 3992
Illicit substances, 293
Illicit uses, 292
Imexon, 991
Imidazole, 412, 862–868, 870, 872–876
Imidazole alkaloids, 863, 872, 873, 878
Imidazole pyruvic acid, 872, 878
Immediate-type hypersensitivity reaction, 4071
Immobilised artificial membrane, 2115
Immobilization, 186, 193, 231
Immobilized cells, 2768
Immobilize plant cells, 105
Immune cells, 1375
Immune-modulating properties, 1621
Immune reactions, 1737
Immune response, 2325
Immune system, 1375, 1443, 3526
Immunoadjuvant, 3215
Immunoaffinity chromatography, 3861
Immunoaffinity column chromatography, 3330
Immunoassay, 692, 1063, 3338
Immunocompetent, 1275
Immunocompromised, 1275
Immunodeficiency, 1513, 2418
Immuno-enhancers, 1414
Immunoglobulin IgG, 3215
Immunomodulator, 3347, 3527
Immunomodulatory, 123, 2681, 3488
Immunomodulatory effect, 3449
Immunopotentiating, 2960
Immunoprecipitation assay, 3485
Immunostimulant, 3507
Immunostimulating properties, 991
Immunosuppressant, 991
Immunosuppressed, 1266
Immunosuppressive, 3642
Immunotoxicity, 3142
Impila, 1386
Implants, 1252
Improved cognition, 2414
Incarvillea arguta, 926
Incarvillea mairei, 926

Increased absorptive surface of the small intestine, 1426
India, 531
Indian Pharmacopoeia Commission (IPC), 3335
Indinavir, 1278
Indirect defences, 150, 2931
Indocybin, 524
Indole, 412, 534
Indole-3-acetic acid (IAA), 1830
Indole alkaloids, 537
Indoleamines, 524
Indole-3-carboxaldehyde (ICA), 29
Induced defense mechanisms, 1568
Inducible defense, 2931
Inducible nitric oxide synthase (iNOS), 1307, 1453, 2183, 3704
Induction/repression, 3820
Infant formula, 2447
Infant gut microbiome, 2442
Infant myeloid leukemia, 2422
Infectious diseases, 1628
Infertility problems, 2421
Infertility syndrome, 2417
Inflammation, 1443, 2624, 2625, 3477, 3523, 3525, 4115, 4117, 4118, 4120, 4127, 4131, 4132, 4134, 4140, 4141, 4147, 4149
Inflammation models, 1510
Inflammatory, 1737, 4119
Inflammatory mediators, 3217
Inflammatory molecules, 2406
Influenza virus, 1310
Infrared spectra, 3472
Inhibition of cell growth, 3448
Inhibitor of apoptosis (IAP), 2200
Inhibitor of drug metabolism, 1400
Inhibitors, 979, 980, 985, 987, 988, 991–994, 1000
Inhibitory concentrations, 3978
Inhibit the 5α-reductase, 2401
Inocybe, 527
iNOS. *See* Inducible nitric oxide synthase (iNOS)
Insecticidal activity, 397, 1253, 3488, 4009
Insecticide, 849, 4070, 4088
Insect resistance, 4070
In silico, 3332
In situ product, 2781
Insulin, 4144
Insulin-like growth factor 1 (IGF-1), 2399
Insulin receptors, 1304
Insulin resistance, 4114
Integerrimine, 1385
Integrated pest management (IPM), 4101, 4102
Interflavanic bond, 1758
Inter-individual differences, 2411
Inter-individual variations, 2416
Intermediate metabolizers, 1378
Intermediates, 56
Intermedine, 1059–1061
International Chemical Weapons Convention, 57
International evaluation, 3149
Internet market, 287
Intestinal bacteria, 2435
Intestinal brush border membrane fluidity, 1406
Intestinal microbiome, 2456
Intestinal microbiota, 2451, 2453
Intestinal microorganisms, 2435
Intestinal mucosa, 1307
Intoxications by herbal preparations, 360
Intracellular redox-sensitive mechanism, 2370
Intramolecular, 985, 1000
Intramuscular, 1379, 1380
Intranasal administration, 1252
Intraocular pressure, 3723
Intraperitoneal injection, 57, 1319
Intrathecal, 1370, 1380
Intravenous, 1319, 1370, 1379, 1380
Intrinsic pathway, 1313, 2199
Introduction, 2048
Inulin, 2454
Invasion capacity, 1458
Invasive malignant cells, 2404
Invasiveness, 1313
Inversely related, 2420
In vitro, 101–104, 108, 111, 917, 1686, 1691, 1704, 1706, 2592, 2596, 2598, 2600, 2602, 2765
  cultivation, 330
  culture, 470, 471, 474, 476
  cytotoxicity, 1512
  data, 2405
  reactions, 1625
  systems, 175
In vivo, 2591–2593, 2596–2599, 2602, 2605
Iochroma, 3467
Ion channels, 3994
Ion-exchange, 2114, 2150
Ion exchange electrolytic technique, 3197
Ionic charges, 1155
Ionic liquids, 1124, 1171
Ionic strength, 2116
Ionization, 2116

Ionone, 2989
α-Ionone, 2887, 2891, 2892
β-Ionone, 3588
Ionotropic receptor, 47
IPC. *See* Indian Pharmacopoeia Commission (IPC)
Ipecac, 132
Ipolamiidoside, 3055
Ipomoea asarifolia, 689
IPP. *See* Isopentenyl diphosphate (IPP); Isopentenyl pyrophosphate (IPP)
Iridodial diastereomer, 942
Irinotecan, 645, 650, 653, 664
biological activity, 664
semisynthesis, 653
Irrigation, 2390
Irritable bowel syndrome, 3656
Ischemia, 1306, 3704, 3722, 3723
Ischemia-reperfusion, 2327
Ischemia–reperfusion injury, 944
Ischemic, 3569
Ischemic brain injury, 3713, 3721
Ischemic damage, 2300
Ischemic heart disease, 3674
Isoamylamine (IAA), 2885
Isoboldine, 430
Isobutylaldehyde, 2887
Isobutylamine (IBA), 2885
Isobutyric acid, 2888
Isocaespitol, 2892
Isocaryophyllene, 2987
3-Isocorynantheol, 611
Isocratic, 2150, 3359
Isocratic elution, 58, 1086, 3378
β-Isocupreidine, 621
Isodehydrodigalloyl units, 1980
Isoeugenol (IEU), 2885, 2905, 2907
Isoflavone reductase, 1671
Isoflavones, 1653, 1729, 1828, 2151, 2155, 2160, 2435, 2456, 2568
Isoflavone synthase, 1655
Isoflavonoids, 1555, 1619, 1629, 1653, 1663, 1722, 1850
glycosides, 1663
Isolation, detection and characterization, 2051
phenolic compounds, 2051
Isoleucine, 851
L-Isoleucine, 365
Isomarchantin, 1918
Isomenthol, 2979
Isomerase, 3588
Isomeric compounds, 1029
Isomerism, 4066
Isomerization, 984, 991, 4024, 4028, 4033
Isomers, 1220, 1223, 1224, 3369, 4066
Isomitomycin A, 985
Isopentenyl, 2787
Isopentenyl diphosphate (IPP), 138, 2696–2698, 2705, 2819, 3175, 3198, 3571, 4038
Isopentenyl diphosphate-dimethylallyl diphosphate-isomerase (IDI), 2705
Isopentenyl pyrophosphate (IPP), 2672, 2735, 2740, 2741, 2943, 4090
Isoperrottetin, 1919
Isopilocarpine, 877
Isoplagilin, 1919
Isoplagiochin II, 1919
Isoprene, 2821, 2974, 3175, 3518
Isoprene rule, 2668, 2669, 3648
Isoprene synthase, 2720
Isoprene unit substituted bibenzyls, 1915
Isoprenoid, 2668, 2763, 2813, 2814, 2867, 2915, 2943
Isoprenoid engineering, 2815
Isopropanol, 1170
Isoquassin, 3782
β-Isoquinidine, 621
Isoquinoline, 412, 1303, 1369
Isoquinoline alkaloids, 132, 163, 237, 1073, 1166, 1170, 1175, 1183
Isorhamnetin, 1829, 2161
Isorhapontigenin, 1906
Isorhapontigenin oligomers, 1910
Isoriccardin, 1918
Isosafrole, 4065
Isosuspensolide F, 3025
Isotanshinone, 3555
Isothebaine, 431
Isothermal or gradient temperature GC, 1075
Isothiocyanate, 3592
Isotrichodermin, 3129
Isotrichodermol, 3129
Isotrichotriol, 3129, 3131
Isounedoside, 3016
Isovaleric acid (IVA), 2885
N-Isovalerylhistamine, 865, 876
Isoxanthohumol, 2343
IspC, 2699
IspG, 2701
IspH, 2701, 2703
Italy, 531
Ithomiines, 157, 162
Itraconazole, 1276
Ivermectin, 4067
IVHD-valtrate, 3049

Ixocarpalactone A, 3470, 3484
Ixocarpalactone B, 3484
Ixocarpalactones, 3470
Ixodes ricinus, 4069, 4070
Ixoroside, 3021
Ixoside, 3023

J
Jaborol, 3469, 3473
Jaborosa, 3467, 3469, 3472
Jaborosa bergii, 3472
Jabrol, 3469
Jabrosalactol 18, 3472
Jabrosalactone 37, 3472
Jabrosalactones 27, 3472
Jabrosalactones 28, 3472
Jabrosa rotacea, 3472
Jacobaea vulgaris, 155
Jacobine, 1061, 1064
Jacoline, 1061
Jaconine, 1061
Jacozine, 1061
Jamaicamide, 41
Jania spp., 59
Japan, 530
Japanese, 2422
Jasminum sambac, 2987
Jasmolactone B, 3032
Jasmolin I, 4063
Jasmolin II, 4063
Jasmonate, 1695, 2800
Jasmonic acid (Ja), 138, 1696, 1699, 1706, 4089
Jatamanin A, 3015
Jatrophane, 3180
Jaundice, 1385
Jetting races, 4074
Jimson weed, 291
Jiofuran, 3031
Jioglutolide, 3029
J774.1 macrophages, 1293
Jones oxidation, 3081
Josciamine, 291
Juglandaceae, 1552
Juice, 2582, 2594–2596, 2598, 2599, 2604, 2605
Jujubogenin, 3648
Julandine, 1455
Juniperus
  J. communis, 2975, 4095
  J. oxycedrus, 4095
  J. sabina, 2983

K
Kaempferol, 1655, 1658, 1729, 1824, 2346, 3501
Kalaazar, 1265
Kalanchoe blossfeldiana, 2951
Kale, 3928
Kalkitoxin, 41
Kangai-1, 2203
Kaposi's carcinoma, 2799
Kappa, 1371
Karenitecin, 666
Karrikin, 3595
Karrikin insensitive 2 (KAI2), 3597
Karyomegaly, 1386
Kaurane, 3179
Kaurene synthase-like (KSL), 3572
KB, 3478
KB-VIN, 3478
K_D, 2148
Keds, 4075
Keloids, 3965
Keratinocyte cell, 3966
Keratolytic, 1962
Ketocarotenoids, 1736
Ketoconazole, 1276
1-Ketol rearrangement, 2700
Ketones (KE), 2885
5-Keto-octanal, 896
5-Keto-octanoic acid, 896
Ketosynthase, 49
Kidneys, 2397, 4117, 4121, 4139, 4149
Kind of tumor, 2404
Kjellmaniella crassifolia (Saccharina sculpera), 2899
Klebsiella pneumoniae, 984, 989
Knockdown, 4070, 4077, 4078
Kopsia griffithii, 1288
Kucha, 956
Kudzu root, 1852
Kutkoside, 3048, 3050
Kynurenic acid, 3699, 3711

L
Labdane, 3182
Labeled resveratrol, 2293
Labiatae, 3467
Lactarius indigo, 2988
Lactase phloridzin hydrolase, 2478
Lactic acid, 1608

Lactone, 1784, 3472
δ Lactone, 3472
Lactone ring, 3470
Lactonifactor longoviformis, 2446
Lactuca, 3530
Ladybirds, 157, 162
Lambda-cyhalothrin, 4070, 4072
Lamiaceae, 3071, 3087, 4089
Lamiide, 3024
Laminariaceae, 2899
Laminariales, 2898–2900
Laminaria spp., 2899
  L. angustata (Saccharina angustata), 2899
  L. digitata, 2899
  L. hyperborea, 2899
  L. japonica (Saccharina japonica), 2899
  L. saccharina (Saccharina latissima), 2899
Laminarin, 1694
Lamiridosins A, 3056
Lamiridosins B, 3056
Land plants, 1545, 3252–3254, 3256–3259, 3263, 3264, 3266
Lanosol, 2893
Lanostanol, 3237, 3243
Lanosterol synthases, 2724, 3243
Lappaconitine, 932, 1507, 1508
Large-scale, 226, 230, 1691, 1706, 2962
Large-scale production, 111, 215, 245
Large-scaling, 107
Lariciresinol, 2450
Larix decidua Mill, 1549
Larnax, 3467
Larvicidal activities, 1211
Laser induced fluorescence (LIF), 1105, 1168, 1170, 1176, 1185
Laserpitium
  L. siler, 2981
  L. zernyi, 2986
Laser trilobum, 2976, 2981
Late hopping, 2343
Latent PCa, 2409
Latex, 2824
Lathyrane, 3180
Laticifer cells, 98
Laudanosine, 443
Laudanum, 1368
Lauraceae, 1558, 3530, 4089
Laurelia sempervirens, 4094
Laurencia
  L. brongniartii, 32
  L. caespitosa (Osmundea hybrida), 2892
  L. cartilaginea, 31
  L. decumbens, 32
  L. dendroidea, 2893, 2906
  L. glandulifera, 2893
  L. majuscula, 31
  L. nipponica, 2893
  L. similis, 32
Laurus nobilis, 4091
Lavandula spp., 4094
  L. angustifolia, 2979
  L. stoechas, 2984
LC/APCI-MS, 3145
LC-ESI-MS/MS, 1026, 3145
LC-FLD, 1137, 1145, 1146, 1150
LC-MS. *See* Liquid chromatography-mass spectrometry (LC-MS)
LC-MS/MS, 2054, 3144, 3145, 3474, 3592
  hybrid systems, 3360
  and LC-ESI-MS, 2053
LD_{50}. *See* 50% Lethal dose (LD_{50})
LD_{50}/ED_{50} ratio, 1508
LDL. *See* Low-density lipoproteins (LDL)
LDLR. *See* Low-density lipoprotein receptor (LDLR)
LdNT1, 1269
LdNT2, 1269
LdRos3, 1275
Leacadine, 991
Leaf-opening factors, 1567
Learned tolerance, 1373
Learning rate, 3654
Ledum palustre, 2999
Legumes-bacterium symbiosis, 1621
Leguminosae, 1554, 1869, 1870, 1877, 1883, 1906, 2388, 3467
Leishmania, 1294, 4125, 4149
  HIV, 1278
  HIV co-infection, 1265
  L. donovani, 1310
  L. infantum, 4071
Leishmaniasis, 816, 1264, 1295, 1319, 4071
Leishmanicidal, 1286
Lepeophtheirus salmonis, 4076
Leprosy, 3477
Leptin, 3812
Lespedezavirgatol, 1934
Lessoniaceae, 2900
Lethal dose, 51
50% Lethal dose (LD_{50}), 1319, 3136, 4099
Leucine, 837
(2*S*)-Leucine, 367
Leucine-rich repeat receptor kinase (LRR-RK), 3856
Leucoanthocyanidin reductase, 1656
Leucoanthocyanidins, 1762

Leucojum aestivum, 1335
Leukemia cell lines, 3561
Leukemias, 1315, 1512
Leukopenia, 993
Leukotrienes, 3679
Levodopa, 1348
Levothyrox, 2422
Lewis lung carcinoma, 988
LH. *See* Luteinizing hormone (LH)
Liberine, 956
Libido, 1374
Lice, 4064, 4069, 4075, 4076, 4083
Licorice, 1874, 3805
LIF. *See* Laser induced fluorescence (LIF)
Life cycle, 704
Lifespan, 2300
Life styles, 2409
Ligand-gated, 600
Light, 230, 2342, 3591
Light saturation, 1601
Lignans, 1557, 2435, 2456
Lignin, 1561
Ligstrohemiacetal B (66) (revised structure of jasmolactone A), 3032
Ligstroside, 3041, 3052
Liliaceae, 1554
Liliopsida, 979
Limonene (LIM), 2787, 2885, 2976, 2977, 2980, 2997, 2999, 3430, 4090, 4096, 4112, 4114, 4115
Limonoids, 3477, 3776
Linalool (LI), 2885, 2905, 2977, 2979, 2993, 2999, 4089, 4090, 4096, 4121, 4128–4130
Linalyl acetate, 2979
Linalyl diphosphate (LPP), 2720
Lindane, 4074
Linear polyprenyl diphosphates, 2708
Linker peptide, 1659
Linognathus vituli, 4069
α-Linolenic acid, 1736
Linum usitatissimum, 3587
Lipidic tissues, 1082
Lipid peroxidation (LPO), 2466, 3653
Lipids, 2321
  deposition, 3811
  membrane, 3981
Lipo-alkaloids, 1517
Lipofuscin, 3933, 3937, 3940, 3943–3948
Lipogenesis, 3814
Lipopeptides, 41, 1292
Lipophilicity, 2109, 3594
Lipophosphoglycan (LPG), 1269
Lipopolysaccharide (LPS), 3964, 4117, 4118, 4134, 4147, 4148
Lipoprotein lipase (LPL), 3808
Liposome, 354
Lipoxygenases (LOXs), 2183
Lippia alba, 2981
Liquidambar formosana, 1990
Liquid chromatography, 1022, 3386
Liquid chromatography coupled to tandem mass spectrometry (LC-MS^2), 52, 63
Liquid chromatography-mass spectrometry (LC-MS), 1052–1055, 1058–1061, 1064, 1137, 1139–1141, 1144–1150, 3337, 3339, 3359, 3360, 3398, 3860
Liquid chromatography-mass spectrometry (LC-MS) system, 324
Liquid-liquid extraction (LLE), 177, 691, 1084, 1106, 1123, 2071, 2112, 3313
Liquid-liquid phase separation, 2033–2034
Liquid-phase extraction, 3354
Liquiritigenin, 1660
Liquor production, 1610
Litter size, 2421
Littoralisone, 3033, 3058
Littorine, 178
Liver, 2397, 4114, 4125, 4126, 4128, 4140, 4149
  enzymes, 3820
  lesions, 1387
Liver cirrhosis, 373
Liverworts, 1920, 3585
LLE. *See* Liquid-liquid extraction (LLE)
L1210 leukemia cells, 982, 983, 993
LNCAP, 3478
LO A and LO B, 1212
LOAEL, 3136, 3137, 3139, 3140
Loasaside, 3016
Lobelane, 334, 337
Lobelanidine, 324, 328
Lobelanine, 318, 324, 337
Lobelanine isomers, 329
Lobelia
  L. dortmanna, 321
  L. inflata, 304, 305, 320–322, 324, 325, 329–332, 336, 337
  L. urens, 321
Lobelia alkaloids, 320, 322
Lobelia piperidine alkaloids, 305
Lobelidine, 328
Lobeline, 304, 318, 323–325, 328, 330–336, 338, 1348
Lobophora variegata, 2895
Local anesthesia, 65

Local anesthetic, 3996
Localization, 1690
Loganiaceae, 590, 725
Loganic acid, 3022, 3047, 3056
Loganin, 90, 109, 3040, 3047, 3053, 3054, 3056
Logoniaceae, 1553
Lolium
  L. arundinaceum, 164
  L. perenne, 864
  L. pratense, 164
Lomentaria catenata, 2891
Longifolene, 3521
Long Term Potentiation (LTP), 2406
Lonijaposide C, 3053
Lophocladia spp., 34
Lophocladine (LO) A, 34
Lophocladine (LO) B, 34
Lotus japonicus, 3587
Low density lipoprotein cholesterol (LDL-C), 3668, 3669, 3672, 3673, 3682, 3683
Low-density lipoprotein receptor (LDLR), 1305
Low-density lipoproteins (LDL), 1305, 2298, 2591, 2595–2597
  oxidation, 3670, 3672, 3683
Lower, 2406, 2421
  blood concentrations of carotenoids, 3453
  incidence, 2409
Low larkspurs, 159, 162
Low-observed-adverse-effect level (LOAEL), 3136
Low-risk products, 4097
Low temperature stress, 1565
LPP. *See* Linalyl diphosphate (LPP)
LPS. *See* Lipopolysaccharide (LPS)
LSD. *See* Lysergic acid diethylamide (LSD)
LTP. *See* Long Term Potentiation (LTP)
L-type calcium channels, 63
Luche reduction, 278
Lukemia cells, 3478
Lumbago, 1305
Lumbar spine, 2414
Lunasia, 723
Lung
  cancer, 2210
  carcinoma, 1316, 1836
  lesions, 373
Lung carcinoma (A-549), 3479
Luotonin A, 658
Lupanine, 383, 1177
Lupin alkaloids, 382
Lupinine, 389
Lupinus, 383
  L. albus, 387, 3584
  L. angustifolius, 387
  L. luteus, 387
  L. mutabilis, 388
Lurtotecan, 666
Lutein (L), 3254, 3256, 3264, 3265, 3369, 3925, 3926, 3928–3931, 3933–3937, 3939, 3941–3944, 3948, 4021, 4024, 4031
Luteinizing hormone (LH), 1374
  production, 2418
  secretion, 2417
  surge is prevented, 2418
Luteolin, 1827
Luteolinidin, 1569
Lutzomyia, 845
Luvunga, 766
Luzonoid glycosides, 3050
Luzonoids A-D, 3050
Lyaloside, 136
Lycium, 3467
Lycium cestroides, 4094
Lycodine, 1242
Lycodoline, 1248
Lycopene, 1730, 1731, 1736, 1836, 2651, 2652, 2787, 2830, 2855–2858, 2860–2870, 2873–2875, 3369, 3875–3910, 4019, 4022, 4023, 4031
Lycopersicon esculentum, 2787, 4098
Lycopodiaceae, 1241, 1243
Lycopodiales, 1241
Lycopodine, 1242
Lycopodium alkaloids, 1239, 1242, 1247, 1257
Lycoposerramine H, 1248
Lycopsamine, 1059–1062, 1064
Lycoriella america, 4094
Lycorine, 484, 1532
Lymphocytes, 991, 4113, 4118
Lymphomas, 1315
Lyngbya wollei, 54, 59
Lyophilization, 4027
Lyophilization (freeze-drying), 3593
Lysergic acid, 1135–1137, 1140–1143, 1146, 1148
Lysergic acid diethylamide (LSD), 542, 693, 1135, 1137, 1140, 1143, 1147, 1149
Lysine, 318, 416, 1455
L-Lysine, 305

M
Maceration, 3290
  cold extraction method, 3667

Macfadyena unguis-cati, 4094
mAChR. *See* Muscarinic acetylcholine receptors (mAChR)
Macrocyclic bisbibenzyls, 1920
Macrocyclic diesters, 363
Macrocyclic musks, 3300
Macrocyclic polyoxide, 988
Macrophages, 3217, 3964
Macula, 3925, 3928–3935, 3937, 3939, 3940, 3942, 3944, 3948
Macular degeneration, 1444
Macular pigment (MP), 3925, 3928–3937, 3941–3944, 3948
Macular pigment optical density (MPOD), 3930–3935, 3937, 3941, 3942
MAD. *See* Mevalonate-dependent (MAD)
Madecassic acid (MA), 3961
Madecassol®, 3961
Madecassoside (MS), 3961
Madurastatin B1, 986
Maduropeptin (MDP), 988
MAE. *See* Microwave-assisted extraction (MAE)
Magic mushrooms, 526
Magnoflorine, 11, 20
Magnoliaceae, 1558
Makaluvamine C, 257
Makaluvamine D, 257
Makaluvamine F, 255
Makaluvamines A, 257
Malaria, 626, 816, 3100, 3101, 3105, 3114, 3783, 3795
MALDI. *See* Matrix-assisted laser desorption/ionization (MALDI)
Male babies, 2418
Male behavior, 2421
Males react more sensitive than females, 373
Malonate/acetate pathway, 1545
Malondialdehyde (MDA), 3682
Malondialdeide (MDA), 3699, 3704, 3705, 3718, 3721
Malonyl-CoA, 61, 1761, 1825, 1925
Malt kilning, 2347
Malts, 2334, 2343, 2346, 2350
Malus domestica Borkh, 1566
Malvaceae, 802
Malvidin, 2467
6-MAM, 1082
Mammalian cell culture, 1652
Mammalian predators, 2390
Mammary adenocarcinoma, 994
Mammary epithelial cells, 3566
Mammary gland adenoma, 2421
Mammary gland tumor, 991
Management, 543
Mandelin, 535
Manduca sexta, 4096
Mangifera indica, 1553
Mangiferin, 1553
Mannich cyclisation, 50
Mannosyl transferases (MTs), 1270
Manzamine-type, 1293
MAO. *See* Monoamine oxidase (MAO)
MAPKs. *See* Mitogen-activated protein kinases (MAPKs)
Mappicine, 647
Mappicine ketone, 647, 652
Marchantin, 1918
Marchantinquinone, 1936
Marine invertebrates, 980
Marine organisms, 978
Marine pyrroloiminoquinone alkaloids, 253
Marine sponges, 253, 980, 986, 987, 1291
Market trends of nutraceuticals, 2574
Markofane, 992–993
Marquis, 535
Martefragin (MRF) A, 29, 1212
Martensia
  M. denticulate, 31
  M. fragilis, 29
Martensine A (MRT A), 29, 1212
Martensine B (MRT B), 29
Masculinization, 2418
Massadine, 268
Mass spectral fragmentation, 178
Mass spectrometric detection, 2047
  GC/MS, 3144
  phenolic acids, 2047
Mass spectrometric/spectrometry (MS), 535, 1038, 1051, 1057, 1060, 1086, 1125, 2052, 2080, 2121, 2512, 2514, 3195, 3359, 3397, 3473, 4030
  detectors, 1024, 1076
  EI, 2053
  GC-MS, 2052
Matairesinol, 2450
Maté *(Ilex paraguariensis)*, 955
Matricaria recutita, 2985
Matrigel, 1445
Matrine, 1456
Matrix-assisted laser desorption/ionization (MALDI), 3398
  mass spectrometry, 3339
  TOF MS, 2055
  TOF MS spectra, 2056
    phenolic acids, 2056

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), 3401
Matrix effects, 1062, 1147, 2321
Matrix metalloproteases (MMPs), 2201
MAX1, 3588
MAX2, 3596
Maxi chloride channels, 4067
Maysin, 1826
MDA-MB-231 cells, 3485
mDP. *See* Mean degree of polymerization (mDP)
MDR. *See* Multidrug resistance/resistant (MDR)
Mean degree of polymerization (mDP), 2267
Mechanical cold pressing, 4090
Mechanism of action, 1515, 3488
Mechanism of ati-tumor ativities, 3485
Mechanism of burning and painful sensation, 1427
Meconic acid, 434, 441
Meconium, 1083
Medicagenic acid, 3207, 3219
Medicago
  M. sativa, 3595
  M. truncatula, 3587
Medical survey, 2410
Medicarpin, 1569
Medicosma, 734
Mediterranean diet, 2362, 3607
Medium optimization, 229
Medium pH, 229
MEEKC. *See* Microemulsion electrokinetic chromatography (MEEKC)
Mefloquine, 629
MeJA/MeJa. *See* Methyl jasmonate (MeJA/MeJa)
MEKC. *See* Micellar electrokinetic chromatography (MEKC)
Melaleuca
  M. alternifolia, 2980, 4099
  M. quinquenervia, 4094
  M. viridiflora, 2980
Melanins, 1562
Melanocyte proliferation, 1414
Melanoma, 1316, 1836, 3483
  B16F10, 3480
  SKMEL-28, 3480
Melatonin, 2583, 2590, 2593, 2594, 2598
Meliaceae, 850
Melicope, 720
Melissa officinalis L., 2978, 2979, 4128
Memantine, 1244
(7+5)-Membered bicyclic system, 943
Membrane estradiol receptors (mERs), 2399
Membrane fluidity, 2326
Membrane separation, 4032
Memory, 2414, 2415, 3643, 3655, 3703, 3707, 3709, 3713, 3717, 3720–3722
  enhancer, 3219
  function, 3219
Meniere's syndrome, 588
Menispermaceae, 749
Menopausal symptoms, 1858
Menopause, 2408
Mentha genus, 3990
p-Menthane-3,8-diol, 4089
Mentha spp., 2780, 2956, 4094
  M. aquatica, 3000
  M. arvensis, 4122
  M. piperita, 4091, 4122, 4128
  M. pulegium, 2981, 2982, 2998, 4099
  M. spicata, 2978
  M. x piperita, 2980
1-*p*-Menthene-8-thiol, 2994
Menthofuran, 2821, 2982, 2998, 3000, 4099
Menthol, 2734, 2735, 2979, 2980, 4089, 4090, 4121–4124
(+)-Menthol, 2676
Menthone, 4090
Mentzeloside (Deutzioside), 3016
MEP. *See* 2-*C*-Methyl-D-erythritol-4-phosphate (MEP)
MEP/DOXP pathway, 2734, 2741
Meroterpenes, 2737
mERs. *See* Membrane estradiol receptors (mERs)
Mesaconitine, 1170, 1508
Mesembrine, 1168
Meso-zeaxanthin (MZ), 3925, 3926, 3928–3931, 3937, 3941, 3944, 3948
Meta-analysis, 2408, 3669, 3676, 3705, 3709, 3717, 3718, 3723
Metabolic, 1686, 1688, 1691, 1695
  conversion, 2292
  costs, 1570
  engineering, 6–11, 14, 21, 88, 101, 108, 109, 111, 215, 232, 235–238, 240, 243, 245, 1593, 1626, 1650, 1686, 1691, 1703, 1707, 1837, 2826, 2954, 3102, 3105–3106, 3108
  flux, 232, 233, 236
  networks, 2456
  node, 1633
  pathways, 1718, 4069
  toxication of PAs, 367
  toxication process in the liver, 367
  transformation, 2398

Metabolic activation, 370
Metabolic syndrome (MetS), 3808, 4114
Metabolism, 544, 1272, 1317, 2321, 2411, 2438, 2814, 3616
Metabolism of 1,2-saturated pyrrolizidine alkaloids, 372
Metabolite/father compound ratios, 1099
Metabolomic, 2453
Metabolon, 1661
Metagenomic, 2445
Metal cofactors, 4044
Metallacyclopropane mechanism, 2704
Metallic ions, 1808
Metamphetamine abuse, 338
Metastasis, 3525, 3527, 3562
Metatranscriptomic, 2452
Meteloidine, 350
Methadone, 1082
Meth A fibrosarcoma, 988
Methamphetamine abuse, 334
Methamphetamines, 334, 912
Methanethiol, 2887, 2892, 2897, 2899
Methicillin-resistant *Staphylococcus aureus* (MRSA), 3429–3430
Methiin, 3666, 3667
7-Methoxyapigeninidin, 1569
(E)-4-Methoxycinnamoyl, 3210
8-*O*-*E*-p-Methoxycinnmoyl harpagide, 3058
Methoxy-5-deoxystrigol, 3595
Methoxygaertneroside, 3054
1β-Methoxygardendiol, 3047
5-Methoxyluteolinidin, 1569
1β-Methoxymussaenin A, 3046
4-Methoycinnamoyl, 3207
Methylamine (MA), 2885
Methylanthraniloy, 821
Methylation domain, 51
1-Methyl-aziridine, 979
Methyl bromide, 4100
1-*O*-Methylcachinol, 3051
Methylcatalpol, 3051
Methylcholanthrene, 1315
N-Methylconiine, 893
2-*C*-Methyl-ᴅ-erythritol-4-phosphate (MEP), 2671, 2698, 2742, 2818, 2828, 2915, 2943, 3571, 4038
N-Methyl-Δ^1-pyrrolinium cation, 182, 344
Methylecgonine, 203, 351
3-*O*-Methylellagic acid 4′-*O*-β-ᴅ-glucopyranoside, 2164
Methylenedioxy group, 936
Methylephedrine, 1219
Methyl-erythritol 4-phosphate (MEP), 90
Methylfurfural (MFU), 2885
3-Methyl–2H-azirine-2-carboxylic acid, 979
Methyl jasmonate (MeJA/MeJa), 92, 94, 98, 106, 107, 109, 1690, 1696–1699, 1703, 1706, 1707, 2784, 2954, 2956, 2958, 4045
Methylliberine, 956
Methylmercaptan, 2899
2-Methyl-mercapto-propylamine (MMPA), 2885
N-Methyl-mitomycin, 984
N-Methylmitomycin A, 984
Methyl orange, 1063
N-Methylpelletierine, 305, 306, 337
2-Methyl-2-pentanal, 2887
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 3699, 3710, 3718
N-Methyl porfiromycin, 983, 984
Methylpseudoephedrine, 1219
N-Methylputrescine oxidase, 182
Methylpyrazines, 1610
Methylscutelloside, 3051
Methyltransferase, 1655, 1670
N-Methyltryptamine, 540
Methyluric acids, 954, 956, 961
Methylxanthines, 954, 1178, 1180, 1182
7-Methylxanthosine synthase, 959
Metopolophium dirrhodum, 4094
Metrodorea, 736
Mevalonate-dependent (MAD), 2741
Mevalonate pathway, 2696, 2697, 2734, 2741, 2763, 2803, 3175, 4090
(R)-Mevalonic acid, 2697
Mevalonic acid (MVA), 2818, 2828, 2991, 3518, 3529, 3574, 4038
Mevalonic acid (MVA) pathway, 2671, 3101, 3102
M-3-G. *See* Morphine-3-glucuronide (M-3-G)
M-6-G. *See* Morphine-6-glucuronide (M-6-G)
MGT. *See* Multigene transfer (MGT)
MHC. *See* Minimum hydrotrope concentration (MHC)
MIC. *See* Minimum inhibitory concentration (MIC)
Micellar electrokinetic chromatography (MEKC), 1104, 1177
Micellar incorporation, 3446
Michaels additions, 3072, 3075, 3077
Microangiopathy, 3969
Microbial, 1704
  biosynthesis, 1626
  biotransformation, 919

Microbial (*cont.*)
  cell factory, 1625
  pathogens, 3976
  production, 3–21
  system, 5, 21
Microbiome, 2435, 2479
Microbiota, 2472, 2475, 2479–2481
Micrococcus luteus, 986, 989
Microcrystin LR, 1835
Microemulsion electrokinetic chromatography (MEEKC), 1179
Microencapsulation, 2773
Microglia, 2289, 2624, 2625
Micromelum, 752
Micromonospora, 984
Micronesia, 980
Micronutrient, 1732
Micropropagation, 3197
microRNAs, 2205
Microsomal enzymes, 968
Microsporum gypseum, 4125
Microtubules, 588, 2957, 2959
Microwave-assisted extraction (MAE), 1016, 2028–2032, 2037, 2069, 3331
Microwave irradiation, 276, 3331
Microwaves, 3613
Migraine attacks, 697
Migrant studies, 2409
Migration, 1446, 3560
Milk, 361, 4080
Miltefosine, 1275
Miltiradiene, 3573
Miltirone, 3573
Minabeolides, 3467
Minabolides-1, 3480
Mineralocorticoids, 3808
Minimal lethal dose, 47
Minimal mental scores, 2410
Minimal risk level (MRL), 4080
Mini Mental State Examination (MMSE), 1253
Minimum hydrotrope concentration (MHC), 3331
Minimum inhibitory concentration (MIC), 2676
Minthostachys mollis, 4094
Miosis, 1373
Miraziridine A, 986–988
Miscarriages, 2418
Mist bioreactor, 188
Mites, 4064, 4069, 4070, 4075, 4076, 4083
Mitiromycines, 983
Mitochondrial dysfunction, 3715
Mitochondria maintenance, 2406
Mitogen-activated protein kinases (MAPKs), 2187, 3570, 3748, 3749
Mitomycines, 985, 986
Mitosane ring, 985
Mitsui Petrochemical Industries, 2769
Miyabenol, 2281
MMPs. *See* Matrix metalloproteases (MMPs)
MMSE. *See* Mini Mental State Examination (MMSE)
Mobile phase, 3315, 3328, 3329, 3336, 3385
Moderate soy-food intake, 2416
Modern biotechnology, 1737
Modest reduction of PSA, 2413
Modular pathway engineering (MOPE), 3574
Modulation in membrane dynamics, 1425
Modulation of dopaminergic transmission, 1207
Mogolicain A, 1999
Molecular
  absorption, 1086
  biology, 1294
  deficit, 1477–1478
  distillation, 4033
  indicators, 1602–1603
  ions, 325, 3397
  markers, 2804
  mechanism, 3883, 3886
  modeling, 1246
  simulation, 3332
  weight, 3398
Molecularly imprinted polymers (MIPs)-based extraction, 1123
Molluscicidal, 816
Monitored, 2416
Monnieria, 737
Monoacylglycerol (MAG) lipase, 3425
Monoamine oxidase (MAO), 8, 10, 17, 21, 1352, 3699, 3710, 3711, 3718
Monoamine oxidase-A, 1309
Monoamine oxidase inhibitory, 1352
Monoclonal antibody, 45
Monocrotaline, 1061–1064
Monocyclic, 3369
Monocyte-macrophage, 1590
Monocytic leukemia cells, 3560
Monodesmoside, 3227, 3233
Monodesmosidic, 3233
Monoesterified xanthophylls, 3373
Monoglycerides, 994
Monohydroxy pigment (MHP), 3376
Monolithic, 2114–2116, 2125
Monomeric phenanthrenes, 1923
Monomers (+)-catechin, 2256
Monostroma

M. fuscum, 29
M. nitidum, 2902
Monostromataceae, 2902
3-Monosubstituted tropanes, 347
Monoterpene alcohols, 2918
Monoterpenes, 2780, 3993, 4089
Monoterpenoid alkaloids, 929
Monoterpenoid indole alkaloids (MIAs), 220
Monoterpenoids, 2720, 2735, 2946, 2956
Mono-triterpenes, 2670
Monotropein, 3023
Mood disorders, 2414
Moraceae, 1551
More convincing results, 2411
mOR-EG, 4008
Mormon, 2409
Morphine, 6, 11–13, 429, 430, 434, 1073, 1169, 1170, 1172, 1181, 1317, 1369–1371, 1373–1377, 1379, 1380, 3426, 3647
Morphine biosynthetic pathway, 428
Morphine-3-glucuronide (M-3-G), 1376
Morphine-6-glucuronide (M-6-G), 441, 1376
Morroniside (isolated as 7α/7βmixture), 3028
MPTP. *See* 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)
MRF A. *See* Martefragin A (MRF A)
MRL. *See* Minimal risk level (MRL)
MRM. *See* Multiple reaction monitoring (MRM)
MRT A. *See* Martensine A (MRT A)
MS. *See* Mass spectrometric/spectrometry (MS); Multiple sclerosis (MS)
MS/MS, 332
mTOR signaling, 2196
MTS viability assay, 3480
Mu, 1371, 1373, 1377
Mucosicola, 2444
Multianalyte methods, 1149
Multi-component defense response, 1569
Multidrug resistance/resistant (MDR), 317, 335–337, 1310, 2214, 3188
Multidrug-resistant tumor, 338
Multiflorine, 385
Multigene transfer (MGT), 2833
Multimerized transcriptional enhancers, 2953
Multiple congenital contracture, 901
Multiple reaction monitoring (MRM), 1060, 1061, 1147, 3592
Multiple reaction monitoring (MRM) mode, 1027
Multiple sclerosis (MS), 2624, 3426
Multiple-stage MS, 3316
Multiple-Step Engineering, 235
Multiple targets, 1295
Multistage mass spectrometry, 3239
Multi-target approach, 1257
Multitargeting phytochemicals, 2225
The Mu-1 opioid receptor subtype (OPRM1), 1377
Murashige and Skoog, 2783
Murraya, 740
Musca domestica, 4075
Muscarine, 1343
Muscarinic acetylcholine receptors (mAChR), 1248, 1333
Muscarinic receptor, 3710
Muscarinic receptor ligands, 1343
Muscle, 64, 4080
CK, 3820
contraction, 2906, 2907
paralysis, 47
Mushrooms, 979
Musk compounds, 3299
Musks, 3293
Mussaenosidic acid, 3022
Mutagenic activities, 978
Mutagens, 4119
Mutants, 1666
Mutasynthesis, 239, 1664
Mutation, 2858, 2860, 2861, 2868, 2870
Mutualist, 2925
MVA. *See* Mevalonic acid (MVA)
Mycelial networks, 2646
Myc factor, 3595
Mycology, 3125–3127
Mycorrhizal dependency, 2647
Mycorrhizal inoculation, 2651, 2652
Mycorrhizal symbioses, 2645
Mycotoxins, 2782, 2975
Mycotoxins like aflatoxins, 374
Myenteric plexus, 4069
Myocardial infarction, 3668, 3678, 3683
Myotonia, 4067
Myrcene, 4120
Myricetin, 1828
Myristica fragrans, 2980, 2984, 4089
Myrtaceae, 3467, 4089
Myrtopsis, 733
Myrtus communis, 2979
Myxobacterium, 990
Myxol glycoside, 3254, 3258, 3267, 3268
Myxol 2′-glycoside, 3256
Myzus persicae, 4094

N

N^{α}-acetylhistamine, 865
NACE-MS, 1034
Na^{+} channels, 1515
N^{α}-cinnamoylhistamine, 865
N^{α}-cinnamoyl-N_{π}-methylhistamine, 866
Nacional, 1601
NADPH, 3712
NADPH-dependent reductase, 893
Nagelamide, 268
Na^{+}/K^{+}ATPase, 3746–3751, 3753
Naloxone, 1373
N^{α}-methylhistamine, 864
N^{α}, N^{α}-dimethylhistamine, 864
Naphthalene dioxygenase, 1670
1-Naphthoic acid, 988
Naphthoquinones, 1552
Naphthylisoquinoline, 1286
2,7-Naphthyridine derivatives, 34
Narceine, 434, 440, 1073
Narciprimine, 1533
Narcissus, 1335
Narcotine, 1073, 1169, 1170, 1181
Narcotoline, 442
(2S)-Naringenin, 1658
Naringenin chalcone, 1762
Naringin, 2570
Native precursors, 1636
Native producers, 1640
Natural, 1961
  bases, 691
  compounds, 1391
  and hybrid biosynthetic pathways, 1640
  perfumes, 3290
  product modification, 1625
  products, 978, 980, 982, 984, 987, 990–1002
  pyrethrins, 4063
  sweeteners, 3194
  and unnatural flavonoids, 1633
Naucledal, 3031
Nausea, 1374, 1380
N-1-β-D-ribofuranosylmakaluvamine I, 255
NBT-272, 3785
NCI-H460 cell lines, 3479
N^{6}-(D^{2}-isopentenyl)adenine, 958
N^{6}-(D^{2}-isopentenyl)adenine-type cytokinins, 956
Near-infrared spectroscopy (NIRS), 4031
Necic acids, 370
Necrosis, 373, 1385, 3478
Nectriaceae, 165
Negative inotropic effect, 1511
Nemaliales, 2887
Nematolepis, 734
Neocarzinostatin producer, 990
Neodictyoprolenol, 2895
Neoflavenes, 1555
Neoflavonoids, 1555, 1620
Neoisomenthol, 2979
Neolignans, 1557
Neomarchantin, 1918
Neomenthol, 2979
Neonatal T, 2419
Neopetrosia, 1291
Neophysalin, 3468
Neopinone, 430
Neoplasm inhibitor, 985, 991, 992, 995, 1000
Neoquassin, 3347, 3782
Neoraputia, 740
Neosartorya, 807
Neosaxitoxin (neoSTX), 53, 54
Neosenkirkine, 1385
NeoSTX. *See* Neosaxitoxin
Neo-tanshilactone, 3556, 3564
Neotyphodium, 689
Neotyphodium coenophialum, 164
Neovascularization, 1448, 2300
Neoxanthin, 3390
Nepalensinols, 1933
Nepenthaceae, 1552
Nepetalactone, 2982, 4089
Nephropathy, 4117, 4149
Neral, 2979
NERICA. *See* New Rice for Africa (NERICA)
Nerol, 2979
Nerolidol, 2682, 4124, 4125
Nerolidyl diphosphate (NPP), 2723
Nerve-action current, 4097
Nerve-action current firing, 4097
Nerve cell, 3646
Nerve conduction, 4005
Nerve membrane, 4066
Nervous system, 4065
Nervous tissues, 4069
Netherlands, 533
Neuralgic pain, 1507
Neurite elongation, 3967
Neuroblastoma, 57, 1208
Neurodegeneration, 1526
Neurodegenerative, 1814
  diseases, 1257, 1331, 1333, 1334, 1355, 1621, 2278, 3627, 3703, 3727
  disorders, 3488
Neurofibrillary, 2799
Neurofibrillary tangles (NFT), 3712, 3716

Neuroinflammation, 2619, 2623–2625, 2630
Neuroinflammatory processes, 2291
Neurological effects, 393
Neuromodulator, 1206, 1208
Neuromuscular junction, 47
Neurons, 2406, 2619, 2623, 2624, 2627–2629, 3645, 3967, 3996
Neuropathic pain, 3427
Neuropathies, 4079
Neuropeptides, 3709
Neuroprotection, 3428, 3701
Neuroprotective, 123, 1249, 1940, 2406, 3627, 3966
  activity, 3627
  effects, 1257, 2482, 3220, 3710, 3718, 3727
  properties, 2291
Neuropsychological, 3654
Neurothrophic activity, 3219
Neurotoxic, 41
Neurotoxicity, 4096, 4097
Neurotoxins, 51, 898, 3714
Neurotransmitters, 599, 3709, 3710
Neurotransmitter serotonin, 534
Neurotrophic, 2406
Neurotrophic factors, 2620
Neurotropic fungi, 527
New Rice for Africa (NERICA), 3595
NFCS. *See* Nonfat cocoa solids (NFCS)
NF-kB. *See* Nuclear factor kappa B (NF-kB)
Nicandra, 3467
Nicandra physaloides, 3469
Nicandrenone, 3469
NICI ionization, 1076
Nicolaou's, 2801
Nicotiana, 1345
Nicotiana tabacum, 979, 3587
Nicotine, 151, 159, 160, 163, 1345, 1355, 4122, 4123
Nicotine-induced toxicity, 3645
Nicotinic acid, 416
Nicotinic receptor ligands, 1345
Nicotinic receptors (nAChRs), 47, 334, 1248, 1333
Nictitating, 899
Niemann-Pick C1-like 1 (NPC1L1), 3444, 3446
Nigella sativa, 2981
Nijmegen-1, 3597
Ninhydrin, 915
Nitidine, 1170
Nitrareaceae, 787
Nitric oxide (NO), 1832, 2363, 3672, 3674, 3682, 3686, 3699, 3704, 3712, 3713, 3721, 3965
Nitrite oxide (NO), 3217
Nitrogen-containing compounds, 2667
Nitrogen-phosphorus detection (NPD), 1057, 1064, 1076
Nitro musks, 3300
Nitrone functionality, 936
Nitrosodiethylamine, 1315
Nitto Denko Co., 2770
N-methyl D-aspartate receptor (NMDA), 3699, 3711, 3716
N-methyl-D-aspartic acid (NMDA) receptor, 1373, 1374, 4006
  antagonism, 1249
  antagonist, 1246
N-methyl-4-dimethylallyltryptophan, 699
NMR. *See* Nuclear magnetic resonance (NMR)
NO. *See* Nitric oxide (NO); Nitrite oxide (NO)
NOAEL. *See* No observed adverse effect effect-levels (NOAEL)
Nocardia farcinica, 1635
Nociceptive, 1371, 1374
Nodulation, 3595
Nodulation signaling pathway1 (NSP1), 3596
Nodule formation, 1566
NOE correlations. *See* Nuclear Overhauser effect (NOE) correlations
NOESY experiments, 938
Noladin, 3424
(2E,6Z)-Nonadienal, 2887
(Z,Z)-3,6-Nonadienal, 2898–2900
Nonahydroxyterphenoyl (NHTP), 1986
Nonanal, 2891, 2892
Nonanoic acid, 2887, 2892
1-Nonanol, 2891, 2892
Nonaqueous capillary electrophoresis (NACE), 1032, 1174
Nonconventional extraction, 1015
(E)-2-Nonenal, 2891, 2892, 2898–2902, 2906
(E)-2-Nonenol, 2898–2900
Non-enzymatic browning, 1783
Nonfat cocoa solids (NFCS), 2319
Noninsulin-dependent, 3806
Nonmammalian species, 4076
Non-MVA, 3571
Non-natural analogs, 1664
Non-ribosomal peptide synthetases (NRPSs), 42, 699
Non-small cell lung cancer, 3562
Nonsteroidal anti-inflammatory drugs, 1510

Non-toxic necines, 370
No observed adverse effect effect-levels (NOAEL), 2291–2292
Nootkatone, 2991, 3001
NO production, 2290
Noradrenaline, 588, 3709, 4068
NORA programme, 3541
Norbelladine, 484
Norbornane, 3472
Norcoclaurine 6-*O*-methyltransferase (6OMT), 8–11, 21
Norcoclaurine synthase (NCS), 8–10, 21
Norcodeine, 1377
Norditerpenoid alkaloids, 932
Norephedrine, 1219, 1221, 1223, 1224
Norepinephrine, 598
Norethisterone, 2420
Norflurazon, 2787
Norguaianolide, 3074
Norharman, 1352
Norlaudanosoline, 8–11, 19, 21
Nor-lobelane, 334, 337, 338
Norlobelanidine, 329
Norlobelanine, 318, 324, 325, 328, 337
Norlobeline, 329
Normal fetal fibroblast (MRC-5) cells, 3480
Normal-phase high performance liquid chromatography (NP-HPLC), 1086
Normolipidemic, 3673, 3679, 3683
Normorphine, 1376
Normoxic, 3965
Nornicotine, 1346
Norpseudoephedrine, 1219, 1221, 1223, 1224
nor-Sesquiterpenoids, 2989
Noscapine, 434, 441, 1172, 1446
Nostocales, 58, 63
Nostoxanthin, 3256, 3265, 3266
Notch-1 signaling, 3485
Nothapodytes foetida, 647
Nothapodytine A, 647
NOx. *See* Oxides of nitrogen (NOx)
N-oxidation, 370
N-(4-oxodecanoyl) histamine, 866
NPAA. *See* *N*-propinoyl aspartic acid (NPAA)
NPD. *See* Nitrogen-phosphorus detection (NPD)
NP-HPLC. *See* Normal-phase high performance liquid chromatography (NP-HPLC)
n-3 polyunsaturated fatty acids, 3444
NPP. *See* Nerolidyl diphosphate (NPP)
N-propinoyl aspartic acid (NPAA), 3333
N-retinylidene-*N*-retinyl-ethanolamine (A2E), 3937, 3947, 3948
Nrf–2. *See* Nuclear factor E2 p45-related factor (Nrf–2)
NRPS. *See* Non-ribosomal peptide synthetases (NRPSs)
N,S-acetal moiety, 260
NSE/DTE triad, 2719
NSP2, 3596
Nuclear factor, 337
Nuclear factor E2 p45-related factor (Nrf-2), 2190
Nuclear factor kappa B (NF-kB), 1316, 1453, 1870, 1872–1875, 1881, 1883, 1889, 2187, 2291, 2626, 2679, 3089, 3090, 3704, 3713, 3715, 3750, 3751, 3966, 3967
Nuclear magnetic resonance (NMR), 178, 1137, 2123, 2802, 3359
  spectra, 3476
  spectroscopy, 1037, 2082, 2119, 3239
Nuclear Overhauser effect (NOE) correlations, 933
Nucleophiles, 978
Nucleoside diphosphate kinase, 1664
Nucleotide-activated sugars, 1664
Nucleus accumbens, 1373, 1375
Nude athymic mice, 2402
Nutraceuticals, 1736, 1740, 2451, 2491–2495, 2501, 2503, 2505, 2511, 2583, 2590, 2605, 2642, 2764
  properties, 2644
  value, 2653
Nutrition, 2434, 3902
Nutritional, 1959
  conditions, 1688
  value, 2855
Nutrition claims, 2505, 2519–2521
Nuzhenide, 3052
Nyctinasty, 1566
Nylandtia, 3206

O

Oakmoss, 3299
Obesity, 2495, 2496, 3477, 4114, 4148
Obligate parasites, 3585
$1'''$-*O*-β-*O*-glucosyl formoside, 3052
Obsessive-compulsive disorders (OCD), 526
OCD. *See* Obsessive-compulsive disorders (OCD)
Oceanapia, 799
Ochrophyta, 2893–2901

Ochrosia elliptica, 598
Ocimum
  O. basilicum L, 2946, 4091
  O. canum, 4090
  O. suave, 4095
Octadecane, 2892
5-Octadecene, 2894
(2E,4E)-Octadienal, 2887
1-Octanol, 2892
1,3,5-Octatriene, 2892
(E)-2-Octenal, 2891, 2892, 2902, 2906
2-Octene, 2897
1-Octenol, 2892
1-Octen-3-ol, 2891, 2892, 2902
(E)-2-Octenol, 2891, 2892
1-Octen-3-one, 2889
Octopamine, 4096
N-Octyl bicycloheptene dicarboximide, 4065, 4077
Ocular, 1813
Ocular blood flow, 3723, 3724
O. cuma, 3592
Odorant binding proteins (OBPs), 4096
Oestrogen, 1861
Oestrogenic activity, 1852
Oestrone, 1546
Off-flavor, 2348
6-OHDA. *See* 6-Hydroxydopamine (6-OHDA)
O. hederae, 3594
Oil cells, 2822
Oil glands, 2824
Oil soluble constituents, 3665
Old yellow enzyme, 701
Oleander, 295
Oleandrin, 3747–3751
Oleoresin, 2822
Olfactometric cues, 2920
Olfr73, 4008
Oligogalacturonides, 1694
Oligomeric and polymeric proanthocyanidins, 2267
Oligomers, 2256
Oligostilbenes, 1906
Omega fatty acids, 1736
Omics
  metabolomics, 2513, 2514
  proteomics, 2513, 2514
  transcriptomics, 2513, 2514
Omnopon, 1100
Oncogenes, 189, 2952, 3527
Oncogenicity of resveratrol, 2291
Oncomelania, 846
Oncostatic agent, 992
Onjisaponin A, 3215
Onjisaponin B, 3215
Online preconcentration, 1185, 1189
Onychomycosis, 4008
Oomycetes, 2805, 2806
Open-chain glycsoides, 3242
Open-chain saponins, 3233
Open-chain steroidal glycosides, 3227, 3233–3236, 3243, 3244
Open-chain steroidal saponins, 3232, 3233
Open-column chromatography (OCC), 3371, 4027
Open-field test, 3968
Open tubular/capillary columns, 1074
OPG. *See* Osteoprotegerin (OPG)
Ophioscorodon, 3665
Opioid-like activity, 135
Opioid receptors, 1371, 1373, 1508
Opium, 1368–1370
  alkaloids, 1172
  plant, 1111
OP poisoning, 1252
OPRM1. *See* The Mu-1 opioid receptor subtype (OPRM1)
Optimization, 226, 229, 3354
Optimization of the enzyme sources, 1636
ORAC. *See* Oxygen radical absorbance capacity (ORAC)
Oral, 1319, 1370, 1371, 1376, 1377, 1380
  administration, 3647
  dosage, 3969
  fluids, 1077
  intake, 2412
Oral contraceptives (OCs), 2751
Orange oil, 4101
Orbitrap, 1101
Orbitrap mass spectrometers, 58
ORCA3, 109, 110
Orchidaceae, 805
Oreophoetes, 788
Organic farming, 4102
Organoleptic, 1959, 3198
Organophosphorus, 4065, 4066, 4079
Organosulpfur compound, 3666
Oricia, 738
Oriciopsis, 741
Orientaline, 431
Orientalinone, 431
Origanum
  O. majorana, 2980, 2983, 2991
  O. vulgare, 2976, 4091, 4098, 4116, 4133
Oripavine, 446

Orius insidiosus, 4098
Orixa, 722
Ornithine, 365, 417
L-Ornithine, 344
Ornithine decarboxylase, 182
Ornithonyssus sylviarum, 4072
Orobanchaceae, 3585
Orobanche, 3585
Orobanche minor, 3591
Orobanchol, 3585
Orobanchol series, 3589
Orobol, 1667
Oroidin alkaloids, 265
Orotic acid, 1664
Orsaponin, 3235
Ortho-chloranil, 1063
Orthologous/homologous, 698
Orthostatic hypotension, 697, 1373
Oruwacin, 3027
Oryzaephilus surinamensis, 4094
Oryzalin, 198
Oryza sativa, 1569, 3587
Oscillatoria, 47
  O. formosa, 42
  O. nigro-viridis, 1293
Oscillatoriales, 59
Oscine, 349
Osteoblastic differentiation, 2405
Osteoblastic proliferation, 2405
Osteoblasts, 2405
Osteopenia, 2413, 2418
Osteoporosis, 1374, 1858, 2405, 2447, 4117
Osteoporotic fractures, 2409
Osteoprotegerin (OPG), 2405
Ostericum sieboldii, 4094
OSW-1, 3235, 3243, 3244
Ouabain, 57, 3745, 3747–3750
Outbreak, 3125, 3144
Ouvrardianine A, 927
Ouvrardianine B, 934
Over-expression, 232, 235–237, 1704
Oviposition stimulants, 1570
Oxaliplatin, 2215
Oxazolidine ring, 938
Oxetane, 2801
Oxidation, 2335, 2342, 3711, 3925, 3933, 3937, 3943, 3944, 3946, 3947, 4024
β-Oxidation, 1273
Oxidation of ethanol to acetaldehyde, 2269
Oxidative, 4117
  damage, 3646, 3668
  pentose phosphate pathway, 1571
  phosphorylation, 3716
  stress, 135, 1249, 2371, 2473, 2592–2596, 2598, 3526, 3644, 3707, 3711–3713, 3719, 4114, 4127, 4132, 4134, 4138
Oxidized low-density lipoprotein (Ox-LDL), 3218, 3681
Oxidoreductase, 699
2,3-Oxidosqualene, 3242
5-Oxo-5-deoxystrigol, 3587
2-Oxoglutarate-dependent dioxygenases (2OGD), 1826
2-Oxo-3-(5-imidazolyl)-propanol, 872
7-Oxoorobanchol, 3587
2′-Oxoquininone, 633
3-Oxo-un-4-decenyl, 2894
Oxygen, 226, 230, 2343
  limitation, 186
  in wine, 2267
Oxygen radical absorbance capacity (ORAC), 2318
Oxymethyl bridge, 3352
Oxyresveratrol, 1906
Oxyresveratrol oligomers, 1911
Ozone, 1698, 2929
Ozone-depleting chlorofluorocarbons, 2682

P
p53, 1313
P450, 2829
PA. *See* Propylamine (PA)
Pachydictyol A, 2895
Packed columns, 1074
Paclitaxel, 1652, 2216, 2674, 2737, 2783, 2829, 2957, 3165, 3167–3169
Paederoside, 3050
Pagoside, 3044
Pain management, 66
Pain of sciatica, 1507
Pain reliever, 1425
Palatoschisis, 901
Palau'amine, 268
Paleatin, 1919
Palliative care, 1380
Palmatine, 6, 12
Palmitoylated, 2399
Palmitoylethanolamide (PEA), 3424
Panaelus, 527
Panaeolina, 531
Panaeolus, 531
Panax, 2764
Panax ginseng, 2766, 2947, 2952
Panicum miliaceum, 3585
Panonychus ulmi, 4098

Papain, 980, 987, 2342
Papaver alkaloids, 1369, 1370
Papaverine, 434, 440, 1073, 1170, 1172, 1181, 1369, 1370
Papaver somniferum L., 1073, 1172, 1349, 1446
Papilio xuthus, 1570
PA poisoning, 374, 1389
Paracelsus, 1368
Paralytic shellfish poisoning (PSP), 53, 957
Paralytic shellfish toxins (PSTs), 53, 972
Paralyze, 4077
Paraminabea, 3467
Paraoxon, 51
Paraquat, 4044
Parasitological, 1294
Parasympatholytic agents, 1014
Parathyroid hormone (PTH), 2405
Paraxanthine, 959
Paraxanthine (1,7-dimethylxanthine), 955
P. arenaria, 3209
Paresthesia, 1516, 4065–4067
Parkinson, 332, 589, 848, 3488
Parkinson's disease (PD), 175, 697, 1207, 1333, 1471, 1473, 1480–1482, 1526, 1527, 2619, 2623, 3699, 3718
Paromomycin, 1275, 1279
PARP. *See* Poly(ADP-ribose) polymerase (PARP)
Parthenin, 3092, 3094
Parthenocarpy, 1591
Parthenocissin A, 2153, 2162
Parthenolide (PRT), 3083, 3529
Party pills, 289
PAs. *See* Pyrrolizidine alkaloids (PAs)
PASs. *See* Potentially allergenic substances (PASs)
Pathogen-associated molecular patterns, 1569
Pathogenic bacteria, 978
PA toxicity in humans, 373
Pattern recognition receptors, 1569
Pausinystalia, 589
Paw edema, 3655
PC-3, 3478
P. carajaensis, 868–871
PCR. *See* Polymerase chain reaction (PCR)
PD. *See* Parkinson's disease (PD)
PDR1, 3590
PEA. *See* Phenylethylamine (PEA)
p4E-BPI, 3485
Pectinases, 1609
Pediculus capitis, 4094
PEG. *See* Poly(ethylene glycol) (PEG)
Peganum, 830
Peganum harmala, 1291
Pelargonidin, 1664, 2467
Pelargonium
  P. graveolens, 290, 2982
  P. odoratissimum, 2979
Pelea, 738
Pelletierine, 304–306, 337
Pelletization, 2350
Pellets, 2335
Pellicularia sasaki, 980
Penethylisoquinoline, 461–476
Penetration, 3762
Penicillium, 834
Penicillium griseoroseum, 1639
Penicillium spp., 1554
Peninsularinone, 3785
Pentadecanal, 2887, 2891, 2892, 2902, 2904
Pentadecane, 2892
Pentadecanol, 2891, 2892
Pentadecene, 2891, 2892
Pentaflavonoids, 1621
Pentamidine, 1274, 1289
Pentane, 3371
2-Pentanol, 2902
Pentavalent antimoniate, 1273
1-Penten-3-ol, 2902
2-Penten-1-ol, 2902
Pentobarbital, 4067
Pentostam, 1286
13-(3-Pentyl-2-aziridinyl)-5,8,11-trideca-trienoic acid, 994
Peonidin, 2467
PEPCK. *See* Phosphoenolpyruvate carboxykinase (PEPCK)
Pephytoene diphosphate, 2717
Peptic ulcers, 1444
Peptide leader sequences, 1660
Peptide madurastatin A1, 986
Peramenolide A, 3480
Peramine, 164
Perception of bitterness, 2265
Perfumes, 3289
Perhydropurine, 54
Perhydropurine alkaloid, 42
Periaqueductal gray matter, 1371
Pericardial fluid, 1082
Pericarpium papaveris, 1100
Peridinin, 3254, 3256, 3264
Peridroma saucia, 4095
Periglandula
  P. ipomoeae, 689
  P. turbineae, 689

Peril alcohol, 2997
Perilla argata, 2981
Perillaldehyde, 2976, 2981
Perillyl alcohol, 4115, 4139, 4140
Peripheral neurons, 64
Peripheral neuropathies, 4079
Periplaneta
  P. americana, 4094, 4096
  P. orientalis, 4094
Periplocaceae, 787
Periprocedural care, 66
Permeability, 3791, 3981, 3982
Permeabilizing agents, 2771
Permeation, 3759, 3762
Permethrin, 4063, 4068, 4070–4072, 4075–4078
Peroxidases, 94, 2184, 4113, 4114, 4135
Peroxides, 982
Peroxisome proliferator-activated receptor (PPAR), 4118, 4128, 4134, 4147
Peroxisome proliferator-activated receptor (PPAR) agonists, 3809
Peroxisome proliferator-activated receptor γ (PPARγ), 4117
Peroxyl radical (HOO•), 3673
Perpivaloyl glucosides, 3050
Perrottetin, 1919
Persistent pollutants, 2410
Perulactone B, 3470
Peruvoside, 3750
Perylene, 1564
Peschiera van heurkii, 1288
Pestalotiopsis, 2802
Pesticides, 2780, 4049, 4125
Pest management, 4095
Pet animals, 4071, 4077
Petasites japonicus, 979
Petiveria, 1553
Petroleum, 4065
Petroleum ether, 3371
Petroselinum sativum, 4091
Petunia, 1722
Petunia hybrida, 3590
Petunidin, 2467
Peumus boldus, 2998, 4099
PGE$_2$. *See* Prostaglandin E$_2$ (PGE$_2$)
P-glycoprotein (P-gp), 2214
PGOCA. *See* Phenyl glycine-*o*-carboxylic acid (PGOCA)
P. grandiflorus, 870
Phagocytic cells, 3523
Phagocytosis, 3523
Phakellin, 270
Phalaris arundinacea, 158, 162
Pharmaceuticals, 1100, 4049
  formulations, 3336, 3337
  industry, 132, 2650
  properties, 3517
Pharmacodynamics, 1376
Pharmacogenetics, 1377
Pharmacokinetics, 4068
Pharmacology/pharmacological, 541, 1014, 3657
  active, 3214
  activities, 978, 1002, 1507, 3488
  effects, 481, 1504
  importance, 3476
  interactions, 2482
  properties, 65, 121, 979
Phebalium, 735
Phelodendron, 735
Phenanthraquinones, 1923
Phenanthrene alkaloids, 1073
Phenanthrenes, 1369, 1923
Phenobarbital, 2786
Phenolics, 2013–2037, 2261, 2435, 2667, 3501, 3977, 3981
Phenolics acids, 1548, 2438, 2475, 2476, 2528–2534, 2537, 2538, 2540, 2542–2545, 2547–2552, 2554, 2555
Phenolics compounds, 2528, 2529, 2531, 2533, 2534, 2540, 2543, 2544, 2549, 2552, 2557, 2583, 2584, 2589, 2590, 2595, 2598, 2600, 2601, 2603, 2604
  benzoic acids, 2095, 2098
  extraction, 2095
  flavonoids, 2095
  hydrolysis, 2096
  hydroxycinnamic acids, 2100
  LC-MS, 2097
  LC-UV/LC-DAD, 2096
    flavonoids, 2096
    phenolic acids, 2096
  phenylpropanoids, 2095, 2098
Phenols (PH), 2886–2893, 2895–2904, 2907, 3665
Phenothrin, 4065, 4075
Phenoxodiol, 2192, 2218
Phenserine, 1341
L-Phenylacetylcarbinol, 919
Phenylalanine, 909, 1688, 1825
L-Phenylalanine, 918
Phenylalanine ammonia-lyase (PAL), 1654, 1688, 1825
Phenylalanine ammonia-lyase activity, 1565
Phenylalkylamines, 913

Phenylethylamine (PEA), 28, 1206
2-Phenylethylamine (PEA), 2885
Phenylethylamine (PEA) derivatives, 1205
Phenyl glycine-*o*-carboxylic acid (PGOCA), 3333
Phenyliodonium bis(trifluoroacetate) (PIFA), 260
Phenylisoserine, 2801
Phenylketonuria, 1207
Phenylpropanoids, 4, 14, 1549, 1653, 1685, 1688, 1691, 1705, 1707, 1720, 1955, 4002, 4003, 4089
  acids, 1629
  pathway, 1722, 1850
Phenylquinolines, 832
Pheomelanins, 1564
Pheromones, 2920, 3181, 4121
Philadelphicalactone A, 3484
Philadelphicalactone B, 3484
Philipanche, 3585
Philotheca, 733
Phlebotomus, 845
Phlegmarine, 1242
Phlegmariurus squarrosus, 1245
PHLI. *See* Polyhalogenated indoles (PHLI)
Phlobaphene pigments, 1838
Phlomiol, 3045
Phlomis brunneogaleata, 1290
Phloretin, 1566, 2153, 2164
3-Phloroeckol, 1560
Phloroglucinol, 1548, 1765
Phloroglucinolysis, 2269
Phlorotannins, 1559
Phloyoside 1, 3045
Pholcodine, 1100
Pholiota, 528
Phorbol, 3180
Phorbol-12-myristate-13-acetate (PMA), 2213
Phormidium, 47
Phosphatidate phosphohydrolase (PAP), 3669
3′-phosphoadenosine 5′-phosphosulfate (PAPS), 1829
Phosphoenolpyruvate (PEP), 14, 15
Phosphoenolpyruvate carboxykinase (PEPCK), 3809
Phosphoenolpyruvate synthetase (PEPS), 15
Phosphoglucomutase, 1664
Phospholipase, 2676
Phospholipase-2 (PLA2), 2679
Phospholipase A2 (iPLA2), 3966
Phospholipid bilayer, 2471
Phospholipids, 993, 1277
Phosphoribosyltransferase (PRT), 1269
Phosphorylation, 537
Photodiode array, 3592
Photodiode array detector, 3378
Photomorphogenesis, 3585
Photooxidation, 1100
Photoprotection theory, 1568
Photoprotective, 3925, 3933
Photoreceptors, 3925–3930, 3937, 3939–3941, 3943, 3944, 3946, 3947
Photosensitive, 44
Phthalates, 3293, 3302
Phycotoxins, 53
Phyllopertha, 806
Phyllophora
  P. crispa, 28
  P. nervosa, 29
  P. nervosa (Phyllophora crispa), 2890, 2904
Phyllophoraceae, 2890
Phyllospora comosa, 2898
Phylogenetic, 544
Phylogenetic analysis, 63
Phylogenetic distribution, 61
Physalins, 3468, 3473, 3476
  A, 3468
  B, 3468, 3478, 3485
  D, 3478
  F, 3478
  P, 3468
  U, 3478
Physalis, 3467
  P. alekengi, 3468
  P. angulata, 3479, 3485
  P. minima, 3468, 3477, 3479
  P. philadelphica, 3477
Physalism minima, 3477
Physcion, 1554
Physcomitrella patens, 3585
Physical environmental conditions, 103
Physical factors, 104, 1692, 1693, 2783
Physostigma venenosum, 1340
Physostigmine, 1340, 1342, 1527–1529
Phytoalexins, 1569, 1685, 1687, 1693, 1695, 1831, 2388, 2782
Phytoanticipins, 3503
Phytochemicals, 2069, 2075, 2084, 2470, 2507–2511, 2642, 2643, 2653, 2768, 2771, 2942, 3206, 4002
Phytoecdysteroids, 3828–3832, 3835, 3836, 3838–3840, 3842–3845
Phytoene, 2715, 2717, 3390
Phytoestrogenic, 2448

Phytoestrogens, 1850, 2437, 2440, 2453, 2571, 2643, 2648
Phytofluene, 3390
Phytohormone-derived anticancer drugs, 3864
Phytohormones, 230
Phytol, 2733, 2734, 2737, 2744, 3176
Phytolacca dioica, 4094
Phytonutrients, 1740
Phytosterol oxides (PSOs), 3451–3452
Phytosterols, 2416, 2422, 2568
  bioavailability, 3441
  compared to phytostanols, 3442
Phytotoxic, 3488
Phytotoxicity, 1941
pH-zone-refining, 2150
PI. *See* Pinene (PI)
Picea, 1557
Picea abies, 3000
Picea abies (L.) Karst, 1549
Piceatannol, 2282, 2289, 2297, 2298
Piceatanol, 1906
Piceatanol oligomers, 1911
Piceid, 1692, 1696, 1698, 1699, 1703, 2279, 2283, 2286, 2350, 2351
Picein, 1549
Picloram, 2787
Picrasin K, 3348
Picroside 1, 3048, 3056
Piepunine, 940
PIFA. *See* Phenyliodonium bis (trifluoroacetate) (PIFA)
Pig ascarids
Pigments, 2260, 2466, 2468, 2763, 2861, 2866, 2869, 2870, 4018, 4022
Pilocarpidine, 869
Pilocarpine, 861, 863, 868–870, 872–878, 1343
Pilocarpus, 728, 863, 867, 868, 870–877, 1343
  P. jaborandi, 869, 870
  P. jaborandi, 868
  P. microphyllus, 868–871, 873–875, 877, 878
  P. pennatifolius, 868, 869, 871, 872, 875, 878
  P. trachyllophus, 868, 869, 871
Pilosinine, 869
Pimenta racemosa, 4121
Pimpinella affinis, 2986
PIN. *See* Prostatic intraepithelial neoplasia (PIN)
Pinellia, 1219, 1221
Pinene (PI), 2887, 2888, 2892, 2896, 2898, 2899, 2901, 2902, 2905, 2907, 4040
(2*S*)-Pinocembrin, 1658
Pinocytosis, 2471
Pinoresinol, 2438
(+)-Pinoresinol, 1557
Pinus spp., 1557, 4094
  P. contorta, 2986
  P. massoniana, 3000
Piper, 305
  P. longum, 311, 312, 337
  P. nigrum, 304, 309, 312, 313, 337, 4116
  P. officinarum, 314, 337
Piperaceae, 772, 1558
Piperanine, 311, 337
Piperettine, 337
Piperidine, 320, 336, 412
Piperidine alkaloids, 304–306, 324, 330–332, 337
Piperine, 304, 309, 311–315, 317, 318, 336, 337, 1352
Piperitone, 2895, 2981
Piperlonguminine, 315, 337
Piperonyl butoxide, 4066, 4077
Piracetam, 4123
Pisum sativum, 3587, 3596
Pitohuis, 159, 162
Pituitary gland, 2421
P. japonica, 3210, 3219
pKa, 2472
pKa values, 1165
PKC. *See* Anti-protein kinase C (PKC)
Plagilin, 1919
Plagiochin, 1918
Plagiolin, 1920
Plains larkspur, 159
Planar chromatography, 1020
Planar compounds, 2387
Planar configuration, 1834
Plant(s), 228, 231, 237, 240, 242, 689, 971, 978, 979, 1002, 2417, 2942, 3828–3831, 3836, 3839–3845
  alkaloids, 215, 219, 220, 232, 240
  cell and organ cultures, 2777
  cell bioprocessing, 224
  cell cultivation, 226
  cell cultures, 215, 220, 226, 228, 230, 231, 243, 2768, 2770
  cell technology, 215, 245
  genetic transformation, 1582
  genotype, 2644
  growth regulators, 2763
  hormones, 3585
  mycorrhizal infection, 1566

phenolic compounds, 2049
phenolics, 1546, 1953
regeneration, 2951
resistance, 1805, 3862, 3864–3865
secondary compounds, 2771
secondary metabolites, 1619
tissue culture, 3109–3110
in vitro systems, 185
volatiles, 3983
Plantation systems, 1602
Plant-based natural antimicrobials, 3976
Plant-derived alkaloids, 215
Plant-derived foods, 1953
Plant protection products (PPP), 4088, 4101
Plant-to-bacterium signal, 2390
Planusin, 1919
Plaque, 3677, 3681, 3682, 3686
Plaque formation, 2287
Plasma, 2324
concentrations, 2294, 2323, 2388
concentrations vary, 2400
steady-state levels, 2398
Plasmodium, 846, 3786
Plasmodium falciparum, 626, 1310, 2960
Plastids, 2832, 2856, 2861, 2865, 2867, 2870, 3588
Plastoquinones, 2695
Platelet, 1307
Platelet activating factor (PAF), 3704, 3715, 3720–3723, 3726, 3727
Platelet aggregation, 2299, 2326, 3664, 3677, 3678, 3680, 3686
Platelet-derived growth factor, 1450
Platydesma, 734
Pleiospermium, 764
P388 leukemia, 982, 988, 996
Plinia cerrocampanensis, 2986
Plocamiaceae, 2891
Plocamiales, 2891
Plocamium
P. angustatum, 2891
P. vulgare (Plocamium cartilagineum), 2891
P-loop, 64
Plumbagnaceae, 1552
Plumbago capensis, 1552
Plumenoside, 3027
Plumeria type iridoid, 3054
Plunge dipping, 4074, 4079
Pluteus, 532
PMA. *See* Phorbol-12-myristate-13-acetate (PMA)
p38 MAPK, 2192
P815 mastocytoma, 988
P450 monooxygenase, 581
PMTDI. *See* Provisional maximum tolerable daily intake (PMTDI)
Pneumotoxic, 360
P nutrition, 2646
Poaceae, 725, 3590
Podostemaceae, 1553
Pogostemon cablin, 2988, 2998
Pogostemon spp., 4091
Poison antidote, 1511
Poison Control Centre, 295
Poison hemlock, 884
Polianthes tuberose, 2766
Polyamines, 1732
Polyamine spermidine, 991
Polybromomethanes, 2890, 2896, 2897, 2902
Polycyclic musks, 3300
Polygala, 3206, 3220
P. aureocauda, 3218
P. crotalarioides, 3213
P. micrantha, 3213
P. ruwenzoriensis, 3207
P. tenuifolia, 3209, 3219
Polygalaceae, 1553
Polygonaceae, 805, 1554
Polyhaloacetone, 2889
Polyhalobut-3-en-2-ones, 2888
Polyhalogenated indoles (PHLI), 34, 1212
Polyidaceae, 2890
Polyides rotundus, 28, 2890
Polyketide, 833
acetate/malonate pathway, 1546
pathway, 1545
synthase:type III, 1654
Polyketide synthases (PKS), 42, 49, 61, 1654
Polyketoacid, 893
Polymerase chain reaction (PCR), 2803
Polymeric pigment, 2265
Polymorphisms, 1377, 2627
Polyphenolic compounds, 1619, 2369
Polyphenol-inducing medium, 1692
Polyphenol oxidases, 1564, 1607, 1764, 1782
Polyphenols, 1546, 1585, 2015–2026, 2030–2033, 2035, 2037, 2063, 2075, 2080, 2146, 2147, 2150–2152, 2156, 2158, 2160, 2161, 2163, 2164, 2176, 2177, 2335, 2342, 2362, 2442, 2453, 2467–2469, 2472, 2642, 2647
in olive, 2059
polyphenolic, 2467

Polyphenon E, 2214
Polyploid, 473–474
Polyploidization, 199
Polyploidy, 197
Poly(ADP-ribose) polymerase (PARP), 3528
Polyps, 2420
Polysaccharides, 2266, 3501, 3978
Polysiloxanes, 1075
Polysiphonia
  P. morrowii, 28, 2893, 2903
  P. sphaerocarpa (Neosiphonia sphaerocarpa), 2893
  P. tripinnata, 28, 2893, 2904
  P. urceolata, 28
  P. urceolata (Polysiphonia stricta), 2893
Polyterpenes, 2824
Polyterpenoids, 2670, 2915
Polyunsaturated lipids, 1737
Polyvinylpolypyrrolidonec (PVPP), 2342
Pomace, 2600, 2604
Poncirus, 773
Poorer semen quality, 2421
Poor metabolizers, 1377
Populus
  P. balsamifera, 1549
  P. deltoides, 2986
Porfiromycin, 983, 984
Porphyra
  P. columbina, 2886
  P. tenera (Pyropia tenera), 2887
Portal vein thrombosis, 1387
Portisins, 1787
Posaconazole, 1277
Postharvest processes, 2318
Post-harvest treatment, 1601
Post-hyperpolarization, 4097
Postmenopausal, 2408, 2419, 3708
Post-menopausal women, 2448
Postmortem toxicology, 1111
Post-traumatic stress disorder (PSTD), 1471
Potassium channels, 41, 2365, 3996
Potentially allergenic substances (PASs), 3293
Potocytosis, 2471
Pour-ons, 4072, 4074, 4076
PPAR. *See* Peroxisome proliferator-activated receptor (PPAR)
PPARγ. *See* Peroxisome proliferator-activated receptor γ (PPARγ)
PPARγ, PPARγ1, and PPARγ2, 3811
PR. *See* Propionic acid (PR)
P. racemosus, 868, 869, 871
P. ramosa, 3592
Preakuammicine, 596
Prebiotics, 2454
Precursor feeding, 1686, 1692
Precursors, 88, 89, 98, 104, 105, 109, 111, 220, 225, 226, 231, 238, 239, 243, 537, 1633, 2804
Predator reproduction, 2390
Preformed antibiotic compounds, 1568
Pregenomic approach, 224
Pre-menopausal, 2408, 2412, 2419
Prenylchalcones, 1869, 1873, 1874, 2343
Prenyldiphosphate, 2828
Prenylflavanones, 2345
Prenylflavonoids, 2151, 2162
Prenylnaringenin, 2345, 2346
8-Prenyl naringenin (8-PN), 2385
Prenyltransferases, 2708, 2818, 2819
Presenegenin, 3207, 3220
Presqualene diphosphate, 2715
Pressurized liquid extraction, 1017
Preulactones, 3470
Preventive, 2413
  effect, 2402
  intervention, 2225
pRi, 2774
Prianosin B, 261
Primaquine, 629
Primary infection, 704
Primary metabolites, 4, 1544
Prismatomerin, 304
PRO. *See* Propional (PRO)
Proanthocyanidins, 1559, 1604, 1719, 1755, 2334, 2335, 2343, 2568
Probiotics, 2454
Procedure, 3373
Processed food, 4024
Processed-soy, 2402
Processed tubers, 1517
Process optimization, 226
Proconvulsants, 4067
Procyanidins, 2259, 2335, 2367, 2588, 2591, 2598, 2603, 2604
Procyanidins and anthocyanins, 2369
Prodelphinidins, 1758, 2335
Product inhibition, 1662
Production, 215, 224, 225, 228–231, 234, 236–238, 240, 243, 245, 4031
Production model, 1602
Productivity enhancement, 226
Progesterone, 2418
Progression and invasion, 2296
Proinflammatory cytokines, 2283, 2289
Prolactin, 1374
Prolactin-inhibiting factor, 697

Proliferation, 1448, 2321, 2404, 3525
Proliferation of smooth muscle cells, 2298
Proliferative effect, 2399, 2402
Proline, 50, 1571
Proline redox cycle, 1571
Prolonged nerve block, 66
Prolyl oligopeptidase (POP), 1309
Promastigotes, 845, 1266, 1290
Promiscuous enzymes, 1666
Promyelocytic cell lines, 3561
Pronase E, 1085
Pro-oncogene MDM2, 2404
Pro-oxidant response, 2370
Propeller, 2777
Propional (PRO), 2887, 2899, 2904
Propionic acid (PR), 2887, 2892, 2897, 2899, 2901–2903
Propylamine (PA), 2890, 2891, 2893, 2904
8-Propyl-10-phenyllobelionol, 329
Proscillaridin, 3750
Proscillaridin A, 3750
Prostacyclin, 1832, 2365
Prostaglandin, 1306, 1590
Prostaglandin biosynthes, 1510
Prostaglandin E_2 (PGE_2), 4119, 4134
Prostaglandin G_2 (PGG_2), 3679
Prostate cancer progression, 2423
Prostate cancers, 1859, 2209, 2297, 2449
Prostate membrane specific antigen (PMSA), 3091, 3092
Prostate serum antigen (PSA), 2404
Prostatic intraepithelial neoplasia (PIN), 2210
Prostephanus americana, 4094
Prostration, 4079
Proteaceae, 176, 346
Protease, 979, 980, 987, 988
Protease inhibitors, 2416, 2422
Proteasome, 3485
Protect against oxidative damage, 1409
Protected, 2408
Protective, 2413
  compounds, 1603
  influence on the integrity of erythrocyte membranes, 1420
Proteinases, 1270
Proteins, 1766, 2335, 2342, 3665, 3668, 3674
  from the ANX family, 3447
  binding, 1376
  engineering, 1626, 1660, 1667
  fusion, 1659, 1672
  kinase C, 4067, 4068
  kinase C phosphorylation, 4067
  omeostatis, 3477
  scaffold, 1672
  secretion, 2405
  storage vacuoles, 1609
  synthesis, 3141, 3144, 3789
Proteolytic activity, 987
Proteus, 984
Proteus vulgaris, 979, 989
Protoberberine alkaloids, 1170, 1176, 1181, 1185
Protocatechualdehyde, 2163
p-Protocatechualdehyde, 1548
Protocatechuic, 1548
Protocatechuic acid, 2475, 2476, 2480
Protodioscin, 3228–3230, 3244
Protogracillin, 3229
20(S)-Protopanaxadiol (PPD), 3502
20(S)-Protopanaxatriol (PPT), 3502
Protozoal, 3093
Protozoans, 1279
Provisional maximum tolerable daily intake (PMTDI), 3150
Provitamins A, 1730, 3369
Przewaquinone, 3554
PSA. *See* Prostate serum antigen (PSA)
Psathyrella, 532
Pseudans, 824
Pseudoalkaloids, 947
Pseudoconhydrine, 892
Pseudoephedrine, 1174, 1179, 1219–1221, 1223, 1225, 1232
Pseudojujubogenin, 3648
Pseudomonas, 163, 828
Pseudomorphine, 442
Pseudonocardia, 830
Pseudopelletierine, 305, 306, 337
Pseudotropine, 346
Pseudoxandra sclerocarpa, 1287
Psilocin, 524
Psilocybe, 528, 532
Psilocybe mushrooms, 529, 540
Psilocybin, 524, 542
Psilocybine, 524
pS6K, 3485
Psoroptes ovis, 4075
P. spicatus, 869–871
PSTD. *See* Post-traumatic stress disorder (PSTD)
Psychedelic, 545
Psychoactive, 126, 4150
Psychoactive effects, 4117, 4142, 4143
Psychollatine, 138
Psychological, 542
Psychomotor activity, 2621
Psychomotor effect, 1207

Psychostimulant abuse, 334
Psychotomimetic, 541
Psychotria, 121
Psychotria klugii, 1289
Ptaquiloside, 2989
Ptelea, 722
PTEN, 2191
Pteridium aquilinum, 2989
Pterocarpans, 1569
Pterocladia
  P. capillacea (Gelidium capillaceum), 2888
  P. lucida, 2888
Pterocladiaceae, 2888
Pterocladiella capillacea, 2888, 2906
Pterodon polygalaeflorus F., 4118
Pterogyne nitens Tul., 1459
Pterogynidine, 1459
Pterostilbene, 2280, 2297, 2298
PTK. *See* Anti-protein tyrosine kinase (PTK)
Puccinia coronata, 164
Pueraria lobata, 1852
Puerarin, 1852
Puffer fish, 54, 59, 970
Pulchelloside 1, 3045
Pulegone, 2981, 2998, 3000, 4099, 4149, 4150
Pulse pressure (PP), 3675
Pumiloside, 659
Punica, 305
Punica granatum, 304, 306, 337, 2001
Purgative, 3477
Purification, 2015, 2032–2036, 2071, 3238, 3369
Purines, 412
  alkaloids, 222, 1609
  catabolic pathway, 961
  of primary metabolism, 59
Purple bacteria, 3254, 3257, 3258, 3268, 3269
Putative target, 1268
Putrescine, 365, 1732
Putrescine *N*-methyltransferase, 182
PVPP. *See* Polyvinylpolypyrrolidonec (PVPP)
P2X receptors, 4004
Pyranoacridone, 821
Pyranocoumarins, 1551
Pyranoquinolines, 789
Pyrazines, 1610
Pyrethrin I, 4062, 4063
Pyrethrin II, 4062, 4063, 4077
Pyrethrins, 4062, 4063, 4065, 4072, 4075–4077, 4079, 4083
Pyrethroids, 4063, 4064, 4074, 4076, 4078–4080, 4083
Pyrethrum, 4079
Pyridine alkaloid, 159
Pyriproxyfen, 4077
Pyrocatechol, 4065
Pyrogallol, 4065
Pyronotropane alkaloids, 353
Pyrrole, 1385
Pyrrolidine, 412
Pyrrolidine alkaloids, 237
Pyrrolidinium, 1290
Pyrrolizidine, 412
Pyrrolizidine alkaloids (PAs), 154–158, 161–163, 166, 293, 360, 419, 1049, 1179, 1191
Pyrus communis L., 1548
Pyruvic acid, 913
Pythium debaryanum, 980
Python Biotech, 2784

Q

QESAR. *See* Quantitative electronic structure activity relationship (QESAR)
Qian Ceng Ta, 1243
Q-LIT. *See* Quadrupole-linear ion trap (Q-LIT)
QSAR analysis, 946
Q-TOF. *See* Quadrupole time of flight (Q-TOF)
Quadrangularin A, 2153, 2162
Quadrupole-linear ion trap (Q-LIT), 1125
Quadrupole time of flight (Q-TOF), 1125
Quantitative electronic structure activity relationship (QESAR), 3352
Quantitative ^{1}H NMR, 1037
Quantitative structure activity relationship (QSAR), 3351
Quantum dots, 1036
Quassia, 3780
  Q. amara, 3781
  Q. indica, 3778
Quassin, 3347, 3778
Quaternary salt of cinchona alkaloids, 621
Quebrachine, 596
QuEChERS, 1054
Quercetagetin, 1829
Quercetagetin glycosides, 2164
Quercetin, 1655, 1663, 1824, 2155, 2156, 2158, 2161, 2224, 2346, 2439, 2568, 3501
Quercetin glycoside, 1728
Quinamine, 611
Quinazoline, 717
Quincoridine, 623
Quincorine, 623

Quinic acid conjugates, 1549
Quinicine, 611
Quinidine, 611, 617, 629
Quinine, 608, 611, 630
bark, 630
bitter taste, 631
constitution, 615
reductase, 3483, 3484
reductase inhibition, 3477
Quinolactacins, 834
Quinoline, 153, 412, 717, 1283
Quinoline alkaloids, 1178
Quinolizidine alkaloids (QAs), 152, 153, 157, 158, 160, 161, 166, 382, 1165, 1176, 1180, 1188, 1189
Quinolizidine ring, 382
Quinone reductase (QR), 2181, 3483
Quinones, 983, 2896
Quinonoidal base, 2468
Quitenine, 619
Quorum-sensing, 825
QW motifs, 2725

R

R65 (risk phrase), 3542
Radical scavenging, 2466
Radio-receptor assays, 57
Radiosensitizing, 3483
Radiotherapy, 3483
Ragwort, 155, 156
Ranaconitine type, 932
Random coupling model, 1561
Randomized controlled trial, 3942, 3948
RANK, 2405
Ranunculaceae, 802, 925
Raphidiopsis, 47, 49
Rapid, 1103, 2400
Raputia, 738
Rational modification, 1633
Raulinoa, 736
Rauscher leukemia virus, 991
Rauwolfia, 578, 589
Ravenia, 730
Raw garlic *per se*, 3666
2*R* configuration, 1756
R-dysidazirine, 980
Reactive acyl derivatives, 1077
Reactive oxygen species (ROS), 1308, 1811, 1832, 2321, 2466, 2572, 2621, 2917, 3485, 3562, 3618, 3670, 3715, 3716, 3721, 3925, 3933, 3943, 3946–3948, 4114, 4127, 4138, 4144
Reaxys, 3071
Recirculation phenomenon, 2398
Rectal, 1370, 1380
Red algae, 28
Red clover, 2413
Red grapes, 1719
Redox-sensitive mechanism, 2366
Reduced glutathione (GSH), 701
Reduced mitosis, 2403
Reduces proliferation, 2400
Reduces the incidence of hot flushes, 2407
Reduction, 2411, 3704, 3709–3713, 3718–3723, 3725, 3726
of BTM, 2414
of GST1, 3453
in hot flushes, 2407
of PSA, 2413
Reductionist approach, 1294–1295
Red wines, 1782, 1993, 2262, 2369
Reed canarygrass, 158, 159, 162
Refractive index (RI), 2735
Regional variations, 2410
Regulatory genes, 235
Rehmaglutin C, 3030
Rehmaglutin D, 3047
Reinioside C, 3218
Relatively safe compounds, 2683
Remijinine, 614
Renal excretion, 2293
Renewable substrates, 1631
Repellent effects, 4095
Repellents, 4049, 4077, 4100
Repetitive firing, 4067
Reproductive toxicity, 1412
Reserpine, 589, 596
Resilience, 2442
Resin ducts, 4042
Resiniferatox, 3187
Resistance against herbivores, 971
Resmethrin, 4072, 4077, 4078
Resolving agents, 607
Respiratory depression, 1373
Respiratory failure, 47
Respiratory reflexes, 333
Respiratory stimulant, 304, 332, 338
Restenosis, 2799
Resveratrol, 1582, 1728, 1903, 1906, 2162, 2279, 2282, 2286, 2296, 2299–2302, 2350, 2351, 2568, 2589, 2592–2594, 2596, 2598, 2601, 2603–2605
monoglucuronides, 2294
oligomers, 1906
receptor, 2296

Resveratrol-3-glucuronide, 2295
Resveratrol-3-sulfate, 2294, 2295
Retention time, 3336
Reticuline, 6–12, 17, 19, 21, 430, 447
Retina, 3723, 3925–3939, 3941, 3945, 3946, 3948
Retinal pigment epithelium (RPE), 3925, 3927–3929, 3937, 3939–3941, 3943–3947
Retinoids, 2675, 2695
Retinol, 3369
Retinopathy, 1450
Retroaldol-aldol mechanism, 2700
Retrochalcones, 1869, 1874, 1875
Retrograde amnesia, 292
Retronecine, 1058, 1064
Retro-Prince reaction, 3075, 3076
Retrorsine, 1061, 1063, 1385
Reversed phase
  cation exchange, 1101
  liquid chromatography, 1073
Reverse-phase high performance liquid chromatography (RP-HPLC), 691, 1086, 3359
Rewiring of native pathways, 1641
Rf, 3355
Rf value, 3329, 3335, 3336
Rhamnaceae, 1554
Rhamnocitrin, 1824
Rhamnose, 2467
Rhein, 1554
Rheumatic diseases, 1834
Rheumatism, 1305
Rheumatoid, 1964
Rheumatoid arthritis, 1444, 2960, 3964
Rhipicephalus, 4070
Rhipicephalus sanguineus, 4070
Rhizobia colonization, 3585
Rhizobium, 2390
Rhizoma coptidis, 1170, 1185
Rhizoperta dominica, 4095
Rhizophoraceae, 176
Rhizosphere, 163
Rhodomelaceae, 2891–2893
Rhodomela confervoides, 2893
Rhodophyllis membranacea, 34
Rhodophyta, 2886–2893
Rhodymeniaceae, 2891
Rhodymeniales, 2891
Rhopalosiphum padi, 160, 4094
Rhyzopertha dominica, 4091
Riccardin I, 1918
Riccardin II, 1918
Rice endosperm, 1732
Rice-lamina inclination bioassay, 3858
Rice-lamina inclination test, 3853
Ricin, 296
Ricinoleic acid, 1148
Ricinus communis, 296
Rimonabant, 3427
Ringer solution, 2907
Ripeness, 1766
Ripening of tomato fruits, 2787
Ri-plants, 2950, 2956
Ri-regenerants, 2961
Risk of bone fracture, 2416
Ri-T-DNA, 2948
Rivastigmine, 1244, 1341, 1342
RMS4/*MAX2*/*D3*, 3596
RMS5/*MAX3*/*D17(HTD1)*, 3596
RMS1/*MAX4*/*D10*/*DAD1*, 3596
RNAi. *See* RNA interference (RNAi)
RNA interference (RNAi), 238, 1719, 1729
Roadside drug screening, 1099
Rod photoreceptors, 3928, 3930
Rods, 3926, 3930, 3931, 3933, 3940, 3944–3947
ROESY, 3241
Rolandra fruticosa, 3530
rolB, 1704, 1707
Role as cancer preventive agents, 3448
The Role of cytokinins in planta, 969
The Role of purine alkaloids in Planta, 965
Rol genes, 2774, 2948
Root architecture, 3585
ROOTec bioactives Ltd, 2779
Root exudates, 1566
ROS. *See* Reactive oxidative stress (ROS); Reactive oxygen species (ROS)
Rosaceae, 1548, 1551
Rosa gallica, 2979
Rosefuran epoxide, 2982
Rose geranium oil, 290
Rose oxide, 2982
Rosin, 4039
Rosmarinic, 1964
Rosmarinic acid, 1571, 2150
Rosmarinus officinalis, 2984, 4091, 4121, 4128, 4140
ROS molecules. *See* Reactive oxidative stress (ROS) molecules
ROS scavenging, 1211
Rotenoids, 2151
Rotenone, 4077
Rough-mouthfeel, 1780
Route of administration, 2470

Routes of penetration, 4094
RP-8, 2780
RP-HPLC. *See* Reverse-phase high performance liquid chromatography (RP-HPLC)
Rstrogenic, 2408
Rubaceae, 1554
Rubanones, 619
Rubiaceae, 124, 804, 1551
Rubiaceous, 413
Rubitecan, 653, 666
Rubus idaeus, 1549
Ruminants, 4070, 4074
Ruta, 729
Rutaceae, 731, 4089
Rutin, 1728, 1831
Rutinosides, 2480
Ruzicka's rule, 2696

S
S62 (safety phrase), 3542
SA. *See* Salicylic acid (SA)
Sabinene hydrate, 2977, 2983
Sabinol, 2983
Saccharomyces cerevisiae, 978–979, 1627, 1705, 2804, 2828
S-adenosylmethionine (SAM), 59
S-adenosylmethionine-dependent methyl transferase, 61
Safflower, 1736, 1738
Safrole, 2902
Sagewood, 3531
Sakuranetin, 1569
Salicylaldehyde, 1548
Salicylate production, 971
Salicylic acid (SA), 986, 1565, 1695–1697, 1699, 1706, 1953, 4044
Salivary proteins, 2263
Salivation, 51, 4065, 4067, 4079
Salix spp., 1549
S-allylcysteine (SAC), 3665, 3667, 3668, 3674
S-allyl-L-cysteine sulfoxides
  alliin, 3666, 3667
Salmonella, 984
Salmonidae, 4076
Salpichroa, 3467
Salpichrolide G, 3473
Salutaridine, 11, 13, 20, 430, 448
Salutaridinol, 430
Salvianolic acid, 2150, 2163
Salvia spp., 2946
  S. guaranitica, 4094
  S. militiorrhiza, 2782, 2954
  S. officinalis, 2998, 4120, 4121, 4140, 4142, 4143
  S. procurrens, 4094
SAM cycle, 961
Sandfly, 1265
Sanguinaria canadensis L., 1447
Sanguinarine, 445, 448, 1447
Sanguisorboyl unit, 1983
Sanshiside D, 3050
Sanshiside methyl ester, 3050
Santalaceae, 786
Santalum spp., 4089
Santonin, 3079
Santonine, 2906
Sapindales, 821
Sapogenins, 2416, 2422, 3227, 3648
Saponification, 3371, 4027, 4033
Saponins, 2403, 2416, 2422, 2785, 2947, 3501, 3666, 3668, 3806
Saprophytic, 2802
Saquinavir, 1278
SAR. *See* Structure-activity relationship (SAR)
Sarcoma 180 cell line, 985, 986, 993, 996
Sarcomelicope, 734
Sarcoplasmic reticulum, 3992
Sargassaceae, 2897–2898
Sargassum spp., 2897
  S. confusum, 2897
  S. erosum (Haraldiophyllum erosum), 2897
  S. globulariaefolium, 2898
  S. horneri, 2897
  S. lophocarpum, 2898
  S. neurophorum, 2898
  S. polyacanthum (Sargassum siliquastrum), 2898
  S. vulgare, 2898
Sargentia, 736
Sarsasapogenin, 3230
Sativolides, 3472
Satureja hortensis, 4091
Saussurea medusa, 1557
Saxidomus giganteus, 53
Saxifragaceae, 851
Saxiphilin, 57
Saxitoxin (STX), 53, 54, 57, 59, 60, 64, 66, 957, 958, 963
S-benzylcysteine (SBC), 3673
Scale-up, 226, 1700, 1703, 1706, 2958
Scandoside methyl ester, 3057
Scavengers of free radicals, 1610
Sceletium, 1168
Sceptrin, 268

Schedule I drugs, 525, 540, 544
Schedule III, 544
Schinus areira, 4098
Schistosoma, 846
Schizanthus grahamii, 1182
Schizonepeta tenuifolia, 4094
Schizophrenia, 1207, 1307, 1491–1492
Schweinfurthins, 1933
Scirpusin A, 2282, 2286
Sclerotia-like cells, 706
Scolopendra, 788
Scopine, 349
Scopolamine, 175, 291, 349, 1013, 1188, 3657, 3710
Scopoletin, 1551
Scopolioside A, 3059
Scorodioside, 3055
Scoulerine, 11, 19, 20, 449
Scrophulariaceae, 851, 1554, 3641
Scrophuloside A4, 3059
Scrovalentinoside, 3059
SCX. *See* Strong cation exchange (SCX)
Scytonema, 58
Scytosiphonaceae, 2900
Scytosiphonales, 2900
Scytosiphon lomentarius (Scytosiphon lomentaria), 2900
Scytosiphon spp., 2900
SDG. *See* Secoisolariciresinol diglucoside (SDG)
SDS. *See* Sodium dodecyl sulfate (SDS)
S-dysidazirine, 980
Sea foods, 53, 2422
Seco-furoquinoline, 805
Secoiridoid, 576
Secoisolariciresinol, 2450
Secoisolariciresinol diglucoside (SDG), 2439
Secologanin, 88–91, 104, 109, 110, 576, 581, 623
Seco-loganin (=loniceroside), 3028
Seco-loganin dimethyl acetal, 3047
Secondary amine, 44
Secondary bicyclic amine alkaloids, 41–42
Secondary growth, 3585
Secondary infection, 704
Secondary metabolism (SM), 2805, 2958
Secondary metabolites, 4, 6, 150, 151, 154, 155, 159, 160, 165, 1544, 1719, 1952, 2583, 2584, 2592, 2593, 2600, 2667, 2927, 2961, 3517
Secondary plant metabolites, 3664
Secondary toxicity, 1311, 1317
Second bean internode bioassay, 3853
Second phases, 2780
β-Secretase, 2286
Secretory cells, 2923
Secretory epithelial cells, 4040
Securidaca, 3206
Securidaca longepedunculata, 3211, 3213
Sedamine, 337
Sedative, 2906, 4115, 4128
  effect, 4115
  hypnotic, 3220
Sedridine, 306, 337
(+)-Sedridine, 306
Seed bank, 3597
Seirococcaceae, 2898
Seizures, 4067, 4079
Selagine, 1241
Selected ion monitoring (SIM), 180
Selected reaction monitoring (SRM), 1146, 1147
Selective adsorbent, 3332
Selective estrogen receptor modulator, 1667
(Z)-Selective prenyltransferases, 2713
Selectivity filter, 64
Seminal vesicle, 2421
Senecio jacobaea, 155
Senecionine, 1052, 1054, 1061, 1062, 1178, 1385
Senecio spp., 1384, 1553
Seneciphylline, 1061, 1062, 1178, 1386
Senegin III, 3217
Senile plaques, 3712, 3716
Sensitivity enhancement, 1105
Sensory analysis, 2267
Sensory characteristic, 2906
Sensory perception, 2264
Sepiapterin reductase, 1209
Sepsis, 1306
S-Eq producers, 2402
Sequence tags, 2954
S-equol (*S*-Eq), 2398
SERCA, 3077, 3090, 3091, 3093, 3095
Sergeolide, 3778
L-Serine, 942
Serine protease, 987, 988
Serjania meridionalis, 4094
Serotonergic (5-HT), 3709, 3711
  neurons, 1516
  system, 1309
Serotonin, 598, 686, 4068
Serpentine, 1126
Sertüurner, 1368, 1373
Sesame oil, 4065
Sesamin, 4065

Sesamolin, 4065
Sesquicannabigerol, 3420
Sesquiterpenes, 2723, 2763, 2782, 2831, 2946, 3081–3083, 3085, 3087–3090, 3094, 3981, 4089
  coumarins, 3532
  cyclases, 3085
  lactones, 2782, 2947, 3076, 3081, 3083, 3086–3090, 3094, 3529
Sesquiterpenoids, 2589, 2593, 2736, 3081–3083, 3520
Sesqui-triterpenes, 2670
Sesterpenes, 2670
7th day Adventists, 2409
Severinia, 723
Sex differentiation, 2418
Sex hormone binding globulins, 2398
SFE. *See* Supercritical fluid extraction (SFE)
SFRP2, 2194
Shampoos, 4078, 4083
Shapiro, 3081
SHC. *See* Squalene/hopene cyclase (SHC)
Shear stress, 193, 2777
Shelf life, 3978
Shellfish, 53
Shikimate, 88, 90
Shikimate pathway, 2314, 4090
Shikimate/phenylpropanoid pathway, 1546
Shikimic acid, 536, 1857
Shikimic acid pathway, 1545
Shikonin, 2766
Shoot cultures, 186, 2776
Short-chain alcohol dehydrogenase, 61
Short-chain dehydrogenase, 701
Short plasma residence time, 2423
Short-term memory, 3707, 3722
Shower dipping, 4074
Shuangyiping, 1245
Sialogogue, 877
Sida cordifolia, 1219, 1221
Siddha system, 3477
Side effects, 543
Side-groups
  ethoxy, 1667
  halide, 1667
  hydroxy, 1667
  methoxy, 1667
Siderophore, 986
Sieboldine A, 1248
Signaling cascades, 4051
Signaling factors, 1736
Signaling pathways, 2619, 2623, 2626
Signal transduction, 3526
Silatecan, 653, 666
Silenced, 2805
Silibinin, 2224
Silica gel, 691, 2342, 3328
  60F254, 3334, 3335
  GF plates, 3336
Silver thiosulfate, 2800
Silylation, 179
Simaba, 3780
Simalikalactones, 3354, 3778
Simaroubaceae, 769, 3346
SIM mode. *See* Single ion monitoring (SIM) mode
Simple, 1915
  diffusion, 2472
  fermentation technique, 1626, 1641
  phenols, 1548
Sinapic acids, 1549
Sinapyl alcohol, 1561
Sinensal, 2985
Single ion monitoring (SIM) mode, 1027
Single reaction monitoring (SRM), 1060
Single-step engineering, 233
Singlet oxygen, 1832
Sinomenine, 1187, 1448
Sinomenium acutum, 1187
Sinomenium acutum Rehder and Wilson, 1448
Sinusoidal obstruction syndrome, 1387
Siphonaxanthin, 3254, 3256, 3257, 3265
Sirtuin, 2301
Sirtuin activation, 2301
Sissotrin, 1663
Sitamaquine, 1277
Site-directed mutagenesis, 1666
Site 1 toxins, 64
Sitophilus
  S. americana, 4095
  S. oryzae, 4091
  S. zeamaïs, 4094
Sitosterol, 3237, 3243
β-Sitosterol, 3666
Sitotroga cereallela, 4095
Size exclusion chromatography, 2071
Sjögren’s syndrome, 877
SkD. *See* Simalikalactone D (SkD)
Skimmia, 722
Skin, 2262, 2583, 2584, 2588–2590, 2592, 2601, 2604, 3628
  ailments, 3477
  protection, 3628
Skin + fat, 4080
Skp2 suppression, 3485
Skytanthine-type alkaloid, 930

Slackia, 2444
Slash pine, 4047
Sleep deprivation, 1471, 1473, 1484–1485
Slovanolides, 3075
SmCPS, 3574
Smilagenin, 3230
SmKSL, 3574
Smoking cessation, 334
Sodium channel blocker, 54
Sodium channels, 3994, 4065–4067, 4069
Sodium cholate, 1180
Sodium dodecyl sulfate (SDS), 1177, 1191
Sodium stibogluconate, 1273
Sodium tanshinone IIA sulfonate (STS), 3570
Soft coral, 3467
Solanaceae, 176, 291, 344, 979, 1169, 1345, 1551, 3467, 3476, 3590
Solanaceous, 413
Solanacol, 3587
Solanacyl acetate, 3587
Solanum, 2856, 2862, 2863, 2865, 2873
Solanum lycopersicum, 1585, 3587
Solenopotes capillatus, 4069
Solid carcinomas, 1314
Solid phase extraction (SPE), 177, 324, 691, 1051, 1053–1056, 1058, 1060, 1084, 1139–1141, 1143, 1150, 2033–2037, 2068, 2071, 2112
Solid phase microextraction (SPME), 1019, 1084, 2072
Solid tumors, 1454
Solieriaceae, 2890
Solieria robusta, 2890
Solubility, 890, 1663, 2109, 3370
Soluble expression, 1660
Solvents, 2016–2027, 2030–2037, 3370
  extraction, 2017–2022, 2027, 2031–2033, 2037, 3290, 4027
  system, 2147, 2148, 2150, 2161, 2163
Somaclonal variation, 191, 2770
Somatic embryogenesis, 3198
Somatostatin, 3709
Sophora flavescens, 1165, 1639
Sorangium cellulosum, 990
Sorbitol, 2781
Sorghum bicolor, 1569, 3585
Sorghum spp., 1548
Sorgolactone, 3585
Sorgomol, 3586
Soxhlet extraction, 2069, 2111
Soy, 2413, 2414, 2422, 2437
  concentrates, 2411
  extracts, 2411
  isoflavone, 2449
  protein, 2403
  sauce, 2391
Soya saponins, 2416
Soy-based food supplements, 2411, 2413, 2414
Soy-based infant formulas, 2391, 2418, 2422
Soybeans, 1736, 1852
Soy-germ, 2415
Soy phytochemical concentrate (SPC), 2214
Sparteine, 151, 158, 383
Spasmodic cough, 3477
Spasmolytic, 2906
Spathelia, 752
Spathulenol, 2988
Spatial, 2414
  memory, 2285
  working memory, 2415
Spay-bioreactor, 2778
SPC. *See* Soy phytochemical concentrate (SPC)
SPE. *See* Solid phase extraction (SPE)
Special isoprene rule, 2669
Specific doses, 2423
Specific symptoms, 2423
Spectral generalization of withanolides, 3472
Spectrophotometric methods, 2073
Sperm count, 2420, 2421
Spermidine, 1732
Spermine, 1732
Sperm production, 2418
Sphacelariaceae, 2900–2901
Sphacelariales, 2900–2901
Sphaerococcaceae, 2890
Sphaerococcus coronopifolius, 2890
Sphaerotrichia japonica (Sphaerotrichia divaricata), 2896
Spherisorb ODS2, 3393
Spheroidene, 3270, 3271
Spheromastigote, 845
Spinacea oleracea, 864
Spinacia oleracea, 3584
Spindle cell sarcoma 45, 992
Spinosin, 2163
Spiranoid δ-lactone, 3472
Spiranthera, 735
Spirilloxanthin, 3268, 3270, 3271
Spirilloxanthin pathways, 3268, 3270, 3271
Spirostanols, 3227–3230, 3232, 3239, 3241, 3244
  glycsoide, 3230
  saponins, 3227, 3228

Spleen disorders, 3477
SPME. *See* Solid phase microextraction (SPME)
Spodoptera littoralis, 4095
Sporadic AD, 3716
Sporochnaceae, 2901
Sporochnales, 2901
Sporochnus comosus, 2901
Sporopollenin, 1592
Spray reagents, 692
Squalene, 2715
Squalene/hopene cyclase (SHC), 2724
Src/PI3-kinase/Akt, 2366
SRM. *See* Selected reaction monitoring (SRM)
SRT501, 2221
Stable flies, 4075, 4076
Staphylococcus
  S. aureus, 979, 980, 989, 1310, 2906, 4124, 4149
  S. epidermidis, 4126
STAT, 2195
Static extraction, 1017
Statine, 986, 987
Status, 2407
Stauranthus, 740
Steam distillation, 3290, 3667, 3978, 4090
Stearidonic acid, 1737
Steatosis, 1385
Stegobium paniceum, 4091
Stemmedine, 584
Stemona, 1175
Stemona alkaloids, 1183
Stephaniae tetrandrae, 1179
Stephania tetrandra S. Moore, 1449
Stephanine, 431
Stephanitis pyri, 4094
Stepholidine, 449
Sterculiaceae, 725
Stereocenters, 947
Stereochemistry, 3241, 3242
Stereoisomerism, 1014
Stereoisomers, 912, 2109, 3647
Stereospecific conversion, 1670
Stereospecificity, 4069
Steroidal, 412
  alkaloids, 3237
  lactones, 3468, 3473, 3488
Steroid hormone receptors, 3508
Steroids, 2733–2735, 2737–2741, 2746–2749, 2751, 2753, 2755–2757, 2763, 3666, 3670
Sterol, 2739
D24,25-Sterol methyltransferase (SMT), 1268
Sterol regulatory element-binding protein-1 and-2, 2741
Stevia, 3198
Steviol, 3197
Stevioside, 3197, 3199
Stierle, D., 2801
Stigmasterol, 3237, 3243
Stigmatella, 829
Stilbenes, 1553, 1629, 1809, 2350, 2589, 2592
  monomers, 2282
  synthase, 1583, 1654, 1688, 1689
Stilbenoids, 1654, 1901, 1903, 2277, 2296, 2297
Stilbenoids in wine, 2282
Stilbericoside, 3016
Stirred system, 2777
Stirred tank, 1693, 1700, 1701
Stirred tank bioreactor, 188
Stoichiometric-based modeling, 1671
Stomoxys calcitrans, 4069
Stratum corneum (SC), 2681, 3759
Streptococcus faecalis, 979
Streptomyces spp., 728, 1293, 1627
  S. ardus, 983
  S. avermitilis, 1638
    S. ceolicolor, 1638
    S. lividans, 1638
    S. venezualae, 1638
  S. caespitosus, 983, 985
  S. carzinostaticus, 990
  S. coelicolor CH999, 990
  S. fradiae SCF5, 984
  S. globisporus, 990
  S. griseofuscus S-42227, 987
  S. lavendulae, 984
  S. sahachiroi, 988
  S. sandaensis 6897, 981
  S. verticillatus, 983
Streptomycetes MD 398-Al, 980
Stress, 1471, 1473–1475, 1479, 1485–1487, 1493, 2589, 2591–2596, 2605, 3644, 3711, 3719, 3720, 4117
  factors, 1688
  hormone, 2956
Strictosamide, 136
Strictosidine, 88, 92, 93, 98, 104, 109–111, 576, 581
Strictosidine synthase, 623
Strictosidinic acid, 136

Striga
S. asiatica, 3584
S. lutea, 3584
Strigol, 3584
Strigolactones (SLs), 3584
Strigol series, 3589
Strigyl acetate, 3585
Strobiline, 353
Strong cation exchange (SCX), 1056, 1058
Strophanthidin, 3750
Stropharia, 528, 533
Structural type, 3320
Structure-activity relationship (SAR), 1205, 1667, 3351, 3479
Structure-based design, 1247
Structure-fragmentation relationship (SFR), 3474
Strychnine, 590
Strychnine-like effect, 4068
Strychnos nux-vomica L., 590, 1191, 1451
sts genes, 1688, 1690, 1692, 1704, 1705
STX. *See* Saxitoxin (STX)
Stylissadines, 264
Subcellular compartments, 98
Subcutaneous, 1370, 1380
Suberin, 1562
Subfertile couples, 2420
Subhirsine, 354
Submerged cultivation, 706
Submerged culture, 545
3-Substituted-4-benzyltropane alkaloids, 352
3-Substituted-6,7-epoxytropanes, 349
Substrate
feeding, 1666
requirements, 1667
specificity, 1666
specificity of NAT, 1208
Subtrifloralactone K, 3484
Subtrifloralactone L, 3484
Subtrifloralactones A, 3484
Subtrifloralactones F, 3484
Succinate dehydrogenase, 61
Sucrose, 1692, 1698, 1699, 1701, 2837
Sudan, 531
Sugar absolute configuration, 3242
Sugars, 1807, 2108, 2837
Suicidal germination, 3597
Sulfatation, 2397
Sulfenic acids, 3666, 3674
Sulfhydryl, 3674
N-Sulfocarbamoyl, 63
N-Sulfocarbamoyl toxins, 54, 55
Sulfotransferase, 2475
Sulfoxide, 4065
2-Sulfoxymethyl-3-thiomethyl-4,6-dibromo-1 *H*-indole, 33
Sulfur compounds (S), 2886, 2887, 2892–2894, 2896, 2897, 2899, 2901–2904, 2907
Sulfur-containing bromoindole alkaloids (SBRI), 32, 33
Sulfur dioxide, 3666
Sumithrin, 4072, 4077
Summation methods, 1058
Supercritical fluid chromatography (SFC), 3144
Supercritical fluid extraction (SFE), 1018, 1123, 2022, 2024–2026, 2032, 2037, 2069, 2111, 3291, 3371
Superoxide, 1832
Superoxide anion (O^{-2}), 2370, 3712
Superoxide dismutase (SOD), 2184, 3710, 3713, 3718, 3963, 4113, 4127
Suprafacial proton transfer, 2707
Surveillance, 3125, 3145, 3148
Survival, 2321
Survivin expression, 3561
Suspension cultures, 1861, 2800
Sustainable agriculture, 2647
Sustainable development, 4102
19-*S*-vindolinine, 1126
Sw-620, 3485
Sweat patches, 1099
Sweetening agent, 3185
Sweet itch, 4071
Sweet lupins, 157
Swerilactone A, 3056
Sweroside, 3028
Swertiamarin, 3028
Swine, 4076
Swinglea, 761
Swollen shoot, 1602
Symbiosis, 2390
Symbiotic communities, 690
Sympathomimetic, 912, 1225, 1226, 1229
activity, 1205
potency, 1209
Symphytine, 1059, 1061
Synaptic plasticity, 1474–1477, 1485–1487
Synaptosomes, 1515
Synephrine, 290, 1220, 1228–1230
Synergism, 3541, 3983
Synergistic effects, 1455
Synergists, 4065, 4077
Synthetic biology, 1626, 3108, 3575
Synthetic perfumes, 3291
Synthetic pyrethroids, 4063, 4083
Systematics-guided investigation, 3575

Systems biology, 2435, 2837
Systolic blood pressure (SBP), 3674–3676, 3682
Syzygium aromaticum, 2998

T

TA. *See* Terpenyl acetate (TA)
TA-associated receptor 1 (TAAR1), 1206
Tabersonine, 94, 97, 104, 105, 108–110, 579, 584, 587, 588, 1126
Taccaceae, 3467
Tagetes minuta, 4098
Tall fescue grass, 1142
Tanacetum vulgare, 2983, 4095
Tanager birds, 157
Tandem mass spectrometry, 3316, 3398
Tannic acid, 2342
Tannin-anthocyanin reaction, 2266
Tanning, 1608
Tannins, 1559, 2588
Tannins perception, 2264
Tannoids, 2342
Tanshen, 3553
Tanshindiol, 3554
Tanshinone I, 3553
Tanshinone IIA, 3554
Tanshinones, 2782, 3553
Taonia atomaria, 2895
Taraxacum, 3528
Targeted gene disruption, 3131, 3133
Target tissues, 2295
Taspine, 1448
Tau-fluvalinate, 4063, 4076
Tauopathies, 2289
Tau protein, 2284
Taurine, 4069
Taxa-4(20), 11(12)-dien-5α-ol-*O*-acetyltransferase (TAT), 2803
Taxadiene, 2804
Taxanes, 2766, 2799, 2957
Taxoids, 3161, 3162, 3170
Taxol, 2695, 2815, 2829, 3159–3170, 3186
Taxotere, 3187
Taxus, 2766
  T. baccata, 2948
  T. brevifolia, 2799, 2801, 2948
  T. media, 2957
TBA. *See* Tribromoanisole (TBA)
T-DNA. *See* Transfer-deoxyribonucleic acid (T-DNA)
T-domain, 703
TDP-D-allose, 1636
TDP-glucose synthase, 1670
TDP-L-rhamnose, 1636
TE. *See* Terpinolene (TE)
TECA. *See* Titrated extract of *Centella asiatica* (TECA)
Tecomoside, 3021
Temozolomide, 2216
Tempeh, 2391, 2410
Temporary immersion system, 197
Tenebrionidae, 985, 4095
Tension-headaches, 65
Teonanacatl, 526
Teratogenic, 360, 900
Terpenes (TERPs), 2567, 2667, 2735, 2886–2888, 2890–2907, 3289, 3519, 3759, 3760, 3762, 3980, 4021
  aldehyde, 2957
  biosynthetic activity, 4051
  lactones, 3517
  origin, 3977, 3981
  trilactones, 3701, 3716
    diterpene trilactones, 3701
Terpenes/terpenoids, 2961
Terpene synthases (TPSs), 2717, 2820, 2836
Terpenoid indole alkaloids (TIAs), 94
Terpenoids, 4, 2733–2735, 2814, 2815, 2915, 3194, 4089
  biosynthesis, 3854
  indole alkaloids, 89, 92, 98, 101, 103–105, 107, 108
Terpenyl acetate (TA), 2902
γ-Terpinene, 2976
Terpinen-4-ol, 2991, 4089, 4099
Terpineol (TOL), 2887, 2896, 2902, 4047
Terpinolene (TE), 2887, 2888, 2890, 2901, 2903, 2905
α-Terpinyl cation, 2721
TERPs. *See* Terpenes (TERPs)
Testicular size, 2418
Testosterone, 1374, 2413
Tetrabenazine, 1351
Tetrabromo-2-heptanol, 2889
2,3,4,6-Tetrabromo-1*H*-indole, 32
2,3,5,6-Tetrabromo-1*H*-indole, 32
2,3,4,6-Tetrabromo-1-methyl indole, 32
2,3,5,6-Tetrabromo-1-methyl indole, 32
Tetrabromo-2-nonanol, 2889
1,1,3,3-Tetrabromo-2-nonanone, 2889
Tetradecanal, 2887, 2891, 2892, 2904
Tetradecane, 2892
Tetradecanoic acid, 2894
Tetradecanol, 2891, 2892
Tetradecene, 2891, 2892

Tetradium, 724
Tetraflavonoids, 1621
Tetrahydrofuran (THF), 1175, 3371
Tetrahydronaphthalene-1-ol (TNO), 3566
Tetrahydroneotanshinlactone (TNT), 3566
Tetrahymena pyriformis, 336
Tetramethrin, 4066, 4077
(1R*,2S*,3R*,5S*,8S*,9R*)-2,3,5,9-Tetramethyltricyclo-[6.3.0.01,5] undecan–2-ol, 2893, 2906
(1S*,2S*,3S*,5S*,8S*,9S*)-2,3,5,9-Tetramethyltricyclo-[6.3.0.01,5] undecan-2-ol, 2893, 2906
Tetrandrine, 1179, 1449
Tetrapetide, 987
Tetraploid transformed roots, 202
Tetraterpenes, 2670
Tetraterpenoids, 2915
Tetrodotoxin, 64, 957, 958, 963, 3996
Tetrodotoxin biosynthesis, 965
Textural profiles, 2265
Thalidomide, 1444
Thamnosma, 733
Thamnosma rhodesica, 1290
Thapsia, 3070, 3073, 3090
Thapsigargin, 3072, 3073, 3077, 3079, 3085, 3086, 3090, 3091, 3093, 3094
Theacrine (1,3,7,9-tetramethyluric acid), 956
Theaflavins, 1782
Thearubigins, 1782
Thebaine, 429, 434, 438, 1073, 1169, 1170, 1172, 1181, 1369
Theobroma cacao (cacao), 956, 1178
Theobromine (3,7-dimethylxanthine), 954–956, 1178
Theobromine synthase, 959
Theonella aff. *mirabilis*, 986, 987
Theophylline, 961, 1178, 1181
Theophylline (1,3-dimethylxanthine), 955
Therapeutic, 543, 978, 985, 3840, 3844, 3845
The Therapeutic indices, 543, 1251, 1508
Therapeutic target, 1459
Therapeutic value, 2649
Thermogenesis, 1225, 1229
Thermogenic influence, 1423
Thermoinhibited seeds, 3597
Thin-layer chromatography (TLC), 180, 915, 1020, 1055, 1056, 1064, 1137, 1139, 1142, 1143, 3144, 3314, 3328–3330, 3334, 3335, 3376
Thioesters, 50
2-Thiomethyl-3-sulfoxymethyl-4,6-dibromoindole, 33
3-Thiomethyl 2,4,5,6-tetrabromo-1*H*-indole, 32
3-Thiomethyl 2,4,6-tribromo-1*H*-indole, 32
Thiowithanolide, 3482
Three-dimensional structure, 64
Three dose-related levels (acute, sub-acute and chronic), 373
Three-phase partitioning (TPP), 3333
L-Threonine, 365
Thresholds, 2347
Thrombocyte, 3682
Thrombosis, 3670, 3677, 3683
Thrombospondin, 1444
Thromboxane A_2 (TXA_2), 3679
Thromboxane-B_2 (TXB_2), 3679
Thujanol, 2983
Thuja occidentalis, 2983, 2984
β-Thujaplicin, 2781
Thujones, 2983, 2999, 4089, 4142–4144, 4146
Thylakoids, 98, 100
Thymidine, 991
Thymidylate synthase (TS), 1270
Thymidyldiphosphate, 1670
Thymol, 2977, 2980, 2997, 2998, 4096, 4137, 4138
Thymoquinone, 2981
Thymus
  T. serpyllum, 4091
  T. vulgaris, 2977, 2979, 2983, 4090
Thyroid hormone, 3652
Thyroid hormone receptors, 2422
TIAs. *See* Terpenoid indole alkaloids (TIAs)
Ticks, 4064, 4069–4072, 4075–4078, 4083
Ticorea, 734
Tigliane, 3180
Tiglic aldehyde, 2887
3-Tigloyloxy-6-propionyloxy-7-xydroxytropane, 203
Time-of-flight mass spectrometry (LC/TOF-MS), 3144
T7 inducible plasmid, 1659
Tinuclear metal cluster, 2719
Tissue distribution and elimination, 1414
Tissue inhibitor of MMPs and TIMP2, 2202
Titrated extract of *Centella asiatica* (TECA), 3961
293T kidney epithelial cells, 1293
TL, 3144
TLC. *See* Thin-layer chromatography (TLC)
TL-DNA, 2948
TLR4. *See* Toll-like receptor 4 (TLR4)
TMA. *See* Trimethylamine (TMA)

T$_{max}$, 2398
T. media Hicksii, a hybrid *T. media* Hicksii, a hybrid, 2800
TMS ethers. *See* Trimethylsilyl (TMS) ethers
TNF. *See* Tumor necrosis factor (TNF)
TNFα. *See* Tumor necrosis factor alpha (TNFα)
Tobacco, 159, 160, 979, 1346, 1347, 1355, 4122, 4123, 4142, 4148
Tobacco alkaloids, 1183
Tocopherols, 2567, 3386
Tocotrienols, 2567
TOCSY. *See* Total correlation spectroscopy (TOCSY)
Toddalia, 720
Tofu, 2391, 2409, 2410
Tolerability of hupA, 1256
Toll-like receptor 4 (TLR4), 3569
Tolserine, 1341
Tomato, 2650, 2651, 2854–2860, 2862–2865, 2867–2874
Tomato fruit, 1728
Tonyu, 2391
Topoisomerase I, 661
Topoisomerases, 1271
Topotecan, 645, 650, 652, 665
  biological activity, 665
  semisynthesis, 652
Total cholesterol (TC), 3668–3670
Total correlation spectroscopy (TOCSY), 3240
Total ion chromatogram, 325
Total phenolic content (TPC), 2073–2075, 2528–2537, 2540–2542, 2544–2547, 2549, 2550, 2552–2554
Total synthesis of quinine, 607–608
Toxicity, 543, 1209, 1210, 1220, 1226–1230, 1233, 1516, 1671, 2291, 2328, 3628, 4095
Toxicity of purine alkaloids in animals, 969
Toxicokinetic properties, 4068
Toxicokinetics, 4068
Toxicological characteristics, 1516
Toxicological studies, 1249
Toxicology, 3165, 3168, 3199
Toxic poisons, 1504
Toxin binding site 1, 64
TPC. *See* Total phenolic content (TPC)
TPP. *See* Three-phase partitioning (TPP)
TPSs. *See* Terpene synthases (TPSs)
Trace elements, 3665, 3666
Tracheal muscle, 3991
Trachomas, 1319
Traditional, 2391
  and alternative medicines, 3519
  breeding, 1737
  communities, 132
  medicine, 1507
TRAIL, 2199, 2217
TRAMP, 2210
Transacetylase, 827
Transamination reaction, 925
Trans conformation, 1756
Transcriptional activities, 2399
Transcriptional level, 2825
Transcriptional regulators, 236
Transcription factors, 88, 103, 108–111, 139, 235, 243, 245, 1690, 1729, 1838, 2385, 2834, 3133, 4114, 4115, 4118, 4148
Transcriptome, 2953
Transcriptomic, 220, 243
Transdermal administration, 1251
Transdermal drug delivery (TDD), 2681, 3758, 3759
Transesterification, 4027, 4033
Trans-e-viniferin, 1925
Transfer-deoxyribonucleic acid (T-DNA), 189
  activation tagging, 2962
  insertion, 2952
Transformed, 2785
Transformed lines, 2868
Transforming growth factor-α, 2198
Transforming growth factor beta 1, 1451
Transgene, 1719
Transgenic cell culture, 110
Transgenic hairy root, 193
Transgenic hairy root cultures, 109, 110
Transgenics, 1728, 1736, 1815
  plant, 3106, 3112, 3113
  yeast, 110, 242
Transient receptor potential (TRP), 4122, 4123, 4134, 4137, 4146–4148
  ion channels, 4122
  TRPM8, 3992
  TRPV1, 3426
Transient receptor potential (TRP) channels, 3991, 4003, 4123
Transintestinal cholesterol efflux (TICE), 3446
Trans-isomers, 4066, 4069
Transketolase (TKT), 15
Transmembrane proteins, 2471
Trans-pinane, 4098
Trans-pinocarveol, 2984
Transplastomic, 2833
Transporters, extracellular, 1672

Trans-R, 1706, 1707
Trans-resveratrol, 1569, 1685
Trans-stilbene, 1553
Trans-zeatin, 958
Trans-zeatin-type cytokinins, 956
TR-DNA, 2948
Trechonaetes, 3467
Treemoss, 3299
Trehalose, 2646
Tri5, 3130, 3131
Tri101, 3131
Trialeurodes vaporariorum, 4094, 4095, 4098
Tribolium
  T. castaneum, 4091, 4095
  T. confusum, 985, 4095
Tribromoanisole (TBA), 2893, 2902
1,3,3-Tribromo-2-heptanone, 2889
trans-1,3,3,-Tribromo-1-hepteneoxide, 2889
2,3,6-Tribromo-1*H*-indole, 32
2,4,6-Tribromo-1*H*-indole, 32
3,5,6-Tribromo-1*H*-indole, 32
2,3,5-Tribromo-1-methyl indole, 32
2,3,6-Tribromo-1-methyl indole, 32
3,5,6-Tribromo-1-methylindole, 32
1,2,3-Tribromo-(1-octene-3-ones), 2889
Trichilia connaroides, 2988
Trichoderma harzianum, 1639
Trichodiene, 3129
Trichomes, 2835, 2921
Trichomonas vaginalis, 1310
Trichothecene chemotypes, 3127
Trickle-bed bioreactor, 193
Tricyclene, 2984
Tricyclic diterpenoidquinones, 3556
Tridecanal, 2887, 2892, 2900
Tridecanol, 2891, 2892
Tridecene, 2892
Triethylamine, 3385
Triflavonoids, 1621
Trifolium, 1553
Trifolium pratense, 3586
Tri gene, 3130–3133
Trigger substances, 1568
Triglyceride (TG), 3668, 3669, 3676
Trigonelline, 1354
3,4,5-Trihydroxybenzoic, 1963
4,5,7-Trihydroxyisoflavone, 2222
3,5,4′-Trihydroxy-trans stilbene, 2223
Trimeric tropane alkaloid, 355
(E)-3,4,5-Trimethoxycinnamoyl, 3211
Trimethylamine (TMA), 2887. 2889–2891, 2893, 2894, 2896, 2897, 2899–2904
Tri-methylhalosilanes, 1076
6,10,14-Trimethyl-2-pentadecanone, 2891, 2892
Trimethylsilylation, 1076
Trimethylsilyl (TMS) ethers, 1057, 3592
Trinitario, 1601
Trinuclear magnesium cluster, 2710
Triohima A, 3031, 3048
Triohima B, 3048
Triohima C, 3048
Tripeptidyl residue, 703
Triphenanthrene, 1925
Triple quadrupole, 58
Trisdesmoside, 3227
Trisdesmosidic, 3233
Tris(2,2′-bipyridil)ruthenium(III), 1087
3,6,7-Trisubstituted tropanes, 349
Triterpenes, 2763, 3666, 3963
  lactone, 2953
  saponins, 2770, 3207
Triterpenic glycosides, 2951
Triterpenoids, 2737, 2744, 2915, 2955, 3854
  aglycone, 3806
  glycosides, 2947
  saponins, 2952, 3312, 3641
Triticum aestivum, 4098
Tropane, 412
Tropane alkaloids, 222, 237, 344, 1165, 1175, 2774
Trophic levels, 154, 156, 165, 166, 2937
Tropical pines, 4047
Tropine, 346
Tropinone reductase, 346
Tropinone reductase I (TR-I), 184
Tropinone reductase II (TR-II), 184
3-Tropoyloxy-6-acetoxytropane, 203
3-Tropoyloxy-6-hydroxytropane, 349
TRP. *See* Transient receptor potential (TRP)
TRP channels. *See* Transient receptor potential (TRP) channels
Trpene cyclases, 2717
True alkaloids, 218
Trypanosoma, 845
Trypatamines, 544
Trypomastigote, 845
Tryponacidal, 3488
Trypsin, 987, 988
Tryptamine-4,5-epoxide, 540
Tryptamines, 135, 534, 837
Tryptophan, 417, 533, 576, 837, 1451
L-Tryptophan, 685
Tryptoquialanine, 840
Tryptoquivaline, 840
Tsetse flies, 4072

TS-syndrome, 4065
T syndrome, 4065
TTFCA, 3968
T2 toxin, 3125, 3130–3333
Tube formation, 1445
Tubocapsanolide A, 3485
Tubocapsicum, 3467
Tubulin, 588
Tumor, 336, 1313, 1442, 3478, 3482, 3483
  angiogenesis, 1211
  cell, 2403
  cell lines (A549, SK-OV–3, SK-MEL-2 and HCT15), 3480
  growth, 1447
Tumorigenesis, 2404
Tumor necrosis factor (TNF), 2679, 3524
Tumor necrosis factor alpha (TNFα), 1451, 1870, 1873, 1879, 1881, 3218, 3529, 3559, 3963, 4117, 4119, 4120, 4131, 4134, 4140, 4141, 4145
Tumors, 918, 3489
Turpentine, 2668, 2974, 4039
Two-dimensional, 2124
Two-dimensional HPLC, 2108
Txs, 2784
αα-Type, 2710
Type and sequence of monosaccharide, 3320
Type 2 diabetes mellitus (T2DM), 3808
ε-Type enzyme, 2717
Type I IDI, 2705
Type II diabetes and obesity, 1211
Type II IDI, 2707
Type II poisoning syndrome, 4067
Type II pyrethroids, 4065
Type I pyrethroids, 4065
Tyramine (TYR), 28, 29, 1207
Tyria jacobaeae, 154, 156
Tyrolobibenzyls, 1916
Tyrosinase, 15, 17
Tyrosine, 6, 15, 17, 417, 1446
L-Tyrosine, 6, 8, 14–17, 484
Tyrosine hydroxylase (TH), 15
Tyrosine kinase, 2192
TyrR, 15, 16, 21

U

Ubiquinone (UQ), 2695, 2737
Ubiquitinated protein, 3485
Ubiquitine-proteasome pathway, 3485
Udoteaceae, 2903
UDP. *See* Uridine diphosphate (UDP)
UE. *See* Ultrasonic extraction (UE)
UHPLC. *See* Ultra high performance liquid chromatography (UHPLC)
Ulcer, 3482, 4125, 4131
Ulothrichaceae, 2901
Ulotrichales, 2901
Ultrafiltration (UF), 2072, 3197
Ultra high performance liquid chromatography (UHPLC), 1058, 1059, 1061, 1087, 1144, 1148
Ultra high performance liquid chromatography (UPLC), 3593
Ultra-performance liquid chromatography (UPLC), 1023, 3861
Ultrarapid metabolizers, 1378
Ultrasonication, 3333
Ultrasonic extraction (UE), 2027–2028, 2037
Ultrasound-assisted extraction, 1124, 2069
Ultraviolet (UV), 90, 106, 107, 3328, 3472
  absorption, 3592
  B radiation, 2930
  detection, 1024
  light, 1688, 1690, 1697, 1698, 1700
  treatment, 2773
Ultraviolet visible (UV–vis), 2118, 2128
  detector, 3387
  spectrophotometry, 3314, 3377
Ulva
  U. lactuca, 2902
  U. pertusa, 2902, 2904, 2906, 2907
  U. rigida, 2902
Ulvaceae, 2901–2902
Ulvales, 2901–2902, 2906
Ulvaria obscura, 29
Umbelliferae, 3533, 4089
Umbelliferone, 1551
Umbelliprenin, 3533
Uncaria, 121
Unclear, 2416
Undaria pinnatifida, 2898
1,4-Undecadien-3-one, 2894
Undecanal, 2891, 2892
Undecanol, 2891, 2892
3-Undecanone, 2894
Undecaprenyl diphosphate (UPP) synthases, 2713
1,3,5-Undecatriene, 2894
2,4,6-Undecatriene, 2894
1-Undecen-3-one, 2894
Undifferentiated cell cultures, 2943
Ungeremine, 1337
United States Pharmacopeia (USP), 3664
Unonopsis buchtienii, 1284

Unpredictability, 238, 244
Unsaturated fatty acids, 2471
uPA. *See* Urokinase-type plasminogen activator (uPA)
uPAR, 2201
UPLC-qToF/MS, 1026
UPLC-TOFMS, 3360
UPP synthases. *See* Undecaprenyl diphosphate (UPP) synthases
Uridine, 991
Uridine diphosphate (UDP)
  galactose, 1669
  glucose, 1664
  glucose consumption pathways, 1633
  glucose dehydrogenase, 1664
  glucuronic acid, 1664
  glucuronosyltransferase, 2475
  glycosyltransferase, 1663
  rhamnose, 1669
  xylose, 1669
Uridine triphosphate, 1664
Urinary disorders, 3477
Urocanic acid, 872, 878
Urokinase-type plasminogen activator (uPA), 2201
Ustilago maydis, 1564
Uterine contraction, 695
Uterotrophic, 2417
UV. *See* Ultraviolet (UV)
UV-vis. *See* Ultraviolet visible (UV–vis)

V
Vaccinium spp., 1548
Vacuoles, 94, 100, 972, 1764
Vacuum liquid chromatography, 3329
Vakognavine, 939
Valencene, 2991, 3001
n-Valeraldehyde (VA), 2886
Valeric acid, 2899
Valine, 835, 991
(2*S*)-Valine, 367
Valoneoyl group, 1979–1980
Valoneoyl unit, 1980
Valtrate, 3049
Vandyl sulphate, 2784
Vanillic, 1958, 1963, 2346
  acid, 1954, 2476
  and syringic acids, 1548
Vanillin, 1549, 4089
Vanillin assay, 2074
Vanillin-sulfuric acid reagent, 3328
Vanilloids, 4003
van Urk's reagent, 692
Varroa mite, 4076
Vascular, 2406
  function, 2299
  health, 2373
  incidents, 2410
  protection, 2362, 2366
  smooth muscle, 3993
  streak disease, 1602
  tone, 2363
Vascular endothelial growth factor (VEGF), 1442, 2197, 2204
Vasoconstrictor, 2327, 3993
Vasodilatation, 2406, 3993
Vasodilatatory, 3622
Vasodilating, 3677
Vasodilation, 2327
Vasopressin, 3709, 3719
Vasoprotective factors, 2373
Vasorelaxant, 694, 3677, 3681
Vasorelaxing, 2363
Vassobia, 3467
Vegetarian diet, 2418
VEGF. *See* Vascular endothelial growth factor (VEGF)
Veno-occlusive disease (VOD), 373
Veno-occlusive liver disease, 1387
Venous hypertension, 3968
Venous-lymphatic disorders, 3968
Ventricular arrhythmias, 945, 1516
Ventricular tachycardia (VT), 3683
Vepris, 724
Veratridine, 57
Verbenaceae, 4095
Vermifuge, 3477
Vernonia, 3529
Vertebrate hosts, 1266
Verticine, 1171, 1189
Verticinone, 1171, 1189
Vertigo, 3724
Very Fast Death Factor (VFDF), 42
Very low density lipoprotein (VLDL-C), 3669
Vestibular, 3724, 3725
Veterinary
  applications, 4072
  medicine, 4061
Vigna unguiculata, 3585
Vimentin expression, 3485
Vinblastine, 580, 587, 588, 1122, 1183, 1454
Vinca, 580, 1353, 1355, 1454
Vincamine, 580, 587–589, 1353
Vincanol, 1353
Vinca rosea L., 1454

Vincristine, 580, 587, 588, 1122, 1454
Vindoline, 580, 584, 587, 588, 1123
Vinflunine, 1129
Viniferin, 2280, 2289
(+)-ε-Viniferin, 1935
ε-Viniferin glucoside, 2286, 2287
Vinpocetine, 1353, 1355
Vinyl dithiins, 3678, 3685
4-Vinylguaiacol, 2347
4-Vinylphenol, 2347, 2348
p-Vinylphenols, 2269
p-Vinylpyranoanthocyanin-flavan-3-ol, 2269
4-Vinylsyringol, 2349
Violaxanthin, 3369, 3390, 4021
virE2/*virE1*, 2950
vir genes, 2949
Virgin olive oil (VOO), 3607
Visceral leishmaniasis (VL), 1265
Visoltricin, 1533
Visual function, 3935, 3936, 3942
Visual pigment, 3926, 3927, 3939, 3940
Visual-spatial, 2414
Vitaceae, 1553, 1687, 1906
Vitamin, 2583, 2593, 2595, 2604
  A, 2737, 3369, 3927, 3939, 4018, 4023, 4024
  C, 1732, 2469
  C corn, 1718
  D, 2405, 2414, 2695
  E, 2469
  K1, 3176
Vitis, 1685, 1687, 1689, 1706
Vitisin, 2282, 2289, 2290, 2299
Vitisin B, 2269
Vitis vinifera L., 1583, 2277, 2582, 2584, 2593, 2946
Vitreous humor, 1077, 1082
Voacanga, 587
VOC. *See* Volatile organic compound (VOC)
VOD. *See* Veno-occlusive disease (VOD)
Volatile organic compound (VOC), 2923
Volatiles, 3983
  components, 3977
  mixtures, 3980
  terpenes, 2822
  terpenoids, 2951
Volatility, 2926, 4096
Voltage-dependent chloride channels, 4067
Voltage-dependent sodium channel, 4066
Voltage-gated calcium channels, 4006
Voltage-gated ion channels, 41
Voltage-gated potassium channels, 4005
Voltage-gated sodium, 64
Voltage-gated sodium channels, 63, 4005
Voltage-sensitive sodium channel, 4066
Volvaltrate B, 3049

W

Wagner–Meerwein rearrangements, 943, 2724, 3081, 3085
Walker carcinosarcoma 256, 993
Wall-coated open-tubular columns (WCOT), 1074
Walnuts, 1736
Water insoluble polyphenols, 2327
Water soluble necines, 370
Wave-bioreactor, 2778
Wavelength blue light, 3929, 3931, 3945, 3946
Wavelengths, 3377, 3931–3933, 3936, 3937, 3944–3946
WCOT. *See* Wall-coated open-tubular columns (WCOT)
WHI. *See* Women's Health Initiative (WHI)
White beer, 2348
White clover, 165
Whole cell biotransformation, 1638
Widen, 2423
Wine, 2261, 2277, 2284, 2296, 2468, 2475, 2477, 2582, 2584, 2588–2590, 2596, 2599, 2600, 2604, 2605
  chemistry, 1993
  consumption, 2278
  polyphenols, 2366
  red, 2476
  red wine, 2469, 2470, 2481
Witches broom, 1602
Witchweed, 3584
Withaferin A, 3476, 3479, 3481, 3483, 3485
Withajardine A, 3471
Withajardins, 3471
Withametelinol, 3472
Withametelinone, 3472
Withametelins, 3471
Withanergia, 3467
Withangulutin B, 3478
Withania, 2786, 3467
Withania somnifera, 3467, 3476, 3477, 3481, 3482, 3485
Withanolide D, 3481
Withanolide E, 3472
Withanolides, 3467, 3469, 3473, 3476, 3477, 3479, 3480, 3482, 3485, 3488
Withanone, 3484

Withaphysacarpin, 3484
Withaphysalins, 3468
Withaphysalins A, 3468
Withaphysalins E, 3468
Withaphysalins M, 3478
Withaphysalins O, 3478
Without fertility disruption, 2421
Wittig reaction, 3079
WNT5a, 2404
Wnt signaling, 2194
Women's Health Initiative (WHI), 2407
World Anti-Doping Agency (WADA), 290
World Health Organization (WHO), 1370, 1380, 3101
Wort, 2335
Wound healing, 1444, 3965

X
Xanomeline, 1344
Xanthid crabs, 59
Xanthine, 961
Xanthine oxidase, 968
Xanthium strumarium, 3528
Xanthohumol, 2154, 2162, 2343, 2346, 2452
Xanthones, 1553
Xanthophylls, 1730, 1732, 2787, 2860, 2868, 3369, 3925, 3928–3931, 3934, 3935, 3937, 3948, 4019, 4020, 4022, 4031
Xanthosine, 959
Xanthoxylin, 1549
Xanthoxylum spp., 1549
Xanthylium cation, 1784
Xenobiotics, 2450, 2472
Xeno-estrogens, 2386
Xenohormetic compounds, 2572
Xerostomia, 599, 877
Xiphophora gladiate, 27
Xylose, 2467

Y
Yamogenin, 3227, 3229, 3230
Yeast, 1705, 1707
Yeast extract, 4044
Yew, 2799
Yohimbine, 589, 596

Z
Zabrotes subfasciatus, 4091
Zanthoxylum, 721
Zanthoxylum limonella, 2998
Zapotidine, 864
Z-β-ocimene, 2979
Z-3-bromo-2-heptenoic acid, 2889
Z-3-bromo-2-nonenoic acid, 2889
Zearalene, 3125
Zeatin, 2737
Zeaxanthin (Z), 3369, 3925, 3926, 3928–3931, 3933–3937, 3939–3944, 3948, 4021
Zeinoxanthin, 3378
Zeta potential, 1155
Zetekitoxin AB, 54
[Z]-3-hexen-1-ol, 4096
Zieridium, 734
Zingiberaceae, 786
Zingiberene, 2985
Zingiber officinale, 2985
Zonocerus variegatus, 4094
Zooecdysteroids, 3828
ZR751, 3478
ZT-1, 1252, 1256
Zygophyllaceae, 800